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**Evaluating long-term consequences of adolescent
antipsychotic exposure**

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Abstract

Antipsychotic drugs (APDs) are being used increasingly to treat a variety of conditions in adolescence. Accordingly there has been a dramatic increase in the prescription of APDs to adolescents over the past twenty years. Adolescence is an important postnatal developmental period in which major maturation changes occur in both brain structures and multiple neurotransmitter systems. Therefore, adolescence may represent a period of sensitivity to environmental perturbations including exposure to such pharmacological agents. The specific neurobiological consequences of APDs on the adolescent brain are however still poorly understood. The aim of the work presented in this thesis is to investigate how APD administration in adolescence can affect the immature adolescent brain, using a rodent model of adolescence.

In this thesis, I examined chronic risperidone treatment (1.3 mg/kg/day for 21-22 days) in adolescent rats (postnatal day (PND) 36-PND56/57) with respect to short- and long-term neurobiological changes. Short-term alterations were measured either during or proximal to chronic treatment. Long-term effects were measured after a lengthy drug-free interval of 36 – 60 days. Risperidone-induced neurobiological effects in adolescents were compared with those in adult rats (PND80-PND100/101) treated with the same risperidone regimen. Risperidone was chosen for detailed examination given this is the atypical APD that is most commonly prescribed to adolescents in the clinic. Behavioural effects were assessed using two well-validated tests for APD action, namely suppression of the conditioned avoidance response (CAR) and the horizontal bar test for catalepsy. Risperidone-induced changes in brain structures and metabolism of the nucleus accumbens (NAc) were examined with clinical comparable methods namely magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (^1H MRS), respectively. Neurochemical alterations and gene expression in the NAc and the striatum were assessed with high performance liquid chromatography (HPLC) and real-time polymerase chain reaction respectively.

My data reveal that, *during* chronic risperidone treatment, adolescent rats were less sensitive to risperidone-induced increases in catalepsy (when tested in horizontal bar test) and escape failures (when tested in CAR paradigm), both of which are striatum-dependent behaviours. By contrast, adult rats were observed to develop a progressive increase in these behaviours during chronic risperidone treatment. Accompanying these behavioural findings, increased levels of dopamine metabolites were observed selectively in the striatum of rats treated with risperidone in adolescence. Increased dopamine metabolites represent increased turnover of dopamine and/or increased dopamine availability, which both suggest increased dopaminergic signalling. Increased dopamine neurotransmission in adolescent-treated rats may overcome the behavioural effects of dopaminergic blockade by risperidone. When assessed after an equivalent drug-free interval of 36 days, rats

previously treated with risperidone in adolescence were less sensitive to catalepsy induced by a challenge dose of risperidone, compared to those previously treated in adulthood. In fact, rats with prior adult risperidone exposure developed a sensitization-like catalepsy response to this challenge. However, no accompanying neurochemical correlates were identified in the striatum in both age groups at this time of assessment.

Unlike these cataleptic outcomes, rats with prior adolescent risperidone treatment were more sensitive to risperidone-induced changes in CAR, a NAc-dependent behaviour, compared to those with prior adult risperidone treatment. These behavioural changes in CAR were detected after a lengthy drug-free interval of 60 days. A challenge dose of risperidone induced a sensitization-like *suppression of previously acquired* CAR selectively in rats treated with risperidone in adolescence. In these same rats, a decreased expression of 5-hydroxytryptamine 2A (5HT_{2A}) receptors and catechol-o-methyl transferase (COMT) was observed in the NAc, the major brain region associated with APD-induced *suppression* of avoidance. Blockade of D2 receptors is critical for CAR suppression, whereas blockade of 5HT_{2A} receptors potentiates this effect. Given 5HT_{2A} receptors, and therefore presumably 5HT_{2A} function, was downregulated in adolescent risperidone-exposed animals, this may help to explain why CAR *suppression* was sensitised in this group. *Acquisition* of CAR was also assessed in another cohort of *previously untrained* rats after a lengthy washout from chronic risperidone treatment. I found that rats with prior adolescent risperidone exposure had a retarded ability to acquire CAR when assessed as adults. However, there were no accompanying changes in the levels of monoamines or their metabolites in the NAc. This risperidone treatment also did not induce any short-term alterations in NAc metabolites or long-term alterations in brain structures in either age group. Whether this CAR learning deficit was specific to adolescent risperidone exposure or if it reflects a broader learning impairment remains unknown.

In summary, through a comparative examination of the same risperidone regimen in adolescent and adult rats, I have identified short- and long-term behavioural and gene expression changes selective to adolescent exposure. These findings indicate that risperidone treatment can alter maturation changes and induce long-term effect on the adolescent brain. The findings of this thesis provide supporting evidence that the adolescent brain differs markedly from the adult brain in response to risperidone. Furthermore, these findings indicate that adolescent APD prescription practices should directly not follow adult findings or guidelines. Given risperidone is the most commonly prescribed atypical APD to adolescents, these findings may prove clinically relevant.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer-reviewed publications

1. **Aung Aung Kywe Moe**, Nyoman D. Kurniawan, Suzanne Alexander, Xiaoying Cui, Thomas H. J. Burne, Darryl W. Eyles. “Long-lasting change in conditioned avoidance response and accumbal gene expression occurs selectively with risperidone treatment in adolescence” – *Neuropharmacology* 2016 Apr 2016; 108:264-274. doi: 10.1016/j.neuropharm.2016.04.035.
2. **Aung Aung Kywe Moe**, James G Scott, Thomas H. J. Burne, Darryl W. Eyles. “Neural changes induced by antipsychotic administration in adolescence: A review of preclinical studies” – *Journal of Psychopharmacology* 2016 Aug;30(8):771-94. doi: 10.1177/0269881116654776.

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This publication on risperidone was incorporated as a part of Chapter 3.

Contributor	Statement of contribution
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Darryl W. Eyles	Wrote and edited the paper (20%)

Contributions by others to the thesis

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Lists of abbreviations

Abbreviation		Definition
¹ H MRS	–	Proton magnetic resonance spectroscopy
3-MT	–	3-Methoxytyramine
5-HT	–	5-hydroxytryptamine or serotonin
5-HIAA	–	5-Hydroxyindoleacetic acid
AMPA	–	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APDs	–	Antipsychotic drugs
CAR	–	Conditioned avoidance response
CLZ	–	Clozapine
COMT	–	Catechol-o-methyl transferase
Cr	–	Creatine
DAT	–	Dopamine transporter
DOPAC	–	3,4-Dihydroxyphenylacetic acid
EPS	–	Extrapyramidal side effects
GABA	–	Gamma-amino butyric acid
GAPDH	–	Glyceraldehyde 3-phosphate dehydrogenase
HAL	–	Haloperidol
HPLC	–	High performance liquid chromatography
HVA	–	Homovanillic acid
IP	–	Intraperitoneal injection
MAO-A/B	–	Monoamine oxidase A/B
MIA	–	Maternal immune activation
MRI	–	Magnetic resonance imaging
NAA	–	N-acetylaspartate
NAc	–	Nucleus accumbens
NMDA	–	N-methyl-D-aspartic acid
NOR	–	Novel object recognition
NVHL	–	Neonatal ventral hippocampal lesion
PCP	–	Phencyclidine
PFC	–	Prefrontal cortex
Poly I:C	–	Polyriboinosinic-polyribocytidylic acid
PND	–	Postnatal days
PPI	–	Prepulse inhibition

RIS	–	Risperidone
RT-PCR	–	Real-time polymerase chain reaction
SC	–	Subcutaneous injections
SD rats	–	Sprague Dawley rats
SEM	–	Standard error of the means
STR	–	Striatum
VEH	–	Vehicle
VTA	–	Ventral tegmental area
WHO	–	World Health Organization

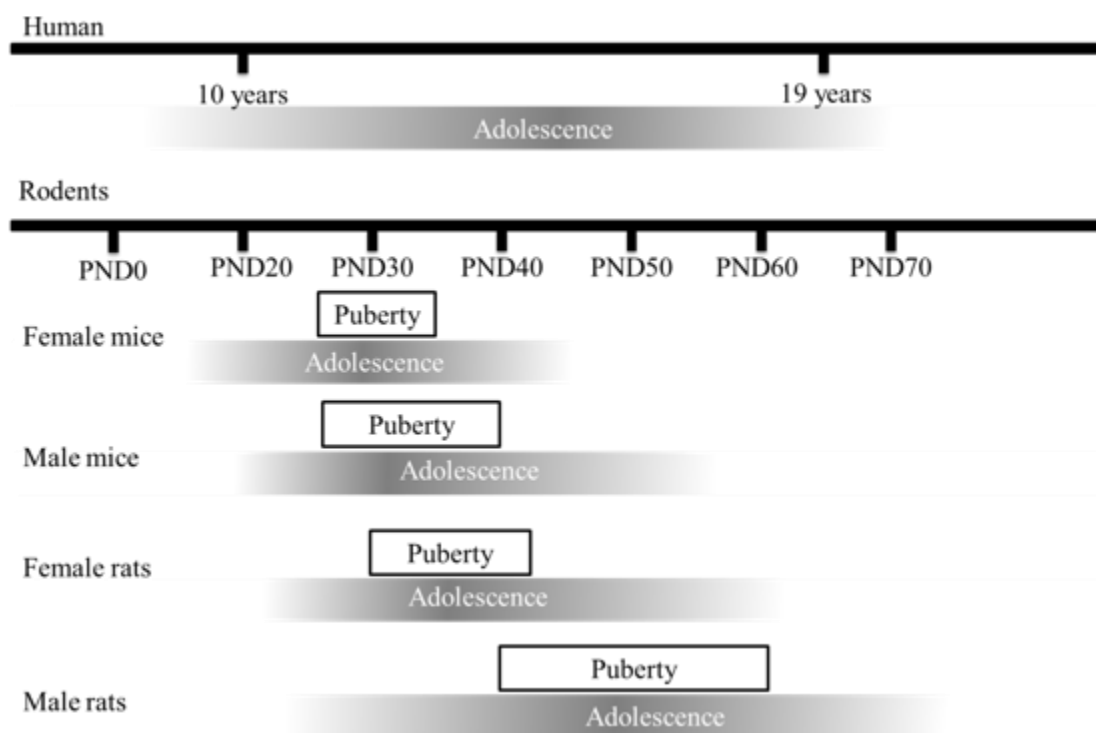
Chapter 1. General introduction

1.1. Adolescence and brain maturation changes

Adolescence represents a period of major changes in brain and behaviour. According to the definition by the World Health Organization (WHO), adolescence spans the period between 10 and 19 years of age (http://www.who.int/maternal_child_adolescent/topics/adolescence/dev/en/). During this period, several major remodelling processes take place in the brain in addition to well-known behavioural and hormonal changes (Dahl 2004a). For example, in human subjects, both longitudinal and cross-sectional studies have reported adolescence-specific maturation changes in grey matter volume and cortical structures (Giedd et al. 1999; Gogtay et al. 2004), volume of subcortical structures (Goddings et al. 2014; Raznahan et al. 2014; Wierenga et al. 2014), functional connectivity among different brain regions (Tomasi and Volkow 2014), global and regional cerebral blood flow (Chiron et al. 1992), local cerebral metabolic rates of glucose utilization (Chugani et al. 1987), dopamine receptors (Jucaite et al. 2010; Seeman et al. 1987; Weickert et al. 2007) and dendritic spines (Petanjek et al. 2011; Peter 1979). Detailed reviews of adolescent maturation changes in humans and primates can be found elsewhere (for example, see (Blakemore 2012; Giedd and Rapoport 2010; Keshavan et al. 2014)).

Developmental changes during adolescence are not restricted to humans. Rodents also undergo changes in neurobehavioural, physical and sexual characteristics which resemble those developmental changes in adolescent humans (Spear 2000; 2007). Based on the timing of these ‘adolescence-like’ characteristics including puberty and its related changes, the adolescent period in experimental rodents (Figure 1-1(a)) is generally considered to span from postnatal day (PND) 28 to PND56 while this period can vary with sex and species (Brenhouse and Andersen 2011; McCutcheon and Marinelli 2009; Schneider 2013; Spear 2000; 2007; Yetnikoff et al. 2014). Other researchers have proposed that it can also be as early as PND21 and as late as PND70 (Burke and Miczek 2014; Schneider 2013; Tirelli et al. 2003).

(a)



(b)

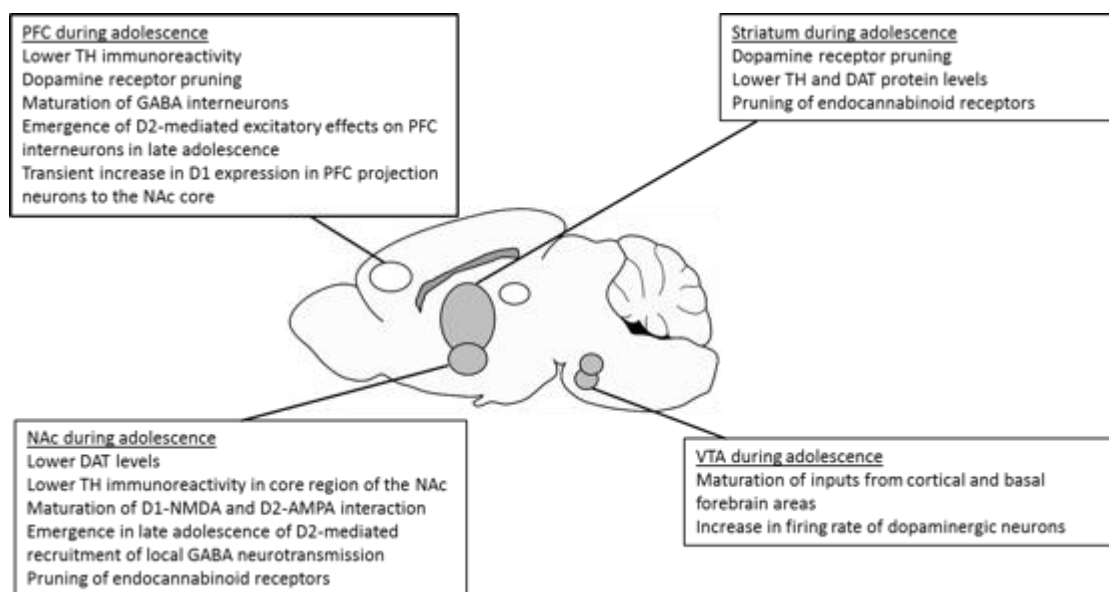


Figure 1-1 Adolescence in rodents and neural maturation changes during adolescence(a) The World Health Organization (WHO) defines the adolescence in human to be between 10 and 19 years. Timing of puberty and the estimated age windows of adolescence in mice and rats of both sexes are shown (Figure adapted after (Schneider 2013)). (b) Invasive neurochemical and neurophysiological investigations in adolescent rodents have shown important changes principally in dopaminergic systems and also in endocannabinoid and gamma-aminobutyric acid (GABA)-ergic systems in major brain regions such as prefrontal cortex (PFC), striatum, nucleus accumbens (NAc) and ventral tegmental area (VTA).

Studies in adolescent rodents have complemented investigations in humans and enabled further identification of multiple neural maturation processes at both structural and functional levels during this period (Figure 1-1(b)); in particular, maturation of dopaminergic (DA) (Andersen et al. 2000; Matthews et al. 2013; McCutcheon et al. 2012; McCutcheon and Marinelli 2009; Tarazi et al. 1998; 1999; Teicher et al. 1995; Tseng and O'Donnell 2007; Yetnikoff et al. 2014) and endocannabinoid systems (de Fonseca et al. 1993; Klugmann et al. 2011) in major brain regions such as striatum, nucleus accumbens (NAc), ventral tegmental area (VTA) and prefrontal cortex (PFC) have been explored. Recently, adolescent maturation of gamma-amino butyric acid (GABA)-secreting interneurons of the PFC has also been reported (Caballero et al. 2014). A recent longitudinal magnetic resonance imaging (MRI) study in rats has also identified a peak in cortical thickness during adolescence and a continued increase in volume of cerebral cortex and striatum and myelination from adolescence to adulthood (until PND60) (Mengler et al. 2014). Levels of striatal metabolites such as N-acetylaspartate (NAA), glutamate and glutamine have also been observed to increase from neonatal period through adolescence to adulthood as examined in a longitudinal magnetic resonance spectroscopy (MRS) study in rats (Morgan et al. 2013). Detailed reviews on maturation of adolescent neuronal systems can be found elsewhere (Brenhouse and Andersen 2011; Lewis 1997; McCutcheon and Marinelli 2009; Spear 2000; Sturman and Moghaddam 2011; Wahlstrom et al. 2010). Given these major remodelling changes in the brain, adolescence has been regarded as a critical postnatal developmental period.

Pharmacological exposures, when given at critical windows, have been hypothesized to be assimilated into normal brain development altering the developmental trajectory, with a subsequent change in mature neuronal function (Andersen and Navalta 2004; Dahl 2004b; Tirelli et al. 2003). Adolescent exposure to antipsychotic drugs (APDs) is of particular importance here given a dramatic increase in prescription of these drugs to adolescents and children over the recent two decades (See Section 2).

APDs are generally classified into two categories: first generation or *typical* APDs and second generation or *atypical* APDs although a consensus for this classification has not been achieved [See reviews for classifications of APDs and their proposed mechanisms (Lieberman et al. 2008; Miyamoto et al. 2005; Miyamoto et al. 2012) and meta-analyses for comparison among different APDs (Leucht et al. 2009; Samara et al. 2014)]. In general, *typical* APDs, for example haloperidol, have high affinities for dopamine D2 receptors, a slow dissociation rate from these receptors and a low affinity for 5-hydroxytryptamine 2A (5HT_{2A}) receptors. As a result of this receptor binding profile, they are associated with an increased likelihood of extrapyramidal side effects (EPS). *Atypical* APDs, for example, clozapine, risperidone and olanzapine, show primary affinities for a

wide range of neurotransmitter receptors including dopaminergic, serotonergic (5-hydroxytryptamine, 5HT), adrenergic alpha (α), muscarinic and histaminergic receptors; have a high ratio of affinity for 5HT_{2A} compared with that for D2 receptors; and fast dissociation from D2 receptors. In adult patients, *atypical* APDs are generally considered to have fewer EPS compared to *typical* APDs. However this notion has been challenged by the findings of Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) schizophrenia trial (Miller et al. 2008). Our current knowledge of neurobiological effects of APDs is largely limited to clinical and preclinical studies in adults. The influence of APDs on the neuronal systems during adolescence is poorly understood. For instance, we do not understand which alterations in the adolescent brain induced by APDs are irreversible, partially or fully reversible nor which exposure window is critical. The use of animal models is crucial here to both complement clinical studies in this age group and permit a detailed interrogation of brain circuits and neurotransmitter pathways (Vitiello et al. 2009).

The neurotransmitters targeted by APDs exist in animals and have a similar maturation profile (Spear 2000; 2007). Well-controlled preclinical studies in adolescent animals can deliver results in a more timely and cost-effective manner than clinical studies. Both short- and long-term outcomes of APDs independent of underlying disease states can also be examined. However, one problem in the interpretation of most preclinical APDs studies is the age of exposure. For example, a number of studies simply report the body weight of the animals utilized. Body weight is an imprecise indicator of age given the large variability of body weights of rats at the same age even from the same colony (McCutcheon and Marinelli 2009). Even in preclinical literature, only a limited number of studies have investigated the outcomes of adolescent APD administration, especially long-term consequences. The majority of studies ignore the age of APD exposure. Here, I critically review preclinical studies of adolescent APD treatment in both neurodevelopmentally normal animals and rodent models of neuropsychiatric disorders. Next I discuss the translational value of these preclinical studies, along with their advantages and disadvantages as well as possible future directions.

1.2. Adolescent APD prescription: A brief overview of the clinical picture

Given the focus here is on the value of *preclinical* studies, I provide only a brief overview of the clinical pharmacoepidemiology of APD use in adolescence. More comprehensive clinical reviews and perspectives on APD prescription in children and adolescents are reported elsewhere (for example, see (Ben Amor 2012; Pringsheim et al. 2011; Ronsley et al. 2015; Schneider et al. 2014; Seida et al. 2012; Vitiello et al. 2009).

An increasing pattern of APD prescription has been observed in patients of all age groups over the previous two decades. In particular, there has been a significant increase in *atypical* APD prescription to adolescents and children, compared to adults, mainly due to lower rates of extrapyramidal side effects compared to *typical* APDs (Karanges et al. 2014; Kaye et al. 2003; Olfson et al. 2012). The increase in APD prescribing to adolescents and children has been reported in the United States (US) (Olfson et al. 2006; Patel et al. 2002; Zito et al. 2003), Australia (Hollingworth et al. 2013; Karanges et al. 2014), France (Verdoux et al. 2015), the United Kingdom (UK) (Kaye et al. 2003), the Netherlands (Kalverdijk et al. 2008), Canada (Ronsley et al. 2013), Israel (Gilat et al. 2011) and China (Song and Guo 2013). Moreover, these studies also report a differential gender distribution of APD prescription. In adolescents and children, a higher proportion of male patients receive APD prescriptions (Hollingworth et al. 2013; Kalverdijk et al. 2008; Olfson et al. 2006; Olfson et al. 2012; Ronsley et al. 2013). The duration of APD administration in youths is also increasing, again with male patients receiving longer duration of APD treatment (Kalverdijk et al. 2008).

Despite the US Food and Drug Administration's (FDA) approval of selected *atypical* APDs such as risperidone, olanzapine and aripiprazole for a limited number of disorders, for example, autism, bipolar disorder and schizophrenia (Ronsley et al. 2015), the majority of APD prescriptions in adolescents and children are for symptom-targeted treatment of behaviour such as aggression, mood instability, violent behaviour or irritability associated with non-psychotic neuropsychiatric disorders (Cooper et al. 2006; Olfson et al. 2006; Olfson et al. 2012; Rettew et al. 2015). Consequently, off-label prescribing constitutes a greater proportion of increased use of APDs in these two populations. In addition, a significant proportion of clinical APD studies in adolescents to date have only investigated short-term tolerability and safety profiles. Very few studies have follow-up periods of more than 1 year (Seida et al. 2012). Information about the efficacy and safety of APDs in adolescents and the existing guidelines also tend to be extrapolated from adult data (Cooper et al. 2006; Correll 2008). Systematic reviews of existing clinical trials in children and adolescents have identified that hyperprolactinemia and metabolic side effects are prominent with *atypical* APD treatment (Martínez-Ortega et al. 2013; Pringsheim et al. 2011). However, the knowledge and understanding of long-term neural outcomes resulting from adolescent APD treatment remains limited and several questions remain unanswered (Patel et al. 2005). Consequently the rise in *atypical* APD prescription to children and adolescents has received significant attention and concern from health care professionals, care-givers and the general public with wide media coverage.

As outlined in the Section 1.1., adolescent brain maturation pathways are highly conserved across species (Spear 2000; 2007). This enables researchers to model adolescent pharmacological

treatment in experimental rodents and investigate the effects of such administration in a strictly-controlled manner. Preclinical APD studies also allow invasive investigations into brain neurochemistry and ultrastructure, which are clearly not possible clinically. Moreover, another advantage of preclinical studies is that the issues of drug-drug interaction, residual drug effect in polypharmacy or sequential drug exposure often confounds interpretation in clinical studies (Andersen and Navalta 2011). Preclinical studies in adolescent rodents can thus allow the investigation of short- and long-term effects of APDs on neural maturation processes with high translational value (Vitiello et al. 2009) although these preclinical studies clearly have certain limitations (See Section 1.4 for discussion on limitations).

1.3. Adolescent APD administration: Findings of preclinical studies

In this section, I critically review and discuss the studies that subjected animals to APDs at postnatal ages corresponding to adolescent human brain development (Brenhouse and Andersen 2011; Burke and Miczek 2014; McCutcheon and Marinelli 2009; Schneider 2013; Spear 2007; Yetnikoff et al. 2014). Studies that examined APD treatment in prenatal period or in animals older than PND60 or that did not clearly describe the age of the animals at the start of treatment were excluded from the review. I choose to discuss the literature on APD treatment in adolescent animals separated on the basis of ‘healthy’ animals (Table 1 and 2) and rodent models of schizophrenia (Table 3) to highlight how the effects of APDs differ, depending on neurobiological status of the animal. The effects of APDs on *control* animals in the studies of rodent models of neuropsychiatric disorders are discussed together with the findings in healthy adolescent animals.

Table 1-1 Short-term outcomes of adolescent APD administration in neurologically intact animals

APD	Dose	Route	Duration	Age of treatment	Species, Strain, Sex	Washout period	Behavioural changes	Neurochemical changes	Metabolic changes	References
HAL	0.05-10 mg/kg	IP	Single administration	Age range from PND18 to PND825	Male SD rats	None	Catalepsy, ptosis and motor effects: PND18, 25 and 32 and 540 rats > PND56 rats	Not studied	Not studied	(Campbell and Baldessarini 1981)
HAL	0.001-30 mg/kg	IP	Single administration	PND18, 30 or 110	SD rats	None	Not studied	HAL-induced changes in DA metabolites in NAc: PND30 rats > PND18 and PND110 rats;	Not studied	(Teicher et al. 1993)
HAL	1 or 2 mg/kg/day	IP	3 or 6 weeks	PND42 to PND62/84	Male C57BL/6 mice	2-4 days	↓ spontaneous alterations in Y maze with both regimens; ↑latency to platform in WM with 6-week exposure	↑striatal D1 and D2 proteins with both regimens; ↑hippocampal D2 proteins only with 6-week treatment; Normal PFC D2 proteins	Not studied	(Xu et al. 2012)

RIS	1 mg/kg/day	Drinking water	140 days	PND28/35 to PND168/175	Male Wistar rats	None; Tests on-drug.	↑PPI only on day 70 of treatment; normal startle response; ↑grooming; Normal locomotion after day 75 and CAR acquisition at day 85	Normal thickness and number of cells(+) for GFAP, Fos, PV, CaBP of PL after 140-day treatment	Not studied	(Castellano et al. 2009)
RIS	0.3 mg/kg t.i.d.	Cookie dough	21 days	PND23 to PND43	Female SD rats	None	↓motor activity on day 12;	↑histamine H1R, NPY and AgRP mRNA levels in mediobasal hypothalamus	↑weight gain, food and water intake starting from day 14 of treatment	(Lian et al. 2015)

RIS	0.3, 1 or 3 mg/kg/day	IP	21 days	PND22 to PND 42	Male SD rats	24 hours	Not studied	<p>↑D1 in NAc and STR with 1 and 3 mg/kg (cf. No change in adults)</p> <p>↑D2 and ↑D3 in mPFC, NAc, STR and hippo e with higher doses (same changes in adults)</p> <p>Normal D3</p>	Not studied	(Moran-Gates et al. 2007)
RIS	0.3, 1 or 3 mg/kg/day	IP	21 days	PND22 to PND 42	Male SD rats	24 hours	Not studied	<p>↓NMDA in both NAc and STR with higher doses (cf. ↓NMDA in STR and Hippo in adults)</p> <p>↑AMPA in mPFC and STR (cf. ↑AMA in STR only in adults)</p> <p>Normal kainate binding</p>	Not studied	(Choi et al. 2009)

RIS	0.3, 1 or 3 mg/kg/day	IP	21 days	PND22 to PND 42	Male SD rats	24 hours	Not studied	<p>↑5HT_{1A} in mPFC and Hippo (cf. ↑5HT_{1A} in mPFC only in adults)</p> <p>↓5HT_{2A} in mPFC (same change in adults)</p>	Not studied	(Choi et al. 2010b)
HAL	0-5 mg/kg	IP, Oral or ICV	Single administration	PND30, 56 or 100	Male SD rats	None	Catalepsy and ptosis by HAL/PPZ: PND30 rats > PND56 and 100 rats regardless of the route	Not studied	Not studied	(Campbell et al. 1988)
PPZ	0-10 mg/kg									
HAL	0.03, 0.1 or 0.3 mg/kg	IP	Single administration	PND22, 40 or 70	Male and female LE rats	None; 1 h after injection	↓locomotion by higher doses in PND40 and PND22 rats; no effect on PND70 rats	Not studied	Not studied	(Wiley 2008)
CLZ	1, 3, 10 mg/kg	IP					↓locomotion by 10 mg/kg only in ♀ PND40 rats			

HAL	0.03, 0.1 or 0.3 mg/kg	IP	10 days	PND40 to PND49	Male and female LE rats	None; 1 h after injection	Motor suppression by both APDs in both sexes; No change in motor effects over 10-day period			
CLZ	1, 3 or 10 mg/kg	IP								
HAL	0.3 mg/kg/day	IP	10 days	PND30 to PND39 or >PND70 to >PND80	Male and female LE rats	None; at 30 min after injection	Catalepsy by HAL: ♂ adolescents < ♂ adults and ♀ of both ages; increasing motor suppression across 10 days in both ages Catalepsy by CLZ: ♀ adolescents > ♀ adults; no difference in ♂;	Not studied	Not studied	(Wiley and Evans 2008)
CLZ	10 mg/kg/day									

HAL	0.5 mg/kg/day	IP	21 days	PND49 to PND69	Female Lister rats	None	Not studied	Not studied	<p>↑Weight gain from day 3 of injection until day 21 (except ZPD);</p> <p>↑intra-abdominal fat and abnormal oestrous cycles (except ZPD);</p> <p>↓uterine weight in HAL- & SUL-treated rats</p>	(Fell et al. 2005)
RIS	0.5 mg/kg/day									
OLZ	4 mg/kg/day									
ZPD	2.5 mg/kg/day									
SUL	10 mg/kg/day									
FLZ	1 mg/kg	IP	21 days	PND22 to PND42	Male SD rats	24 hours	Not studied	<p>↓D1 in mPFC with all 3 APDs; ↓D1 NAc with FLZ; (cf. No change in adults)</p> <p>↑D2 in NAc, STR, Hippo with FLZ and OLZ; ↑D2 in Hippo with</p>	Not studied	(Moran-Gates et al. 2006)
OLZ	5 mg/kg									

CLZ	20 mg/kg twice daily								CLZ; (cf. ↑D2 in mPFC in adults) ↑D4 in NAc, STR with all 3 APDs (same outcome in adults) Normal D3		
RIS	0.3 mg/kg t.i.d.	Cookie dough	20 days	PND 23 PND 52	Male and Female SD rats	2 days	Not studied	RIS: ↓5HT _{2A} in PFC in ♀; ↓D1 in PFC in ♂; OLZ: ↓5HT _{2A} and ↓5HT _{2C} in PFC NAc and striatum in both ♂ and ♀; ARZ: ↓5HT _{2A} in PFC only in ♂;	Not studied	(Lian et al. 2016)	
OLZ	1 mg/kg t.i.d.										
ARZ	1 mg/kg t.i.d.										

5HT = 5-hydroxytryptamine; AgRP = agouti-related peptide; AMPA = 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propionic acid; CaBP = calcium binding protein; CAR = conditioned avoidance response; CLZ = clozapine; CPP = conditioned place preference test; DA = dopamine; FLZ = fluphenazine; GABA = gamma-amino butyric acid; GFAP = glial fibrillary acidic acid protein; HAL = haloperidol; Hippo = hippocampus; ICV = intracerebroventricular; IP = intraperitoneal injection; LE = Long Evans; mPFC = medial prefrontal cortex; NAc = nucleus accumbens; NMDA = N-methyl-D-aspartic acid; NPY = neuropeptide Y; OFC = orbital frontal cortex; OLZ = olanzapine; PCP = phencyclidine; PL = prelimbic cortex; PND = postnatal day; PPZ = perphenazine; PV = parvalbumin; qPCR = real-time quantitative polymerase chain reaction; RIS = risperidone; SC = subcutaneous injection; SD = Sprague Dawley; SUL = sulpiride; VEH = vehicle; WM = water maze test; ZPD = ziprasidone;

1.3.1. APD treatment in ‘healthy’ adolescent animals

1.3.1.1 *Behavioural effects of adolescent APD administration*

1.3.1.1.1. *Locomotor and reward behaviour*

Given the prominent effect of APDs on locomotion, this is the most widely investigated behaviour in adolescent APD studies in animals. Locomotor effects of APDs vary with age, sex, drug, dose, duration of treatment and drug withdrawal.

Early studies reported that, with acute administration, adolescent male rats were more sensitive to motor suppressive effects of haloperidol and perphenazine than adults over the dosage range tested regardless of the route of administration (Campbell and Baldessarini 1981; Campbell et al. 1988; Wiley 2008). Female adolescent rats, in particular PND22, were also sensitive to low dose (0.03 mg/kg) haloperidol-induced motor suppression while this dose actually increased motor activity in adult female rats (Wiley 2008). By contrast, motor suppression by acute clozapine (1, 3 and 10 mg/kg) occurred only in male early-adolescent rats (PND22), without any effect on male rats at PND40 and PND70. Overall, motor suppressive effects of acute administration of APDs appear more prominent in early-adolescent animals, with sex-dependent variation in drug response. By contrast, repeated exposure to either haloperidol or clozapine for 10 days produced less motor suppression in adolescent animals compared to the outcomes in adults (Wiley 2008; Wiley and Evans 2008).

Of more interest, long-term off-drug motor outcomes (i.e. after a drug-free interval from adolescent APD exposure), which may indicate persistent nature of APDs’ effects, have been demonstrated in several recent studies. Adolescent treatment with 0.7 or 2.5 mg/kg haloperidol, a *typical* APD, from PND30 to PND37 induced a long-term increase in adult motor activity at PND80 as measured in circling and open field tests whereas the same regimen at younger (PND20-PND27) and older (PND40-PND47) ages did not lead to such outcomes (Soiza-Reilly and Azcurra 2009). In another study, rats treated subcutaneously with risperidone, an *atypical* APD, from PND14 to PND42, which spans both juvenile and early-adolescent periods in rats, were observed to have spontaneous hyperlocomotion at the 7th day of drug withdrawal (i.e. PND49) (Bardgett et al. 2013). This increased motor activity in both males and females was more robust with a higher dose (3 mg/kg) of risperidone, compared with the lower dose (1 mg/kg), and persisted until PND270. Lack of any significant deficits in T maze spatial discrimination task in the same rats suggests that the adverse effects of juvenile risperidone exposure at least in this study may be restricted to motor activity. Similarly, an increase in adult locomotor activity was reported with adolescent risperidone

administration from PND22 to PND50 and this long-term motor outcome was observed only in males, not in females (De Santis et al. 2016).

After adolescent treatment (PND44-PND48) with olanzapine (1 or 2 mg/kg) or clozapine (10 or 20 mg/kg), an increase in spontaneous ‘baseline’ locomotion developed at PND50 and persisted until adulthood (PND75 and PND90) (Shu et al. 2014a). Another study from the same group has shown that adult rats who had adolescent exposure to risperidone (0.3 or 1 mg/kg/day from PND44 to PND48) showed an increased sensitivity to motor effects of a challenge dose (0.3 mg/kg) of this APD (Qiao et al. 2014a). However, the rats in these studies were examined for the effects of APDs on repeated phencyclidine-induced hyperlocomotion during adolescence. The rats treated with vehicle alone or a combination of vehicle and PCP did not show such abnormal locomotion. Therefore, the observed locomotor abnormalities would be contributed mainly by APD treatment; still the contribution of PCP treatment could not be ruled out.

Long-term adult motor outcomes in *control* animals treated with APDs in adolescence (within studies of rodent schizophrenic models) also provided further evidence of persistent motor abnormalities induced by chronic adolescent APD administration (Table 1-3). Despite variations in APDs, dosage and route used, these studies consistently showed that ‘neurodevelopmentally normal’ *control* animals treated with APDs during adolescence developed locomotor abnormalities in adulthood. When treated with risperidone during adolescence (0.045 or 1.2 mg/kg from PND34 to PND47), *control* rats not exposed to maternal immune activation (MIA) had decreased locomotion both at baseline and after amphetamine (1 mg/kg) challenge in adulthood (Piontkewitz et al. 2011). This long-term locomotor abnormality was selective to high dose (1.2 mg/kg) risperidone exposure. Similarly, another group has reported that non-immune challenged control rats which were treated in adolescence with risperidone or aripiprazole (0.45 and 0.66 mg/kg/day respectively in drinking water from PND35 to PND70) developed abnormal increase in locomotion after saline injection and in the post-stereotypic phase of high dose (5 mg/kg) amphetamine-induced locomotion in adulthood (PND91-92) (Richtand et al. 2011; Richtand et al. 2012). In mouse MIA model, adolescent haloperidol treatment in controls (3 mg/kg/day in drinking water from PND35 to PND65) induced an increased locomotion at baseline and after challenge with saline, amphetamine and MK801, when these mice reached adulthood (PND90-120) (Meyer et al. 2010). In control rats of neonatal ventral hippocampal lesion model, adolescent risperidone treatment (0.085 mg/kg) increased novelty-induced locomotion selectively, without any effect on amphetamine-induced hyperlocomotion (Richtand et al. 2006). Therefore, these studies collectively suggest that long-lasting motor effects can occur in adulthood after adolescent exposure to both *typical* and *atypical* APDs.

In contrast with the findings discussed above, APD-induced long-term locomotor abnormalities were not observed in a limited number of other studies. For instance, rats treated in adolescence with risperidone (1 mg/kg/day for 5 days via subcutaneous injections (SC)) (Qiao et al. 2014a) or haloperidol (0.05 mg/kg/day SC or 0.25 mg/kg/day via osmotic minipumps for 4 weeks) (Gao and Li 2014) did not show any significant locomotor alteration when challenged in adulthood with quinpirole, a D2 agonist. Similarly, adolescent 21-day olanzapine exposure administration (7.5 mg/kg/day via drinking water) did not alter in spontaneous motor behaviour in the open field test in adulthood (Milstein et al. 2013). This study however did not examine psychostimulant induced locomotor response. By contrast, when reward behaviour was assessed in conditioned place preference, rats treated with the same adolescent olanzapine regimen showed significantly higher preference to amphetamine-paired chamber (Vinish et al. 2013). Chronic adolescent clozapine exposure treatment in rats (7.5 mg/kg/day from PND34 to PND47) (Piontkewitz et al. 2009) and in mice (15 mg/kg/day in drinking water from PND35 to PND65) (Meyer et al. 2010) also did not alter baseline or amphetamine-induced locomotion in adulthood. Differences in experimental conditions including drug, dose, route of administration, duration of treatment and method of locomotor assessment (baseline or psychostimulant-induced) may perhaps explain discrepancy in locomotor findings. In general, when given to adolescents at sufficient dose and duration, both *typical* and *atypical* APDs (risperidone and haloperidol in particular) induce long-lasting motor abnormalities in adulthood at baseline or after psychostimulant challenge.

Given the lack of a comparison age group in the aforementioned studies, we indirectly compared these findings with the reported data on adult APD treatment. In adults, chronic treatment with *typical* and *atypical* APDs differentially affects the sensitivity to locomotor and/or stereotypic effects of dopamine-releasing agents. For instance, after withdrawal from chronic treatment with a well-known *typical* APD haloperidol (0.25-1 mg/kg/day for 14-21 days), there were abnormally increased locomotor responses to amphetamine or cocaine (Bédard et al. 2013; Carvalho et al. 2009; Fukushiro et al. 2008; Montanaro et al. 1982; Samaha et al. 2007) and increased stereotypic response to apomorphine (Rupniak et al. 1985; Saldaña et al. 2006). By contrast, chronic exposure to *atypical* APDs such as clozapine, risperidone and olanzapine in adults did not induce such an effect (Bédard et al. 2013; Rupniak et al. 1985; Saldaña et al. 2006; Samaha et al. 2007). Instead, co-treatment with risperidone has been reported to counteract the effects of haloperidol in locomotor response to amphetamine challenge (Carvalho et al. 2009). Similarly, when APD-induced change in reward behaviour was assessed in the operant chambers, chronic treatment in adults with haloperidol, but not olanzapine, significantly increased the lever presses to amphetamine reward (Bédard et al. 2013).

Thus, administration of *typical* APDs induces similar effects on locomotion and reward outcomes in both adolescents and adults whereas chronic exposure to *atypical* APDs induces long-lasting motoric and/or reward effects selectively in adolescents. This differential sensitivity to *atypical* APDs suggests that specific maturation processes of adolescent brain may be more vulnerable to the effects of these drugs. This increased sensitivity of the adolescent brain to *atypical* APDs may have important clinical implications given these *atypical* APDs such as risperidone are the ones most often prescribed to adolescents in clinical practice. The question remains, “What is the underlying neural mechanism(s) that increase(s) the sensitivity of the adolescent brain to long-term motor effects of *atypical* APDs?” In adult exposure, a hyper-responsive dopaminergic system, sometimes referred to as ‘dopamine supersensitivity’ (Montanaro et al. 1982; Samaha et al. 2007), has been suggested to underlie abnormal locomotor responses following chronic exposure to high-potency D2 antagonists such as *typical* APDs. However, this would appear a less likely explanation for the effects of adolescent *atypical* APDs given their affinities for a broad range of different receptors such as 5HT, α and muscarinic receptors. The fact that abnormal locomotion can develop with chronic adolescent treatment with aripiprazole, an *atypical* APD with partial DA agonist function (Shapiro et al. 2003), also questions the central role of ‘dopamine supersensitivity’. Changes in interactions between dopaminergic and glutamatergic pathways which have been implicated in locomotor sensitization induced by psychostimulants such as amphetamine (Vanderschuren and Kalivas 2000) or 5HT systems have yet to be investigated.

What is the target brain region mediating the long-term locomotor effects of adolescent *atypical* APD exposure? Given the role of the NAc in control of locomotor activity (Beninger 1983; Kelly et al. 1975; Staton and Solomon 1984), this brain region may be a target for long-lasting effects of *atypical* APDs given to adolescents. The adolescent NAc undergoes major maturation changes in dopaminergic receptors (Tarazi and Baldessarini 2000; Tarazi et al. 1998) and transporters (Matthews et al. 2013). In addition, functional interaction of dopaminergic (D1 and D2) and glutamatergic receptors (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-D-aspartic acid (NMDA)) in the adolescent cortico-accumbens circuits has not reached the adult level (Huppe-Gourgues and O'Donnell 2012; Huppé-Gourgues and O'Donnell 2012). D2 receptor-mediated modulation of NAc medium spiny neurons also functionally matures from adolescence to adulthood (Benoit-Marand and O'Donnell 2008). Therefore, chronic *atypical* APD treatment in adolescence may perhaps interfere with one or more of these maturation changes in the NAc. There may be some support for these hypotheses. For example, increased D1 and NMDA receptor binding in the NAc was observed selectively with chronic adolescent risperidone treatment at 24 hours after this regimen (Moran-Gates et al. 2007). Some changes, for example, increased D2 binding in the NAc induced by olanzapine treatment appear to persist from adolescence to

adulthood given reports of these changes both proximal to (Moran-Gates et al. 2006) and long after chronic exposure (Vinish et al. 2013). Chronic adolescent olanzapine administration could also induce long-lasting changes in the NAc such as reduced D1 receptor binding, increased D2 receptor binding, decreased evoked DA release (Vinish et al. 2013) and reduced levels of glutamate and GABA (Xu et al. 2015). Nonetheless, the precise neural mechanisms of locomotor/reward effects of adolescent *atypical* APD treatment are still unknown. It is also unclear whether the reported changes in the NAc may also represent downstream or compensatory effects of alterations in neurotransmission in other regions such as the PFC, the VTA or the striatum.

1.3.1.1.2. Conditioned avoidance response (CAR) and catalepsy

CAR is a preclinical behavioural test which enables screening of novel drugs with antipsychotic potential with high predictive validity and reliability (See reviews (Wadenberg 2010; Wadenberg and Hicks 1999)) whereas APD-induced cataleptic response in rodents predicts the potential to induce EPS in humans such as parkinsonism (Hoffman and Donovan 1995; Porsolt et al. 2010; Sanberg et al. 1988). In schizophrenic patients, therapeutic efficacy of APDs is thought to only be obtained when 60-80% of D2 receptors are occupied whereas higher doses inducing >80% D2 occupancy lead to EPS (Kapur et al. 2000). Similarly in the preclinical models, suppression of CAR occurs with 60-80% blockade of dopamine receptors whereas cataleptic response is observed with >80% dopamine receptor occupancy (Wadenberg et al. 2000; Wadenberg et al. 2001b).

In the CAR paradigm, Li and colleagues have examined different APDs in adolescent rats in a series of studies. In this paradigm, repeated daily injections of APDs for 5 days or 4 weeks progressively suppressed the avoidance response in adolescents. After a drug withdrawal period, challenge with the same APD induced a sensitized *suppression* of CAR, i.e. higher blockade of avoidance by a challenge dose occurred in APD-treated rats than in APD-naïve rats. This APD-induced sensitized *suppression* of CAR in adolescence persists until adulthood (PND76-92). This long-lasting increased drug response in CAR has been demonstrated with adolescent treatment with olanzapine (Qiao et al. 2013), risperidone (Qiao et al. 2014a), asenapine (Shu et al. 2014b) and haloperidol (Gao and Li 2014). The exception was clozapine which induces an opposite effect, a tolerance-like state with less suppression of the avoidance response in rats with prior adolescent exposure. Clozapine-induced tolerance response is not long-lasting until adulthood (Qiao et al. 2013). In addition, adult rats previously treated in adolescence with risperidone demonstrated cross-sensitization to olanzapine and cross-tolerance to clozapine in the CAR paradigm (Qiao et al. 2014b).

Sensitization-like effects of APDs on CAR do not seem to be confined to adolescent exposure given similar findings with adult exposure to olanzapine, risperidone, haloperidol and asenapine (Gao and

Li 2013; Mead and Li 2010). However, neurobiological mechanisms underpinning these behavioural changes in CAR, although not thoroughly understood, seem to be different in adolescents and adults. Putative neural circuits involved in acquisition of CAR as well as suppression of this behaviour are discussed in details in Section 6.2. Here I discuss the findings from existing studies related to sensitized suppression of CAR. In adult APD treatment, alterations in DA system may be responsible for sensitized CAR suppression response. Two lines of evidence supporting this theory are: (1) DA neurotransmission in the striatum (Darvas et al. 2011) and the NAc (McCullough et al. 1993; Oleson et al. 2012) plays an important role in the maintenance of CAR and (2) local injection of sulpiride, a D2 antagonist, into the NAc induces suppression of CAR (Wadenberg et al. 1990b). Li and colleagues have also suggested that, in adult APD treatment, abnormal D2 receptor function may underlie sensitization-like CAR response. Through a challenge with 1 mg/kg quinpirole, a D2 agonist, the authors have demonstrated a robust increase in D2-mediated locomotor response in rats treated as adults with risperidone (1 mg/kg/day for 5 days) (Gao and Li 2013).

However studies do not support abnormal D2 function as the sole underlying mechanism for behavioural changes in CAR in animals treated with APDs in adolescence. For instance, heightened locomotor responses to quinpirole were not observed in animals that were treated as adolescents with risperidone (1 mg/kg/day for 5 days) (Qiao et al. 2014a) or haloperidol (0.05 or 0.25 mg/kg/day) (Gao and Li 2014). A recent study also did not find a quantitative change in D2 receptor protein levels in striatum, PFC and hippocampus of adult rats expressing sensitized CAR responses after adolescent asenapine treatment (Shu et al. 2014b). Thus, changes in D2 receptor per se, at least in the striatum, may not be responsible for development of long-term sensitized CAR responses following adolescent APD administration. Functional roles of neuro-receptors in the mesocortico-limbic system such as the NAc have yet to be thoroughly examined. In a recent study, using 5-bromodeoxyuridine (BrdU) incorporation, Li and colleagues have also suggested a possible link between hippocampal cell survival and olanzapine-induced sensitization in CAR in adolescent rats (PND51) at least 2-days after olanzapine exposure (Chou et al. 2015). Yet this finding still needs to be replicated. Given that sensitized CAR responses were observed selectively with repeated injections, but not with continuous delivery through osmotic minipumps (Gao and Li 2014), stress associated with injections and handling as well as pharmacokinetic factors may perhaps play a role in this behavioural outcome. Therefore, several questions remain unanswered and future investigations are required to map out the neural mechanism(s) of APD-induced long-lasting CAR changes in adolescents.

APDs can also induce catalepsy in rodents particularly if given at high doses. Again cataleptic responses to APDs are different in adolescents and adults. Adolescent rats have been reported to be more sensitive to cataleptic effects of *acute* administration of *typical* APDs than adult rats regardless of the route of administration (Campbell and Baldessarini 1981; Campbell et al. 1988; Wiley 2008). By contrast, repeated haloperidol injections (0.5 mg/kg/day) for 10 days induced *less* catalepsy in adolescent male rats (PND30-PND39), compared to adult rats (>PND70) of both sexes (Wiley and Evans 2008). The mechanism for this apparent switch in adolescents from higher sensitivity to catalepsy with acute treatment to lower sensitivity following repeated treatment is still unknown. Blockade of striatal dopamine neurotransmission by APDs is tightly correlated with their cataleptic effect (Wadenberg et al. 2000; Wadenberg et al. 2001b). Therefore, ongoing maturation processes in both pre- and post-synaptic dopamine systems of the adolescent striatum [See, for examples, (Matthews et al. 2013; Teicher et al. 1995)] may perhaps play a role in differential on-drug cataleptic response. Long-term cataleptic responses after a drug-free interval are yet to be investigated with APD administration to adolescents. This is important given the clinical reports of APD-induced movement disorders in the young population (Sikich et al. 2004; Wonodi et al. 2007).

1.3.1.1.3. *Cognition, anxiety and social interaction*

Cognitive outcomes following adolescent APD administration have been examined in preclinical studies. Adult rats treated previously with olanzapine during adolescence (7.5 mg/kg/day from PND28 to PND49) had deficits in working memory (longer time to reach the criteria in delayed non-match to sample task) (Milstein et al. 2013). The same study reported deficits in fear conditioning: olanzapine-treated rats showed abnormal increase in freezing responses to both unpaired conditioned stimulus and context as well as impaired extinction response although the acquisition process was not affected. This adolescent olanzapine regimen was also reported to induce deficits in novel object recognition (NOR) after 10 days of drug-free period i.e. in adulthood (Llorente-Berzal et al. 2012); however risperidone treatment in adolescence (0.5 mg/kg from PND42 to PND56) did not affect NOR at PND 60, i.e. after 4 drug-free days (Zhu et al. 2014). Social interaction responses in adulthood were normal after chronic adolescent treatment with *typical* APD haloperidol (Gao and Li 2014) and *atypical* APDs such as risperidone (Zhu et al. 2014) or olanzapine and aripiprazole (De Santis et al. 2016) regardless of the route of administration.

Adult spatial memory and measures of anxiety appear to be minimally affected by adolescent exposure to *atypical* APDs. Adult rats with prior adolescent olanzapine or clozapine treatment did not show any impairment in either Morris Water Maze (MWM) test or elevated plus maze (EPM) (Milstein et al. 2013; Piontkewitz et al. 2009). However, a recent study reported that adult rats treated with aripiprazole or risperidone treatment in adolescence spent longer time in the centre and

the open part in EPM and these findings were selective to males (De Santis et al. 2016). Reversal learning in T maze in adulthood was also not altered by chronic risperidone administration in juvenile and early-adolescent period (Bardgett et al. 2013). However, chronic adolescent exposure to *typical* APDs seems to have adverse outcomes on these measures. Chronic haloperidol treatment (1 or 2 mg/kg/day for 3 or 6 weeks) in mid-adolescent mice (PND42) could decrease the performance in both Y maze and MWM tests when examined at 2 or 4 days after treatment (Xu et al. 2012). Given the age of mice being 6-week old (i.e. at PND42) at the start of the treatment in this study, we consider these animals to be mid-adolescent although the authors described them as adult mice.

In adult APD treatment, deficits in NOR were observed following chronic exposure to haloperidol, risperidone or clozapine at high dose but not at low dose (Ozdemir et al. 2012; Schröder et al. 2005; Terry Jr et al. 2007b). The findings on cognitive performance in MWM and radial arm maze (8 or 12 arms) are inconsistent, varying with experimental conditions such as dose and duration of APD, withdrawal period and cognitive tests employed. Adverse outcomes have been observed with both *typical* and *atypical* APDs in a few studies (Didriksen et al. 2006; Terry Jr et al. 2007a) although this is far from universal (Hutchings et al. 2013; Ortega-Alvaro et al. 2006; Terry Jr et al. 2007a; Terry Jr et al. 2003). A direct comparison between adults and adolescents with respect to the effects of APDs on cognition has been hampered by experimental variations such as drug, dose and duration of treatment, withdrawal period and cognitive test used. Thus, well-designed studies which simultaneously examine the same APD exposure in adolescents and adults are required.

To sum up, it would appear select domains of cognition and measures of anxiety are susceptible to the long-term impact of adolescent APD treatment but the outcomes seem to be drug-dependent. Yet, it is unclear whether these reported cognitive deficits are selective to adolescent APD administration given the lack of a comparison age group in the aforementioned studies. Neural substrates potentially responsible for these APD-induced cognitive deficits are also still unknown. Since the prefrontal cortex, which undergoes extensive maturation during adolescence, plays a central role in cognitive performance (Miller 2000; Ridderinkhof et al. 2004), APD-induced changes in this region may be crucial. Indeed, some studies have reported neurochemical changes in adult PFC following adolescent APD exposure (See Section 1.3.1.3.2). Other brain regions such as hippocampus which has been implicated in T maze performance [See review (Lalonde 2002)], are yet to be examined for adolescent APD-induced effects.

1.3.1.1.4. Sensorimotor gating

Prepulse inhibition (PPI) of the startle reflex is a behavioural test widely used to examine sensorimotor gating. Deficits in PPI have been reported in neuropsychiatric patients as well as animal models of schizophrenia (Braff et al. 2001; Swerdlow et al. 2001).

Several studies have shown that rats treated with *atypical* APDs such as olanzapine, clozapine and risperidone during adolescence have normal PPI when tested either at 24 hours after APD exposure or even after a lengthy drug washout period (Qiao et al. 2014a; Qiao et al. 2013; Shu et al. 2014a). Similarly, no significant PPI change was observed in adult mice which were treated in adolescence with clozapine (15 mg/kg/day from PND35 to PND65) (Meyer et al. 2010). These findings in adolescents are consistent with data from adult exposure studies, which reported no change in PPI either whilst on drug or 14 days drug-free after chronic treatment with *atypical* APDs such as olanzapine, risperidone and sertindole (Andersen and Pouzet 2001; Terry Jr et al. 2005). Thus, in both adolescents and adults, chronic treatment with *atypical* APDs does not alter sensorimotor gating. However, there has been one report that continuous treatment with risperidone (1 mg/kg/day in drinking water for 140 days) in male Wistar rats from adolescence to adulthood induced a significant increase in PPI at Day 75 of administration (Castellano et al. 2009). Given the prolonged treatment vastly exceeded the duration of adolescence in rats in this study, the observed PPI increase might not be specific to the adolescent exposure. This was confirmed by the authors' findings that changes in PPI and grooming behaviour was not observed at shorter (21 and 35 days) or surprisingly, longer (91 days) time-points of treatment.

In regards to *typical* APDs, chronic administration of haloperidol to adults at low dose (0.08 mg/kg/day via osmotic pumps for 20 days) (Andersen and Pouzet 2001) or high dose (2 mg/kg/day in drinking water for 60-180 days) (Terry Jr et al. 2005) also produced no significant effects on PPI. By contrast, chronic haloperidol treatment in adolescent mice (3 mg/kg/day in drinking water from PND35 to PND65) has been reported to induce long-term reductions in PPI in these same animals when they reached adulthood (Meyer et al. 2010). This finding suggests that the adolescent brain may be more susceptible to PPI-altering effects of *typical* APDs.

Taken together, the existing literature suggests that adolescent exposure to *atypical* APDs produces no long lasting effect on PPI whereas the *typical* APD haloperidol may have long-lasting effect adverse effects. Future studies are required to confirm the detrimental nature of adolescent exposure to *typical* APDs on PPI.

Table 1-2 Long-term outcomes of adolescent APD administration in neurologically intact adolescent animals

APD	Dose	Route	Duration	Age of treatment	Species, Strain, Sex	Washout period	Behavioural changes	Neurochemical changes	References
HAL	0.7 or 2.5 mg/kg/day	IP	8 days	PND20-27, PND30-37 or PND40-47	Male SD rats	33-53 days	↑circling velocity, ↑motor activity at PND80 selectively with PND30-PND37 exposure;	Normal striatal D2 receptor on both radioligand binding and qPCR	(Soiza-Reilly and Azcurra 2009)
HAL	0.05 mg/kg/day	SC	28 days	PND44 to PND72	Male SD rats	7 days	SC >> minipumps in CAR blockade during chronic exposure;	Not studied	(Gao and Li 2014)
	0.25 mg/kg/day	Osmotic minipumps					↑CAR suppression by HAL challenge at PND80-82 only with SC route. Normal social interaction and quinpirole (0.5 or 1 mg/kg)-induced locomotion with both regimens		

RIS	1 or 3 mg/kg/day	SC	29 days	PND14 to PND42	Male and female LE rats	7 days	↓weight which normalized at 3 rd day of drug withdrawal; ↑spontaneous locomotion from PND49 until PND270; Normal reversal learning in T maze	Not studied	(Bardgett et al. 2013)
RIS	0.5 or 1 mg/kg	SC	5 days	PND40 to PND 44	Male SD rats	~ 35 days	↑CAR suppression by RIS challenge at PND80 in 1 mg/kg RIS group; Normal quinpirole-induced hyperlocomotion and PPI;	Not studied	(Qiao et al. 2014a)
RIS ± PCP 3.2 mg/kg	0.3 or 1 mg/kg	SC	5 days	PND44 to PND48			↑suppression of PCP-induced hyperlocomotion by RIS challenge in 0.3 mg/kg group at PND76		
RIS	1 mg/kg/day	SC	5 days	PND40 to PND44	Male SD rats	~ 24 - 30 days	↑PPI only on PND45; Normal learning of CAR with two different conditioned stimuli at PND77-79 Cross-sensitization	Not studied	(Qiao et al. 2014b)

							to OLZ and cross-tolerance to CLZ challenge in CAR at PND80-84		
RIS	0.045 mg/kg/day	IP	14 days	PND34 to PND47	Male Wistar rats	72 days	Not studied	Changes in PFC proteomics at PND120: pathways of mitochondrial function, protein trafficking and cytoskeleton	(Farrelly et al. 2014)
OLZ	7.5 mg/kg/day	Drinking water	21 days	PND28 to PND49	Male LE rats	70-220 days	<p>↓learning in delayed non-match to sample test;</p> <p>No deficit in spatial memory but ↑swimming speed on WM;</p> <p>↑contextual freezing on fear conditioning test;</p> <p>Normal OFT and EPM</p>	<p>Region-specific alterations in dendritic spine pruning;</p> <p>↓D1 receptor and ↑GABA_A receptor binding in medial PFC and OFC;</p> <p>↑D2 receptor binding in medial PFC;</p>	(Milstein et al. 2013)

OLZ	7.5 mg/kg/day	Drinking water	21 days	PND28 to PND49	Male LE rats	~ 135 – 210 days	↑Preference for amphetamine-paired chamber on CPP; No change in body weight	↓D1 and ↑D2 receptor binding and ↓stimulus-evoked DA release in the NAc core	(Vinish et al. 2013)
OLZ	7.5 mg/kg/day	Drinking water	21 days	PND28 to PND49	Male LE rats	40 days	Not studied	↓GABA and ↓glutamate in NAc at PND90-120	(Xu et al. 2015)
ASE ± PCP 2 mg/kg	0.05, 0.1 or 0.2 mg/kg/day	SC	5 days	PND43 to PND47	Male SD rats	28 days	↑CAR suppression by asenapine challenge with 0.1 or 0.2 mg/kg ASE ↓locomotion with 0.1 mg/kg ASE challenge	Normal D2 receptor and ΔFosB proteins in striatum, PFC and hippocampus	(Shu et al. 2014b)

OLZ	1 or 2 mg/kg	SC	5 days	PND43 to PND47	Male SD rats	~ 33 days	↑avoidance suppression by OLZ challenge at PND48, 76 and 92	Not studied	(Qiao et al. 2013)
CLZ	10 or 20 mg/kg						↓avoidance suppression by CLZ challenge only at PND48, not at PND76 and 92. Normal PPI		
OLZ ± PCP3.2 mg/kg	1 or 2 mg/kg	SC	5 days	PND44 to PND48	Male SD rats	~ 2 days or 29 days	↑spontaneous locomotion at PND50, 75 and 90 PCP-induced hyperlocomotion: ↑suppression by OLZ challenge at PND76 and 91, not at PND51;	Not studied	(Shu et al. 2014a)
CLZ ± PCP3.2 mg/kg	10 or 20 mg/kg						↓suppression by CLZ challenge at PND51 and 76, not PND91. Normal PPI		

ARZ	1 mg/kg t.i.d. [#]	Cookie dough	28 days	PND22 to PND50	Male and Female SD rats	22 days	♂ - ↑motor activity in OFT with RIS; ↑centre and open part duration with all 3 APDs; ↑climbing and floating with OLZ;	Not studied	(De Santis et al. 2016)
OLZ	1 mg/kg t.i.d. [#]						♀ - ↓climbing and ↑floating in FST with OLZ and RIS; normal motor and EPM test.		
RIS	0.3 mg/kg t.i.d. [#]						Normal social interaction in both sexes		

AgRP = agouti-related peptide; ASE = asenapine; CaBP = calcium binding protein; CAR = conditioned avoidance response; CLZ = clozapine; CPP = conditioned place preference test; DA = dopamine; EPM = elevated plus maze; GABA = gamma-amino butyric acid; GFAP = glial fibrillary acidic acid protein; HAL = haloperidol; ICV = intracerebroventricular; IP = intraperitoneal injection; LE = Long Evans; NPY = neuropeptide Y; OFC = orbital frontal cortex; OFT = open field test; OLZ = olanzapine; PCP = phencyclidine; PL = prelimbic cortex; PPZ = perphenazine; PV = parvalbumin; qPCR = real-time quantitative polymerase chain reaction; RIS = risperidone; SC = subcutaneous injection; SD = Sprague Dawley; VEH = vehicle; WM = water maze test; ZPD = ziprasidone;

3-step increase over 7 days from starting doses to achieve the stated target doses.

1.3.1.2 Brain structural effects of adolescent APD administration

Only a limited number of preclinical studies have examined the effects of adolescent APD treatment on brain structures.

At gross regional structural level, adolescent treatment with high doses of risperidone (1.2 mg/kg/day IP from PND34 to PND47) was shown to reduce whole brain volume in adulthood (PND120), along with a small reduction in hippocampal volume (Piontkewitz et al. 2011). Other brain structures such as striatum and prefrontal cortex were not examined in this cross-sectional MRI study given the focus of this study on schizophrenia-related structural phenotypes. In addition, voxel-wise changes in brain volume or alterations in shape or geometry of brain regions were not examined in the study by Piontkewitz et al. given the use of manual segmentation method. Another study has reported that prolonged exposure to risperidone (1 mg/kg/day in drinking water) for 140 days from adolescence to adulthood did not induce a significant change in cortical thickness of the prelimbic cortex or the number of cells positively stained with glial fibrillary acidic protein (GFAP), parvalbumin or calbindin in this brain region (Castellano et al. 2009). Another study by Piontkewitz et al. showed that adolescent clozapine treatment (7.5 mg/kg/day from PND34 to PND47) did not induce any long-lasting brain structural change (Piontkewitz et al. 2009).

A longstanding change in whole brain volume with only 2 weeks of risperidone treatment in adolescence (Piontkewitz et al. 2011) raises a question on the selective vulnerability of the adolescent brain structures given that, in adults, a prolonged administration (≥ 8 weeks) is required to induce reductions in whole brain volume. For example, adult rats treated with either haloperidol or olanzapine (2 or 10 mg/kg/day respectively via osmotic mini-pumps) developed a reduction in volumes of whole brain and cortex only at 8 weeks of treatment, but not at 4 weeks, while other structures such as striatum, hippocampus and corpus callosum were unaffected (Vernon et al. 2011). Structural changes induced by APD treatment in adults have also been reported to be dependent on dose, duration of treatment and withdrawal. High doses of haloperidol (2 mg/kg/day) delivered via osmotic minipumps for 8 weeks could induce more robust structural changes (increased striatal volume and decreased cortical volume) than low doses (0.5 mg/kg/day) and the observed brain structural changes were reversible with normalization after 8 weeks of drug-free interval (Vernon et al. 2012). Increased striatal volume has been reported in adult rats when examined after both 4 (haloperidol at 1 mg/kg/day) and 8 months (haloperidol and clozapine at 1 and 20 mg/kg/day respectively) of continuous treatment (Andersson et al. 2002). By contrast, risperidone (1 mg/kg/day for 4 or 8 months) did not induce any change in caudate putamen volume in adults at any time point.

At the ultrastructural level, pruning of dendritic spines from adolescence to adulthood seems to be affected by adolescent olanzapine treatment (7.5 mg/kg/day from PND28 to PND49), with region-specific changes in dendritic architecture such as abnormal dendritic spine density in the medial and orbital prefrontal cortex, the NAc and the dentate gyrus (Milstein et al. 2013). For example, immediately after termination of treatment in adolescence, dendritic spine density (both apical and basal dendrites) in the mPFC layer 3 of olanzapine-treated rats was lower than that of vehicle-treated rats. By contrast, on reaching adulthood, olanzapine-treated rats had higher dendritic spine density in the same region than vehicle-treated controls. These findings suggest that adolescent treatment with atypical APDs can alter developmental trajectory of dendritic architecture. In addition, this study by Milstein et al. also highlights the importance of longitudinal assessment.

In summary, among the small number of studies examining the structural outcome, two studies have reported brain structural changes at the levels of both ultrastructure and gross regional volume induced by adolescent treatment with *atypical* APDs. It appears that the structural outcomes may be dependent on brain region, drug, dose and route. Further studies are also required to replicate the structural findings with adolescent exposure to risperidone and olanzapine as well as to investigate the underlying mechanisms. Several questions are yet to be answered. For example, it is not known whether long-standing changes in cortical or striatal volume, in addition to whole brain, can occur with chronic adolescent APD treatment. The former brain structure is important since major maturation changes occur in cortical structures of adolescent brain (Giedd et al. 1999; Gogtay et al. 2004) and the impact of APDs on these maturation changes is still largely unknown. Studies in adult rats have suggested a possible reversal of APD-induced brain structural changes after a prolonged drug-free interval (Vernon et al. 2012). The question as to whether brain structural effects of adolescent APD treatment are reversible still needs to be addressed while existing studies have indicated possible long-lasting effects (Milstein et al. 2013; Piontkewitz et al. 2011). Although studies in adults suggest a need for prolonged exposure to detect a significant structural change, investigators should also be aware that the duration of adolescence in rodents, as discussed in Section 1.1, is a maximum of 5-6 weeks and any extended treatment longer than this duration [for example, (Castellano et al. 2009)] will not be able to tease apart the adolescent exposure-specific findings. Changes in geometry and shape of brain structures have been reported with chronic APD treatment in adults in addition to volumetric changes (Crum et al. 2016). The outcomes of adolescent APD treatment on brain morphometry are still to be investigated. Given the limitations of traditional manual segmentation approach for volumetric analysis, future studies should also employ voxel-based morphometry or tensor-based morphometry analysis to achieve a more sensitive measurement of voxel-wise changes. Investigation of mechanisms of APD-induced structural changes can also be a good future direction. For example, in adult rats with APD-induced

reduction in anterior cingulate cortex, increased density of neurons and astrocytes have been reported, without any significant alteration in the total number of these cells (Vernon et al. 2014). It is still unknown whether adolescent APD treatment can induce similar changes in neuronal and astrocytic density.

1.3.1.3 Neurochemical effects of adolescent APD administration

In this section, I examine neurochemical changes (i) proximal to adolescent APD exposure i.e. within a few days after termination of APD exposure and (ii) long after exposure i.e. weeks or months after APD exposure. The majority of the studies examining neurochemical outcomes after adolescent APD exposure focus on changes within the striatum, the NAc and the PFC.

1.3.1.3.1. Neurochemical effects proximal to adolescent APD administration

One earlier study showed early-adolescent rats (PND30) were more sensitive to acute haloperidol-induced changes in DA metabolites compared to juveniles (PND18) and adults (PND110) (Teicher et al. 1993). This higher sensitivity in adolescence was selective to the NAc. In the striatum and the frontal cortex, PND18 rats showed the highest sensitivity to haloperidol-induced metabolite changes.

Alterations in several neural receptors immediately after cessation of chronic treatment with different APDs in adolescents have been demonstrated in a series of studies from Tarazi's group (Choi et al. 2009; Choi et al. 2010; Moran-Gates et al. 2006; Moran-Gates et al. 2007). Through retrospective comparison of adolescent and adult exposure to similar doses of APDs, these studies have identified neuro-receptor changes which are selective to adolescent or adult treatment and those common to both ages. For example, adolescent risperidone administration (3 mg/kg/day for 21 days) selectively increased radioligand binding of both accumbal and striatal D1 and hippocampal 5HT_{1A} receptors, while decreasing accumbal NMDA receptor binding. By contrast, a reduction in hippocampal NMDA receptor binding was observed only with adult risperidone treatment (Moran-Gates et al. 2007). Chronic olanzapine treatment (5 mg/kg/day for 21 days) in adolescents downregulated D1 receptor binding in the medial PFC while the same regimen in adults upregulated medial PFC D2 receptors and hippocampal D4 receptors (Moran-Gates et al. 2006). Other neuro-receptor changes such as increased D2 and D4 binding in the NAc and the striatum were observed with both adolescent and adult exposure to risperidone and olanzapine. Another study in mice has also reported that the protein levels of striatal D1 and D2 receptors and hippocampal D2 receptors were elevated, without a significant change in the PFC dopamine receptors, at 3-6 days after chronic haloperidol exposure in mid-adolescence (1 or 2 mg/kg/day for 3 or 6 weeks from PND42) (Xu et al. 2012).

In addition to neuro-receptors, the effects of adolescent APD treatment on presynaptic glutamate neurotransmission have been examined. In the PFC of rats treated with risperidone or paliperidone in adolescence (both at 0.01 mg/kg/day from PND35 to PND55), the basal glutamate levels on microdialysis were normal when examined 1-3 days after exposure to these drugs, i.e. at PND56-58. However, the same rats showed a blunted response to MK801-induced increases in extracellular glutamate in the PFC (Roenker et al. 2011). This study suggests that glutamatergic neurotransmission might have been suppressed at least proximal to adolescent APD exposure. Despite these neurochemical changes, adolescent risperidone administration (0.5 mg/kg/day from PND42 to PND55 via gastric gavage) was reported not to alter immune function in certain brain regions. At PND65, following treatment with this APD, no significant change in Iba-1 labelled activated microglia was observed in the cerebral cortex, the hippocampus and the thalamus of risperidone-treated rats (Zhu et al. 2014).

The studies reviewed above are informative about short-term neural changes proximal to adolescent exposure to different APDs. Nonetheless, important questions on the chronicity and persistence of the observed neural changes have yet to be addressed.

1.3.1.3.2. Neurochemical outcomes that persist long after adolescent APD withdrawal

When examined in adulthood following adolescent olanzapine administration (7.5 mg/kg/day from PND28 to PND49), changes in both postsynaptic (a reduction in D1 receptor binding and an increase in D2 receptor binding) and presynaptic (a decrease in induced DA release) dopaminergic system of the NAc have been observed (Vinish et al. 2013). Moreover, a significant decrease in D1 receptor binding in both medial and orbital prefrontal cortices (MPC and OPC) and an increase in D2 receptor binding in MPC and GABA_A receptor binding in OPC and MPC were also observed in adult rats treated in adolescence with the same olanzapine regimen (Milstein et al. 2013). Similarities of findings on dopamine receptors (a reduced D1 binding in MPC and increased D2 binding in NAc) at both proximal to (Moran-Gates et al. 2006) and long after chronic adolescent exposure as in the above two studies suggest that these changes may indeed persist from adolescence to adulthood. Moreover, chronic olanzapine treatment in adolescence has also been reported to induce a long-lasting reduction in levels of glutamate and GABA in the NAc (Xu et al. 2015).

While functional changes in the NAc induced by adolescent APD exposure are evident from the literature, neurochemical changes in other brain regions, such as the PFC and the striatum, are still far from clear. As shown in a recent proteomic study, adolescent risperidone treatment from PND34 to PND47 induced changes in PFC protein profile at PND120 (Farrelly et al. 2014). Proteins with roles in regulation of cell death, protein trafficking, cytoskeleton, vesicle-mediated transport and

mitochondrial function, were shown to be altered, potentially reflecting a long-term change in brain metabolism remodelling. To date, a few studies have reported normal striatal dopamine D2 receptor levels following adolescent APD administration. When examined as adults at PND120, rats with prior adolescent haloperidol treatment (0.7 or 2.5 mg/kg/day from PND30 to PND37) showed normal striatal D2 receptor levels as measured by radioligand binding and real-time polymerase chain reaction (RT-PCR) (Soiza-Reilly and Azcurra 2009). The protein levels of D2 receptor and Δ FosB proteins in the striatum, the PFC and the hippocampus in adulthood (PND77) were also not altered by adolescent asenapine treatment (0.05, 0.1 or 0.2 mg/kg/day from PND43 to PND47) (Shu et al. 2014b). However, the striatum to date has not been examined thoroughly with adolescent exposure to other commonly prescribed *atypical* APDs such as risperidone and olanzapine.

Long-lasting changes in neurogenesis in the dentate gyrus also appear to develop with adolescent APD exposure. Following adolescent risperidone treatment (0.045 mg/kg from PND34 to PND47), both an increase in BrdU-positive cells and a decrease in number and percentage of cells double-labelled with BrdU, a cell proliferation marker, and NeuN, a neuronal marker, have been reported in the dentate gyrus at PND72 (Piontkewitz et al. 2012).

Taken together, the NAc appears to be particularly labile to adolescent APD treatment, with long-lasting alterations in dopaminergic, GABAergic and glutamatergic systems. These APD-induced alterations may perhaps reflect the locomotor readouts of adolescent APD administration. This is not surprising since the adolescent NAc undergoes several maturation changes as discussed earlier. A more detailed examination with techniques to detect functional change is still needed for other regions such as the striatum, PFC and VTA which undergo important changes in adolescence. For instance, a recent report indicates that the dopamine synthesis capacity of adolescent dorsal striatum is still immature with lower levels of TH and DAT proteins, compared to adult counterparts (Mathews et al. 2009; Matthews et al. 2013). The effects of adolescent APDs on this major maturation change have not been investigated yet. Moreover, adolescent APD-induced changes in the 5HT system, which has important interactions with DA [see reviews (Di Giovanni et al. 2008; Di Matteo et al. 2008)], are still to be investigated. This is relevant given *atypical* APDs such as olanzapine and risperidone, which are most frequently prescribed to adolescents, have high affinities for 5HT receptors (Schotte et al. 1996).

1.3.1.4 Metabolic effects of adolescent APD administration

In rodents, the metabolic effects of APD treatment appear to be more robust in females [See review (Van Der Zwaal et al. 2014)]. The preclinical findings in female adolescent rodents are in line with the clinical reports that adolescent patients are susceptible to the metabolic effects of APDs. As reported by Fell and colleagues, chronic 21-day treatment with both *typical* and *atypical* APDs in

adolescent female hooded Lister rats induced abnormal metabolic and endocrine effects (Fell et al. 2005). A significant increase in intra-abdominal fat, weight and abnormal estrous cycles were observed immediately after cessation of treatment with all APDs studied (risperidone 0.5 mg/kg, olanzapine 4 mg/kg, haloperidol 0.5 mg/kg, sulpiride 10 mg/kg). The exception was 2.5 mg/kg ziprasidone which did not induce a significant metabolic effect. In another recent study, chronic 21-day risperidone treatment of adolescent female rats was shown to significantly increase both food and water intake, and weight gain starting after Day 12 of treatment. The authors proposed that upregulation of histamine H1 receptor, neuropeptide Y and agouti-related peptide in mediobasal hypothalamus in these adolescent female rats would be the underlying mechanism for weight gain-inducing effects of risperidone (Lian et al. 2015).

To date, one preclinical study has investigated long-term metabolic outcomes of adolescent APD exposure. Following adolescent olanzapine administration, long-term abnormalities in triglyceride levels at PND75 have been reported in both males and female rats (Llorente-Berzal et al. 2012). Interestingly, no significant change in triglyceride was observed immediately after cessation of adolescent treatment. The findings of this study suggest that APD-induced metabolic changes may have a delayed emergence during the drug withdrawal.

In summary, a limited number of preclinical studies have examined short- and long-term metabolic effects and possible underlying mechanisms of adolescent APD treatment. Given a high prevalence of metabolic outcomes in the young population in clinical practice, more studies are required to better understand APD-induced long-term metabolic effects and associated mechanisms and ways to prevent them. In preclinical studies, olanzapine has been reported to disturb gut microbiota in rodents along with producing metabolic side effects (Davey et al. 2012) and this effect of olanzapine has been reported to be corrected with antibiotic cocktail therapy (Davey et al. 2013). Thus, a possible future direction can be investigation of effects of adolescent APD treatment on the gut microbiota in addition to those on central nervous system.

1.3.2. Adolescent APD treatment in animal models of neuropsychiatric disorders

Although not yet recommended as a standard practice, early intervention with APDs to prevent the onset of clinical psychosis has been trialed in individuals who satisfy research criteria for being “at-risk mental state” (for example, see (McGlashan et al. 2006; McGorry et al. 2002)). To complement these clinical studies, several preclinical studies have examined the outcomes of early adolescent APD treatment in the animal models of schizophrenia. In this section, the effects of adolescent APD treatment on these model animals will be presented whereas the outcomes of the same adolescent APD regimens on the respective control animals have been presented under specific domains in the previous section.

1.3.2.1 Maternal immune activation models (MIA)

Maternal infection and its consequent immune activation response during critical stages of pregnancy is a significant risk factor for development of schizophrenia in the offspring (Brown 2006; Brown and Patterson 2011). In rodents, maternal infection and immune activation (MIA) can be modelled in two primary ways (Brown 2011): (1) injection of pregnant dams with viral mimic polyriboinosinic-polyribocytidylic acid (Poly I:C) (for example (Shi et al. 2003)) or bacterial lipopolysaccharide (LPS) (for example, (Romero et al. 2007)) and (2) direct inoculation with an infectious agent (for example, (Fatemi et al. 2002)). The offspring of immune-activated dams have been shown to have schizophrenia-like phenotypes changes in behaviour, brain structures and neurochemistry which are relevant to schizophrenia.

In the rat Poly I:C model, adolescent treatment with clozapine (7.5 mg/kg) (Piontkewitz et al. 2009) or risperidone (0.045 or 1.2 mg/kg) (Piontkewitz et al. 2012) prevented the adult onset of altered phenotypes such as structural brain abnormalities (enlarged lateral ventricles and reduced hippocampal volume) and behavioural deficits in latent inhibition (LI), reversal learning and amphetamine-induced locomotor response. Adolescent risperidone treatment (0.045 mg/kg) reversed Poly I:C-induced reduction in calretinin-positive cells and parvalbumin-positive neurons in the dentate gyrus at PND72, with partial rescue of vascular abnormalities at PND100. However, in both MIA and non-MIA control rats that received adolescent risperidone treatment, BrdU-positive cells significantly increased in adult dentate gyrus at PND72 while both number and percentage of cells doubled with BrdU and NeuN decreased (Piontkewitz et al. 2012).

As adults, Poly I:C-exposed rats showed an abnormal increase in basal extracellular glutamate concentration in the PFC at PND55-58. This altered glutamate level was prevented by adolescent treatment with risperidone or paliperidone (both at 0.01 mg/kg/day from PND35 to PND56) (Roemaker et al. 2011). The same group has reported that amphetamine-induced locomotor abnormalities at PND91-92 in Poly I:C rats can be ameliorated by adolescent treatment with risperidone or paliperidone (Richtand et al. 2011) or aripiprazole (Richtand et al. 2012).

In a mouse MIA model, similar protective effects of APDs were also reported in another study that examined adolescent treatment with 3 mg/kg/day haloperidol or 15 mg/kg/day clozapine (Meyer et al. 2010). In Poly I:C mice, adolescent haloperidol treatment prevented the adult onset of behavioural deficits in LI and abnormal locomotor sensitivity to amphetamine and MK-801, but this treatment could not correct Poly I:C-induced PPI reductions. By contrast, adolescent clozapine treatment prevented the adult onset of Poly I:C-induced deficits in PPI and LI without any protective effect on the abnormal locomotor response to psychostimulants.

In summary, these studies suggest that APD treatment prevents the progression of selected behavioural, brain structural and neurochemical changes in adolescent MIA rodents at risk of developing phenotypes relevant to schizophrenia. It is still largely unknown how adolescent APD treatment can prevent these changes induced by MIA although APDs have been suggested to possess anti-inflammatory properties [see review (Drzyzga et al. 2006; Kato et al. 2011)]. However, a recent preclinical study has suggested that chronic APD treatment per se can alter immune responses, by demonstrating increased density and amoeboid reactive morphology of activated microglia in brain regions such as hippocampus, striatum and anterior cingulate cortex after chronic treatment with haloperidol and olanzapine in adult naïve rats (Cotel et al. 2015). By contrast, an earlier study did not find any significant change in activated microglia with chronic risperidone treatment in adolescent rats (Zhu et al. 2014). Therefore, it remains unanswered how APDs exert their immunoregulatory effects and whether the underlying neuropathological status can determine APDs' action on the immune response.

Table 1-3 Outcomes of adolescent APD administration in animal models of neuropsychiatric disorders

Model	APD	Dose	Route	Duration	Age of treatment	Species, Strain and Sex	Effects on model animals	Effects on corresponding control animals	References
MIA by IV 4 mg/kg poly I:C on GD15	RIS	0.045 or 1.2 mg/kg/day	IP	14 days	PND34 to PND47	Male Wistar rats	No effect on body weight Prevention of adult structural abnormalities (↑ventricles and ↓hippocampus) and loss of LI, abnormal rapid reversal and hypersensitivity to AMPH	No effect on body weight ↓whole brain volume ↓locomotion after saline challenge	(Piontkewitz et al. 2011)
	RIS	0.045 mg/kg/day					↑BrdU-labelled DG cells at PND72; ↓ cells (%) double-labelled with BrdU and NeuN at PND72 Prevention of poly I:C induced ↓ CR(+) cells and PV(+) cells	↑BrdU-labelled DG cells at PND72 ↓ cells (%) double-labelled with BrdU and NeuN at PND72	(Piontkewitz et al. 2012)

MIA by IP 8 mg/kg poly I:C on GD14	RIS	0.045 mg/kg/day	Drinking water	35 days	PND35 to PND70	Male and female SD rats	Prevention by RIS and PAL of abnormal locomotor response to 1 mg/kg AMPH on PND91; No effect on response to 5 mg/kg AMPH on PND92;	↑locomotion on saline challenge at PND91 and ↑post-stereotypic locomotion with 5 mg/kg AMPH at PND92 with RIS exposure; No change with PAL treatment.	(Richtand et al. 2011)
	PAL	0.05 mg/kg/day							
MIA by IP 8 mg/kg poly I:C on GD14	RIS or PAL	0.01 mg/kg/day (both at same dose)	Drinking water	21 days	PND35 to PND55	Male SD rats	Reversal of Poly I:C induced ↑ basal glutamate level of PFC at PND55-58	No change in PFC basal glutamate with both drugs; ↓MK-801 induced PFC glutamate levels with both RIS & PAL at PND55-58	(Roemaker et al. 2011)
MIA by IV 4 mg/kg poly I:C on GD15	CLZ	7.5 mg/kg	IP	14 days	PND34 to PND47	Male Wistar rats	Prevention of adult structural abnormalities (↑ventricles and ↓hippocampus), loss of LI and abnormal sensitivity to AMPH at PND90-120+	No effect on brain structures, locomotion and Morris water maze performance at PND90-120+	(Piontkewitz et al. 2009)

MIA by IV 4 mg/kg/ poly I:C at GD15	OLZ	1 or 2 mg/kg	SC	5 days	PND44 to PND48	Male and female SD rats	<p>↑ avoidance suppression by OLZ challenge on PND51;</p> <p>Normal hippocampal BrdU(+) cells at both 2 and 9 days post-BrdU injection;</p> <p>(+)correlation between hippocampal BrdU(+) cells at 2 days post-BrdU injection and change in avoidance suppression from PND44 to PND45;</p>	<p>↑avoidance suppression by OLZ challenge on PND51;</p> <p>Normal hippocampal BrdU(+) cells at both 2 and 9 days post-BrdU injection;</p> <p>(-) correlation between hippocampal BrdU(+) cells at 9 days post-BrdU injection and change in avoidance suppression from PND44 to PND51;</p>	(Chou et al. 2015)
MIA by IV 2 mg/kg poly I:C on GD9	HAL	3 mg/kg	Drinking water	30 days	PND35 to PND65	Male and female C57BL/6 mice	prevention of adult deficits in LI and sensitivity to AMPH and MK801, not PPI sensitivity	↓PPI, ↑locomotion (at baseline, after saline, AMPH and MK801 challenge)	(Meyer et al. 2010)

	CLZ	15 mg/kg					prevention of adult PPI and LI deficits; no effect on psychostimulant sensitivity	minimal effect except ↑ startle response	
	Fluox	20 mg/kg					prevention of PPI deficits and AMPH sensitivity; no effect on LI and MK801	LI deficits and abnormal sensitivity to MK801	
MIA by IP 8 mg/kg poly I:C on GD14	ARZ	0.66 mg/kg/day	Drinking water	35 days	PND35 to PND70	Male and female SD rats	Correction by FLUOX and ARZ of abnormal locomotor response to 1 mg/kg AMPH on PND91;	↑locomotion on challenge with saline, not with 1 mg/kg AMPH on PND91;	(Richtand et al. 2012)
	FLUOX	10 mg/kg/day					No change on response to novelty or saline or 5 mg/kg AMPH at PND92	No effect on locomotor response to 5 mg/kg AMPH at PND92	
Bilateral ibotenic acid injection (10 µg/µl) into VH on PND7	RIS	0.045 or 0.085 mg/kg	IP	22 days	PND35 to PND56	Male SD rats	Prevention of (1) ↑locomotion to novelty by both doses, (2) AMPH-induced hyperlocomotion and (3) ↑ nocturnal locomotion by 0.045 mg/kg but not 0.085	↑locomotor response to novelty No effect on the response to AMPH and nocturnal locomotion	(Richtand et al. 2006)

Bilateral LPS injection (10 µg/µl) into VH on PND7	RIS ± Minocycline 40 mg/kg/day	0.05 mg/kg	Injections (route not specified)	14 days	PND42 to PND55	Male SD rats	Both regimens: Reversal of deficits in social interaction, NOR and PPI at PND58-63 and ↑activated microglia in CCx, hippocampus, thalamus at PND65.	No abnormal findings with either RIS or RIS+minocycline	(Zhu et al. 2014)
Maternal deprivation x 24 hours at PND9	OLZ	7.5 mg/kg/day	Drinking water	21 days	PND28 to PND49	Male and female Wistar rats	Correction of deficits in stress-induced corticosterone response at PND70-71 only in females; partial correction of ↓CB1 receptor levels in MD rats; No effect on NOR and PPI	↓discrimination index in NOR; ↓plasma triglyceride levels on PND75 in both males and females	(Llorente-Berzal et al. 2012)

10 mg/kg PCP SC on PND 2, 6, 9, 12	RIS	0.84 mg/kg/day	Drinking water	63 days	PND35- PND97	Male Wistar rats	Correction of ↓GSH and ↓γGCL, ↓GPx, ↓GR in CCx and hippocampus ↓GSH and ↑γGCL in STR No effect on altered GPx in STR and GR in thalamus and STR, SOD in all regions	↑GSH and ↓GPx in thalamus ↓SOD in CCx, Hippocampus, thalamus, and STR ↓lipid peroxidation in Cortex	(Stojković et al. 2012)
							Correction of ↓bone trabeculae in femur No effect on ↓spine bone density, ↓peri- epididimal and retroperitoneal fat	↓peri-epididimal and retroperitoneal fat ↑weight gain at PND100	(Petronijević et al. 2013)
Neontal lesion of dopamine neurons by intracisternal 100 µg 6- OHDA on PND3	OLZ	5 mg/kg/day	Drinking water	42 days	PND45- PND86	Female SD rats	No effect on supersensitive behavioural response to repeated PCP treatment	Not studied	(Moy et al. 2004)
	CLZ	30 mg/kg/day							
	HAL	2.5 mg/kg/day							

AMPH = amphetamine; BrdU = 5-5-bromo-2'-deoxyuridine; CAR = conditioned avoidance response; CCx = cerebral cortex; CLZ = clozapine; FLUOX = fluoxetine; GD = gestational day; HAL = haloperidol; IP = intraperitoneal; IV = intravenous; LI = latent inhibition; LPS = lipopolysaccharide; MD = maternal deprivation; MIA = maternal immune activation; NOR = novel object recognition; OLZ = olanzapine; PAL = paliperidone; PPI = prepulse inhibition; RIS = risperidone; VEH = vehicle; VH = ventral hippocampus; (-) – negative; (+) – positive;

1.3.2.2 Neonatal ventral hippocampal lesion (NVHL) models

NVHL is another widely accepted neurodevelopmental model of schizophrenia, with post-pubertal emergence of increased locomotor responsiveness to stress, novel environment, and amphetamine (Lipska et al. 1993) and deficits in PPI (Lipska et al. 1995). Treatment with risperidone in adolescence from PND35 to PND56 corrected amphetamine-induced hyper-locomotion at PND57 in NVHL rats, with partial effects on novelty-induced and nocturnal locomotor abnormalities (Richtand et al. 2006). This effect was observed with 0.045 mg/kg risperidone, not with higher doses 0.85 mg/kg, suggesting that optimal dosing may be required to ameliorate a specific behavioural deficit.

In another model of neonatal ventral hippocampal lesion, this time using the bacterial inflammatory agent LPS, adolescent treatment with risperidone (0.5 mg/kg/day from PND42 to PND55) alone or together with minocycline (40 mg/kg/day) via gastric gavage has been studied (Zhu et al. 2014). Immediately after cessation of the treatment, the behavioural deficits (impairment in social interaction, novel object recognition and PPI) and an increase in activated microglia in cerebral cortex, hippocampus and thalamus as identified by labelling with ionized calcium binding adaptor molecule 1 (Iba-1) were reversed in LPS-exposed animals again suggesting neuroprotective effects of APDs in adolescent rodents at risk of schizophrenia-related phenotypes.

1.3.2.3 Maternal deprivation (MD) models

Maternal deprivation (MD) has been used to model neuropsychiatric disorders including schizophrenia in rodents. MD for 24 hours in early postnatal period (PND9) has been shown to induce schizophrenia-like behavioural abnormalities such as deficits in PPI and LI, abnormal sensitivity to amphetamine and apomorphine (Ellenbroek and Riva 2003). The outcomes of adolescent APD treatment in the MD model have also been studied (Llorente-Berzal et al. 2012). In this study, adolescent olanzapine treatment (7.5 mg/kg/day) reversed stress-induced corticosterone abnormalities in MD-exposed females. This treatment induced only partial reversal of CB1 cannabinoid receptor abnormalities in the hippocampus without any significant effect on deficits on novel object recognition.

1.3.2.4 Other models of neuropsychiatric disorders

Perinatal/neonatal treatment with phencyclidine (PCP) constitutes another animal model of schizophrenia with adult onset of behaviours relevant to positive symptoms and cognitive deficits [for example, see (Mouri et al. 2007; Wang et al. 2001)]. Neonatal PCP treatment also induces deficits in glutathione and antioxidant mechanisms in the cortex and the hippocampus at adulthood (Radonjić et al. 2010). Adolescent treatment with risperidone has been reported to reverse these

deficits (Stojković et al. 2012). In addition to conventional APDs, adolescent treatment with novel APDs such as metabotropic glutamate receptor modulators has been examined in this model. Exposure to these agents at adolescence prevents adult onset of behavioural deficits in novel object recognition (Clifton et al. 2013) and PPI (Kjaerby et al. 2013).

Neonatal lesion of dopaminergic neurons by intracisternal injection of 6-hydroxydopamine (6-OHDA) on PND3 induced supersensitive behavioural responses to repeated PCP treatment in adulthood (>PND60) (Moy and Breese 2002). In this model, 6-week treatment with olanzapine, clozapine or haloperidol in adolescence did not correct the abnormal sensitized behavioural response to PCP. Only lengthy treatment with olanzapine for 10 months was able to reverse this sensitivity (Moy et al. 2004).

1.3.2.5 Summary: Adolescent APD treatment in animal models

Preclinical studies of adolescent APD treatment in rodent models of neuropsychiatric disorders indicate that this treatment can provide beneficial outcomes in models where brain development has been altered. Surprisingly, the low number of available preclinical studies has concentrated on a limited range of models. The outcomes of adolescent APD treatment have not been explored in genetic models (for example, DISC1 knock-out (KO), dysbindin KO, reelin KO and neuregulin1 and ErbB4 KO), repeated NMDA receptor antagonist models (for example, phencyclidine, dizocilpine or MK801), repeated amphetamine models or gestational methylazoxymethanol acetate (MAM) model. In the rodent models investigated, adolescent APD treatment could prevent or delay the adult onset of certain deficits in behaviour, such as amphetamine-induced locomotor response and LI, changes to brain structures such as ventricles and hippocampus, and neurochemistry such as reduced PFC glutamate. These preclinical findings have been supportive for the early intervention approach. However, recent meta-analyses of clinical trials on early intervention and the commentaries have challenged the roles of APDs for at risk individuals (Amos 2014; Preti et al. 2014; Stafford et al. 2013; Van Der Gaag et al. 2013). Indeed, this is supported in some preclinical studies. Meyer and colleagues have also reported that early intervention with APDs in adolescence can ameliorate only selected behavioural deficits whilst creating adverse phenotypes in control animals (Meyer et al. 2010). This was discussed previously in Section 1.3.2 and is detailed in Table 1-3. The exception was adolescent clozapine treatment which did not induce any adverse outcomes, at least in the studies reviewed above.

As discussed in Section 1.2, APDs are most often prescribed to adolescents for behavioural symptoms while only a subset of adolescent APD prescription accounts for treatment of either early onset schizophrenia or at-risk mental status individuals. Therefore, it raises the question as to whether or not the existing models of neuropsychiatric disorders focussing on schizophrenia may

have face, construct or predictive validity for the spectrum of behavioural disorders that APDs are prescribed for in the young population. In addition to model animals of neuropsychiatric disorders, studies in neurobiologically intact adolescent rodents are therefore required to further investigate mechanisms by which APDs produce specific behavioural and/or neurochemical change. This aim will be more difficult to be achieved in studies of adolescent APD treatment in animal models of neuropsychiatric disorders, in which neurobiological changes are still not thoroughly understood. From the basic understanding of how a specific behavioural or neurochemical change is produced by APDs in an intact adolescent brain, a mechanistic insight can also be achieved as to improve understanding of alterations in neurodevelopmentally aberrant brains, critical windows of intervention and ways of early efficient amelioration.

1.4. Conclusion

As examined in the previous sections, preclinical studies have provided valuable insight into short- and long-term outcomes of adolescent APD exposure. In the case of the neurobiologically altered brain as encountered in animal models of neuropsychiatric disease, the administration of APDs during adolescence has been shown to reduce or even abolish *certain* disease-related neurobiological or behavioural phenotypes. This encouraging result provides justification for further studies aimed at establishing the exact neurobiological targets of such interventions. It is hoped that such future studies may translate directly to the clinic.

However, APD treatment in healthy neurologically intact adolescent animals also has adverse behavioural, structural and neurochemical consequences both proximal to and long after chronic administration. Behavioural measures such as locomotion and reward behaviour, CAR and working memory appear to be susceptible to long-term effects of adolescent APD exposure while adverse metabolic outcomes have also been demonstrated in rodent studies. In particular, the adolescent brain appears to be more susceptible to the effects of *atypical* APDs, compared to the adult brain. Chronic exposure to *atypical* APDs in adolescence can induce long-standing changes in locomotor and reward behaviour. No such effects are observed in adult animals chronically exposed to *atypical* APDs. Given *atypical* APDs such as risperidone and olanzapine are most frequently prescribed to adolescents (Hollingworth et al. 2013; Olfson et al. 2006; Olfson et al. 2012), results from preclinical studies may prove informative for clinicians.

The neural substrates behind how *atypical* APDs preferentially affect adolescent animals are still unclear. Neurochemical changes induced by adolescent *atypical* APD treatment as reviewed above have provided potential leads for the underlying mechanisms. Changes in dopaminergic, GABA-ergic and glutamatergic systems of different brain regions have been reported mainly proximal to

chronic adolescent APD exposure. Recently, a few studies have reported persistent changes in these systems even after a lengthy washout period from chronic adolescent APD exposure (Milstein et al. 2013; Vinish et al. 2013; Xu et al. 2015) (Figure 1-2). In particular, long-standing neurochemical alterations in the NAc appear to be important in mediating the reported behavioural changes. Yet, it is unclear which neural maturation pathway in the adolescent NAc is more sensitive to chronic APDs. In addition, reported changes in the NAc may perhaps represent the downstream effects of alterations in neural pathways in other brain regions such as the PFC or VTA. Careful examination of these target regions with functional measures is thus needed in addition to those techniques detecting quantitative change.

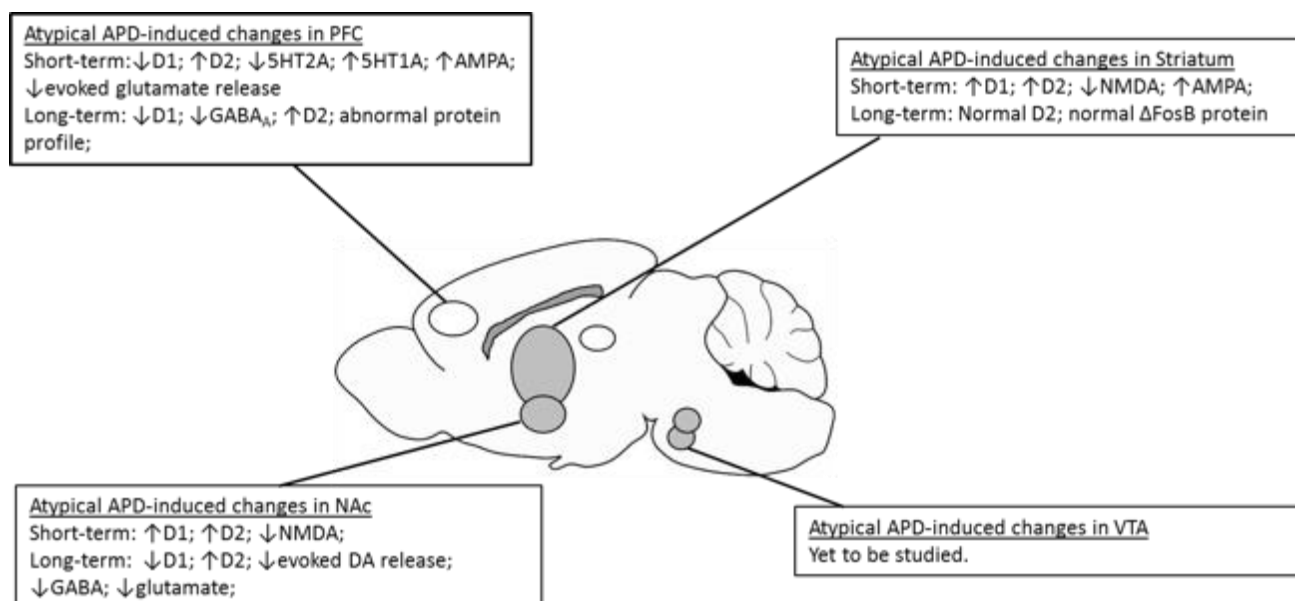


Figure 1-2 Summary of the neurochemical changes induced by chronic adolescent treatment with atypical APDs (risperidone, olanzapine and clozapine) in major brain regions. Short-term changes refer to the findings observed proximal to the atypical APD exposure (i.e. within a few days) and long-term changes to those after a prolonged drug-free interval (i.e. weeks). AMPA – α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NAc – nucleus accumbens; NMDA – N-Methyl-D-aspartic acid; PFC – prefrontal cortex; VTA – ventral tegmental area.

Use of translationally relevant neuroimaging techniques such as functional magnetic resonance imaging (fMRI) and proton magnetic resonance spectroscopy in both clinical and preclinical studies may prove important in the future to longitudinally examine neurochemical alterations induced by adolescent APD treatment. These techniques have matured significantly over the recent years, enabling the research community to examine the trajectory of drug-induced neural activation changes longitudinally from adolescence (during ongoing treatment) to adulthood (after drug-free interval). Robust capability of these techniques to longitudinally monitor brain activity in rodents has been demonstrated in several studies (Moreno et al. 2006; Schobel et al. 2009; Weber et al. 2006). Whole brain mapping of APD-induced neural changes using fMRI or pharmacological MRI [See reviews (Martin and Sibson 2008; Schrantee and Reneman 2014)] can reveal systems-level alterations. Moreover these techniques can help identify adolescent APDs' target brain regions, which in animals could be complemented by other functional measures such as radioligand binding, microdialysis, fast scan cyclic voltammetry or electrophysiology. Behavioural tests on the same animals will also help identify the neural correlates of short- and long-term functional changes.

Another area of interest may be to determine which stage of adolescence (early, mid or late (Burke and Miczek 2014; Tirelli et al. 2003)) is more critical to the effects of APDs. For example, studies examining social isolation or defeat have shown that early adolescence is a more vulnerable window to these environmental factors (Bingham et al. 2011; Makinodan et al. 2012). To some extent this is now being considered for the long-term effect of haloperidol (Soiza-Reilly and Azcurra 2009). Atypical APDs such as risperidone and olanzapine, the most frequently prescribed drugs in adolescents (Hollingworth et al. 2013; Olfson et al. 2006; Olfson et al. 2012), have not been examined in this regard.

The majority of the studies reviewed here utilized male animals, perhaps due to higher rate of APD prescription to male patients in the clinic. Preclinical studies show that metabolic outcomes of adolescent APD administration are more prominent in female rats (Fell et al. 2005; Lian et al. 2015; Van Der Zwaal et al. 2014), in agreement with the clinical reports (Seeman 2009). However, gender differences in other neural outcomes of adolescent APD treatment have often been ignored in most studies. A recent study has elegantly demonstrated the need for inclusion of both males and females, by showing long-term differential behavioural outcomes in males and females treated with the same APD regimens in adolescence (De Santis et al. 2016). Future studies should further investigate sex-dependent outcomes of adolescent APD treatment.

Since all currently used APDs target dopamine receptors existing studies have concentrated on dopamine-enriched brain regions such as the striatum and the NAc. Despite evidence of important maturation changes in other regions such as PFC and VTA these have not been as thoroughly examined for adolescent APD-induced changes. Alterations in these regions may provide insight into reported sub-cortical neurochemical alterations or mechanisms related to cognitive performance influenced by adolescent exposure to APDs. Given the symptom-targeted approach of clinicians, polypharmacy (APD plus other psychotropic drugs such as antidepressants or methylphenidate) is common in children and adolescents (Rettew et al. 2015). The outcomes with APD polypharmacy have still to be examined in preclinical studies.

Preclinical studies with antidepressants are instructive here. Using the same antidepressant regimen in adolescent and adult rodents, these studies have shown differential age-dependent long-term outcomes in behaviour, neurochemistry and drug-induced brain activity, demonstrating the sensitive targets of the adolescent brain (for example, (Homberg et al. 2011; Iñiguez et al. 2010; Karanges et al. 2011; Klomp et al. 2012; Oh et al. 2009)). However, to date no studies that have thoroughly and systematically examined chronic APD exposure treatment in adolescents and adults although the importance of the comparison age group in adolescent studies has been highlighted (Fuhrmann et al. 2015; Spear 2007). Since the existing guidelines for APD prescription in adolescents are largely based on adult findings, comparative examination of APD treatment in adolescents and adults would help identify which maturation processes during adolescence are more vulnerable to APDs compared to adults.

Another critical issue in preclinical adolescent studies is the choice of a clinically relevant treatment with APDs with regards to route, dose and duration of exposure. As can be observed from Section 1.3 and Table 1-1 and 1-2, different studies have utilized different doses, routes and durations of APD treatment. Such experimental variations have impeded a direct comparison of the findings across different laboratories. The route of administration has been suggested to be an important determinant of certain behavioural and neurochemical changes. For instance, as demonstrated by Gao and colleagues, chronic adolescent haloperidol administration could induce differential long-term behavioural outcomes in CAR depending on whether the treatment was delivered via subcutaneous injections or osmotic minipumps (Gao and Li 2014). Subcutaneous or intraperitoneal injections appear to induce more robust long-term effects on CAR and locomotion. However, this route has a drawback that sustained blood levels of APDs cannot be achieved due to the rapid metabolism of rodents (Kapur et al. 2003) and that handling and restraint can induce stress in the animals. Studies examining acute single dose treatment in adolescent animals may have little translational value to the clinic since the clinical use of APDs in children and adolescents is seldom

of this short duration. Given that the duration of APD prescription in adolescents and children is on an increasing trend (Kalverdijk et al. 2008), it may be more translationally relevant to examine chronic or subchronic administration in preclinical studies. The majority of preclinical studies examining the outcomes of APD treatment have determined what dose is clinically relevant based on DA receptor occupancy as advocated by Kapur and colleagues (Kapur et al. 2003). Given the diversity of the disorders that APDs are prescribed for and given the nature of symptom-targeted treatment in adolescents, the dosing approach based on DA receptor occupancy may not be the most optimal. To mimic the clinical scenario of dose titration to response of the patients, a possible optimal approach can be to administer a range of doses of APDs, instead of one dose, to adolescent rodents and determine the outcomes. As discussed in details elsewhere (Spear 2000; 2007), pharmacokinetic factors such as age-related differences in drug metabolism and their possible contribution to the observed outcomes should also be taken into consideration in adolescent APD studies. For example, adolescent mice have been reported to have lower levels of cocaine in plasma and brain than adult mice following acute single dose administration of the same dose (20 mg/kg); however, this difference in plasma and brain levels was not observed following chronic treatment (McCarthy et al. 2004), suggesting a role of chronicity of treatment in determining pharmacokinetics or metabolic adaptation. To the best of my knowledge, no study to date has compared pharmacokinetics in adolescents and adults with both acute and chronic administration of APDs. Measurement of achieved plasma and brain drug concentration and dopamine receptor occupancy during ongoing treatment may help address this issue. Therefore, a careful consideration to these factors must be undertaken in preclinical studies of chronic adolescent APD administration.

In conclusion, investigations of APD treatment in adolescent rodents may indeed prove useful in understanding the neurobiology behind this early treatment. Preclinical studies have provided evidence that, in neurodevelopmentally intact brains, chronic adolescent APD treatment may have a long-lasting impact on behaviour, brain structure and neurotransmission. Future studies should be designed to understand how dosage, chronicity and critical windows of adolescent APD administration can contribute to long-term neurobiological outcomes including secondary adverse effects. Studies using newer advanced imaging capabilities may allow us to understand the trajectory of APD-induced alterations in adolescent brain maturation. Future studies will thus expand our scientific knowledge of APD administration to adolescents and sensitive targets of the adolescent brain. It is imperative that we understand the effects of APDs on the adolescent brain given the increasing APD use in this age group.

1.5. Aims and significance of this thesis

The main goal of this thesis was to identify structural, molecular and behavioural targets within the adolescent brain which can be altered by chronic APD exposure. The main focus was on risperidone which is the most commonly prescribed atypical APD prescribed to adolescents although I have also examined other APD namely clozapine and haloperidol. Here I have examined risperidone treatment in neurodevelopmentally normal adolescent outbred rats and compared my findings with the same treatment in adults.

Specific aims of my thesis are as follows.

- (1) To identify short-term behavioural changes selective to adolescent APD treatment *during* chronic treatment and long-term behavioural alterations after a lengthy drug-free interval in comparison with the same treatment in adults.
- (2) To examine short-term neurochemical changes proximal to (at 24 hours after) adolescent APD treatment and long-term neurochemical alterations after a drug-free interval in comparison with the same regimen in adults
- (3) To examine short-term neurometabolic changes *during* chronic treatment and long-term neurometabolic alterations after a drug-free interval in adolescents in comparison with the same exposure in adults using ^1H MRS
- (4) To examine long-term brain structural changes with risperidone treatment in adolescence in comparison with the same treatment in adulthood, using MRI

Chapter 2. General methods

2.1. Subjects

Male Sprague Dawley (SD) rats were used in all experiments. All procedures were approved by the University of Queensland Animal Ethics Committee and followed the guidelines of the National Health and Medical Research Council of Australia. Rats were pair-housed in Macrolon cages (390 mm x 235 mm x 160 mm) with Sani chip bedding (Able Scientific) and wire lids in a temperature (21 ± 1 °C) and lighting (lights on at 06:00 h and off at 18:00 h) controlled room. All rats were given *ad libitum* access to food and water throughout the whole experiment. Behavioural training and testing were conducted during the light phase of the diurnal cycle.

2.2. Preparation of drugs

All APDs used (haloperidol, clozapine and risperidone (Sigma Aldrich)) were dissolved in 1% acetic acid in water and further diluted in sterile 0.9% normal saline (pH adjusted to 5.7–5.9), to make up to desired volume. Drug concentrations were spectrophotometrically confirmed, using UV absorbance with a known molar absorption coefficient of each APD. The vehicle solution (VEH) was 1% acetic acid diluted with 0.9% normal saline at pH 5.7–5.9. APDs and vehicle were administered to the rats through once-daily intraperitoneal (IP) (1 ml/kg) for 21/22 days except haloperidol which was administered through subcutaneous (SC) injection. Rats were weighed daily approximately 30 min before drug administration. Approximately 60–80% D2 receptor occupancy is required in order to acutely impair CAR behaviour (Natesan et al. 2007; Wadenberg et al. 2001b). The doses of APDs in this study were chosen to achieve such a level of clinically relevant D2 occupancy in SD rats (Kapur et al. 2003; Natesan et al. 2008; Natesan et al. 2006a) and disrupt avoidance responses (Natesan et al. 2008; Natesan et al. 2006a; Wadenberg et al. 2001b) (Also see Chapter 3). Intermittent delivery via IP/SC injection was chosen instead of continuous delivery via osmotic minipumps or oral administration as the delivery route for the following reasons: (1) Delivery through osmotic minipumps has been reported to induce breakthrough dopamine supersensitivity and treatment failure during ongoing treatment (Samaha et al. 2007). (2) SC injections have been reported to induce more robust effects on CAR suppression than osmotic minipumps in both adolescent (Gao and Li 2014) and adult (Samaha et al. 2008) rats. (3) Oral administration may have unpredictable absorption and therefore unstable pharmacokinetic profile.

Table 2-1 Molar absorption coefficients (ϵ) and wavelengths (λ) of absorption of APDs used

APD	Lambda max (λ) (nm)	Epsilon (ϵ) ($M^{-1} cm^{-1}$)	Solvent	Reference source
Risperidone	238 nm	15300	0.1M HCl	Courtesy of Jansen pharmaceutica
Clozapine	297 nm	10500	Ethanol	Merck Index online
Haloperidol	247 nm	13300	1.0M HCl	Merck Index online

2.3. Behavioural tests

2.3.1. Conditioned avoidance responding (CAR)

2.3.1.1 CAR apparatus

Rats were trained and tested in eight identical two-way shuttle boxes, which were individually housed in sound-attenuating cubicles and equipped with 16 photobeam sensors, automatic guillotine door, tone generators and stainless steel grid floor (MedAssociates, USA). The grid floor was connected to aversive stimulus generator to give a mild footshock (0.6 mA) as the unconditioned stimulus (US). The chambers and shock generator were controlled by MED-PC IV software in a computer. A background noise of approximately 68 dB was provided from the fan in the sound-attenuating chamber.

2.3.1.2 CAR training and testing

CAR training was conducted with 40 continuous trials per session per day for 5/7 consecutive days. Following habituation to the CAR boxes for 10 min, each trial began with the presentation of the conditioned stimulus (CS) (80 dB white noise) generated by the centrally located speaker. Movement of the rat into the other chamber within 10 seconds (s) of CS presentation terminated the CS and an avoidance response was recorded. If the rat did not move into the other chamber during the first 10 s of CS presentation, CS was accompanied by US for another 10 s. Movement during this next 10 s of CS-US presentation terminated both CS and US and an escape response was recorded. If the rat failed to make a crossing during the entire 20 s period, both CS and US were terminated and an escape failure was recorded. The inter-trial interval varied randomly from 20 to 40 seconds (Natesan et al. 2006b; Wadenberg et al. 2001b). The avoidance performance in each rat was calculated as the percentage of avoidances (% avoidance) out of the total trials in the session

(either 20 or 40 trials depending on experiment). Escape failure response was also expressed similarly as a percentage. Motor activity was recorded as the number of chamber crossings.

In CAR tests, rats were first injected with APD and avoidance response was examined in 20/40 CS-US trials at 20 min, 90 min, 240 min and 24 hr or 60 min after injection depending on the experiment.

(a)



(b)

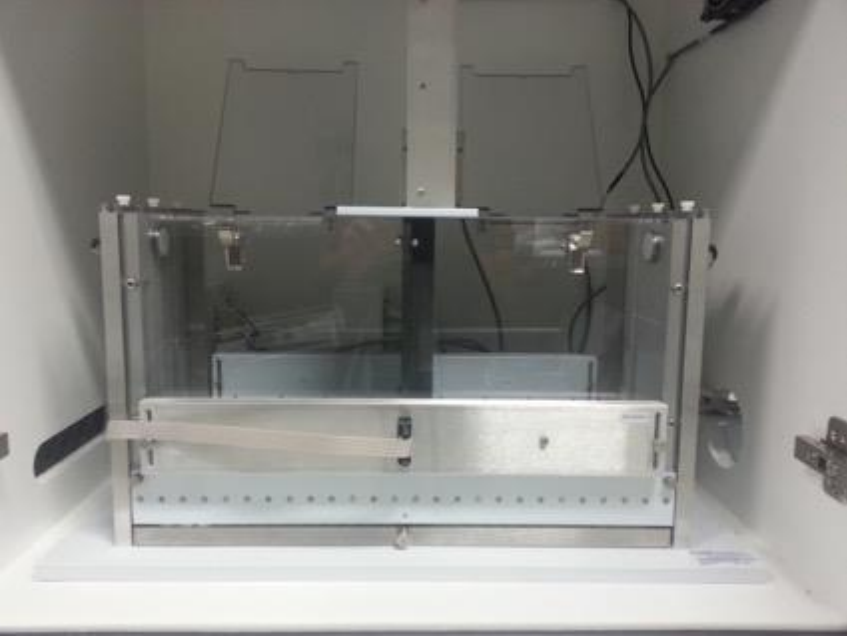


Figure 2-1 CAR chambers used in my study (a) Eight sound-attenuating chambers, aversive stimulus generators and the controlling computer were shown. (b) A two-way shuttle box inside a sound-attenuating chamber was shown.

2.3.2. Catalepsy test

2.3.2.1 Bar apparatus

Horizontal bar test to examine risperidone-induced cataleptic response was performed using catalepsy chamber which was built in-house (35 cm x 20 cm x 25 cm). This chamber had a grid floor and a horizontal bar (1 cm in diameter) with adjustable height (10 cm for adolescents and 13 cm for adults) (Figure 2-1).

2.3.2.2 Procedure

One hour after injection with vehicle or risperidone, rats were individually placed into bar apparatus. The forepaws of rats were gently placed on the horizontal bar and the time rats stayed on the bar was video-recorded. If rats voluntarily removed their paws from the bar, they were placed back on to the bar after a waiting time of 10 seconds. Each rat was examined for a maximum duration of 3 minutes (180 s) or a maximum of 12 trials of placing the forepaws on the bar. The purpose of 12 trials as opposed to single trial determination was to minimize the variability in behavioural response induced by handling stress (Chinen and Frussa-Filho 1999; Wiley 2008).

Videos were coded and analysed in Media Player Classic Home video viewer. The analyst was blind to treatment. The duration rats stayed with both of their forepaws on the bar (time on-bar) was noted in millisecond resolution. The average duration of time on-bar out of 12 trials in each test was calculated for each animal.



Figure 2-2 Catalepsy chamber and video camera used in my study

2.3.3. Open field test (OFT)

2.3.3.1 OFT apparatus

OFT was performed in four black plexiglass chambers (45 cm x 45 cm x 30 cm) which were equipped with infrared beam transmitters and receivers. The locomotor data collected from infrared beam units were transmitted to activity monitor and collection software (Activity Monitor, MedAssociates).

2.3.3.2 Procedure for OFT

Immediately after the completion of catalepsy test (approximately 65 minutes after injection with risperidone or vehicle), rats were placed in OFT chambers. Locomotor activity was recorded for 30 minutes. Total distance travelled (cm), total ambulatory count and total vertical count were recorded.



Figure 2-3 Locomotor activity boxes equipped with infrared beam detectors and the controlling computer

2.4. High performance liquid chromatography (HPLC)

After completion of behavioural tests, rats were sacrificed with an overdose of pentobarbitone sodium (Lethabarb, Virbac) and striatal and accumbal tissues (Paxinos and Watson 2005) collected and immediately frozen in liquid nitrogen and store at -80°C until use. Brain tissues were quickly weighed (wet weight) and homogenised on ice in a minimum volume of 0.1 ml of 0.1M perchloric acid and 50 mg/ml deoxyepinephrine (internal standard for catecholamines), using an ultrasonicator probe (Vibra-Cell, Sonics & Materials, Inc. CT.). After centrifugation at 13,000 rpm for 5 minutes, supernatant from each sample was collected and filtered through 0.2 μm nylon filter. Next, 10 μl of each sample was injected into a HPLC system and dopamine, serotonin, noradrenaline and their metabolites (dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyrarmine (3-MT) and 5-hydroxyindoleacetic acid (5-HIAA)) measured. The HPLC system consisted of an degasser, autosampler and an isocratic HPLC pump (Model 1100, Agilent Technologies, Inc. CA), a Sunfire C18 column, 4.6 mm x 150 mm, 5 μm ; (Waters Corporation, MA) and a Coulochem III (ESA Laboratories, Inc. MA) electrochemical detector. The mobile phase consisted of a 12% acetonitrile / 75mM potassium dihydrogen phosphate buffer containing 25 μM EDTA and 1.7mM octane sulfonic acid adjusted to pH 4.13 with phosphoric acid. Flow rate was 1.2ml/min. Detector settings were as follows: conditioning cell (Model 5020, ESA Laboratories, Inc. MA) at +350mV; analytical cell (Model 5014B, ESA Laboratories, Inc. MA) with the first and second electrodes maintained at -150 and +250 mV, respectively. Data were processed offline with Chemstation software (Rev B.01.03, Agilent Technologies, Inc. CA). The amount of catecholamines and their metabolites were expressed as pictogram per milligram (pg/mg) wet tissue, after correction for the dilution.

2.5. Plasma corticosterone assay

Saphenous blood samples were collected in EDTA tubes from rats under mild restraint. Blood samples were centrifuged at 8000 rpm for 10 min at 4°C and plasma collected and stored at -20°C until use. Plasma corticosterone levels were determined by an in-house liquid chromatography tandem mass spectroscopy (LC-MS/MS) assay. The system consisted of a Shimadzu Nexera[®] UPLC system with a Phenomenex Kinetex[®] 1.7 μ XB-C₁₈ 100 \AA (50x2.1mm) column attached to an ABSciex QTrap-5500[®] triple-quadrupole mass spectrometer. Briefly, in 96-well plates, 20 μl of plasma samples and standards were mixed with 10 μl each of internal standards (500 nM corticosterone-[²H₄] in 1:1 acetonitrile : water) and 10 μl of 1M ZnSO₄ and 600 μl of extraction solvents (9:1 ethyl-acetate : acetonitrile). After centrifugation at 500 rpm for 20 min, 500 μl each of sample mixture was transferred to another clean 96-well plate, evaporated to dryness at 55°C for 20 min in the vacuum concentrator and reconstituted in 50 μl of 1:1 methanol : water. 20 μl sample

extract was then injected in a 384-well plate and assayed overnight. A gradient elution method at 0.5mL/min was used with the mobile phases A= 0.1% aqueous formic acid and B= 0.1% formic acid in 9:1 acetonitrile : water. The mixture was increased from 50%B to 95%B over 2 min, held at 95%B for 0.5 min and then returned to 50%B for 1 min. This resulted in a retention time of 1.2 min. The mass-spectrometer detection was by way of positive-mode, scheduled multiple reaction monitoring with electrospray ionisation. The mass spectrometer parameters were as follows: for corticosterone: $m/z= 247.1 \rightarrow 329.1$, declustering potential (DP)=100V, exit potential (CXP)=12, collision energy (CE)= 23V; for corticosterone- $[^2H_4]$: $m/z= 351.1 \rightarrow 333.0$, DP=100, CXP=15, CE=23. Calibration standards over the range 1000 – 10nM and quality controls at three levels were prepared in stripped plasma. Differential quality control samples were prepared by spiking rat serum with 75nM of corticosterone. A $\pm 15\%$ acceptance criterion was applied to all quality controls.

2.6. Real-time polymerase chain reaction (RT-PCR)

After completion of behavioural tests, rats were euthanised with an overdose of pentobarbitone sodium (Lethabarb, Virbac). Brains were rapidly dissected on ice and either NAc (both core and shell) or striatum (left hemisphere) (Paxions and Watson 2005) collected in RNALater solution (Invitrogen), kept at 4°C overnight and stored at -80°C until RNA was extracted. Briefly, total RNA was extracted from each tissue sample using QIAzol and RNeasy Mini Kit (Qiagen, Australia). For each sample, 0.9/1.0 μg of RNA per 21 μl reaction was reverse-transcribed to cDNA with SuperScript IV First-Strand Synthesis System (Invitrogen). RT-PCR was performed in 12 μl reaction volume on a Roche LightCycler 480 (Roche Diagnostics, Australia), using SYBR Green in 384-well plates. The PCR conditions were as follows: denaturation at 95°C for 5 min followed by 40 cycles of amplification (95°C for 10 s, then 60°C for 20s, then 72°C for 20 s). Relative expression of the target genes (threshold cycle, Ct) was normalized to that of endogenous control, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ΔCt) (Schmittgen and Livak 2008). The primer sequences of the target genes (dopaminergic, serotonergic and GABAergic markers) in this study are listed in the table in Appendix A. All PCR experiments were performed twice. Gene expression data was only considered if significant changes were observed in both experiments. A universal control sample was included in each PCR plate to enable across-age group comparison of $2^{-\Delta\text{Ct}}$ values.

2.7. Structural magnetic resonance imaging (MRI)

MRI scans were performed either longitudinally (PND36, PND57, PND79 and PND120 in Experiment 1A, Chapter 3) or cross-sectionally (PND120 in Experiment 1B, Chapter 3). Rats were anaesthetized with isoflurane (5% for induction and 1.5–1.8% for maintenance with 1.5 L/min

oxygen). The respiratory rate was maintained at 45-60 breaths per minute by adjusting the level of isoflurane as required and the body temperature at 37°C with circulating warm water through the animal bed. Coronal two-dimensional T2-weighted fast spin echo images were acquired at horizontal bore Bruker Biospec 9.4Tesla MRI scanner with Paravision 5.1/6.0 (Bruker, Germany) using with rapid acquisition with relaxation enhancement sequence (RARE) with the following parameters: effective echo time (TE) – 40 ms; repetition time (TR) – 4000 ms; field of view (FOV) – 35 mm x 35 mm; matrix – 256 x 256; in-plane resolution – 0.14 x 0.14 mm; slice thickness – 500 µm; number of slices – 38; RARE factor – 8; number of averages – 10; scan time – 21 minutes. MRI images were coded and the analyst blinded to treatment. A manual segmentation method in the open-source OSIRIX software by a single rater was used to identify the regions of interest (ROI) in both hemispheres. Our laboratory has good experience with this method to detect robust changes in brain volumes in animal model of schizophrenia (Harms et al. 2012). ROIs analysed included whole brain, cerebral cortex, prefrontal cortex, striatum, hippocampus and lateral ventricles using well-established criteria from a rat brain atlas (Paxinos and Watson 2005) and following published methods (Piontkewitz et al. 2011; Vernon et al. 2011). Volume of each ROI was computed by summation of the areas from all respective slices multiplied by slice thickness from both hemispheres. Statistical analysis was performed on regional volumes with or without correction for total brain volumes.

2.8. Proton magnetic resonance spectroscopy (¹H MRS)

2.8.1. Data acquisition

¹H MRS data were acquired together with structural MRI data in Chapter 3. In Chapter 5, only ¹H MRS data were acquired given the focus on brain metabolites in this Chapter. After induction of anaesthesia with 5% isoflurane, rats were mounted on MRI-compatible animal bed. Respiratory rate was maintained at 45-60 breaths per min by adjusting the level of isoflurane as required (1.5-1.7%) with an O₂ flow rate at 1.2 L/min and body temperature at 37°C with circulating warm water through the animal bed.

T2-weighted axial and sagittal localizer images were acquired using the following TurboRARE sequence (TE – 40 ms; TR – 3000 ms; average – 2; repetition – 1; RARE factor – 8; matrix – 256 x 128 for sagittal and 256 x 256 for axial; FOV – 35 mm x 20 mm; 23 x 1 mm slices for axial and 16 x 1 mm slices for sagittal). A voxel of interest (6 mm x 2 mm x 2 mm for adults and 5.5 mm x 1.8 mm x 2 mm for adolescents (PND36)) was placed bilaterally over the NAc (Figure 2-4) following the published literature (Xu et al. 2015). The NAc was chosen for ¹H MRS given the reported role of this brain region in behavioural tests used in the current thesis, namely CAR (Wadenberg et al.

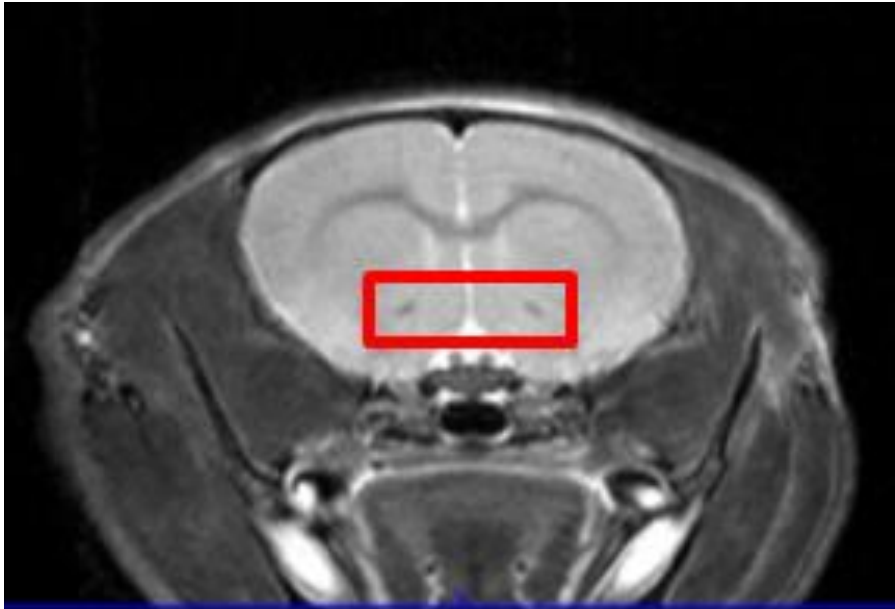
1990a) and locomotion (Kelly et al. 1975), and given reported long-term changes in GABA and glutamate in this brain region with adolescent olanzapine treatment (Xu et al. 2015). Adjustment of first-order, second-order and third-order shims over the voxel was performed with MAPSHIM procedure. A non-suppressed reference water signal from the voxel was obtained for assessment of linewidths and as a metabolite concentration reference. A point-resolved spectroscopy sequence (PRESS) was used to obtain water-suppressed metabolite spectra from the NAc with the following parameters: TE – 9.9 ms; TR – 2500 ms; averages – 356; repetition – 1/15. Chemical shift selective (CHESS) method was used for suppression of the water signal. PRESS was chosen over MEGA-PRESS given that PRESS allows detection of multiple metabolites of interest including N-acetylaspartate (NAA), glutamate and GABA whereas MEGA-PRESS can only detect GABA. 1024 complex points were collected over a spectral/sweep width of 5597.0 Hz/14 ppm and a final 50% linewidth of 13.09 ± 0.13 Hz or 0.032 ± 0.0003 ppm.

2.8.2. Data analysis

^1H MRS data were processed on TOPSPIN and analysed in Linear Combination of Model spectra (LCModel version 6.3-1J) software (Provencher 1993), using the reference basis sets with the same data acquisition parameter. Figure 2-5 shows an example of LCModel fitting of ^1H MRS data in my study.

Given that Cramer-Rao Lower Bounds (CRLB) or %SD ≤ 20 has been reported as acceptable level of quantification reliability (Provencher 2001), metabolites with CRLB or %SD >20 were rejected from the analysis unless otherwise described. The concentration of individual metabolites was expressed as a ratio to total creatine (Cr + PCr) following the guidelines in LCModel manual. Calculation of ‘absolute metabolite concentration’ with water-scaling was not performed since this approach needs large uncertain corrections for water concentration and relaxation, and possible instrumental attenuation of unsuppressed water signal according to LCModel manual.

(a)



(b)

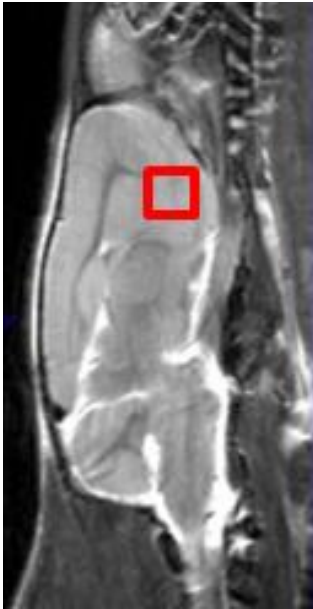


Figure 2-4 Bilateral localization of voxel over nucleus accumbens for both (a) coronal and (b) axial sections.

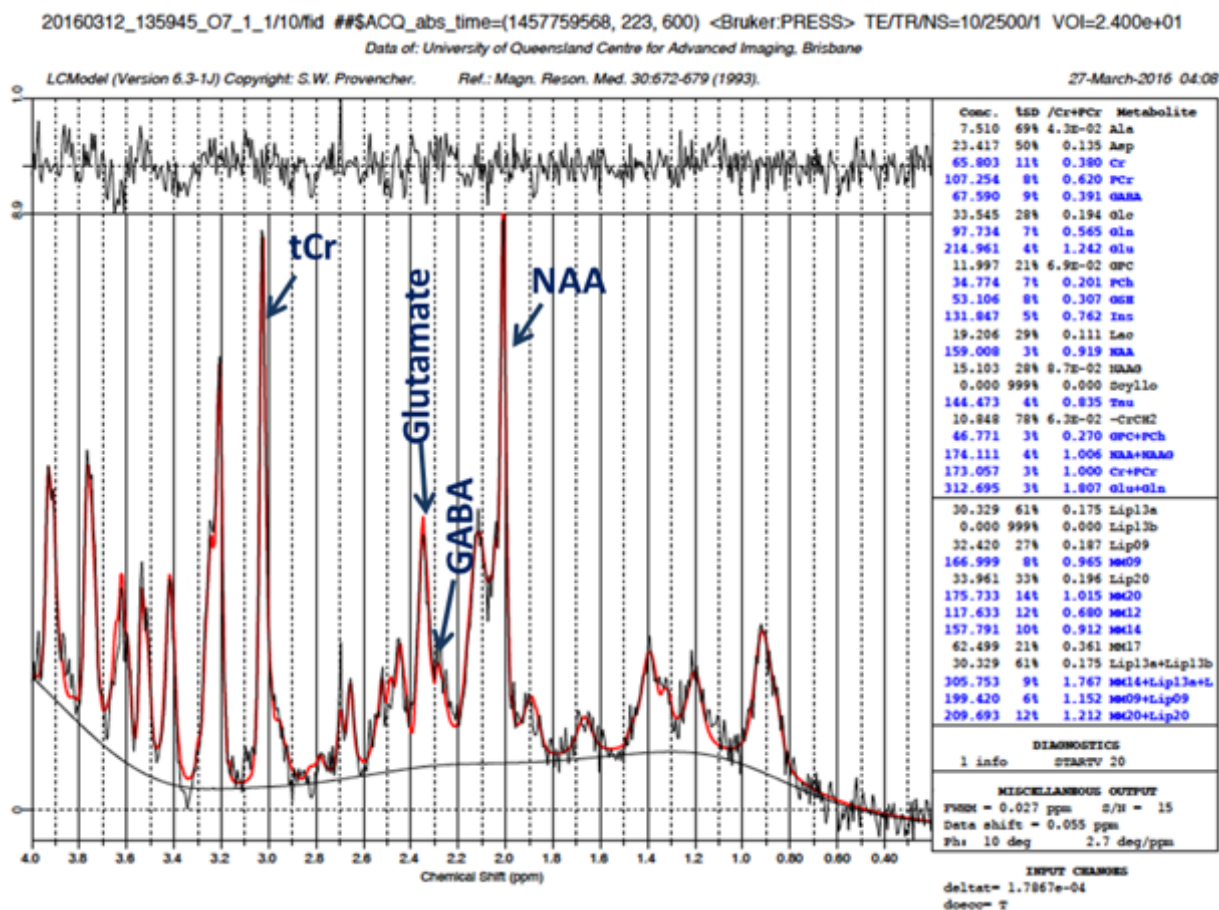


Figure 2-5 An example of LCMoDel fitting of ^1H MRS data. The red line of the plot indicates LCMoDel fit of the data, the thin black line the baseline and the upper plot the residuals i.e. data minus the fit to the data. The columns on the right indicate the metabolites and their concentration. The metabolites with %SD (Cramer-Rao Lower Bounds (CRLB)) below 15 are highlighted in blue. In MISCELLANEOUS OUTPUT in the lower part of the column, full width at half-maximum (FWHM) is also indicated. Data with %SD >20 and FWHM >0.1 ppm are rejected from analysis. tCr = total creatine (Cr + PCr).

Chapter 3. A comparative examination of APD-induced short- and long-term behavioural, structural and neurochemical changes in adolescents and adults with a focus on risperidone

3.1. Introduction

As discussed in Chapter 1, studies in both humans and rodents have shown that important maturation processes are occurring during adolescent brain development. Such data has led to the proposal that adolescence can be conceived as a period of vulnerability to the onset of psychiatric disorders (Paus et al. 2008). In addition, it has been hypothesized that psychopharmacological agents, when given in adolescence, can alter the trajectory of brain maturation, subsequently inducing persistent changes in neural function in adulthood (Andersen 2003; Andersen and Navalta 2004; Fuhrmann et al. 2015; Spear 2007).

Exposure to antipsychotic drugs (APDs) in adolescence is important in this regard given a dramatic rise in prescription of these drugs to adolescents over the past twenty years. APDs target multiple neurotransmitter systems (for example, see (Kapur et al. 2000; Lieberman et al. 2008)) and can induce structural alterations in certain brain regions (Gur et al. 1998; Lieberman et al. 2005). Given that neural systems targeted by APDs are still maturing in adolescence, it seems plausible that such systems may be permanently affected by adolescent APD exposure. However, the long-term effects of chronic APD treatment on immature adolescent brains are poorly understood. This state of affairs is receiving increasing attention and concerns continue to be raised regarding the safety of adolescent APD use (Arango et al. 2004; Ben Amor 2012; Correll 2008; McKinney and Renk 2011). Comprehensive preclinical studies will help to both clarify the long-term neurobiological consequences of such exposures and begin to address whether such concerns are warranted (Vitiello et al. 2009).

In preclinical studies, conditioned avoidance response (CAR) is a well-validated behavioural test widely used for screening of pharmacological compounds with APD potential (Wadenberg 2010; Wadenberg and Hicks 1999). Adolescent APD treatment *suppresses* CAR both during chronic/repeated treatment and after a drug-free interval of 2-3 days or several weeks. Like psychomimetics, APDs would also appear to produce drug sensitisation when assessing CAR. Treatment in adolescence with both atypical APDs such as olanzapine (Qiao et al. 2013), risperidone (Qiao et al. 2014a) and asenapine (Gao and Li 2013) and typical APDs i.e. haloperidol (Gao and Li 2014) have all been shown to induce a sensitization-like CAR *suppression* response to a later challenge dose of these same APDs after drug washout. Practically this is observed as a higher suppression of avoidance response by the challenge dose in rats with prior exposure to APD compared to APD-naïve rats. This sensitized response has been reported at both short term i.e. approximately after 2 days of drug-free interval and at long term i.e. after 25-30 days of drug

washout. By contrast, adolescent clozapine treatment has been shown to induce tolerance i.e. lower suppression of avoidance by a challenge dose in rats with prior clozapine treatment compared to that seen in clozapine-naïve rats (Qiao et al. 2013). However, in these studies, the duration of treatment with the atypical APDs was brief, being only for 5 days (the aforementioned study with haloperidol was for 28 days). Therefore the duration of treatment may not mimic the clinical scenario of a more chronic APD exposure in adolescents. In addition, given a lack of a comparison age group in these studies, it is unknown whether the behavioural changes in the CAR paradigm were selective to adolescent treatment. Despite the reports in the literature that sensitization-like response to a challenge dose of APD can develop with adult APD treatment (for example, (Gao and Li 2013; Mead and Li 2010)), experimental discrepancies such as different ages at the time of CAR training and testing have impeded direct comparison between adolescent and adult studies.

The underlying neural mechanism for how APDs induce sensitization in the CAR paradigm following adolescent treatment is still unknown. In adult APD treatment, an increase in D2 receptor neurotransmission has been suggested as a possible mechanism at least for sensitized CAR response (Gao and Li 2013). These investigators have demonstrated an increased locomotor response to quinpirole, a D2 agonist, in rats with sensitized CAR suppression following adult risperidone treatment. However, this mechanism appears to be less likely in the sensitized CAR response following adolescent risperidone treatment since no significant increase in quinpirole-induced locomotor response was observed in adolescents (Qiao et al. 2014a). In addition, it is still unknown which major brain region is responsible for sensitized responses in the CAR. The NAc has been reported to play a central role in the maintenance of avoidance response (McCullough et al. 1993; Oleson et al. 2012). Intra-NAc injection of APD has also been reported to induce suppression of avoidance in a similar manner to systemic injection of APD (Wadenberg et al. 1990b). Therefore, the NAc seems the likely brain region. However no study to date has thoroughly examined the neurochemical correlates of such behaviour in the adolescent or adult NAc after APD treatment.

Given reports of brain structural changes with APD treatment in clinical studies (Gur et al. 1998; Ho et al. 2011; Lieberman et al. 2005) and preclinical studies in macaque monkeys (Dorph-Petersen et al. 2005) and in adult rats (Vernon et al. 2011), it is plausible that early exposure to APDs may also affect adolescent brain structural maturation. This hypothesis has been supported by a preclinical study which showed a reduction in whole brain volume in adulthood following adolescent risperidone treatment in 'neurodevelopmentally normal' rats (Piontkewitz et al. 2011). However, this study did not examine important brain structures such as PFC and striatum. Therefore further studies are still required to further investigate APD-induced structural changes.

The aim of the current study was to test the hypothesis that chronic adolescent exposure to APDs, especially risperidone, the atypical APD most commonly prescribed to adolescents (Hollingworth et al. 2013; Olfson et al. 2012; Ronsley et al. 2013), can induce long-lasting changes in brain structure, function and neurochemistry when compared with the same exposure in adults. I compared chronic APD treatment in adolescents and adults with regards to changes in (1) the behaviour in the CAR paradigm, (2) brain structures and (3) neurochemistry which may possibly underlie the behavioural change. I first established that the selected doses and routes of three common APDs (risperidone, clozapine and haloperidol) were behaviourally active in the CAR paradigm in adults. Next I screened these three APDs for their short- and long-term effects on the CAR and brain structures in adolescents, in comparison with adults. Following up on the behavioural finding, I examined risperidone treatment in adolescents and adults in greater detail.

3.2. Pilot experiment: Examination of acute single doses of APDs in adults

The aims of this pilot experiment were:

- (1) To determine the dose and route of selected APDs and
- (2) To choose the optimal time point for examination of APD-induced suppression of avoidance response.

3.2.1. Materials and methods

3.2.1.1 Subjects

Adult male SD rats (12-15 weeks old) were utilized in this experiment. Rats were pair-housed in Macrolon cages with Sani chip bedding and wire lids in a temperature (21 ± 1 °C) and lighting (lights on at 06:00 h and off at 18:00 h) controlled room. All rats were given *ad libitum* access to food and water throughout the whole experiment. Behavioural training and testing were conducted during the light phase of the diurnal cycle.

3.2.1.2 CAR training

Rats (n = 26) were trained in 40 CS-US trials per session per day for 5 consecutive days. At the end of the training, 20 rats of 26 (77% success rate) reaching the criteria of $\geq 70\%$ avoidance were selected and assigned to receive a *single* injection of vehicle, haloperidol, risperidone, or clozapine.

3.2.1.3 CAR test with single dose administration of APDs

One day after CAR training, intact CAR was confirmed in all rats prior to injection (0 min test). Next, rats were injected with either vehicle (IP, n = 10), 0.05 mg/kg haloperidol (SC, n = 4), 1.3 mg/kg risperidone (IP, n = 6) or 15 mg/kg clozapine (IP, n = 4) and drug response was examined in

another 4 sessions of 20 CS-US trials at 20 min, 90 min, 240 min and 24 hours respectively after the injection. Avoidance, escape failures and crossings were recorded in each CAR test session.

3.2.1.4 Statistical analysis

Data were analysed with repeated measures one-way ANOVA with Dunnett's post hoc tests. Statistical significance was defined as $p < 0.05$. Avoidance and chamber crossing data were expressed as mean \pm SEM. Escape failure data were expressed as median \pm semi-interquartile range.

3.2.2. Results

Significant *suppression* of avoidance response by all three APDs was observed between 20 and 90 min after injection (Figure 3-1). By 240 min, the drug effect had started to wear off and by 24 hours after injection, rats had regained their pre-APD avoidance levels. Repeated measures ANOVA confirmed the finding with a significant main effect of time ($F_{4,80} = 34.3$, $p < 0.001$), drug ($F_{3,20} = 62.4$, $p < 0.001$) and time x drug interaction ($F_{12,80} = 7.2$, $p < 0.001$). Post-hoc tests further confirmed the significant suppression of CAR by all three APDs at 20 and 90 min after injection (all $p < 0.05$).

Chamber crossings (Figure 3-2) were also suppressed by APDs (significant main effect of time ($F_{4,80} = 6.71$, $p < 0.001$) and time x drug interaction ($F_{12,80} = 2.38$, $p = 0.011$) but no significant effect of drug ($F_{3,20} = 2.394$, $p = 0.099$); repeated measures one-way ANOVA). Further analysis showed that crossings were significantly lowered by both clozapine and risperidone, but not by haloperidol, at 90 minutes after injection (both $p < 0.05$).

As shown in Table 3-1, the three APDs did not significantly induce escape failures at any time point examined (significant main effect of time ($F_{4,80} = 3.158$, $p = 0.018$) but no main effect of drug ($F_{3,20} = 1.023$) or time x drug interaction ($F_{12,80} = 1.093$), both $p > 0.05$).

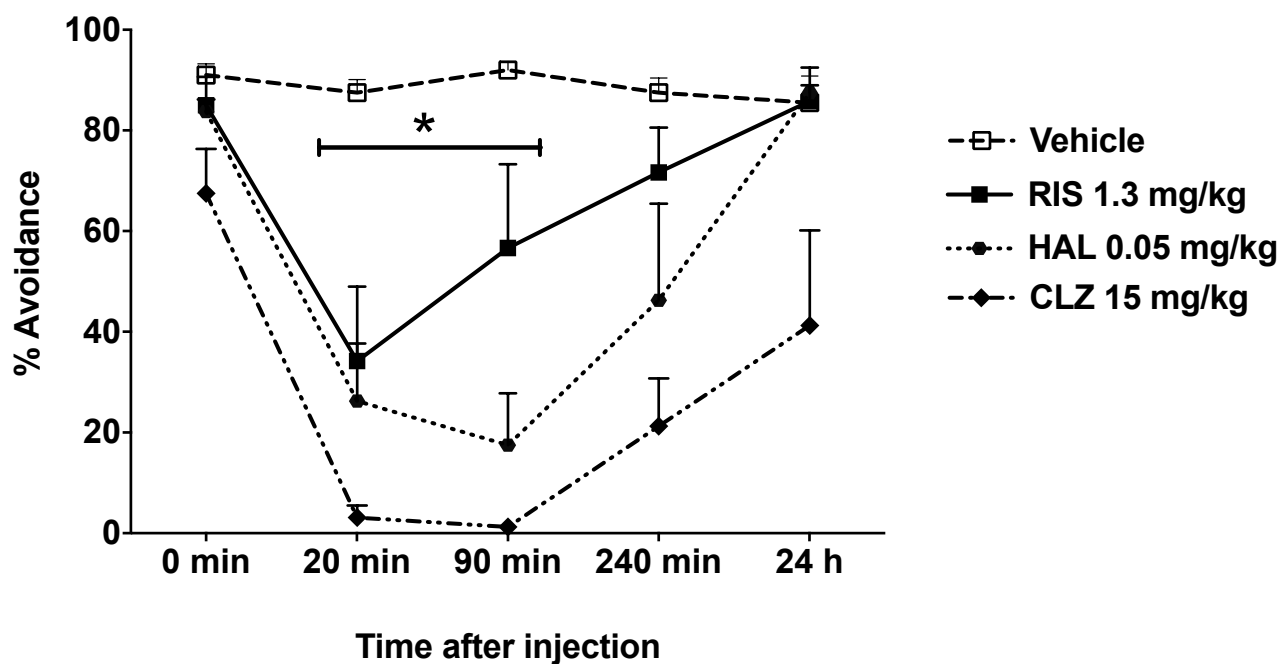


Figure 3-1 Avoidance suppression by acute administration of single doses of risperidone, clozapine and haloperidol. Error bars – mean \pm SEM. $n=10$ for VEH, $n=6$ for RIS, $n=4$ each for HAL and CLZ. * $p < 0.05$ VEH vs all APDs. VEH – vehicle; RIS – risperidone; HAL – haloperidol; CLZ – clozapine

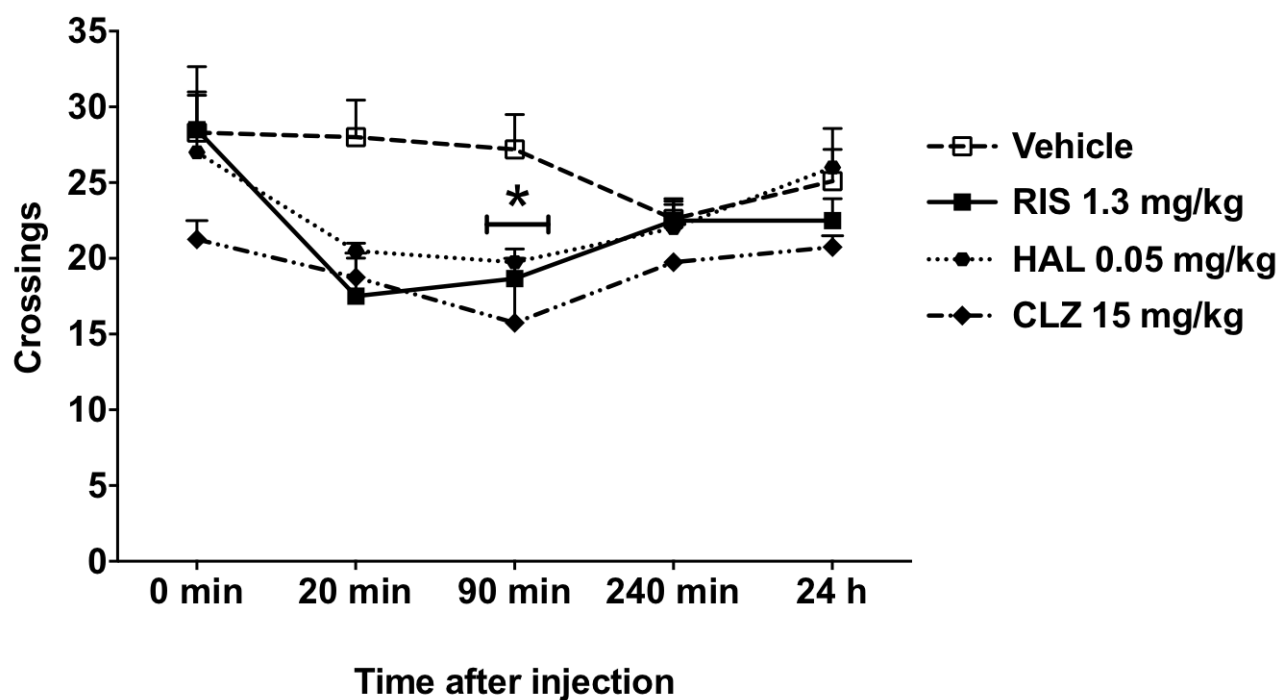


Figure 3-2 Suppression of chamber crossings by acute administration of single doses of risperidone and clozapine, but not haloperidol. Error bars – mean \pm SEM. $n=10$ for VEH, $n=6$ for RIS, $n=4$ each for HAL and CLZ. * $p < 0.05$ for both VEH vs RIS and VEH vs CLZ. VEH – vehicle; RIS – risperidone; HAL – haloperidol; CLZ – clozapine

Table 3-1 No significant increase in escape failures with acute administration of single dose of APDs in adults

APD	%Escape failures				
	0 min	20 min	90 min	240 min	24 h
VEH	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
RIS	0 ± 0	0 ± 1.88	0 ± 0	0 ± 0	0 ± 0
CLZ	0 ± 0	1.25 ± 7.81	0 ± 10.94	0 ± 1.88	0 ± 0
HAL	0 ± 0	0 ± 1.25	2.5 ± 2.5	0 ± 1.88	0 ± 0

Data are expressed as median ± semi-interquartile interval. $n=10$ for VEH, $n = 6$ for RIS, $n = 4$ each for HAL and CLZ. VEH – vehicle; RIS – risperidone; HAL – haloperidol; CLZ – clozapine

3.2.3. Discussion

In this pilot experiment, I established that the selected dose of each APD disrupted the avoidance response in adult rats with the chosen route of administration. Suppression of avoidance was maximal between 20 and 90 minutes after injection, accompanied by suppression of chamber crossings. By 240 minutes, rats returned to their pre-drug avoidance level. This reflects rapid metabolism of APDs in rodents consistent with the reported half-life of APDs being around 1-2 hours (Kapur et al. 2003).

APDs are known to disrupt CAR at doses occupying 60-80% of striatal dopamine D2 receptors (Wadenberg et al. 2000; Wadenberg et al. 2001b). This level of D2 occupancy is also thought to be optimal for antipsychotic drug action (Kapur et al. 2000). At the doses chosen in my experiment (1.3 mg/kg for risperidone, 15 mg/kg for clozapine and 0.05 mg/kg for haloperidol), this level of dopamine D2 receptor occupancy would appear to have been achieved (Kapur et al. 2003; Natesan et al. 2007; Wadenberg et al. 2001b). Higher rates of D2 receptor occupancy are associated with extra-pyramidal side-effects (Natesan et al. 2007; 2008; Natesan et al. 2006a; Wadenberg et al. 2001b). Here I showed that these doses of APDs disrupted avoidance response without inducing any significant increase in escape failures. Therefore these doses would not appear to be achieving > 80 D2 occupancy rates.

These data largely replicated the reported findings in the literature of the effects of acute APDs' action on CAR (Natesan et al. 2008; Natesan et al. 2006a; Wadenberg et al. 2001b), thus confirming the reliability of the CAR paradigm in detecting APD action on the brain (Wadenberg 2010; Wadenberg and Hicks 1999).

In summary, I confirmed that the chosen doses and routes of APD administration could disrupt avoidance response in adult rats. Based on these findings the time to examine CAR in Experiment 1 was determined to be 1 hour after injection in subsequent experiments.

3.3. Experiment 1: Comparative examination of chronic APD treatment in adolescent and adult rats

3.3.1. Experiment 1A: Screening of three APDs for behavioural and structural outcomes in adolescents and adults

In Experiment 1A, chronic 21-day treatment of three well-known APDs (risperidone, clozapine and haloperidol) was examined in adolescents in comparison with the same treatment regimens in adults. APD-induced changes in CAR and in brain structures during ongoing treatment (short-term) and after a drug-free interval (long-term) in adolescents were compared in those at older age windows.

The aims of Experiment 1A are as follows:

- (1) To compare the effects of chronic APD treatment on CAR behaviour in adolescents and adults
- (2) To compare brain structural changes induced by chronic APD treatment in adolescents and adults

To achieve these aims, longitudinal assessment of behaviour and brain structures was performed in the same animals treated with either one of the three APDs or vehicle as adolescents, young adults or adults (a total of 12 subgroups).

3.3.1.1 Materials and methods

3.3.1.1.1. Subjects

Male SD rats arrived at the animal facility in three breeding waves as weaners on PND23. During the 7-day acclimatization period, they were housed in groups of eight in Macrolon cages. After the behavioural training from PND30 to PND34, rats from the same age and drug groups were pair-housed in Macrolon cages with Sani chip bedding and wire lids in a temperature (21 ± 1 °C) and lighting (lights on at 6 am and off at 6 pm) controlled room. All rats were given *ad libitum* access to food and water throughout the whole experiment. Behavioural training and testing were conducted during the light phase of the diurnal cycle.

3.3.1.1.2. Antipsychotic drugs

All drugs and vehicle were administered to the rats through once-daily IP injection (1 ml/kg) for 21 days (between 14:30 h and 16:30 h) except haloperidol, which was given SC. The rats were weighed daily before drug administration. Approximately 60–80% D2 occupancy is required in order to acutely impair CAR behaviour. The APD doses in this study (haloperidol at 0.05 mg/kg/day, risperidone at 1.3 mg/kg/day and clozapine at 15 mg/kg/day) have been shown to have

such a level of 60-80% D2 occupancy in SD rats (Kapur et al. 2003) and disrupt avoidance responses as shown in Pilot Experiment.

3.3.1.1.3. Experimental design

To examine age-related differences in the effects of APDs on the brain, adolescent, young adult and adult animals (See Below) were exposed to one of three APDs (clozapine, risperidone and haloperidol). The timeline of the experiment is depicted in Figure 3-3. The experiment consisted of three main phases as follows.

3.3.1.1.3.1. CAR training and group assignment

First, all rats were trained in the CAR paradigm for 5 days from PND30 to PND34. Only rats with $\geq 70\%$ avoidance (90 out of 120 rats, 75% success rate) on last 2 days of CAR training (PND33 and PND34) were selected. These animals were matched on their performance and assigned into three age groups of 21-day APD treatment at either: adolescence (PND36-PND56); young adulthood (PND58-PND78) or adulthood (PND80-PND100). Each age group had three independent APD treatment groups (clozapine, risperidone and haloperidol, $n = 6$ per drug group) and a control group (CON, $n = 12$). Half of CON received the vehicle solution (VEH, $n = 6$) and the other half never received an injection (NO) to control for the handling effect on the behaviour. These groups were combined in the final analysis as handling effects were minimal. Longitudinal brain MRI was conducted in all groups except for the NO group (See below).

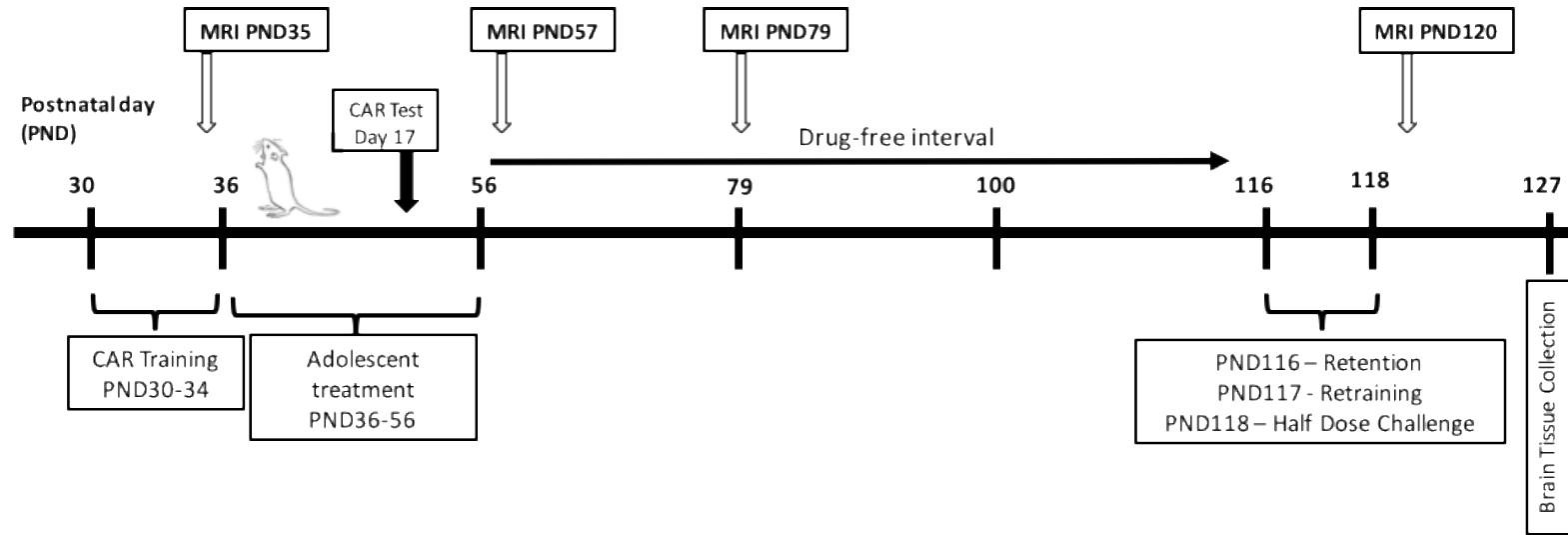
3.3.1.1.3.2. CAR test at Day 17 of chronic treatment

To investigate each APD's action on the CAR *during* chronic treatment, the avoidance response was examined in 40 CS-US trials at 1 hour after APD injection at Day 17 of chronic APD treatment (i.e. at PND52, PND74 and PND96 for adolescent, young adult and adult exposure respectively). CAR testing was performed 1 hour after APD injection as determined by the findings of the Pilot experiment.

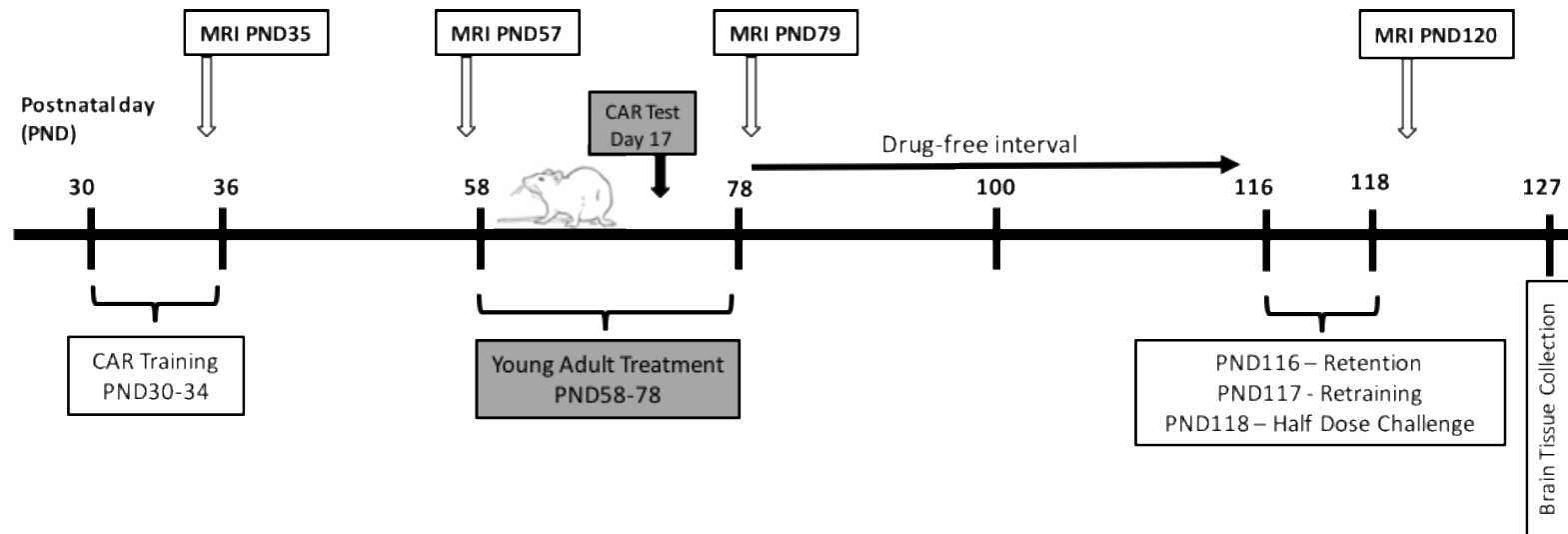
3.3.1.1.3.3. CAR test after a drug-free interval

The final CAR testing after drug washout period was performed over 3 days. On Day 1 (PND116), the retention of avoidance response was examined in all rats with 20 trials of CS-only test (without foot shock). A lower number of trials was used in the retention test to prevent any possible extinction effect. On Day 2 (PND117), the rats were retrained for 40 CS-US trials to regain a high avoidance level prior to drug challenge. On Day 3 (PND118), animals were challenged with a half dose of the APD or vehicle they had been previously chronically exposed to (7.5 mg/kg clozapine, 0.65 mg/kg risperidone, 0.025 mg/kg haloperidol or vehicle) and CAR examined over 40 CS-US trials. On P127, all rats were sacrificed for collection of brain tissue samples.

(a)



(b)



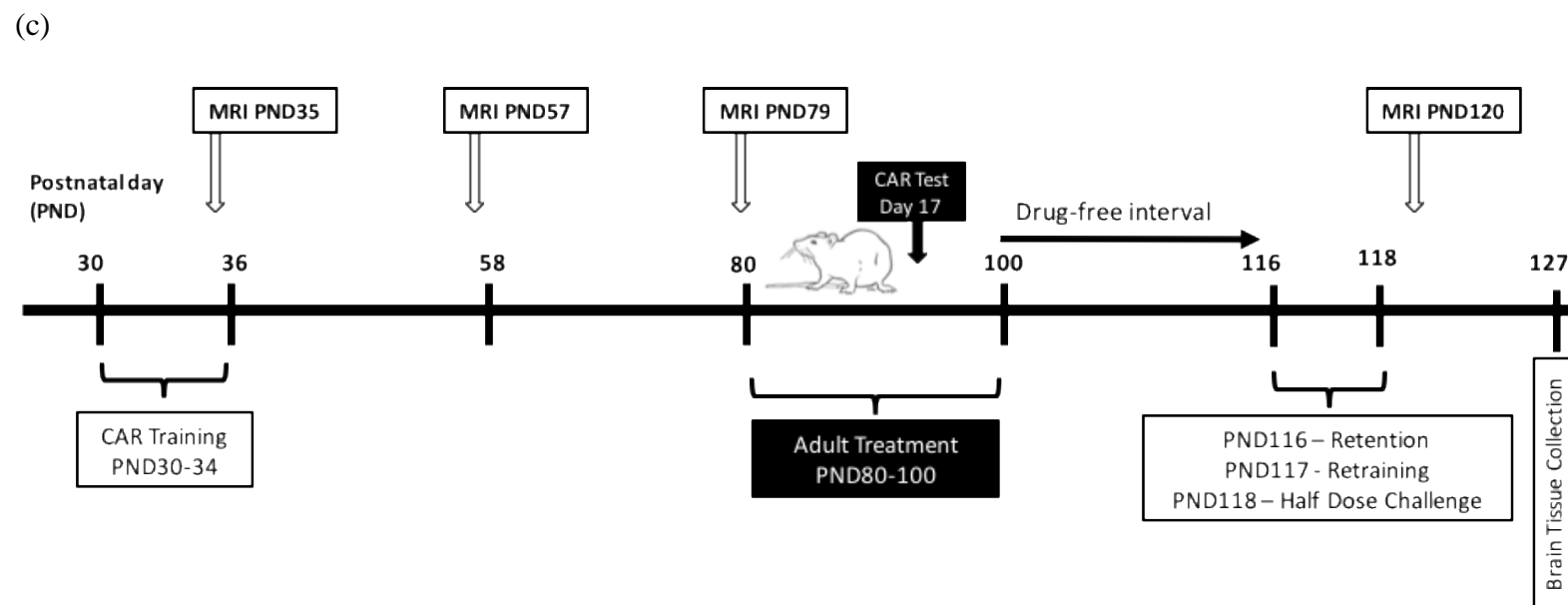


Figure 3-3 Timeline of Experiment 1A. Rats with $\geq 70\%$ avoidance levels after CAR training from PND30-PND34 were divided into three age groups of chronic 21-day antipsychotic exposure: (a) adolescence from PND36-PND56 (white box), (b) young adulthood PND58-PND78 (gray box) and (c) adulthood PND80-PND100 (black box). Avoidance response was examined on Day 17 of drug treatment in each age window (Black arrows, CAR test Day17). After drug-free period, rats from all age groups underwent retention test, retraining and half dose drug challenge test on PND116, PND117 and PND118 respectively. The same rats from all age groups had longitudinal live MRI scans at PND35, PND57, PND79 and PND120 (White Arrows). On PND127, the brain tissues were collected for real-time qPCR.

3.3.1.1.3.4. Longitudinal structural MRI scans and analysis

To examine APD-induced changes in brain structural maturation, longitudinal MRI scans were performed at PND35, PND57, PND79 and PND120 on all rats except those in NO group. Rats were anaesthetized with isoflurane (5% for induction and 1.8–2.2% for maintenance with 1.5 L/min oxygen). The respiratory rate was maintained at 45-60 breaths per minute and the body temperature at 37°C with circulating warm water through the animal bed. Coronal T2-weighted MRI images were acquired with horizontal bore Bruker Biospec 9.4 Tesla MRI scanner with Paravision 5.1 (Bruker, Germany) at Centre for Advanced Imaging, The University of Queensland.

MRI images were acquired using a 40 mm circularly polarized rat head volume coil. Two-dimensional T2-weighted fast spin echo images were acquired using with rapid acquisition with relaxation enhancement sequence (RARE) with the following parameters: effective echo time (TE) – 40 ms; repetition time (TR) – 4000 ms; field of view (FOV) – 35 mm x 35 mm; matrix – 256 x 256; in-plane resolution – 0.14 x 0.14 mm; slice thickness – 500 µm; number of slices – 38; RARE factor – 8; number of averages – 10; scan time – 23 minutes.

MRI images were coded and analyst was blinded to treatment. Manual segmentation method in the open-source OSIRIX software was used to identify the regions of interest (ROI). ROIs analysed included whole brain (WB), cerebral cortex (CCx), prefrontal cortex (PFC) and striatum (STR) using well-established criteria (Paxinos and Watson 2005) and following published methods (Piontkewitz et al. 2011; Vernon et al. 2011). Volume of each region was computed by summation of the areas from all respective slices corrected for slice thickness

3.3.1.1.4. Statistical analysis

Statistical analysis was conducted with SPSS Version 20, using a general linear model. CAR data were expressed as mean % avoidance, % escape failure and number of chamber crossings (n = 6 per APD group and n = 12 for CON for a given age group). CAR data and gene expression data were analyzed with one-way or two-way analyses of variance (ANOVA) with Tukey's post hoc test. Longitudinal MRI data and body weight data were analysed with repeated-measure three-way ANOVA (time x age x drug) and Tukey's post hoc test. To enable comparison among different age groups, avoidance data from Day 17 CAR test and half dose challenge test were plotted and analyzed as % avoidance normalized to the performance of age-matched controls. All data are expressed as mean ± SEM and statistical significance was defined as $p < 0.05$.

3.3.1.2 Results

3.3.1.2.1. Behaviour during chronic treatment

To investigate the behavioural effects *during* chronic APD administration, rats were tested for avoidance response on Day 17 of drug treatment at each age of exposure. Since the avoidance responses of two control groups (VEH and NO) did not differ significantly, they were pooled as a single control group (Table 3-2). Two clozapine-treated rats (one from young adult group and another from adult group) were excluded from the study since they had to be sacrificed due to development of sudden distress with abdominal distension after termination of drug treatment.

3.3.1.2.1.1. Avoidance suppression with chronic APD treatment

Two-way ANOVA on the avoidance yielded significant main effects of drug group ($F_{3,75} = 30.4$, $p < 0.001$) and age ($F_{2,75} = 4.2$, $p = 0.019$) on CAR performance without any significant interaction between drug group and age ($F_{6,75} = 0.8$, $p = 0.57$). Post hoc tests showed that the APD groups differed significantly from CON ($p = 0.01$ for clozapine groups, $p < 0.001$ for both risperidone and haloperidol groups). Moreover, avoidance suppression was significantly lower in all clozapine groups compared with risperidone ($p = 0.026$) and haloperidol ($p < 0.001$) groups. In general, avoidance levels were higher in adolescents than the older ages with this effect being significant between adolescents and adults ($p = 0.007$). Given that the avoidance behaviour varied significantly with age, subsequent analyses of the effects of APDs on CAR were conducted on data normalized to each control group at that age.

The avoidance data as normalized to age-matched controls for individual age groups is depicted in Table 3-2. On separate analysis of individual age groups, a significant main effect of drug was detected at all ages: $F_{3,26} = 8.7$ in adolescents, $F_{3,24} = 8.9$ in young adults and $F_{3,25} = 17.1$ in adults (all $p < 0.001$). Compared with its respective control group, clozapine no longer significantly impaired avoidance after 17 consecutive days of treatment at any age, suggesting tolerance to this drug had developed. By contrast, chronic treatment with risperidone and haloperidol continued to significantly disrupt avoidance in all age windows of exposure ($p = 0.005$ and $p = 0.001$ for adolescent risperidone and haloperidol respectively; $p = 0.03$ and $p < 0.001$ for young adult risperidone and haloperidol respectively; $p < 0.001$ for both adult risperidone and haloperidol).

Table 3-2 CAR performance of APD-treated and control rats at Day 17 of chronic drug treatment.

CAR test on Day 17 of chronic APD treatment				
Age group of chronic APD treatment	Drug Group	Normalized % Avoidance	%Escape Failures	No. of Crossings
Adolescent (PND36-PND56)	CON	100 ± 6.5	0.2 ± 0.2	51.4 ± 3.6
	CLZ	66 ± 18.8	0 ± 0	46.3 ± 2.0
	RIS	39.0 ± 17.2**	16.3 ± 14.8	35 ± 6.0*
	HAL	25.6 ± 9.9****	7.1 ± 5.0	38.5 ± 1.4
Young Adult (NDP58-PND78)	CON	100 ± 10.3	0 ± 0	48.8 ± 3.2
	CLZ	50.2 ± 15.0	0 ± 0	43.7 ± 1.7
	RIS	47.6 ± 21.7*	19.6 ± 13.1	35.8 ± 6.4
	HAL	9.6 ± 2.9****	26.7 ± 6.8*	31 ± 2.8*
Adult (PND80-PND100)	CON	100 ± 11.1	0 ± 0	45.8 ± 1.8
	CLZ	83.7 ± 22.4	0 ± 0	46.8 ± 2.7
	RIS	9.2 ± 4.0****	24.6 ± 16.2	31.2 ± 5.8*
	HAL	4.9 ± 2.5****	39.6 ± 6.2**	26.2 ± 2.6****
Two-way ANOVA	Drug	F _{3,75} = 30.4, p < 0.001	F _{3,75} = 11.6, p < 0.001	F _{3,75} = 16.3, p < 0.001
	Age	F _{2,75} = 4.2, p = 0.019	F _{2,75} = 2.3, p = 0.111	F _{2,75} = 1.7, p = 0.187
	Age x Drug	F _{6,75} = 0.8, p = 0.57	F _{6,75} = 1.3, p = 0.258	F _{6,75} = 0.6, p = 0.74

Data are expressed as mean ± SEM. n = 12 for adolescent and adult CON groups and n = 11 for young adult CON and n = 6 for each drug group except n = 5 for young adult and adult clozapine groups. *p < 0.05, **p < 0.005, ***p < 0.001 relative to respective CON; CON – control; CLZ – clozapine; RIS – risperidone; HAL – haloperidol

3.3.1.2.1.2. *Escape failures with chronic APD treatment*

APDs can also induce catalepsy at sufficiently high doses or with repeated treatment. This effect obviously might impact on apparent avoidance behaviour. Therefore escape failures were also examined (Table 3-2). Two-way ANOVA revealed a significant main effect of drug ($p < 0.001$) but no main effect of age ($F_{2,75} = 2.3, p = 0.111$) or age x drug interaction ($F_{6,75} = 1.3, p = 0.258$). Post hoc tests showed that risperidone and haloperidol groups produced significantly higher escape failures than CON (both $p < 0.001$). There were no escape failures with CLZ.

When the three age groups were examined separately, a significant main effect of drug was observed in young adults ($F_{3,24} = 4.7, p = 0.01$) and adults ($F_{3,25} = 7.3, p = 0.001$), but not in adolescents ($F_{3,26} = 1.4, p = 0.257$). Post hoc tests showed that, relative to the age-matched CON, chronic haloperidol caused a significant increase in escape failures in young adults ($p = 0.019$) and adults ($p = 0.002$), but not in adolescents ($p = 0.85$). Chronic clozapine did not significantly affect escape failures at all three ages.

3.3.1.2.1.3. *Chamber crossings with chronic APD treatment*

Examination of APD-induced suppression of chamber crossings revealed that risperidone and haloperidol groups had significantly lower crossings than CON ($p < 0.001$) while clozapine groups did not ($p = 0.85$) (two-way ANOVA results shown in Table 3-2). This indicates that locomotor impairments might have partly contributed to the apparent CAR suppression by chronic risperidone and haloperidol in all age groups. Next individual age groups were analysed separately.

A significant main effect of drug was observed in all age groups: ($F_{3,26} = 4, p = 0.019$) in adolescents; ($F_{3,24} = 4.5, p = 0.012$) in young adults and ($F_{3,25} = 10.7, p < 0.001$) in adults. Post hoc tests confirmed that, compared to their respective age-matched control, chronic risperidone retarded crossings in adolescents and adults (both $p < 0.05$) but not in young adults. By contrast, chronic haloperidol significantly decreased the crossings in young adults and adults ($p < 0.05$) but had no effect in adolescents. Chronic clozapine did not affect crossings at any age window.

3.3.1.2.2. *Behaviour after a drug-free interval*

After chronic APD treatment, all rats were given a drug-free period before being retested at the same age to minimize the potential confound of differences in final assessment age. This equated to 60 days, 38 days and 15 days for the adolescent, young adult and adult treatment groups, respectively. Assessment of avoidance both during retention (absence of US) on PND116 and retraining (presence of US) on PND117 did not reveal any significant difference for any individual drug at any age of exposure indicating variable drug washout periods were not a factor for any possible difference in subsequent APD re-challenge experiments. On PND118, APD-treated rats

were challenged with the same APD at half of the dose they were chronically exposed to at earlier ages (7.5 mg/kg clozapine, 0.65 mg/kg risperidone or 0.025 mg/kg haloperidol). Half doses were chosen to prevent any floor effect in the likely event of sensitization after drug washout.

3.3.1.2.2.1. Avoidance suppression with half dose challenge

The data from half dose challenge test along with statistics is shown in Table 3-3. Two-way ANOVA yielded a significant main effect of drug ($F_{3,75} = 9.5, p < 0.001$) without any main effect of age ($F_{2,75} = 0.3, p = 0.78$) or of age x drug interaction on CAR ($F_{6,75} = 1.2, p = 0.33$). Post hoc tests confirmed that risperidone and haloperidol rats produced significantly lower avoidance levels compared to CON rats ($p < 0.001$ and $p = 0.001$ respectively). The half dose of clozapine had no effect on CAR. The individual age groups were next examined.

Overall, the rats previously exposed to APDs as adolescents ($F_{3,26} = 6.5, p = 0.002$) and young adults ($F_{3,24} = 3.4, p = 0.033$), but not as adults ($F_{3,25} = 2.1, p = 0.132$), showed a significant main effect of drug in half dose APD challenge. Post hoc tests, however, showed that at this half-dose, only risperidone was capable of significantly impairing CAR and this was significant only in the rats treated previously with risperidone as adolescents ($p = 0.002$), not in those treated as young adults ($p = 0.053$) or adults ($p = 0.55$). Challenge with the other two APDs did not significantly affect avoidance in all age groups ($p > 0.05$).

3.3.1.2.2.2. Escape failures with half dose challenge

Again, two-way ANOVA showed a significant main effect of drug ($F_{3,75} = 4, p < 0.01$) but no main effect of age ($F_{2,75} = 0.4, p = 0.67$) or age x drug interaction ($F_{6,75} = 0.3, p = 0.93$). Posthoc tests confirmed significantly higher escape failures in risperidone groups ($p = 0.007$) compared with controls, but no significant difference was observed for clozapine and haloperidol groups.

When individual age groups were examined however, there was no significant main effect of any drug group at any age (Table 3-3).

Table 3-3 CAR performance with half dose APD challenge after drug washout

Half dose challenge CAR test at PND118				
Age group of prior chronic APD exposure	Drug group	Normalized % Avoidance	% Escape Failures	No. of crossings
Adolescent (PND36-PND56)	CON	100 ± 6.7	0 ± 0	50.3 ± 2.7
	CLZ	84.9 ± 18.1	2.1 ± 2.1	49.8 ± 4.9
	RIS	25.8 ± 16.8**	10.8 ± 9.4	36.8 ± 3.0*
	HAL	50.4 ± 18.8	3.8 ± 2.6	43.8 ± 2.5
Young Adult (PND58-PND78)	CON	100 ± 9.3	0 ± 0	48 ± 2.1
	CLZ	50.3 ± 15.5	0 ± 0	49.2 ± 6.1
	RIS	46.2 ± 17.5	9.6 ± 7.6	42.8 ± 4.7
	HAL	62.4 ± 20.4	0 ± 0	48 ± 5.8
Adult (PND80-PND100)	CON	100 ± 9.5	0.2 ± 0.2	52 ± 3.2
	CLZ	92.7 ± 21.3	0.5 ± 0.5	49.6 ± 4.1
	RIS	71.4 ± 20.6	5.0 ± 5.0	45 ± 4.2
	HAL	46.6 ± 20.6	3.3 ± 2.2	40.2 ± 1.2
Two-way ANOVA	Drug	F _{3,75} = 9.5, <i>p</i> < 0.001	F _{3,75} = 4, <i>p</i> = 0.01	F _{3,75} = 3.8, <i>p</i> = 0.013
	Age	F _{2,75} = 0.3, <i>p</i> = 0.78	F _{2,75} = 0.4, <i>p</i> = 0.67	F _{2,75} = 0.3, <i>p</i> = 0.77
	Drug x Age	F _{6,75} = 1.2, <i>p</i> = 0.33	F _{6,75} = 0.3, <i>p</i> = 0.93	F _{6,75} = 0.8, <i>p</i> = 0.60

Data are expressed as mean ± SEM. n = 12 for adolescent and adult CON groups and n = 11 for young adult CON and n = 6 for each drug group except n = 5 for young adult and adult CLZ groups. **p* < 0.05, ***p* < 0.005 relative to respective CON; CON – control; CLZ – clozapine; RIS – risperidone; HAL – haloperidol

3.3.1.2.2.3. Chamber crossings with half dose challenge

Two-way ANOVA showed a significant main effect of drug ($F_{3,75} = 3.8, p < 0.013$) but no main effect of age ($F_{2,75} = 0.3, p = 0.77$) or age x drug interaction ($F_{6,75} = 0.8, p = 0.60$). Post hoc tests confirmed significantly lower crossings for risperidone-challenged animals ($p = 0.019$) compared to CON but not for clozapine and haloperidol groups.

When individual age groups were examined, there was a significant main effect of drug only in rats previously treated as adolescents ($F_{3,26} = 3.4, p = 0.032$). Post hoc tests showed that crossings were significantly lower only in risperidone-challenged rats from adolescent exposure group ($p = 0.031$) (Table 3-3). Thus again the findings indicate that after a prolonged drug-free interval, only rats with prior adolescent risperidone exposure remained behaviourally sensitive to this challenge with lower dose.

3.3.1.2.3. Brain structural changes with APD treatment

The impact of chronic 21-day APD treatment on the trajectory of structural brain development was examined using longitudinal *in vivo* MRI. In general, all ROIs increased steadily from PND35 through PND79 and plateaued from PND79 to PND120 (Table 3-4). This trajectory of brain development was not significantly altered by chronic APD exposure at any age examined (only significant main effect of time; no significant main effect of drug, age, age x drug interaction or time x age x drug interaction for all ROIs examined). Statistical analysis on either regional volumes normalised to total brain volumes or changes in regional volumes from PND35, i.e. baseline level, also did not show any significant difference induced by APDs at any age. Moreover, correlation analyses did not reveal any significant relationship between volume of target brain structure and CAR performance either at Day 17 of chronic treatment or during half-dose challenge.

Table 3-4 Brain structural trajectory with chronic APD exposure

ROI	Age Group	Drug Group	Age at MRI Scans			
			PND35	PND57	PND79	PND120
WB Volume (mm ³)	Adolescent (PND36-PND56)	VEH	1159.6 ± 23.7	1292.8 ± 17.1	1374.3 ± 4.7	1396 ± 18.5
		CLZ	1160.3 ± 32.2	1236.9 ± 29.9	1351.5 ± 21.2	1366 ± 35.4
		RIS	1167.5 ± 16	1309.9 ± 19.2	1397.1 ± 33.9	1423.4 ± 22.2
		HAL	1192.7 ± 12.8	1301.4 ± 10.8	1362.4 ± 13.4	1405.1 ± 17.9
	Young Adult (PND58-PND78)	VEH	1147.7 ± 23.3	1279.1 ± 25.1	1325.4 ± 23.0	1380 ± 35.6
		CLZ	1229 ± 28.6	1360.1 ± 31.4	1390.2 ± 40.4	1426.8 ± 35.6
		RIS	1225.3 ± 17.5	1366.2 ± 20.7	1384.8 ± 30.4	1456 ± 18.5
		HAL	1220.7 ± 23.4	1340.3 ± 34.1	1410.6 ± 57.3	1431.6 ± 45
	Adult (PNDP80- PND100)	VEH	1203.6 ± 18.9	1332.4 ± 32.1	1398.4 ± 41.3	1428.5 ± 33.4
		CLZ	1179.7 ± 14.6	1314.7 ± 14.9	1366.8 ± 16.1	1384.2 ± 22.3
		RIS	1163.5 ± 38.2	1289.9 ± 34.7	1390.2 ± 21.1	1382.4 ± 42.8
		HAL	1179.8 ± 27.9	1299.3 ± 20.5	1356.9 ± 18.5	1397.3 ± 23.7
STR Volume (mm ³)	Adolescent (PNDP36-PND56)	VEH	87.1 ± 1.4	99.8 ± 1.2	108.5 ± 1.4	110.4 ± 1.9
		CLZ	88 ± 1.8	96.1 ± 2.2	107.1 ± 2.3	107.9 ± 2.4
		RIS	89.2 ± 1.2	103.3 ± 2.3	108.5 ± 1.8	114.1 ± 1.5
		HAL	88.4 ± 0.8	101.5 ± 1	106.2 ± 0.5	109.5 ± 1.2
	Young Adult (PND58-PND78)	VEH	85.5 ± 1.3	99 ± 0.6	105.8 ± 0.3	109.2 ± 1.3
		CLZ	93.6 ± 1.6	105.3 ± 2.5	109.8 ± 2.8	114 ± 3.0
		RIS	90.7 ± 2	106.1 ± 2.2	111.8 ± 1.6	115.1 ± 2.1
		HAL	88.8 ± 1.9	104.4 ± 2.2	111.8 ± 3.5	112.8 ± 2.1
	Adult (PND80-PND100)	VEH	92.2 ± 1.2	105.2 ± 1.9	113 ± 4	114.3 ± 2.8
		CLZ	90.2 ± 0.8	102 ± 1	108.3 ± 1.1	108.2 ± 1.2
		RIS	89.6 ± 2.4	99.9 ± 2.3	110.9 ± 2.9	107.6 ± 3.5
		HAL	90 ± 2.6	101.4 ± 1.5	107.2 ± 1.5	110.4 ± 1.4
CCx Volume (mm ³)	Adolescent (PND36-PND56)	VEH	486.1 ± 8.7	517.4 ± 9.3	545 ± 7.7	542.2 ± 11.6
		CLZ	492.5 ± 4.2	506.1 ± 10.8	534.3 ± 7.5	543.9 ± 14.1
		RIS	499.9 ± 8.5	526.6 ± 10.9	551.9 ± 17.9	567.4 ± 15.6
		HAL	499.4 ± 5.4	526.3 ± 4.6	542.9 ± 9	552.8 ± 8.8
	Young Adult (PND58-PND78)	VEH	480.9 ± 13.2	506.9 ± 11.3	520.8 ± 10	546.3 ± 12.7
		CLZ	498.4 ± 12.5	535.8 ± 12.8	534.7 ± 15.7	574.4 ± 16
		RIS	513 ± 9	542 ± 8.9	552.8 ± 15.6	580.8 ± 13.3
		HAL	504.6 ± 13.8	535.4 ± 16.5	558.2 ± 27.3	569.2 ± 21.3

	Adult (PND80-PND100)	VEH	499.6 ± 8.4	513.1 ± 14.4	543.6 ± 20.2	563.4 ± 20.1
		CLZ	490.2 ± 10.6	502 ± 8.4	535.1 ± 8.3	547.5 ± 13.6
		RIS	481.5 ± 13.6	503.7 ± 15.5	549.7 ± 12.4	551.4 ± 21.1
		HAL	496.9 ± 15.2	506.8 ± 9.9	522.7 ± 6.6	554.4 ± 10.5
PFC Volume (mm ³)	Adolescent (PND36-PND56)	VEH	16.2 ± 0.2	19 ± 0.7	21.6 ± 0.2	21 ± 0.7
		CLZ	16.6 ± 0.2	19.1 ± 0.3	20.3 ± 0.2	21.1 ± 0.7
		RIS	16.9 ± 0.2	19.7 ± 0.5	21.9 ± 0.6	21.7 ± 0.5
		HAL	16.6 ± 0.2	19.9 ± 0.2	21.3 ± 0.8	21.9 ± 0.3
	Young Adult (PND58-PND78)	VEH	16.3 ± 0.3	19.6 ± 0.4	21 ± 0.3	20.8 ± 0.6
		CLZ	17.5 ± 0.3	20.4 ± 0.4	21.6 ± 0.4	21.2 ± 0.3
		RIS	17.5 ± 0.6	20.7 ± 0.7	22.8 ± 1	22 ± 0.5
		HAL	18.1 ± 0.9	21 ± 0.5	22.8 ± 0.8	22 ± 0.4
	Adult (PND80-PND100)	VEH	16.7 ± 0.4	20.3 ± 0.9	22.6 ± 0.9	21.5 ± 0.5
		CLZ	16.9 ± 0.3	18.8 ± 0.8	21.6 ± 1.2	20.5 ± 0.4
		RIS	16.5 ± 0.5	20.4 ± 0.9	22.4 ± 0.7	20.7 ± 0.7
		HAL	16.6 ± 0.3	19.1 ± 0.3	21.6 ± 0.4	20.9 ± 0.7

Data are expressed as mean ± SEM. *n* = 6 for each drug group except *n* = 5 for young adult and adult clozapine groups. *n* = 4 per drug group at PND79 scan due to scanner failure. CCx = cerebral cortex, CLZ – clozapine, HAL – haloperidol, PFC = prefrontal cortex, RIS – risperidone, STR = striatum, WB = whole brain.

3.3.2. Experiment 1B: Further examination of chronic risperidone treatment in adolescents and adults

In the second part of Experiment 1, I increased experimental sample size for the most promising agent, risperidone. Risperidone was chosen for further study for the following reasons: -

(a) Risperidone is the atypical APD most commonly prescribed to adolescents and children in the clinic (Hollingworth et al. 2013; Olfson et al. 2006; Olfson et al. 2012). Therefore, it will be clinically more relevant to examine risperidone administration in adolescent rats.

(b) A preclinical study has reported a long-term change in adult whole brain volume with adolescent risperidone treatment in neurodevelopmentally normal rats (Piontkewitz et al. 2011) but this finding has not been replicated. In addition to the regions examined in Experiment 1A, I also wanted to determine changes in other regions such as hippocampus and ventricles.

(c) The findings of Experiment 1A provided a lead that a long-term change in behavioural response could develop selectively with risperidone treatment in adolescence. I aimed to confirm this finding in a larger sample size and investigate the underlying neural mechanism(s). Justifications for increasing sample size are as follows: with the statistical analyses used in my study, for example, two-way ANOVA (age x drug), a sample size of $n=6$ per group will not provide adequate statistical power and positive predictive power with a possible winner's curse effect (Button et al. 2013).

The aims of Experiment 1B are as follows:

- (1) To confirm that risperidone induces long-term behavioural change in CAR selectively in rats treated as adolescents
- (2) To conduct structural brain studies in a larger sample cohort
- (3) To investigate long-term neurochemical change induced by risperidone treatment in adolescents

In addition to these three specific aims, long-term changes in accumbal metabolites such as GABA, glutamate and NAA were also examined with ^1H MRS in Experiment 1B. Given a recent report of long-term reduction in ^1H MRS levels of GABA and glutamate in the NAc induced by adolescent olanzapine treatment (Xu et al. 2015), the NAc was chosen for examination of metabolites.

3.3.2.1 Materials and methods

3.3.2.1.1. Subjects

As in Experiment 1A, male SD rats that arrived at the animal facility as weaners on PND23 were housed in groups of eight in Macrolon cages. After CAR training from PND30 to PND34, the rats

from the same age and drug groups were pair-housed in Macrolon cages with Sani chip bedding and wire lids in a temperature (21 ± 1 °C) and lighting (lights on at 6 am and off at 6 pm) controlled room.

3.3.2.1.2. Antipsychotic drugs

Risperidone and vehicle were administered to the rats through once-daily IP injection (1 ml/kg) for 21 days (between 2:30 and 4:30 pm). Rats were weighed daily before drug administration.

3.3.2.1.3. Experimental design

In Experiment 1B, chronic risperidone treatment was examined further only in two age groups, adolescents (PND36-PND56) and adults (PND80-PND100) ($n = 6$ per drug for a given age group). Experimental design is the same as in Experiment 1A, with a few modifications. The timeline of the experiment is depicted in Figure 3-4.

3.3.2.1.3.1. CAR training and testing during chronic treatment and after drug-free interval

As in Experiment 1A, rats were trained in a CAR paradigm from PND30 to PND34. Rats with $\geq 70\%$ avoidance (24 out of 30, 80% success rate) on the last 2 days of CAR training (PND33 and PND34) were randomly assigned into either adolescent (PND36-56) or adult (PND80-100) exposure groups. The effect of chronic risperidone on CAR was examined in 40 CS-US trials at 1 hour after injection on Day 17 of the 21-day treatment (i.e. at PND52 and PND96 respectively for adolescent and adult exposures). After a drug-free interval, the rats were examined for retention of avoidance response at PND116 (20 CS-only trials) and retrained at PND117 (40 CS-US trials) and challenged with a *half dose* of risperidone (0.65 mg/kg IP) or vehicle at PND118 (40 CS-US trials).

3.3.2.1.3.2. Examination of structural change after a drug-free interval

In Experiment 1B, MRI scans were performed only at PND120 on all rats since the main aim was to determine risperidone-induced long-term change in brain structures. The imaging parameters and the analysis methodology were the same as in Experiment 1A. In addition to the ROIs analysed in Experiment 1A, hippocampus and lateral ventricles were also analysed using well-established criteria of rat brain atlas (Paxinos and Watson 2005) and following the published methods (Piontkewitz et al. 2011; Vernon et al. 2011).

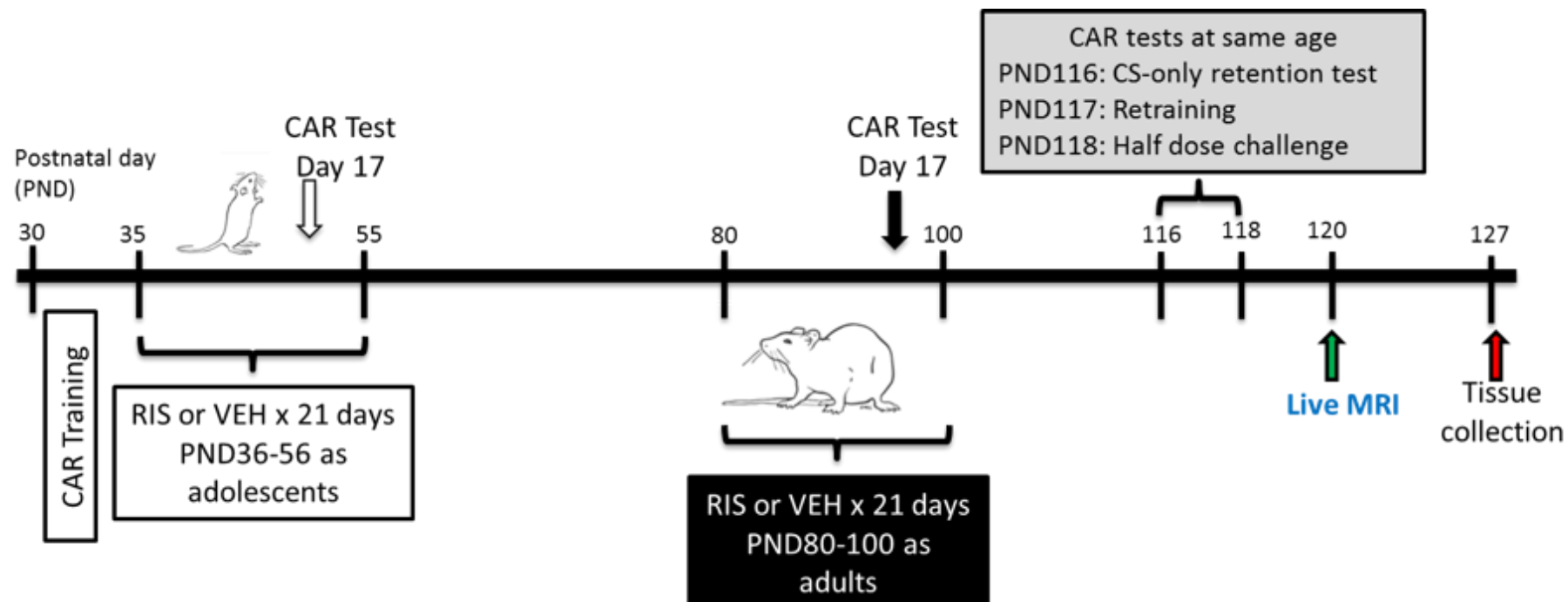


Figure 3-4 Timeline of Experiment 1B. Rats were treated for 21 days with risperidone or vehicle as adolescents (PND36-PND56) or adults (PND80-100). At Day 17 of chronic 21-day treatment, CAR was examined at 1 h after injection. After a drug-free interval, rats from both age groups were tested at same age from PND116 to PND118. Two days later at PND120, structural MRI was performed on all rats. At PND 127, rats were euthanised for collection of brain tissues.

3.3.2.1.3.3. Examination of accumbal metabolites with ¹H MRS after a drug-free interval

Immediately after the structural MRI, single voxel ¹H MRS data were acquired to investigate long-term changes in neural metabolites (glutamate, GABA and n-acetyl aspartate (NAA)) in the NAc. A voxel of interest (6 x 2 x 2 mm³) was placed bilaterally over the NAc. After a fast shimming to improve B0 magnetic homogenization and first, second and third order shimming, a reference water spectrum was acquired. Next, water-suppressed ¹H MRS spectra were obtained from the NAc voxel using PRESS sequence. ¹H MRS data were analysed at LCModel software (version 6.3-1J) (Provencher 1993) and the concentration of metabolites expressed as ratio to total creatine (Cr + PCr).

3.3.2.1.3.4. Examination of neurochemistry in adulthood with RT-PCR

On PND127, following 1-week washout from isoflurane exposure to diminish any possible confounds on neurochemical parameters examined, all rats were sacrificed with an overdose of pentobarbitone sodium (Lethabarb, Virbac). Brains were rapidly dissected on ice and both core and shell regions of the NAc (Paxinos and Watson 2005) collected in RNALater solution (Invitrogen), kept at 4°C overnight and stored at -80°C until RNA extraction procedure. Briefly, total RNA was extracted from each tissue sample using QIAzol and RNeasy Mini Kit (Qiagen, Australia). For each sample, 900 ng of RNA per 21 µl reaction was reverse-transcribed to cDNA with SuperScript IV First-Strand Synthesis System (Invitrogen). RT-PCR was performed in 12 µl reaction on Roche LightCycler 480 (Roche Diagnostics, Australia), using SYBR Green method in 384-well plates. The PCR conditions were as follows: denaturation at 95°C for 5 min followed by 40 cycles of amplification (95°C for 10 s, then 60°C for 20s, then 72°C for 20 s). Relative expression of the target genes normalized to that of endogenous control GAPDH was calculated following the published method (Schmittgen and Livak 2008). Gene expression data was only considered if significant changes were observed in every repeat.

Risperidone-treated and control groups from Experiment 1A and 1B were pooled and examined together. No Injection control groups were not examined in PCR reactions, thus giving n = 12 for both vehicle controls and risperidone groups for a given age. PCR experiments were performed separately for adolescent and adult treatment groups, along with a universal control sample in each PCR plate.

3.3.2.1.4. Statistical analysis

Statistical analysis was conducted with IBM SPSS Version 22. CAR data were expressed as normalized % avoidance (% avoidance normalized to the corresponding age-matched control group's performance), % escape failure and number of chamber crossings (n = 12 for risperidone

group and $n = 18$ for controls for each age group). For chronic exposure in two age groups, CAR data and MRI data were analysed with two-way (age x drug) ANOVA, followed by post hoc tests with Bonferroni correction. Given non-Gaussian distribution of the data, escape failures were analysed with non-parametric independent-samples Mann-Whitney U tests. For change in avoidance response from chronic exposure to half dose challenge, delta Z score analysis of normalized avoidance data was computed with the following formula [Delta Z = (normalized % avoidance on half dose challenge – normalized % avoidance on Day 17) ÷ (normalized % avoidance on half dose challenge + normalized % avoidance on Day 17)]. Since PCR reactions of two age groups were performed separately, the $2^{-\Delta CT}$ values of individual samples were normalized to that of the universal sample and analysed with two-way (age x drug) ANOVA followed by post-hoc tests on each age group. All data were expressed as mean \pm standard error of mean (SEM) except escape failures data which were expressed as median \pm semi-interquartile range following the presentation of the published literature (Wadenberg et al. 2000; Wadenberg et al. 2001b). The level of statistical significance defined as $p < 0.05$.

3.3.2.2 Results

The data of risperidone treatment and control groups from both Experiment 1A and 1B were pooled and presented here.

3.3.2.2.1. Behaviour during chronic treatment

As expected, risperidone suppressed CAR in both adolescents and adults. This effect was numerically lower in adults (Table 3-5). Two-way ANOVA on normalized avoidance data yielded a significant main effect of drug ($F_{1,56} = 78.594$, $p < 0.001$) but the main effects of age and drug x age interaction did not reach statistical significance (both $F_{1,56} = 3.104$, $p = 0.084$). This suppression was independent of age with both age groups showing significant reductions in CAR ($p < 0.001$ for both adolescents and adults).

Risperidone also has the capacity to induce catalepsy which obviously may impact on avoidance behaviour. Catalepsy during CAR would manifest as an escape failure. Therefore escape failures during chronic exposure were also examined. Two-way ANOVA revealed significant main effects of drug ($F_{1,56} = 34.295$, $p < 0.001$), age ($F_{1,56} = 7.844$, $p = 0.007$) and drug x age interaction ($F_{1,56} = 7.975$, $p = 0.007$). Risperidone-treated adolescent rats were not significantly different from their controls ($p = 0.087$). In sharp contrast, rats treated with risperidone as adults had significantly higher escape failures than controls ($p < 0.001$). These findings suggest that escape failures may partly contribute to the CAR disruption by risperidone in adults, but not in adolescents.

Chronic risperidone treatment suppressed chamber crossings in both age groups. Again, this motor-suppressive effect of risperidone appeared lower in adolescents than in adults (significant main effects of drug ($F_{1,56} = 47.205$, $p < 0.001$) and age ($F_{1,56} = 7.873$, $p = 0.007$) but no significant drug \times age interaction ($F_{1,56} = 2.860$, $p = 0.096$) on two-way ANOVA). Examination of two age groups individually showed that risperidone significantly suppressed chamber crossings in both adolescents ($p = 0.002$) and adults ($p < 0.001$). This indicates that locomotor impairments may have partly contributed to the apparent CAR suppression by chronic risperidone in both age groups. All data is displayed in Table 3-5.

Table 3-5 Behaviour during chronic risperidone treatment as adolescents or adults

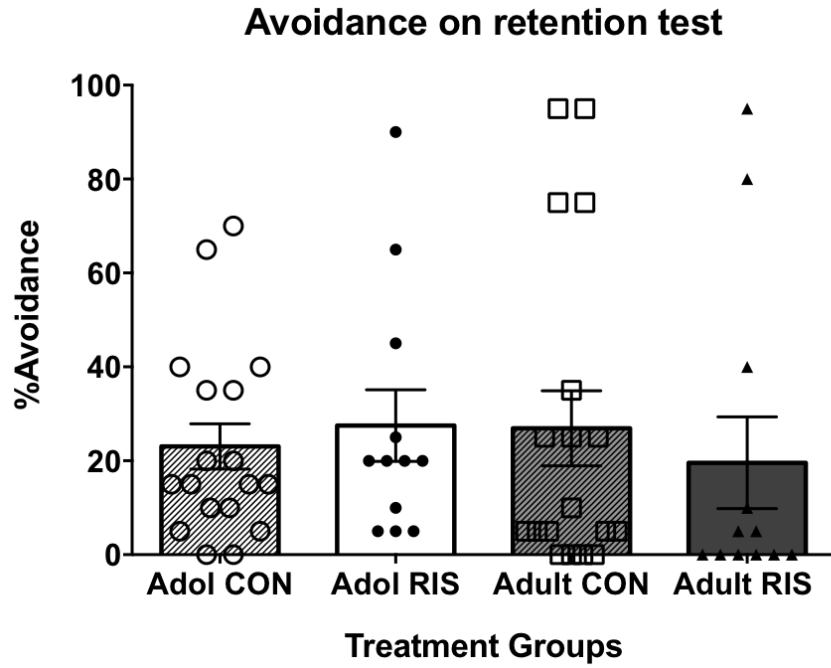
Age	Drug	Normalized %avoidance	%Escape failures	No of chamber crossings
Adolescent	CON	100 \pm 6.46	0 \pm 0	49.39 \pm 2.48
	RIS	36.36 \pm 11.95***	0 \pm 8.44	34.42 \pm 3.81**
Adult	CON	100 \pm 10.35	0 \pm 0	46.17 \pm 1.52
	RIS	4.78 \pm 2.25***	71.25 \pm 41.56 ***	21.42 \pm 4.11***

** $p = 0.002$, *** $p < 0.001$ compared to respective age-matched CON. Avoidance and crossing data are expressed as mean \pm SEM. Escape failure data are expressed as median \pm semi-interquartile range. $n = 18$ for control (CON) groups and $n = 12$ for risperidone (RIS) groups.

3.3.2.2.2. Behaviour after a drug-free interval

After chronic treatment, all rats were given a drug-free period (60 days and 15 days for adolescent and adult treatment groups, respectively) before being retested at the *same age*. CAR assessed during both retention test (absence of US) on PND116 and retraining (presence of US) on PND117, was equivalent for both chronically exposed age groups (Figures 3-5 (a) and (b)). This suggests the variation in drug washout periods did not differentially affect CAR performance prior to subsequent re-challenge experiments.

(a)



(b)

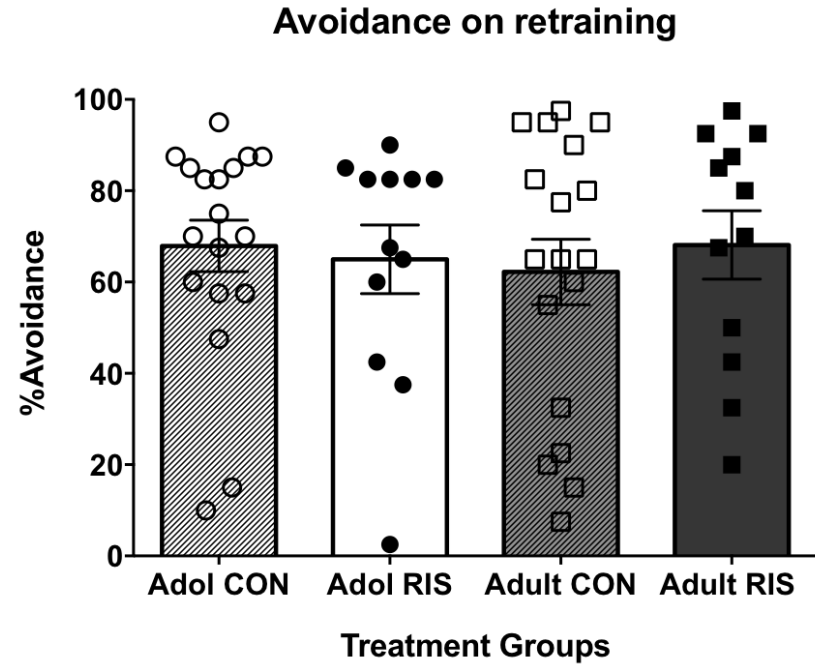


Figure 3-5 Avoidance performance of rats at the retention test on PND116 and retraining on PND117. No difference in avoidance performance at (a) the retention test (in the absence of US) on PND116 and (b) retraining on PND 117 was observed in both adolescent and adult cohorts. Data are expressed as mean \pm SEM. $n = 18$ for CON and $n = 12$ RIS for a given age.

On PND118, risperidone-treated rats were challenged with half of the dose they were chronically exposed to at earlier ages (0.65 mg/kg). CAR was impaired by risperidone challenge in both age groups (main effect of drug ($F_{1,56} = 24.708$, $p < 0.001$); with no main effects of age or drug x age interaction (both $F_{1,56} = 0.813$, $p = 0.371$) on two-way ANOVA). Separate examination of two age groups again showed that the challenge dose of risperidone disrupted CAR in both age groups ($p < 0.001$ in adolescent group and $p = 0.019$ in adult group) (Table 3-6).

Table 3-6 Behaviour after drug-free interval at PND118

Age	Drug	Normalized %avoidance	%Escape failures	No of chamber crossings
Adolescent	CON	100 ± 5.89	0 ± 0	51.39 ± 2.19
	RIS	34.85 ± 13.03***	0 ± 8.13	36 ± 3.12***
Adult	CON	100 ± 10.85	0 ± 0	49.89 ± 2.47
	RIS	54.86 ± 15.11*	0 ± 13.13	38.67 ± 3.39*

* $p < 0.05$, *** $p < 0.001$ compared to respective age-matched CO. Avoidance and crossing data are expressed as mean ± SEM. Escape failure data are expressed as median ± semi-interquartile range. $n = 18$ for control (CON) groups and $n = 12$ for risperidone (RIS) groups.

When the level of CAR suppression *after drug washout* (on half dose) was compared with that seen *during* chronic treatment (Day 17) (on full dose), a significantly different pattern was observed in the two age groups. The response in rats treated as adults was predictable. At challenge, the lower dose of risperidone (0.65 mg/kg) produced less suppression of CAR compared with the full dose (1.3 mg/kg) at Day 17 (Figure 3-6b). By contrast, rats chronically exposed to risperidone as adolescents, when challenged as adults with half dose risperidone, had a similar level of avoidance suppression as chronic full dose (Figure 3-6a). This differential response was confirmed by delta Z score analysis (significant main effects of drug ($F_{1,56} = 7.203$, $p = 0.010$), age and drug x age interaction ($F_{1,56} = 11.014$ and $F_{1,56} = 10.886$ respectively, both at $p = 0.002$) on two-way ANOVA). Further analysis shows that rats with adult risperidone exposure showed significantly higher positive Z score than those with the same risperidone exposure in adolescence ($p = 0.007$).

This pattern was not influenced by the level of escape failures as the half dose challenge induced similar levels of escape failures in rats of both age groups (main effect of drug ($F_{1,56} = 13.518$, $p = 0.001$), no main effect of age or drug x age interaction ($F_{1,56} = 0.001$ and $F_{1,56} = 0.004$ respectively,

both $p > 0.90$) on two-way ANOVA). Examination of individual age groups did not reveal any significant increase in escape failures ($p = 0.059$ for adolescents and $p = 0.079$ for adults). Similarly the number of crossings were significantly suppressed by risperidone challenge in both age groups (a significant main effect of drug ($F_{1,56} = 23.271$, $p < 0.001$), no main effect of age or drug x age interaction ($F_{1,56} = 0.045$ and $F_{1,56} = 0.571$ respectively, both $p > 0.4$) on two-way ANOVA). When the two age groups were examined separately, no age group was selectively affected ($p < 0.001$ for adolescent group and $p = 0.011$ for adult group). All data from half dose challenge test is shown in Table 3-6.

3.3.2.2.3. Long-term structural outcome with chronic risperidone treatment in adolescence or adulthood

To examine the hypothesis that chronic risperidone treatment in adolescence could induce long-lasting structural deficits, we examined the volume of target brain structures with live *in vivo* MRI scans. Table 3-7 shows regional brain volumes in risperidone- or vehicle-treated rats of both age groups. Risperidone treatment for 21 days was insufficient to induce any long-lasting structural change regardless of the age of exposure. Two-way ANOVA of each ROI confirmed this observation, showing no significant main effect of drug, age or age x drug interaction for any of the structures examined (all $p > 0.05$, Table 3-7). Statistical analysis on regional volumes normalised to total brain volumes also did not show any significant difference induced by APDs at any age.

Table 3-7 Brain structural outcome at PND120 with chronic risperidone treatment in adolescence or adulthood

Age	Drug	Volume of ROIs (mm ³)					
		Whole brain	Cerebral cortex	PFC	Striatum	Hippocampus	Lateral ventricles
Adolescent	CON	1382.79 ± 14.49	544.49 ± 7.21	20.46 ± 0.47	82.01 ± 1.07	110.18 ± 1.47	13.85 ± 3.82
	RIS	1384.43 ± 23.80	546.17 ± 11.66	20.53 ± 0.67	82.25 ± 1.12	109.41 ± 1.70	18.49 ± 4.36
Adult	CON	1384.39 ± 24.64	547.93 ± 12.12	20.26 ± 0.73	84.85 ± 1.71	110.07 ± 1.56	10.73 ± 1.94
	RIS	1367.30 ± 24.63	547.01 ± 11.72	20.52 ± 0.59	83.03 ± 2.29	108.69 ± 2.01	14.58 ± 2.58
Two-way ANOVA statistics		Main effects of age, drug and age x drug interaction for each individual ROI – all $F_{1,43} < 0.4$, $p > 0.05$					

All data are expressed as mean ± SEM. $n = 12$ for control (CON) groups and $n = 12$ for risperidone (RIS) groups except $n = 11$ for adult CON. CON – vehicle-treated control; RIS – risperidone-treated; ROI = region of interest

3.3.2.2.4. Long-term change in neural metabolites of the NAc

In Experiment 1B, I examined the levels of glutamate, GABA and NAA in rats that had been treated with risperidone in adolescence or adulthood. All three metabolites of interest can be reliably quantified from ^1H MRS data (all CRLB < 20%, Table 3-8). As shown in Table 3-8, the accumbal levels of glutamate, GABA and NAA at maturity were not altered by risperidone treatment in either adolescence or adulthood (no significant main effect of age or drug or age x drug interaction; all $F < 1.5$, $p > 0.05$).

Table 3-8 Levels of neural metabolites in the nucleus accumbens at PND120 after risperidone treatment in adolescence or adulthood

Age group	Drug Group	Glu/Cr+PCr	GABA/Cr+PCr	NAA/Cr+PCr
Adolescent	CON	1.11 ± 0.044	0.28 ± 0.023	0.84 ± 0.042
	RIS	1.16 ± 0.044	0.28 ± 0.023	0.85 ± 0.042
Adult	CON	1.10 ± 0.048	0.29 ± 0.025	0.82 ± 0.046
	RIS	1.13 ± 0.044	0.32 ± 0.023	0.85 ± 0.042
CRLB		5.04 ± 0.16	15.83 ± 0.78	5.04 ± 0.16
Two-way ANOVA		Main effects of age, drug and age x drug interaction for each metabolite – all $F_{1,19} < 1.5$, $p > 0.05$		

All data (ratio of metabolites to total creatine) expressed as mean ± SEM. $n = 6$ per drug for a given age group except $n = 5$ for Adult CON group. CON – vehicle-treated control; CRLB – Cramer-Rao Lower Bounds; RIS – risperidone-treated;

3.3.2.2.5. Long-term neurochemical change in the NAc

Changes in dopaminergic, serotonergic and GABA-ergic molecules in the NAc (See Table in Appendix A) were examined since this brain region plays a critical role in APD-induced disruption of avoidance response (Wadenberg et al. 1990a). Among neuro-receptors, transporters and enzymes examined significant reductions in 5-hydroxytryptamine-2A (5HT_{2A}) and catechol-o-methyl transferase (COMT) mRNA levels were observed selectively in the rats treated with risperidone in adolescence (Figure 3-7). Two-way ANOVA showed a significant main effect of drug ($F_{1,44} = 5.20$, $p = 0.028$) and age ($F_{1,44} = 23.270$, $p < 0.001$) for 5HT_{2A} mRNA levels and a significant main effect of drug only ($F_{1,44} = 4.21$, $p = 0.046$) for COMT mRNA levels. Age x drug interaction of both for 5HT_{2A} and COMT mRNA levels did not reach statistical significance ($F_{1,44} = 1.05$ and $F_{1,44} = 1.63$ respectively, both $p > 0.05$). Planned comparisons showed that compared to the corresponding controls, rats with prior adolescent risperidone exposure showed a significant downregulation of 5HT_{2A} mRNA levels in the NAc ($p = 0.001$), along with a small but significant downregulation of COMT gene expression ($p = 0.033$). By contrast, in rats treated with risperidone as adults, the gene expression of both 5HT_{2A} and COMT was unaltered (both $p > 0.4$). Gene expression of other markers examined such as tyrosine hydroxylase (TH), D1 and D2 receptors and monoamine oxidase A and B (MAO-A, MAO-B) and glutamic acid decarboxylase 65 (GAD65) was unaffected by the risperidone treatment regimen at both ages (Table 3-9).

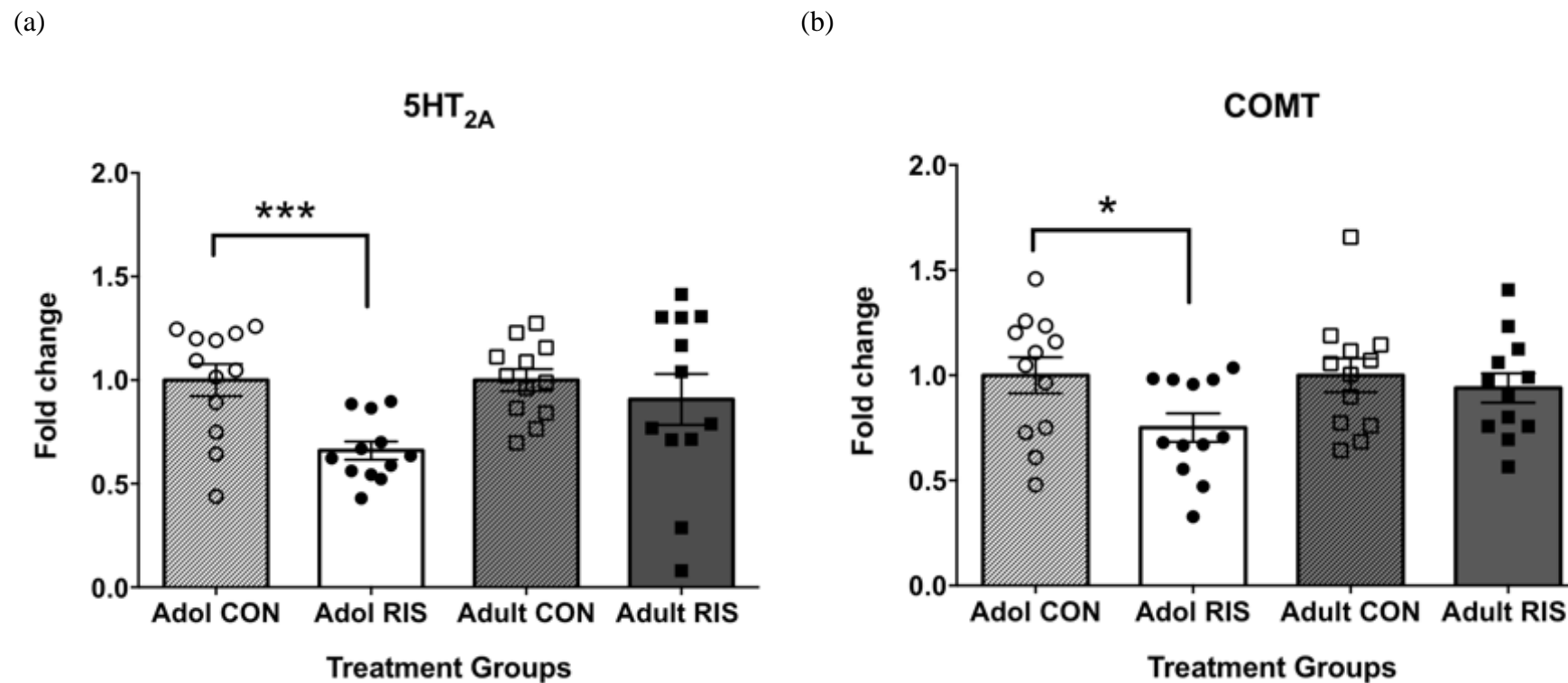


Figure 3-7 Adolescent risperidone treatment produces long-lasting neurochemical alterations in the nucleus accumbens. Rats with prior adolescent risperidone exposure showed a significant downregulation of (a) 5HT_{2A} receptor and (b) COMT mRNA levels. Rats treated with risperidone in adulthood did not show any alterations of 5HT_{2A} receptor and COMT. Data are expressed as mean \pm SEM. $n = 12$ each for vehicle-treated control (CON) and risperidone (RIS) groups for a given age. * $p < 0.05$, *** $p = 0.001$ compared to respective age-matched CON.

Table 3-9 Gene expression of dopaminergic and GABAergic markers in the NAc with risperidone treatment in adolescence or adult

Age	Drug	Fold change of the target genes					
		D1	D2	TH	MAO-A	MAO-B	GAD65
Adolescent	CON	1 ± 0.06	1 ± 0.1	1 ± 0.04	1 ± 0.13	1 ± 0.09	1 ± 0.1
	RIS	0.87 ± 0.06	0.84 ± 0.05	0.99 ± 0.07	0.81 ± 0.08	1.01 ± 0.07	0.98 ± 0.08
Adult	CON	1 ± 0.09	1 ± 0.05	1 ± 0.05	1 ± 0.05	1 ± 0.05	1 ± 0.12
	RIS	0.82 ± 0.1	0.86 ± 0.11	1.01 ± 0.09	0.99 ± 0.12	0.98 ± 0.10	0.87 ± 0.12

All data expressed as mean ± SEM. n = 12 each for vehicle-treated control (CON) and risperidone (RIS) groups for a given age.

3.4. Discussion

In the current Chapter, I established that the selected doses of APDs could disrupt the CAR in adults at the chosen routes of administration. Next, in Experiment 1A, I did a screening of behavioural and brain structural outcomes by subjecting rats to chronic APD treatment at different postnatal age windows. I showed that the same regimens of chronic APD treatment induced differential behavioural outcomes in the CAR paradigm in adolescents and adults, depending on the APD. Further, in Experiment 1B, I examined chronic risperidone treatment in adolescents and adults in larger sample size. I showed that chronic treatment with risperidone and haloperidol induced lower levels of escape failures in adolescents than in adults *during* ongoing chronic treatment. After a drug-free interval, when rechallenged with a low dose of risperidone rats treated with risperidone in adolescence appeared to develop a sensitised behavioural response. Accompanying this behavioural change, a downregulation in 5HT_{2A} receptors and COMT was observed selectively in the NAc of rats treated with risperidone in adolescence.

3.4.1. Behaviour *during* chronic treatment

In Experiment 1, during chronic treatment at three ages, contrasting effects were observed with three drugs, especially for haloperidol, the typical APD with predominant affinity for D2 receptors, risperidone, the atypical APD with high affinity for both D2 and 5HT_{2A} receptors and clozapine, the atypical APD with higher affinity for non-dopaminergic receptors (Miyamoto et al. 2005; Schotte et al. 1996). After 17 days of chronic treatment, risperidone and haloperidol continued to impair avoidance. Since the avoidance suppression of chronic risperidone and haloperidol was generally accompanied by escape failures and reduction in crossings, which are reflective of cataleptic effects of these two APDs, it was speculated that at least in part, the avoidance-suppressive effect of chronic risperidone and haloperidol might be due to these motor and cataleptic effects *during*

chronic treatment. Such motor initiation deficits have been proposed as a behavioural mechanism for APD-induced CAR disruption (Anisman et al. 1982; Fibiger et al. 1975; Posluns 1962).

More importantly, differential age-dependent effects of risperidone and haloperidol were observed with higher levels of locomotor suppression and escape failures with an increase in age. These results must be considered in the light of the fact that an *acute* single dose of any of these APDs did not induce any significant escape failure. Older animals appear more vulnerable to the cumulative locomotor/cataleptic effects of risperidone and haloperidol after repeated daily injection. In agreement with this finding, a sensitization-like cataleptic response, i.e. an increase in cataleptic effect with daily injection, has been reported with repeat haloperidol treatment in adult rats despite the lack of such cataleptic response with the initial exposure to this drug (Banasikowski and Beninger 2012a; Klein and Schmidt 2003; Schmidt et al. 1999). Older animals have previously been shown to be more vulnerable to cumulative locomotor/cataleptic effects of APDs after repeated daily injection (Wiley and Evans 2008). A proper examination of risperidone-induced cataleptic responses in adolescents and adults with either horizontal bar or grid test (Sanberg et al. 1988) will provide more information on age-dependent cataleptic outcomes. These behavioural findings during chronic treatment provided directions for Chapter 4 which would examine cataleptic responses with chronic administration of risperidone in adolescence, in comparison the same regimen in adulthood.

Why would adolescents be less vulnerable to the cataleptic and locomotor impairing effects of risperidone? Adolescence has been reported to be a period of hyper-dopaminergia with dopamine receptors reaching their peak levels of expression (Andersen et al. 2000; Teicher et al. 1995) and midbrain DA neurons achieving their peak levels of firing (McCutcheon et al. 2012; McCutcheon and Marinelli 2009). This relative hyper-dopaminergic state may perhaps overcome any potential for cataleptic/locomotor abnormalities as a result of dopaminergic blockade by risperidone and haloperidol. Alternately, although dopaminergic firing is highly active at this age, presynaptic dopamine innervation of the adolescent dorsal striatum is incomplete compared with adults (Matthews et al. 2013; Stamford 1989). Therefore if the synaptic machinery of the subcortical dopamine synapse is still under development perhaps the anti-dopaminergic effects of risperidone or haloperidol may not be fully experienced. Age-dependent differences in drug metabolism appear unlikely since the expression and function of liver cytochrome P450 enzymes have reached adult levels by PND30 in rats (Johnson et al. 2000).

Tolerance was induced to the CAR suppressing effects of clozapine at all ages. Development of clozapine tolerance with repeated daily treatment was in line with the reported findings in the literature. For example, tolerance developed to 20 mg/kg clozapine developed in a CAR paradigm as early as the third day of daily treatment in adult Wistar rats (Sanger 1985). This finding has been

replicated in recent studies, with clozapine tolerance developing after 3 days of treatment in adult SD rats (Li et al. 2010) and 5 days of treatment in adolescent SD rats (Qiao et al. 2013), which is in excellent agreement with my findings. Clozapine tolerance during chronic treatment can have important clinical implications since the clinical literature has documented several case reports of psychosis relapse (“supersensitivity psychosis”) on sudden clozapine withdrawal or switching from clozapine to other APDs (Ekblom et al. 1984; Eklund 1987; Perényi et al. 1985) or lack of clozapine efficacy on reintroduction after discontinuation (Grassi et al. 1999). While this finding provides a good future direction, further studies with clozapine were not allowed due to ethical reasons: (1) at the time of tissue collection, all clozapine-treated rats (18 out of 18) showed signs of intestinal adhesions and obstructions and (2) two of 18 clozapine-treated animals had to be sacrificed due to distress along with weight loss and abdominal distension.

3.4.2. Behaviour after a drug-free interval

To examine whether three weeks of daily APD exposure would produce persistent effects on neural function, I chose to probe CAR behaviour after re-exposure. The use of a half dose challenge was to prevent any floor effect in the likely event of sensitization after drug washout as suggested in the literature (Qiao et al. 2014a; Qiao et al. 2013; Qiao et al. 2014b).

Predictably, after a lengthy drug washout period, rats with risperidone pre-treatment in adulthood showed lower avoidance suppression by this lower dose compared with the earlier administered full dose. By contrast, rats treated as adolescents responded to half dose challenge at a similar level as they did to chronic full dose risperidone, suggesting an increase in behavioural responsiveness to this drug on re-exposure. Importantly this apparent sensitivity to CAR suppression after drug washout was not confounded by age-related alterations in escape failures and crossings, which were almost exactly the same in both age groups. This finding is in agreement with a recent study in which rats treated as adolescents with risperidone for 5 days were reported to show a sensitization-like CAR response, i.e. an increase in behavioural sensitivity, to a challenge dose of this APD as adults (Qiao et al. 2014a). The selective behavioural sensitivity in rats treated as adolescents is made more notable by the longer drug-washout period in our study (60 days vs. 15 days in adults). Moreover, our supplementary data showing that drug wash-out period had no effect on an animal’s ability to retain or relearn the avoidance response in the behavioural paradigm employed argue against the possibility that rats exposed to risperidone in adolescence were starting from any different baseline. Although sensitization to risperidone in a CAR paradigm has also been reported in adult rats (Gao and Li 2013), this behavioural response was not observed in my study. Variations in our experimental design such as the dose (1.3 mg/kg vs. 1 mg/kg), route (IP vs. SC), duration of

exposure (21 vs. 5 days), age of CAR training (adolescent vs. adult) and nature of CAR paradigms (CS-US test vs. CS-only test) may explain our inability to show this in chronically treated adults.

With respect to haloperidol and clozapine treatment in Experiment 1A, the challenge dose of haloperidol (0.025 mg/kg) suppressed CAR to a level approximately 50% of that seen in all age-matched controls. This dose is approximately equivalent to the reported ED50 dose of haloperidol for CAR suppression (Natesan et al. 2007). This suggests that no significant increase in behavioural sensitivity to haloperidol was induced at any ages after drug washout. Since this second part of our study was designed to assess long-term sensitization to prior APD exposure, the question of whether clozapine *tolerance* persisted after drug-washout could not be addressed due to the use of a *lower* (7.5 mg/kg), not *higher*, clozapine dose as a challenge dose. A challenge with the same (15 mg/kg) or a higher dose would be required to assess this in future studies.

3.4.3. Brain structural outcome and neural metabolism with chronic APD treatment

Chronic 21-day APD administration in both adolescents and adults produced no long-term effect on any brain structure examined, possibly because the duration of treatment was insufficient to induce structural changes. Reduction in whole brain and cortical volumes in *adult* SD rats has been reported to occur after 8 weeks of continuous APD treatment via osmotic minipumps but not after 4 weeks (Vernon et al. 2011). Although differences in route of administration (osmotic minipumps in the study by Vernon et al. and IP in the current study) may impede a direct comparison of the findings, it appears that an extended duration of treatment may be required to induce brain structural changes. Unfortunately any treatment duration longer than 5 weeks would have exceeded the duration of adolescence in rats making it impossible to parse adolescent exposure-specific effects. Still, it remains plausible that ultra-structural changes such as alterations in the dendritic spines can occur (Frost et al. 2010; Milstein et al. 2013).

My findings contrast with those of Piontkewitz and colleagues who reported that adolescent treatment with 1.2 mg/kg risperidone for 14 days led to reduction in adult whole brain volume at PND120 (Piontkewitz et al. 2011). Differences in experimental factors including rat strains (SD vs. Wistar), prenatal handling exposure in the latter's study and adolescent exposure to CAR training in the current study may have confounded comparison. In any case I interpret an absence of any long-term changes in brain volumes in our 21-day risperidone regimen as an indication that my particular regimen may be less toxic than others previously used.

The limitations in interpretation of structural outcomes should also be acknowledged. In this study, manual segmentation of brain regions was performed. This methodology has certain drawbacks: (1) only the regions that have been defined a priori can be analysed; (2) volumetric analysis of brain

regions such as the NAc will be difficult to be achieved reliably; (3) manual segmentation is less sensitive to detect smaller changes in brain volume. Therefore, the use of automated analysis such as voxel-based or deformation-based morphometry (VBM or DBM, for example, see (Lau et al. 2008)) may be able to detect voxel-wise changes in brain structure. A recent study in adult SD rats utilized tensor-based morphometry (TBM) analysis of structural MRI data and showed that chronic 8-week treatment with haloperidol and olanzapine induced inward and outward displacement of dorsal hippocampus respectively while hippocampal volume was not altered (Crum et al. 2016). The use of VBM, DBM or TBM in addition to volumetric analysis will help address the question as to whether adolescent APD treatment can induce similar changes in brain morphometry. Another limitation is a possible role of the drug-free interval in determining structural outcomes. In adult rats, increases in striatal volume and decreases in whole brain volume have been reported to normalize after 8 weeks of drug-free interval (Vernon et al. 2012). Therefore, a more thorough investigation with longitudinal assessment with adequate sample size is still required to determine brain structural changes with adolescent APD treatment.

A recent preclinical study suggested that treatment with the atypical APD olanzapine in adolescence could alter the baseline levels of glutamate and GABA in the NAc at adulthood (Xu et al. 2015). Therefore, in Experiment 1B, I examined the levels of these neural metabolites and NAA in the NAc of mature rats that had been treated with risperidone in adolescence or adulthood. No significant alteration in the levels of accumbal metabolites was observed. It appears that long-term changes in levels of GABA and glutamate in the NAc depend on the type of APD and possibly the route of administration (IP injection in the current study vs via drinking water in Xu's study).

3.4.4. Neurochemistry after a drug-free interval

The NAc is a major locus of APD-induced CAR impairment (Wadenberg et al. 1990b). Dopaminergic neurotransmission primarily within the NAc (McCullough et al. 1993; Oleson et al. 2012) has also been reported to play a critical role in CAR behaviour. Also given the well-known regulation of dopamine by serotonergic systems (Di Giovanni et al. 2008; Di Matteo et al. 2008; Navailles and De Deurwaerdère 2011) and the high affinities of risperidone for 5HT_{2A} receptors (Schotte et al. 1996) I elected to examine gene expression of dopaminergic markers and 5HT_{2A} receptors in the NAc. Here I revealed a significant downregulation in gene expression of 5HT_{2A} receptors and COMT selectively in rats chronically exposed to risperidone as adolescents.

Decreased 5HT_{2A} receptors have been reported in different brain regions shortly after termination of chronic treatment with atypical APDs such as olanzapine, risperidone and clozapine in both adult (Lian et al. 2013; Tarazi et al. 2002; Yadav et al. 2011) and adolescent animals (Choi et al. 2010b). Our findings extend this literature by demonstrating that reductions in 5HT_{2A} receptors in the NAc

can occur long after a drug withdrawal from adolescent treatment. Changes in both mRNA and protein levels of COMT have been reported in the *frontal cortex* of *adult* animals 24 hours after withdrawal from chronic 21-28 day treatment with atypical APDs such as risperidone, olanzapine, clozapine and aripiprazole. The direction of change is inconsistent, with reports of both up- (Chen and Chen 2007; Cheng et al. 2008) and down-regulated (Fatemi and Folsom 2007; Fatemi et al. 2012) levels. To the best of my knowledge COMT expression in the NAc tissues after APD withdrawal has not been examined. Here, I showed that COMT was downregulated in rats with adolescent risperidone exposure even after two months of drug-free period.

What is the functional significance of decreased expression of 5HT_{2A} receptors and COMT in the NAc? Blockade of 5HT_{2A} receptors by a selective 5HT_{2A} antagonist MDL100907 has no effect on CAR performance by itself. However, MDL100907 can enhance the CAR-suppressive effects of both raclopride and haloperidol, which are potent D2 receptor antagonists (Wadenberg et al. 2001a; Wadenberg et al. 1998a). The co-administration of MDL100907 with such potent DA blocking agents recreates the pharmacology of risperidone. Therefore the selective reduction in NAc 5HT_{2A} receptors in the adolescent risperidone-exposed animals may phenocopy the effects of 5HT_{2A} receptor blockade thus enhancing CAR suppression on re-exposure to this APD.

COMT and monoamine oxidase A and B (MAO-A and MAO-B) are enzymes involved in the degradation of mono-amine neurotransmitters such as dopamine, epinephrine and norepinephrine. Given reports that APD-induced downregulation of COMT mRNA levels is accompanied by decreased protein levels (Fatemi and Folsom 2007), it is presumed that NAc COMT protein will also be downregulated in our animals. Such a condition may lead to compromised DA turnover in the NAc although we are wary of such speculation given mono-amine oxidase levels are normal. In any case, given that one outcome of 5HT_{2A} activation is increased dopamine synthesis and release (Navailles and De Deurwaerdère 2011), I speculate this reduction in COMT may be a compensatory process in an attempt to maintain normal NAc DA levels as 5HT_{2A} receptor function is presumably impaired in adolescents exposed to risperidone. Measurement of monoamines and their metabolites by high-performance liquid chromatography (HPLC) along with an assessment of COMT functional activity in the NAc from these animals is therefore now warranted (See Chapter 5).

In summary, the findings of Experiment 1 show that the adolescent brain is highly susceptible to risperidone, with this atypical APD being capable of inducing long-standing changes in behaviour and neurochemistry in the NAc. However, the findings should still be interpreted in the context of certain limitations. First, I have examined CAR as the sole behavioural read-out of risperidone's effects on integrated brain function. Other behavioural tests examining cognitive function, decision-making and risk-taking, the ability to learn a complicated task, reward function, challenge with

other psychomimetic drugs or 5HT_{2A}-dependent behavioural tests such as 5HT_{2A}-agonist induced head shake behaviour (Canal and Morgan 2012; Halberstadt and Geyer 2013) may produce complimentary results. Second, to keep final CAR testing age constant in all groups by necessity, the duration of drug washout period varied. It remains unanswered whether a longer drug-free interval in adult exposure groups may have produced a similar behavioural and neurochemical outcome as in adolescent exposure group. This provided a future direction for Chapter 5 to examine risperidone-induced neurobiological outcomes after an equivalent drug-free interval. Third, other important regions such as PFC, striatum and VTA have not been examined for changes in neurochemistry. It is unknown whether changes in 5HT_{2A} and COMT in the NAc of rats with adolescent risperidone exposure are compensatory to neurotransmission changes in these regions.

Chapter 4. A comparative examination of risperidone-induced cataleptic responses in adolescents and adults

4.1. Introduction

In Chapter 3, I showed that chronic administration of risperidone induced a significantly lower level of escape failures in adolescents than in adults. This finding suggested two possibilities: (1) neural adaptation changes during chronic risperidone treatment are different in adolescents and adults and (2) APD-induced escape failures in the CAR paradigm (total failure to respond to both conditioned stimulus (white noise) and unconditioned stimulus (foot-shock)) possibly reflect cataleptic responses (Wadenberg 2010). Catalepsy in rodents represents as the state in which the animal remains or fails to correct an unusual posture for an extended duration (Sanberg et al. 1988; Wadenberg 1996). Therefore, in Chapter 3, I speculated that a progressive increase in cataleptic response developed after repeated risperidone treatment in adults, but this developed at a lower level when the same treatment regime was used in adolescents.

Supporting this hypothesis, a progressive increase in cataleptic responses in adult rats has been reported with repeated treatment with low dose haloperidol, a typical APD, despite the lack of such a response at the beginning of treatment (Banasikowski and Beninger 2012a; Pezarro Schimmel et al. 2015; Schmidt et al. 1999). Another study has also reported that the cataleptic responses of adolescent male rats progressed at a lower rate than adults during repeated treatment with haloperidol (Wiley and Evans 2008). The findings by Wiley and Evans again suggested a different rate of neural adaptative changes in adolescents in response to ongoing repeated APD treatment. Wiley and Evans also reported that repeated treatment with the atypical APD clozapine could also progressively induce catalepsy in both adolescents and adults; however there was no differential age-dependent effect with clozapine, suggesting that the behavioural outcomes could vary with the receptor affinity profiles of the APD. However, this study did not investigate neurochemical changes that could underlie differential cataleptic responses at these two ages. Therefore, the neural mechanism(s) that predispose(s) adolescents to lower a cataleptic response to APDs is still unknown. Moreover, it is still unknown whether chronic treatment with risperidone, another atypical APD with different neurotransmitter receptor affinity profiles (Schotte et al. 1996), could induce a differential pattern of catalepsy in adolescents and adults.

In rodents, APD-induced cataleptic response models the potential for extrapyramidal side effects (EPS) in humans such as akinesia and rigidity (Hoffman and Donovan 1995; Porsolt et al. 2010). Doses of APDs exceeding 80% striatal dopamine receptor blockade can induce EPS in humans (Kapur et al. 2000) and cataleptic responses in rodents (Natesan et al. 2008; Natesan et al. 2006a; Wadenberg et al. 2000; Wadenberg et al. 2001b). The cataleptic response in rodents is examined with either a horizontal bar or grid test (Sanberg et al. 1988; Wadenberg 1996).

The striatum has been reported to be the major brain region associated with APD-induced catalepsy (Ossowska et al. 1990; Yoshida et al. 1994). A progressive increase in haloperidol-induced catalepsy in adults has also been linked to spike frequency of medium spiny neurons in the striatum (Frank and Schmidt 2004). However, the adolescent striatum is undergoing important maturation changes, especially in the dopaminergic systems with changes in both presynaptic activity (Matthews et al. 2013; Stamford 1989) and postsynaptic receptors (Tarazi and Baldessarini 2000; Tarazi et al. 1999; Teicher et al. 1995). Consequently, chronic APD-induced dopaminergic blockade may not have as much effect on the adolescent striatum as on the adult striatum. This may be an underlying neural mechanism for the differential cataleptic responses induced in adolescents and adults by APDs. Alternately, chronic risperidone treatment may actively alter maturation within the adolescent striatum and consequently alter behavioural responses to this APD. To answer these questions, no study to date has thoroughly examined longitudinal course of behavioural response with repeated risperidone treatment in adolescents, in comparison with the same regimen in adults.

Here in Chapter 4, I aimed to address whether chronic risperidone induces a differential progression of catalepsy in adolescents compared with adults and to understand what neural mechanisms underlie such outcomes. I hypothesized that (1) cataleptic responses induced by chronic risperidone treatment are lower in adolescents than in adults and (2) risperidone-induced increases in dopamine neurotransmission in the immature adolescent striatum underlie a lower cataleptic vulnerability in adolescents.

4.2. Materials and methods

4.2.1. Subjects

Male SD rats arrived at the animal facility either on PND 28 (n= 24 for adolescent cohort) or PND 70 (for adult cohort, n = 24) (n = 12 per drug group for a given age. One rat assigned to the adolescent control group died before the start of experiments, thus giving n = 11 for this group. Rats from the same drug and age groups were pair-housed in Macrolon cages (39 cm x 23.5 cm x 16 cm) with Sani chip bedding (Able Scientific) and wire lids in a temperature (21 ± 1 °C) and lighting (lights on at 06:00 h and off at 18:00 h) controlled room. All rats were given ad libitum access to food and water throughout the whole experiment. Behavioural tests were conducted during the light phase of the diurnal cycle.

4.2.2. Experimental design

Rats were treated with 1.3 mg/kg risperidone or vehicle (IP) for 22 continuous days either as adolescents (PND35-PND56) or as adults (PND80-PND101) (Figure 4-1). On Day 1, 3, 5, 7, 10 and 17 of chronic treatment, rats were examined for cataleptic response 1 h after injection using the

horizontal bar test. This time point was chosen to match the time after drug administration in which escape failures were observed in the CAR in Chapter 3. Immediately after termination of cataleptic test, rats were placed in the locomotor chambers for recording of locomotor activity for 30 min. At 24 h after the last injection i.e. Day 23 of experiment, all rats were sacrificed with an overdose of phenobarbitone (Lethabarb) and brain tissues collected in liquid nitrogen, coded and stored at -80 °C until use.

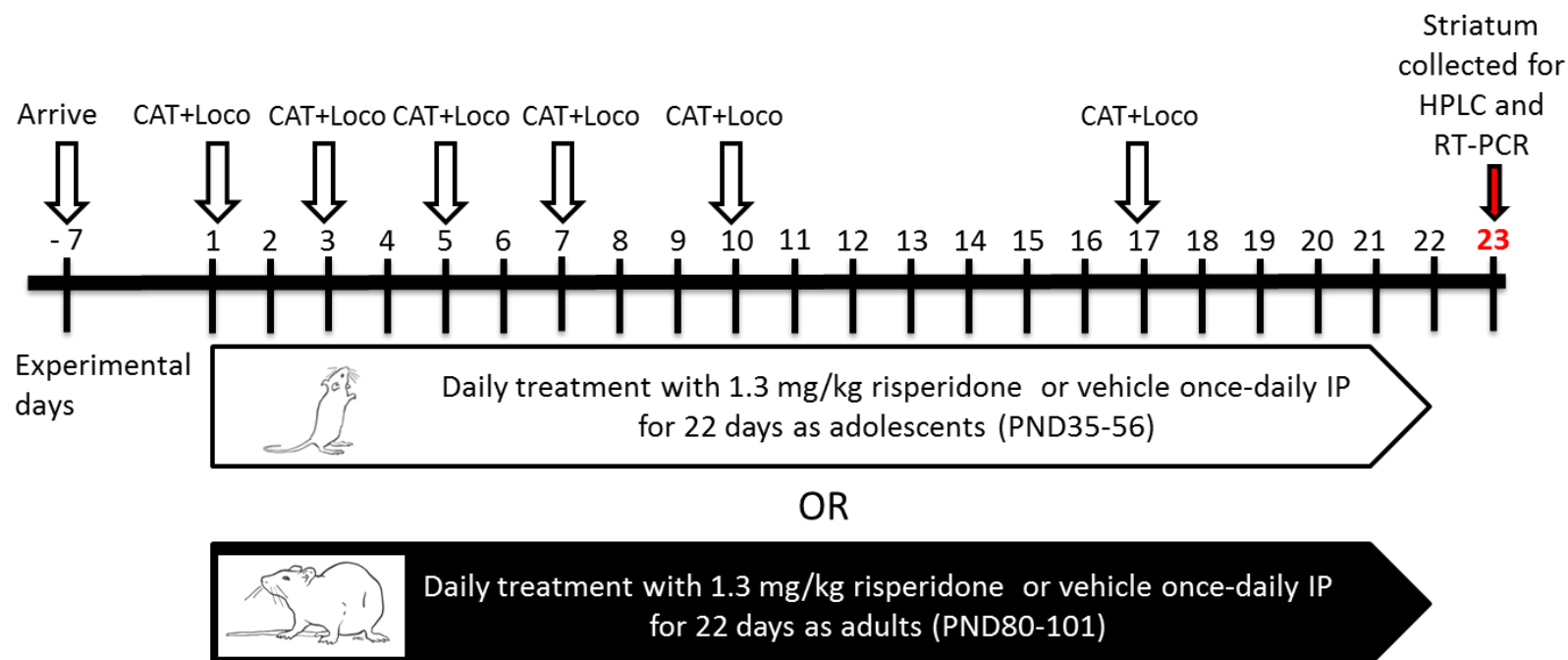


Figure 4-1 Timeline of the catalepsy experiment. Rats treated with risperidone or vehicle either as adolescents (PND35-PND56) or adults (PND80-PND101) were tested for cataleptic responses (CAT) and locomotor activity (Loco) at 1 h after injection at Day 1, 3, 5, 7, 10 and 17 of treatment. At 24 h after the last injection i.e. at Day 23 of experiment, right and left striatal tissues were collected for HPLC and real-time PCR respectively.

4.2.2.1 Bar test for catalepsy

The horizontal bar test was performed using a chamber (35 cm x 20 cm x 25 cm), comprising a grid floor and a horizontal bar (1 cm in diameter) with adjustable height (10 cm for adolescents and 13 cm for adults) (See details in Chapter 2, Section 2.3). At 1 h after injection with vehicle or risperidone, rats were placed individually into bar apparatus. Both forepaws of rats were gently placed on the horizontal bar and the time rats stayed with both forepaws on the bar was video-recorded. If rats voluntarily removed their paws from the bar, they were placed back on to the bar after a waiting time of 10 s. Each rat was examined for a maximum duration of 180 s or a maximum of 12 trials (12 times of placing the forepaws on the bar). Videos were coded and analysed in Media Player Classic Home video viewer. Analyst was blind to treatment. The duration rats stayed with their forepaws (time on-bar) was noted in millisecond resolution. The average duration of time on-bar out of the total number of trials in each test was calculated for each animal.

4.2.2.2 Open field test (OFT) for locomotor activity

Risperidone-induced suppression of spontaneous locomotor activity was tested in four black Plexiglas locomotor activity chambers (45 cm x 45 cm x 30 cm). The chambers were equipped with Activity Monitor (MedAssociates). Immediately after catalepsy test (which took approximately 5 min per rat) i.e. ~ 65 min after injection with risperidone or vehicle, rats were individually placed in the locomotor chambers and locomotor activity was examined for a total of 30 min in terms of total distance travelled (cm).

4.2.2.3 HPLC of striatal tissues

At 24 h after the last injection, all rats were euthanised and their striatal tissues collected and stored at -80 °C until use. Briefly, the right hemisphere striatum was quickly weighed (wet weight) and homogenised on ice in a minimum volume of 0.1 ml of 0.1M perchloric acid and 50 mg/ml deoxyepinephrine (internal standard for catecholamines), using ultrasonicator probe (Vibra-Cell, Sonics & Materials, Inc. CT.). After centrifugation at 13,000 rpm for 5 minutes, supernatant from each sample was collected and filtered through 0.2 µm nylon filter. Next, 10 µl of each sample was injected into a HPLC system (See details in Chapter 2) and dopamine, serotonin (5HT), noradrenaline and their metabolites (dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3-MT) and 5-hydroxyindoleacetic acid (5-HIAA)) and measured. Data were processed offline with Chemstation software (Rev B.01.03, Agilent Technologies, Inc. CA). The amount of catecholamines and their metabolites were expressed as pg/mg wet tissue, after correction for the dilution.

4.2.2.4 RT-PCR of striatal tissues

Briefly, total RNA was extracted from each left striatal hemisphere sample using QIAzol and RNeasy Mini Kit (Qiagen, Australia). For each sample, 1 µg of RNA per 21 µl reaction was reverse-transcribed to cDNA with SuperScript IV First-Strand Synthesis System (Invitrogen). RT-PCR was performed in 12 µl reaction using a Roche LightCycler 480 (Roche Diagnostics, Australia), using a SYBR Green method in 384-well plates. Relative expression of the target genes normalized to that of endogenous control GAPDH was calculated following the published method (Schmittgen and Livak 2008). All PCR experiments were performed twice. Gene expression data was only considered if significant changes were observed in both repeats. PCR experiments were performed with adolescent and adult groups in the same PCR plate along with a universal control sample in each plate. The $2^{-\Delta Ct}$ values of each sample normalized to that of the universal control. This allowed the whole cohort to be compared across-age groups.

4.2.3. Statistical analysis

All data were expressed as mean \pm standard error of the mean (SEM). Catalepsy data (time on bar) were log-transformed for normalization. Behavioural data were analyzed with repeated measures two-way (age x drug) analysis of variance (ANOVA) followed by posthoc Dunnett's tests. Due to a technical problem with the controlling computer, locomotor data from 3 adolescent control animals at Day 3 were lost, thus giving $n = 8$ for this particular time point. HPLC and real-time PCR data were analysed with two-way ANOVA followed by post hoc Dunnett's tests. Pearson's correlation analysis was used to determine the relationship between neurochemical and behavioural data. Statistical significance was defined as $p < 0.05$.

4.3. Results

4.3.1. Cataleptic responses in adolescents vs adults during chronic risperidone treatment

As shown in Figure 4-2, cataleptic responses progressively increased in both adults and adolescents treated with risperidone from Day 1 to Day 17 of chronic treatment, but not in controls. Starting from Day 5, a differential cataleptic outcome was observed; the levels of cataleptic responses were lower in adolescents than in adults [2-way repeated measures ANOVA: significant main effects of Day ($F_{5, 200} = 23.628$), Drug ($F_{1,40} = 96.635$), Day x Drug interaction ($F_{5,200} = 7.823$), all $p < 0.001$; but no significant main effects of Age ($F_{1,40} = 1.186$), Age x Drug ($F_{1,40} = 1.517$), Day x Age ($F_{5,200} = 0.837$) or Day x Age x Drug interaction ($F_{5,200} = 1.346$), all $p > 0.05$].

Further examination at individual testing days showed that risperidone-treated adolescents had significantly lower cataleptic response than their adult counterparts ($p = 0.047$) at Day 10 [2-way ANOVA: significant main effects of Drug ($F_{1,43} = 96.572$, $p < 0.001$) and Age ($F_{1,43} = 4.082$, $p =$

0.05) but no Age x Drug interaction ($F_{1,43} = 2.023$, $p = 0.162$)]. The differences in cataleptic responses between risperidone-treated adolescents and adults approached statistical significance ($p = 0.082$) at Day 5 [2-way ANOVA: significant main effects of Drug ($F_{1,43} = 102.177$, $p < 0.001$) and Age x Drug interaction ($F_{1,43} = 5.241$, $p = 0.027$; no main effect of Age ($F_{1,43} = 0.648$, $p = 0.425$)]. No significant difference in cataleptic responses was observed at other test days. Vehicle-treated adolescents and adults did not show any differences in the time-on bar.

These data support the hypothesis that, with ongoing chronic risperidone treatment, the time course of behavioural responses in adolescents is different from that of adults and the adolescents develop lower catalepsy than adults.

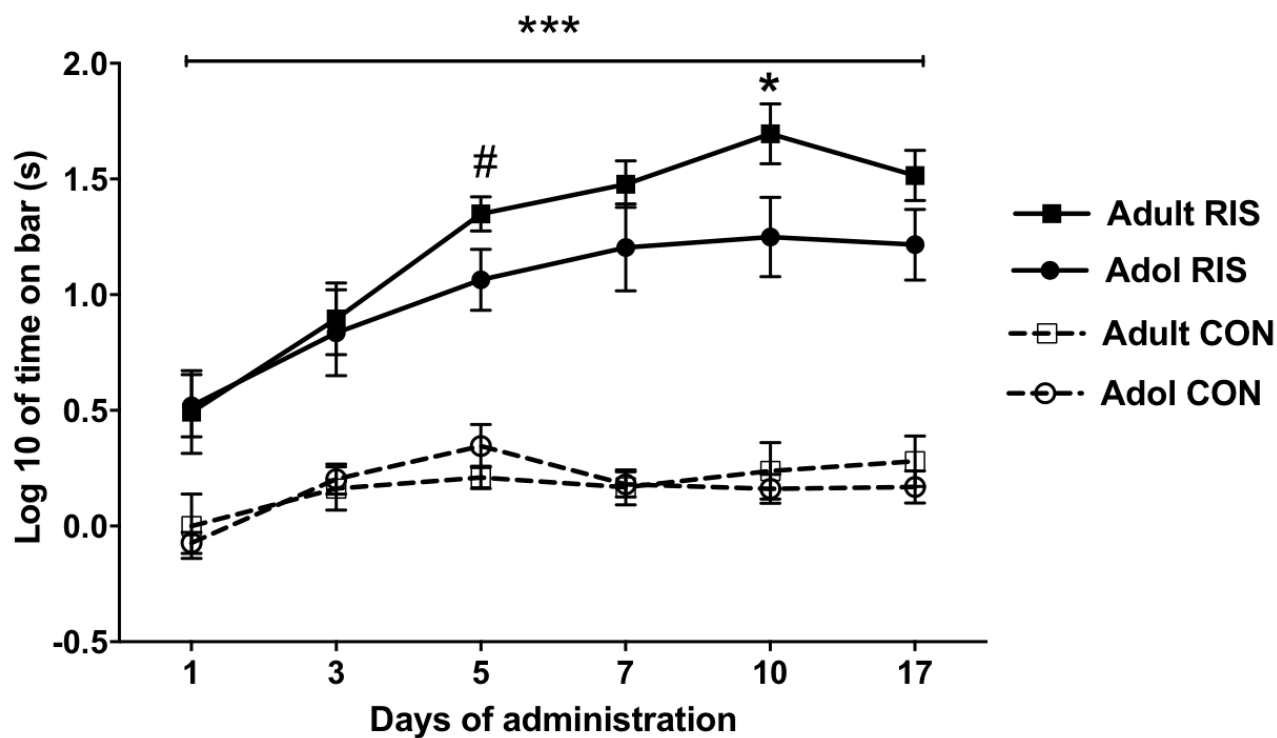


Figure 4-2 Risperidone-induced cataleptic responses were lower in adolescents than in adults. Data expressed mean \pm SEM. $n = 12$ per drug for a given age except $n = 11$ for adolescent CON. *** $p < 0.001$ RIS vs CON; * $p < 0.05$ for adolescent RIS vs adult RIS; # $p = 0.08$ for adolescent RIS vs adult RIS. CON – vehicle-treated controls; RIS – risperidone-treated group;

4.3.2. Locomotor activity in adolescents vs adults during chronic risperidone treatment

Risperidone suppressed locomotor activity in both adolescents and adults starting from Day 1 of administration (Figure 4-3). Again, apparent differences in risperidone-induced locomotor suppression were observed in adolescents and adults [2-way repeated measures ANOVA: significant main effects of Drug ($F_{1,40} = 147.071$, $p < 0.001$) and Day ($F_{5,200} = 5.061$, $p < 0.001$); Age x Drug ($F_{1,40} = 4.021$, $p = 0.052$); no main effects of Age ($F_{1,40} = 0.173$, $p = 0.680$); Day x Age ($F_{5,200} = 2.112$, $p = 0.065$); Day x Drug ($F_{5,200} = 0.574$, $p = 0.720$); Day x Age x Drug ($F_{5,200} = 0.500$, $p = 0.776$)].

Further examination at individual test days confirmed that risperidone-treated animals from both adolescent and adult cohorts had significantly lower locomotor activity than their corresponding controls ($p < 0.001$ for all days). Two-way ANOVA at individual test days showed a significant main effect of age x drug interaction at Day 17 ($F_{1,43} = 5.953$, $p = 0.019$). However, the differences in locomotor activity between risperidone-treated adolescents and adults did not reach the statistical threshold of post-hoc tests (all $p > 0.1$).

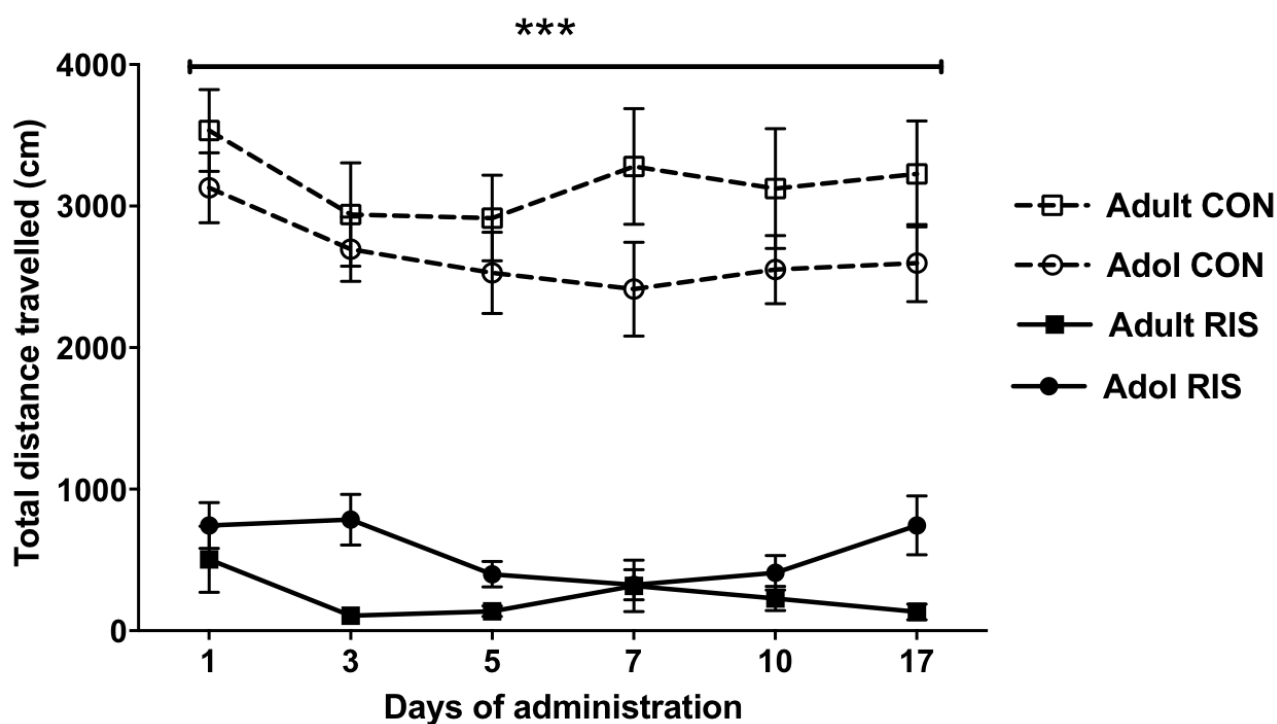


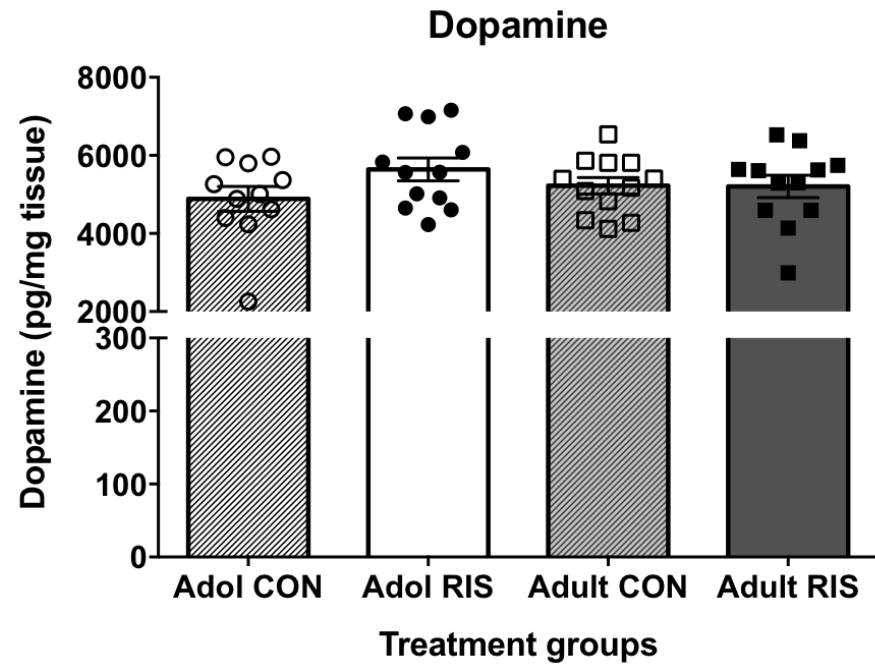
Figure 4-3 Chronic risperidone-induced suppression of locomotor activity in adolescents and adults. Data expressed mean \pm SEM. $n = 12$ per drug for a given age except $n = 11$ for adolescent CON. *** $p < 0.001$ RIS vs CON; CON – vehicle-treated controls; RIS – risperidone-treated group;

4.3.3. Changes in striatal monoamines and metabolites with chronic risperidone treatment in adolescents vs adults

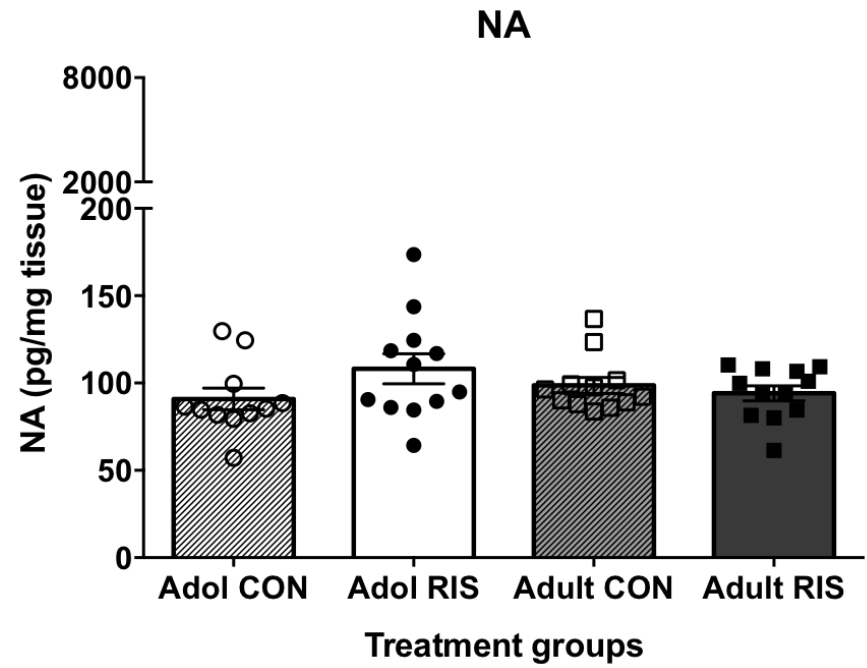
One day after termination of chronic 22-day treatment, risperidone-induced changes in striatal monoamines (dopamine, 5HT and noradrenaline) and their metabolites (DOPAC, HVA, 3MT and 5HIAA) were examined.

Among monoamines (Figure 4-4), no significant alterations were observed for both dopamine [2-way ANOVA: no significant main effect of Age, Drug or Age x Drug interaction, all $F < 1.9$, all $p > 0.1$) and noradrenaline [2-way ANOVA: no significant main effects of Age, Drug and Age x Drug interaction, all $F < 3.1$ all $p > 0.08$]. However, significant differences in 5HT levels were observed between adolescent and adult cohorts [2-way ANOVA: significant main effect of Age ($F_{1,43} = 8.007$, $p = 0.007$) but no main effect of Drug ($F_{1,43} = 1.770$) or Age x Drug interaction ($F_{1,43} = 0.499$), both $p > 0.1$).

(a)



(b)



(c)

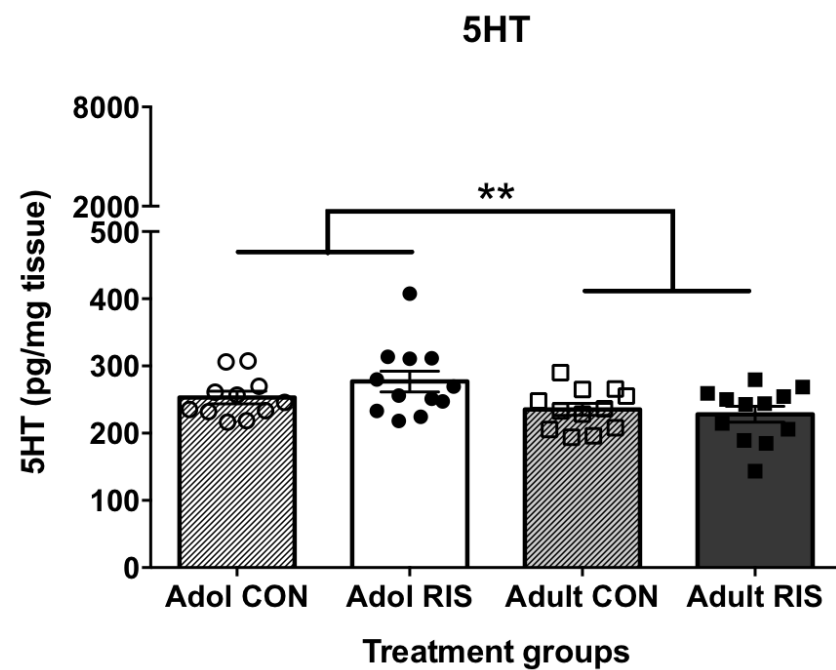


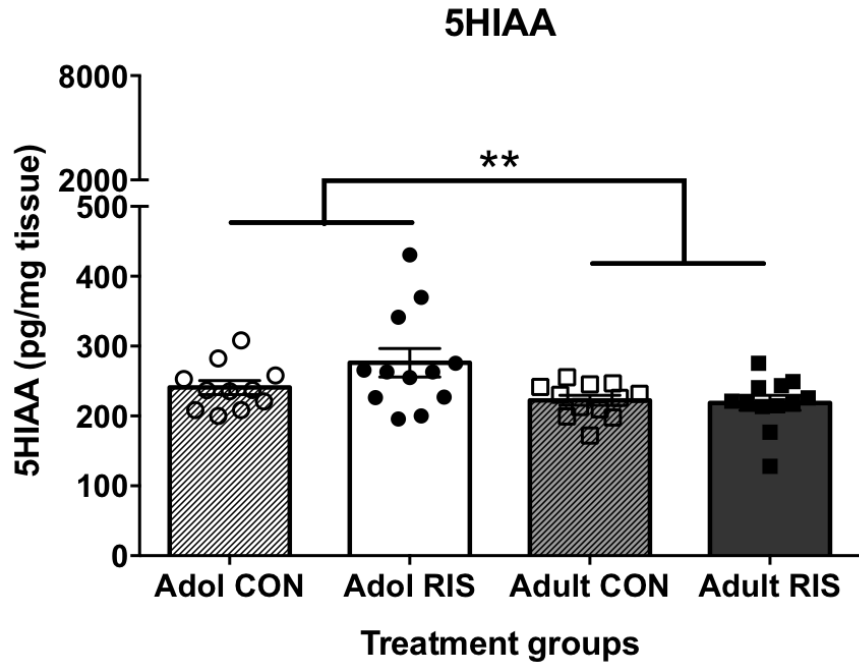
Figure 4-4 Changes in levels of striatal monoamines with chronic risperidone treatment in adolescence or adulthood. Striatal levels of (a) dopamine (b) noradrenaline (NA) and (c) 5HT are shown for both adolescents and adults. Data expressed mean \pm SEM. $n = 12$ per drug for a given age except $n = 11$ for adolescent CON. ** $p < 0.01$ adolescent cohorts vs adult cohorts; CON – vehicle-treated controls; NA – noradrenaline; RIS – risperidone-treated group;

Increases in striatal 5HIAA, the main metabolite of 5HT, followed the increases observed in 5HT. The adolescent cohorts had significantly higher 5HIAA than adult cohorts [2-way ANOVA: significant main effects of Age ($F_{1,43} = 8.232$, $p = 0.006$); no main effect of Drug ($F_{1,43} = 1.455$) or Age x Drug interaction ($F_{1,43} = 2.170$), both $p > 0.1$].

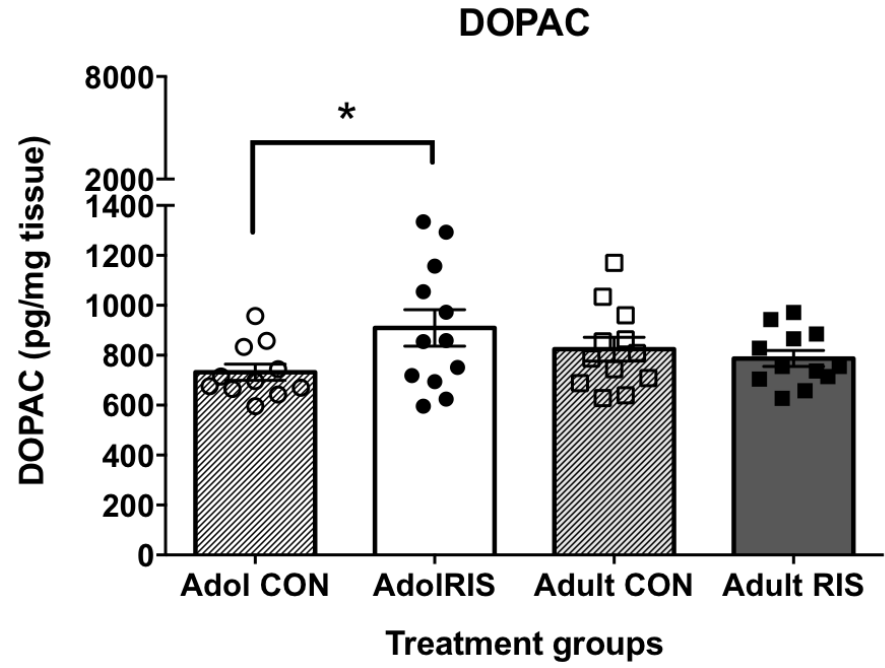
The adolescent risperidone group appeared to show higher levels of dopamine metabolites: DOPAC [2-way ANOVA: significant main effects of Age x Drug interaction ($F_{1,43} = 4.601$, $p = 0.038$) but no main effect of Age or Drug, both $F < 1.7$ and $p > 0.1$]; HVA [2-way ANOVA: significant main effects of Age x Drug interaction ($F_{1,43} = 5.870$, $p = 0.020$) but no main effect of Age or Drug, both $F < 1.7$ and $p > 0.1$]; 3MT [2-way ANOVA: significant main effects of Age x Drug interaction ($F_{1,43} = 5.906$, $p = 0.019$) and Drug ($F_{1,43} = 4.562$, $p = 0.038$); but not main effect of Age ($F_{1,43} = 2.820$, $p = 0.10$)]. On further analysis, the adolescent risperidone group had significant higher levels of DOPAC ($p < 0.05$) and 3MT ($p = 0.007$), compared to adolescent controls. In addition, risperidone-treated adolescent rats had higher levels of 3MT than their adult counterparts and adult controls (both $p < 0.05$). Striatal HVA levels were not statistically different among different groups. Turnover rate of dopamine and 5HT was also examined by comparison of ratios between DOPAC or HVA and dopamine and between 5HIAA and 5HT respectively. However, no significant difference was observed among different groups.

Taken together, these findings of increased dopamine metabolites in risperidone treated adolescents suggested that changes in dopaminergic neurotransmission on a background of higher 5HT and 5HIAA might contribute to some underlying neural mechanism for their diminished cataleptic responses.

(a)



(b)



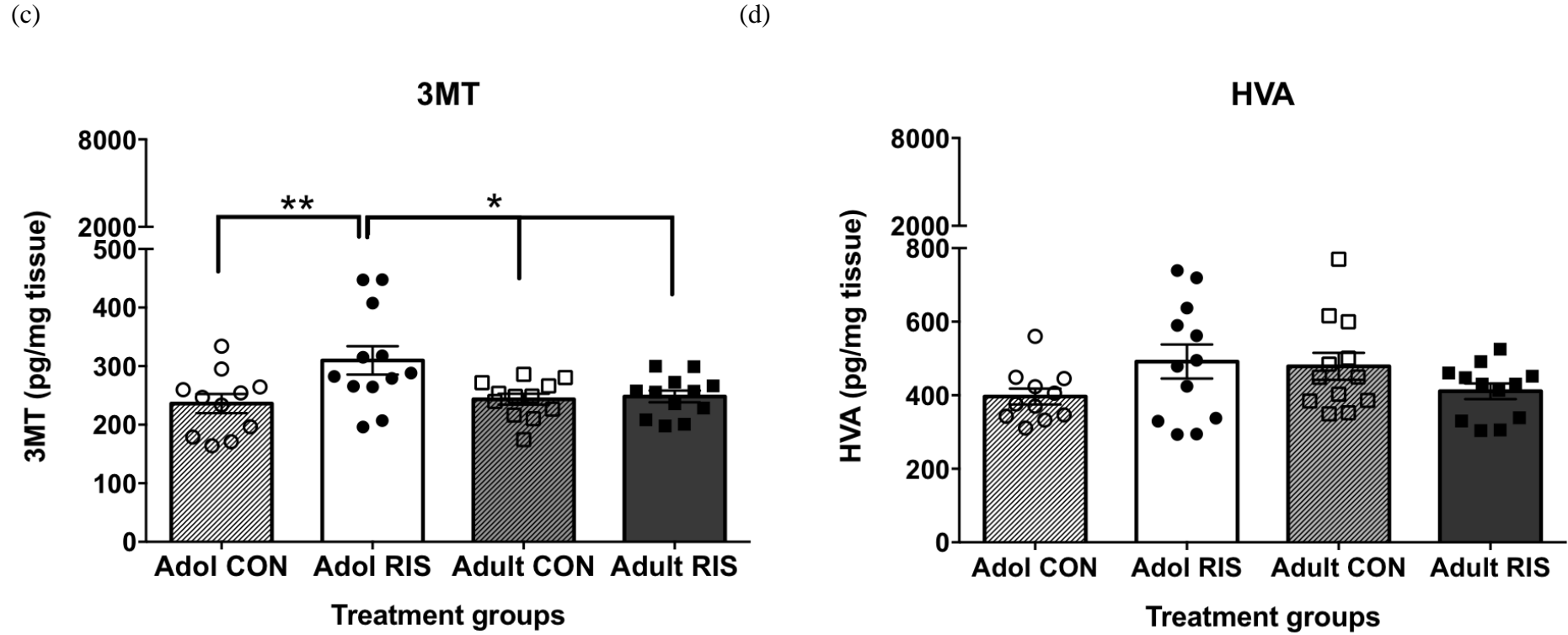


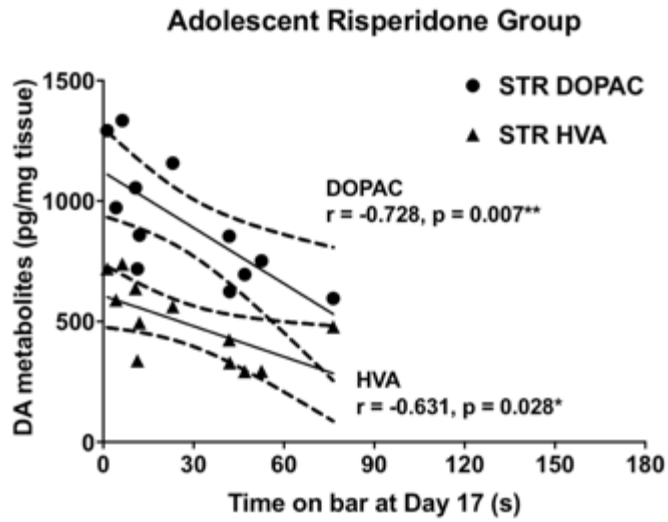
Figure 4-5 Changes in levels of striatal monoaminergic metabolites following chronic risperidone treatment in adolescents or adults. Striatal levels of (a) 5HIAA (b) DOPAC, (c) 3MT and (d) HVA are shown for both adolescents and adults. Data expressed mean \pm SEM. $n = 12$ per drug for a given age except $n = 11$ for adolescent CON. * $p < 0.05$ for adolescent RIS vs other treatment group; ** $p < 0.01$ for adolescent RIS vs other treatment group and for adolescent cohorts vs adult cohorts; 3MT – 3-Methoxytyramine; 5HIAA – 5-Hydroxyindoleacetic acid; CON – vehicle-treated controls; DOPAC – 3,4-Dihydroxyphenylacetic acid; HVA – homovanillic acid; RIS – risperidone-treated group;

4.3.4. Relationship between striatal dopamine metabolites and cataleptic responses at Day 17

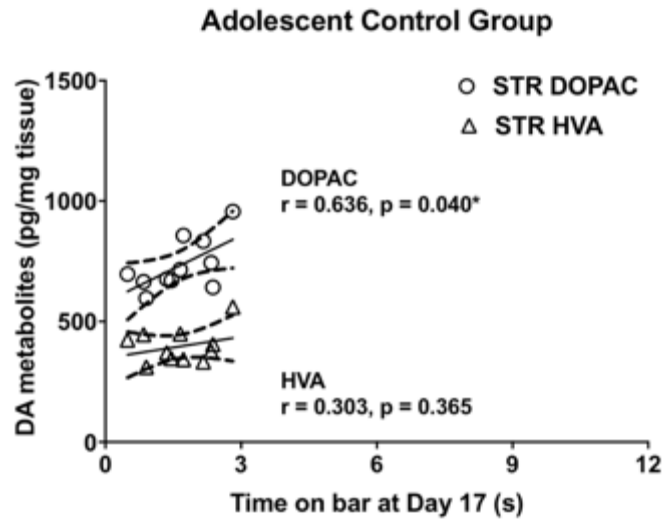
Interestingly, in adolescents treated with risperidone, the levels of the striatal dopamine metabolites: DOPAC ($r = -0.728$, $p = 0.007$) and HVA ($r = -0.631$, $p = 0.028$) *negatively* correlated with the cataleptic responses at Day 17, which was the time of behavioural testing most proximal to that of neurochemical examination (Figure 4-6(a) and (b)). This finding was again selective to adolescent risperidone treatment. No correlation was found in either adult risperidone treated animals or control groups (Figure 4-6(c) and (d)). In adolescent controls, a *positive* correlation between cataleptic scores and striatal DOPAC only ($r = 0.6256$, $p = 0.040$) was observed.

In addition, striatal dopamine levels positively correlated with both DOPAC ($r = 0.818$, $p = 0.001$) and HVA ($r = 0.622$, $p = 0.031$) in adolescent risperidone group and only with DOPAC in their controls ($r = 0.643$, $p = 0.033$). This suggested that in this age group, striatal dopamine availability was closely linked to the levels of dopamine metabolites. However, in adult groups, no correlation was observed between striatal dopamine and its metabolites levels ($r = -0.194$ to 0.425 , all $p > 0.1$).

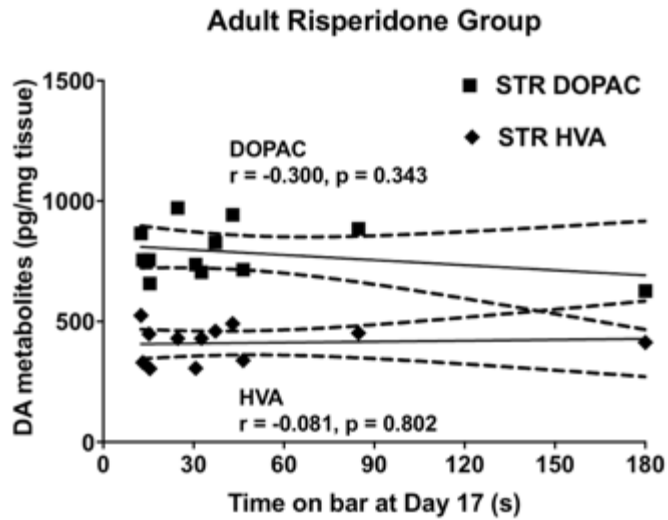
(a)



(b)



(c)



(d)

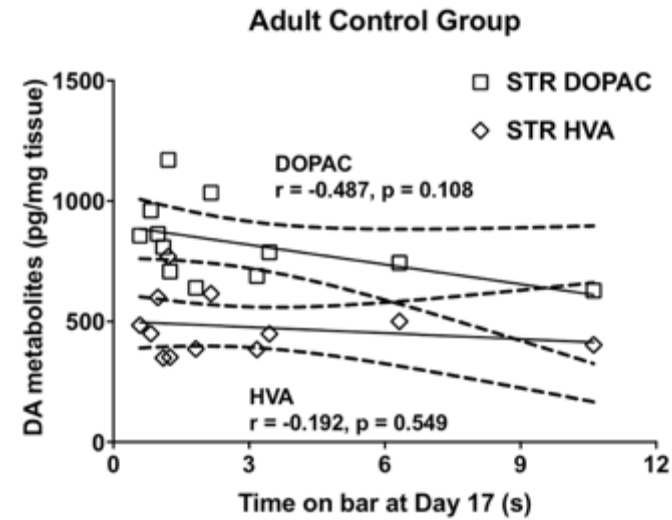
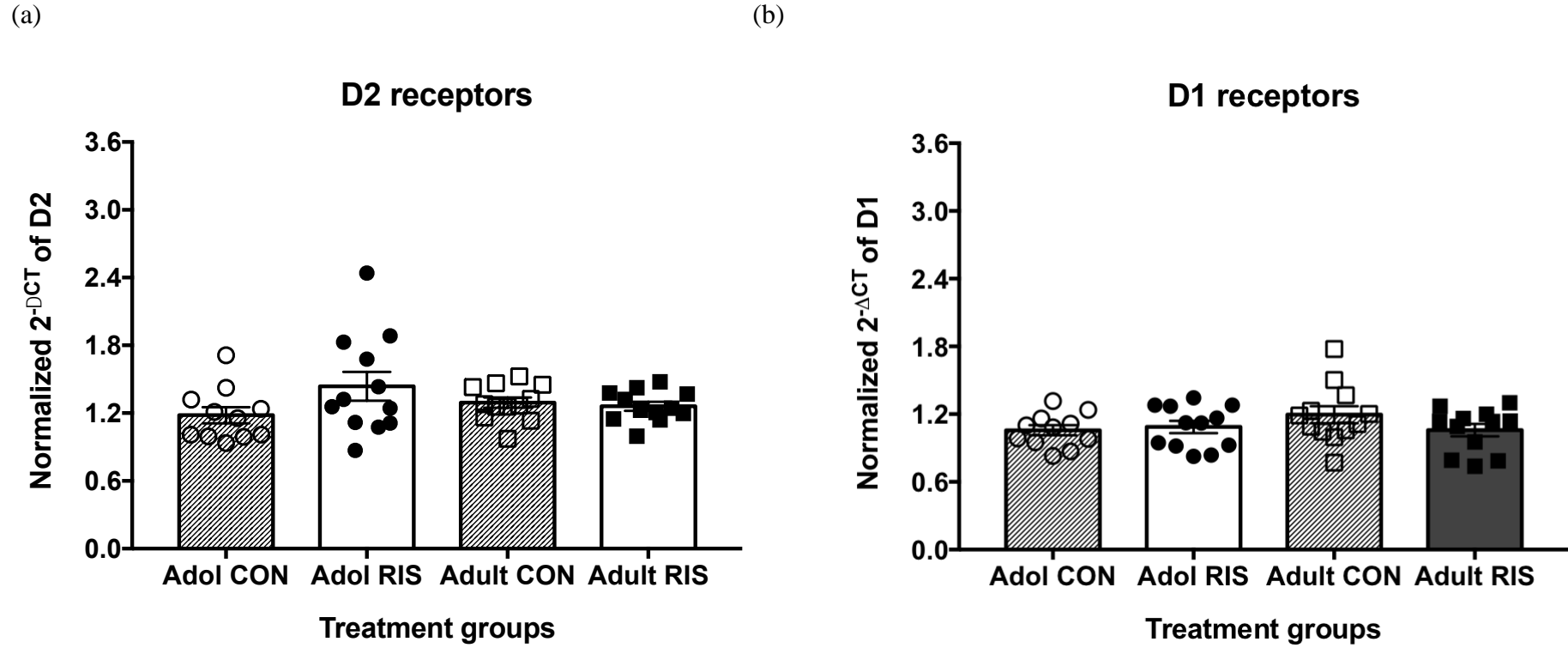


Figure 4-6 Correlation between striatal dopamine metabolites and cataleptic responses at Day 17 in adolescents and adults. (a) Striatal dopamine metabolites negatively correlated with cataleptic responses in adolescent rats treated with risperidone. (b) In adolescent controls, only striatal DOPAC levels correlated with cataleptic responses but this was in the reverse direction. Adult rats treated with (c) risperidone or (d) vehicle did not show any significant correlation between striatal dopamine metabolites and cataleptic responses.

4.3.5. Risperidone-induced changes in dopaminergic receptors and metabolizing enzymes in adolescents and adults

Next I examined whether dopamine receptor expression in the adolescent striatum was altered by chronic risperidone treatment. As shown in Figure 4-7 (a and b), no significant alterations in D2 and D1 receptor mRNA levels were observed in both adolescents and adults (2-way ANOVA for D1: no significant main effect of Age, Drug or Age x Drug interaction, all $F < 2$, $p > 0.1$; 2-way ANOVA for D2: no significant main effect of Age or Drug (both $F < 2$, $p > 0.1$) or Age x Drug interaction ($F_{1,43} = 3.311$, $p = 0.076$).

Given the findings of increased striatal dopamine metabolites in risperidone-treated adolescents, the expression of dopamine-metabolizing enzymes was also examined. As described in Figure 4-7 (c and d), no significant alterations of COMT and MAO-A was observed in both adolescents and adults (2-way ANOVA: no significant main effects of Age, Drug, or Age x Drug interaction for both COMT and MAO-A, all $F < 2.1$, all $p > 0.15$).



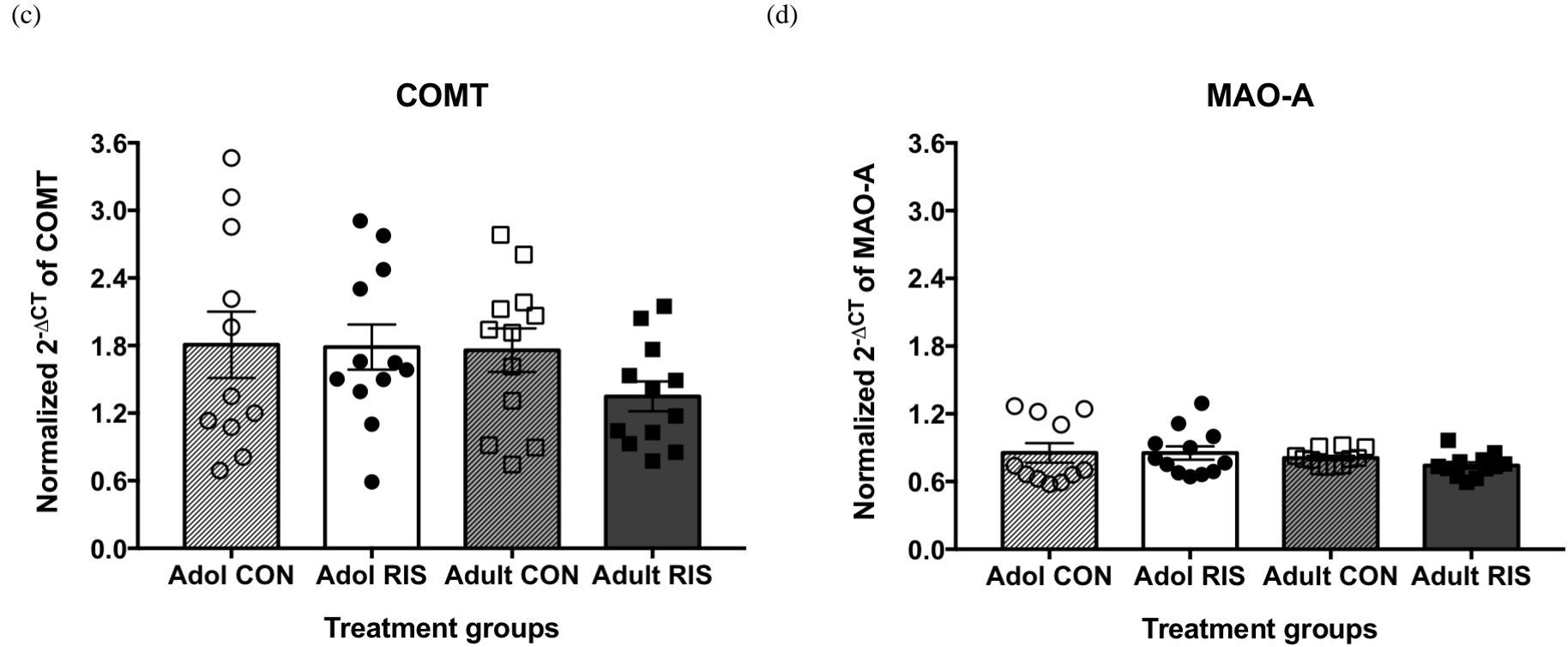


Figure 4-7 Risperidone induced no alteration in gene expression of dopamine receptors and dopamine-metabolizing enzymes in adolescents and adults. No significant alteration was observed in gene expression of (a) D2 and (b) D1 receptors, (c) COMT and (d) MAO-A with risperidone treatment in adolescence or adulthood. Data expressed mean \pm SEM. $n = 12$ per drug for a given age except $n = 11$ for adolescent CON. CON – vehicle-treated controls; COMT – catechol-o-methyl transferase; MAO-A – monoamine oxidase A

4.4. Discussion

The main findings of Chapter 4 were: (1) compared to adults, adolescents developed a lower progression of cataleptic responses during chronic risperidone treatment; (2) striatal levels of dopamine metabolites namely DOPAC and 3MT were elevated selectively in rats treated with risperidone in adolescence; (3) a relationship between striatal dopamine metabolite levels and cataleptic responses was identified in adolescents treated with risperidone.

4.4.1. Differential cataleptic responses induced in adolescents and adults

As predicted, risperidone induced an increasing level of cataleptic responses in both adolescents and adults from Day 1 to Day 17 of chronic treatment. The cataleptic responses in adolescents and adults were almost indistinguishable at the beginning of treatment. However, starting from Day 5, adolescents showed lower levels of cataleptic responses than adults and this difference in cataleptic responses became significant at Day 10. These findings are consistent with a previous preclinical report which showed a lower rate of progression of cataleptic responses in male adolescent rats compared with male adults during 10-day treatment with a typical APD haloperidol (Wiley and Evans 2008). These data explain the lower levels of escape failures in risperidone treated adolescents relative to adults during CAR testing during chronic treatment in Chapter 3. These data further support the hypothesis in Chapter 3 that disruption of avoidance responses by chronic risperidone in the CAR paradigm is partly contributed to by escape failures/cataleptic responses. Moreover, it is still unknown whether the observed outcome in catalepsy persists after a drug-free interval or whether the adolescents are more vulnerable to long-term catalepsy than adults. Following up on these questions, I examined long-term cataleptic response in rats treated with risperidone in adolescence or adulthood in (See Chapter 5).

Several studies have consistently shown that, in adult rats, repeated treatment with haloperidol, a typical APD with high affinity for dopamine D2 receptors, induces a progressive increase in catalepsy, a behavioural response often referred to as ‘catalepsy sensitization’ (Banasikowski and Beninger 2012a; Banasikowski and Beninger 2012b; Frank and Schmidt 2004; Klein and Schmidt 2003; Lanis and Schmidt 2001; Pezarro Schimmel et al. 2015; Riedinger et al. 2011; Schmidt et al. 1999). These studies have also suggested a link between haloperidol-induced catalepsy sensitization and extra-pyramidal side-effects in humans such as akinesia and rigidity. Haloperidol-induced catalepsy sensitization in adult rats has also been explained through the phenomenon of conditioning i.e. repeated pairing of haloperidol-induced decreased dopaminergic activity and bar testing environment (Klein and Schmidt 2003; Schmidt et al. 1999), or inverse incentive learning (Pezarro Schimmel et al. 2015). Within this framework, the repeated pairing of haloperidol-induced hypodopaminergia and catalepsy test environment in adult rats has been proposed to lead to

conditioning effects and catalepsy sensitization. This idea was also supported by lack of catalepsy sensitization in the rats from these prior studies that underwent the same number of both daily haloperidol injections and catalepsy tests but only when the haloperidol injection was given *after* catalepsy test i.e. no pairing of hypodopaminergia with context. Here, I have extended this literature by demonstrating that repeated treatment with risperidone, an atypical APD with high affinity for both D2 receptors and 5HT_{2A} receptors (Schotte et al. 1996), also induces a sensitization-like cataleptic response in adults but at a lower level in adolescents as chronic treatment progresses. The findings of lower catalepsy progression in adolescents during chronic treatment with haloperidol (Wiley and Evans 2008) and risperidone (Current study) indicate that the adolescent brain is rather different from the adult brain in short-term behavioural responses to the same APD regimen.

Consistent with a lower catalepsy sensitization in adolescents in this study, *locomotor* sensitization to repeated treatment with psychostimulants has also been reported to be lower in adolescents, compared to adults – this lower *locomotor* sensitization in adolescents has been reported with cocaine (Collins and Izenwasser 2002; Frantz et al. 2007), nicotine (Collins and Izenwasser 2004), Δ^9 -tetrahydrocannabinol (Δ^9 -THC), 3,4-methylenedioxymethamphetamine (MDMA) and ketamine (Wiley et al. 2008a) (but see (Schramm-Sapyta et al. 2009). What neural mechanism(s) mediate(s) lower sensitivity of adolescents to APD-induced catalepsy sensitization or psychostimulant-induced locomotor sensitization? Although still not thoroughly understood, some experimenters have implicated the role of neural changes in dopaminergic system in the striatum (Laviola et al. 2001) and dopaminergic and serotonergic systems in the striatum and the NAc (Collins and Izenwasser 2002). Here at least with risperidone-induced catalepsy sensitization, I speculated that neurotransmission changes in the striatum would mediate differential cataleptic responses in adolescents and adults (See below).

4.4.2. Striatal neurochemical changes in adolescents and adults following chronic risperidone treatment

The striatum is the major brain region associated with APD-induced catalepsy (Ossowska et al. 1990; Yoshida et al. 1994); Blockade of >80% striatal dopamine receptors in the striatum by high doses of APDs induces cataleptic responses (Natesan et al. 2007; 2008; Natesan et al. 2006a; Wadenberg et al. 2000; Wadenberg et al. 2001b). Additionally spike frequency of the medium spiny neurons in the striatum increases with the development of haloperidol-induced catalepsy sensitization (Frank and Schmidt 2004). The adolescent striatum however is still maturing (See Chapter 6 for detailed discussion). The adolescent striatum has reduced presynaptic dopamine availability (Matthews et al. 2013; Stamford 1989) and a higher number of dopamine receptors

which are subsequently pruned to lower levels in the adult (Tarazi and Baldessarini 2000; Tarazi et al. 1999; Teicher et al. 1995). Therefore, I hypothesized that the striatum would be the brain region associated with differential cataleptic responses in adolescents and adults.

Here, I showed that striatal levels of DOPAC and 3MT, metabolites of dopamine, were elevated selectively in risperidone-treated adolescents. More importantly, the levels of striatal dopaminergic metabolites (DOPAC and HVA) negatively correlated with the cataleptic responses at Day 17, which was the most proximal period to the time of neurochemical examination. Again this was selective to risperidone-treated adolescents. However, I did not observe any risperidone-induced alterations in the gene expression of dopamine-metabolizing enzymes, COMT and MAO-A.

What is the mechanistic link between risperidone-induced catalepsy and observed neurochemical changes in striatum? An increase in dopaminergic metabolites may reflect an increase in either turnover or availability of dopamine. Turnover of dopamine could be increased by elevated levels of dopamine-metabolizing enzymes (COMT, MAO-A and MAO-B) or their enzymatic activity. Given these enzymes were unaltered at least at the level of gene expression, the observed increase in dopaminergic metabolites would not suggest increased metabolism. Instead, increased dopamine metabolites could result from increased availability of dopamine itself in the system. This hypothesis was supported by a tight positive correlation between the levels of dopamine and those of DOPAC and HVA in risperidone-treated adolescents. Tentatively I suggest that risperidone-treated adolescent rats had an increase in dopamine availability, which consequently could overcome catalepsy-inducing effect of dopaminergic blockade by risperidone. However, I did not observe a significant elevation of dopamine in these rats. Still this finding does not exclude the possibility of increased dopamine availability during behaviour. Examination of dopamine release using a technique such as microdialysis would better address this issue.

Lower progression of risperidone-induced cataleptic responses in adolescents may also be contributed to by changes in other neurotransmitter systems such as 5HT. For instance, increasing 5HT activity is known to counteract catalepsy induced by haloperidol or other dopaminergic antagonists (Elliott et al. 1990; Hicks 1990; Wadenberg and Ahlenius 1995; Wadenberg and Hillegaart 1995; Wadenberg et al. 1999). The finding that adolescents had a higher level of 5HT and 5HIAA than adults suggests that this may be one reason why adolescents experience lower APD-induced catalepsy.

In addition to changes in dopaminergic and 5HT systems discussed above, risperidone-induced alterations in other neurotransmitter systems such GABA and glutamate may also play a role in determining cataleptic responses in adolescents. For example, it has been reported that

administration of NMDA or AMPA antagonists immediately after cataleptic tests can reduce haloperidol-induced catalepsy sensitization (Riedinger et al. 2011). Therefore, further investigations into changes in glutamatergic systems may also be warranted. Another outstanding question is related to a potential role of age-related pharmacokinetic factors in differential cataleptic responses. Examination of serum levels of risperidone and its metabolite 6-hydroxyrisperidone can also provide more information as to whether a comparable level of drug concentration is achieved in adolescents and adults. I also observed no change in dopamine receptor expression. A more sensitive technique such as radioligand binding which can also give a read-out of functional receptor activity may provide more information on risperidone-induced changes in dopamine receptors (Moran-Gates et al. 2007).

To sum up, here in Chapter 4, I showed that chronic risperidone induced a lower progression of catalepsy in adolescent rats, compared to adult rats. Accompanying this behavioural outcome, risperidone-treated adolescents had elevated levels of striatal dopamine metabolites, which correlated negatively with their cataleptic response at Day 17 of chronic treatment. I propose that increased availability of striatal dopamine as suggested by increased dopamine metabolite levels was a potential mechanism for lower cataleptic levels in adolescents. Together, the findings provide further support that the adolescent brain differs from the adult brain in short-term behavioural and neurochemical responses to chronic treatment with risperidone.

Chapter 5. A comparative examination of risperidone-induced changes in accumbal NAA levels and long-term behaviours in adolescents and adults

5.1. Introduction

N-acetylaspartate (NAA), a well-studied molecule in neuropsychiatric diseases (Moffett et al. 2007), is synthesized from acetyl coenzyme-A and aspartate by n-acetyltransferase 8 (NAT8L) or NAA synthase (Niwa et al. 2007), which is mainly located in mitochondria of neurons (Patel and Clark 1979). Therefore, NAA is mainly of neuronal origin and changes in NAA levels are thought to reflect neuronal function, viability or density (See review by (Rae 2014)). While NAA changes have been observed in different regions of schizophrenic patients (See systematic reviews and meta-analyses by (Kraguljac et al. 2012; Steen et al. 2005)), potential confounds of APD treatment on these observations are still in debate; some studies have reported an increase or correction of NAA levels with APD treatment (Bertolino et al. 2001; Fannon et al. 2003; Szulc et al. 2005; Szulc et al. 2013); other studies have observed no significant changes with APDs (Bustillo et al. 2008; Bustillo et al. 2010). Examination of NAA levels with APD treatment in ‘normal’ rodents without psychopathological effects can help address this issue.

NAA findings from preclinical APD studies in *adult* rats have also been inconsistent with studies reporting increases (Harte et al. 2005; McLoughlin et al. 2009) or no change (Bustillo et al. 2006; Bustillo et al. 2004; Lindquist et al. 2011) in different brain regions following chronic APD treatment. On-drug changes in NAA levels, that is, changes after an injection of APD and time course profiles of these changes, have not been investigated in an existing study. My preliminary data from a pilot experiment (Appendix C) suggested that acute administration of 1.3 mg/kg risperidone could induce differential changes in NAA levels in the NAc of adolescents and adults; accumbal NAA levels were observed to increase only in adolescents approximately 20-25 min after risperidone administration. This preliminary finding raises the following questions: (1) Does risperidone treatment induce an increase in NAA in the NAc selectively in the adolescents? (2) How does the NAA level in the NAc change from acute (Day 1) to chronic (Day 22) risperidone treatment in adolescents or adults? Thus, to answer these questions in this Chapter, I utilized a translationally relevant technique named proton magnetic resonance spectroscopy (^1H MRS), which enables *in vivo* measurement of brain metabolites (Agarwal and Renshaw 2012; Michaelis et al. 2009). On ^1H MRS spectrum, NAA is detected as a peak at 2.008 ppm (See review (Rae 2014)). In this Chapter, I aimed to address limitations in the pilot ^1H MRS experiment by using a larger sample size and a more optimized ^1H MRS acquisition technique. In addition to NAA, I also aimed to investigate other detectable metabolites such as glutamate, glutamine and GABA given their well-known roles in both metabolism and neurotransmission (Rae 2014).

In Chapter 4, adolescents were observed to develop lower levels of catalepsy compared to adults, *during* chronic treatment with risperidone. This finding raises another question as to whether the

observed differential short-term cataleptic responses in adolescents and adults can persist after a drug-free interval. Given that long-term cataleptic response induced by adolescent risperidone treatment has not been investigated thoroughly in the literature, one aim of Chapter 5 was to investigate the nature of cataleptic outcome induced by risperidone treatment in adolescence or adulthood.

As introduced in Chapter 1 and 3, conditioned avoidance response (CAR) is the behavioural test widely used in screening of novel compounds with APD potential (Wadenberg 2010; Wadenberg and Hicks 1999). In addition, the ability to *acquire* the CAR is frequently investigated in studies of fear and anxiety (for example, see (Choi et al. 2010a; Lázaro-Muñoz et al. 2010)). In Chapter 3, all rats from adolescent and adult cohorts received CAR training up to a criterion of $\geq 70\%$ avoidance during early adolescence (PND30-PND34). Given that the CAR is resistant to extinction (See reviews by (Kapur et al. 2006; Moutoussis et al. 2007)), these rats *re-acquired* the CAR in adulthood (PND117) rapidly with only one session of retraining (See Chapter 3, Section 3.3.2.2.2.). However, it is still unknown whether learning of the CAR at adulthood can be altered by prior adolescent risperidone treatment in *previously untrained* rats. In a recent preclinical study, adolescent olanzapine treatment was shown to *decrease* the rate of learning to the criterion in a delayed non-match to sample task and *increase* context-dependent freezing in adulthood (Milstein et al. 2013). While this data suggests a possible long-term deficit in learning a particular task, it was still unanswered whether this deficit was selective to adolescent treatment given a lack of comparison age group. In addition, the existing preclinical studies in rodents have investigated *acquisition* of the CAR in adults only *during* chronic treatment with risperidone (Castellano et al. 2009; Drago et al. 1997). No study to date has examined the long-term effect of adolescent risperidone treatment on *first-time* CAR learning. Therefore, in the current chapter, I aimed to address the question on the impact of adolescent risperidone treatment on CAR learning at maturity.

Another outstanding question in Chapter 3 is related to the role of drug washout period in determining long-term behavioural and neurochemical outcomes. Different drug washout periods in adolescent and adult cohorts (60 vs 14 days respectively) in Chapter 3 could possibly have contributed to the differential behavioural and neurochemical outcomes observed at the time of assessment. Indeed, the existing literature has suggested that the interval between termination of drug treatment and behavioural and/or neurochemical assessment influences outcome measures [See review by (Spear 2007)]. Therefore, in this Chapter, I aimed to investigate whether any differential long-term behavioural/neurochemical outcomes could still be induced in adulthood following an equivalent drug-free interval from chronic risperidone treatment either as adolescents or adults.

To summarise, the aims of the experiment in Chapter 5 are as follows:

- (1) To examine whether chronic risperidone treatment induces differential changes in NAA levels of the NAc in adolescents and adults;
- (2) To examine whether differential cataleptic responses seen in adolescents and adults during chronic treatment (Chapter 4) persist after a drug-free interval;
- (3) To examine whether prior chronic risperidone treatment affects *CAR acquisition* at adulthood in animals who have not previously been trained;
- (4) To examine whether the drug-free interval is a factor in sensitized *CAR suppression* in rats with adolescent risperidone exposure (Chapter 3);
- (5) To examine long-term neurochemical changes in the striatum and the NAc after a drug-free interval from chronic risperidone treatment.

5.2. Materials and methods

5.2.1. Subjects

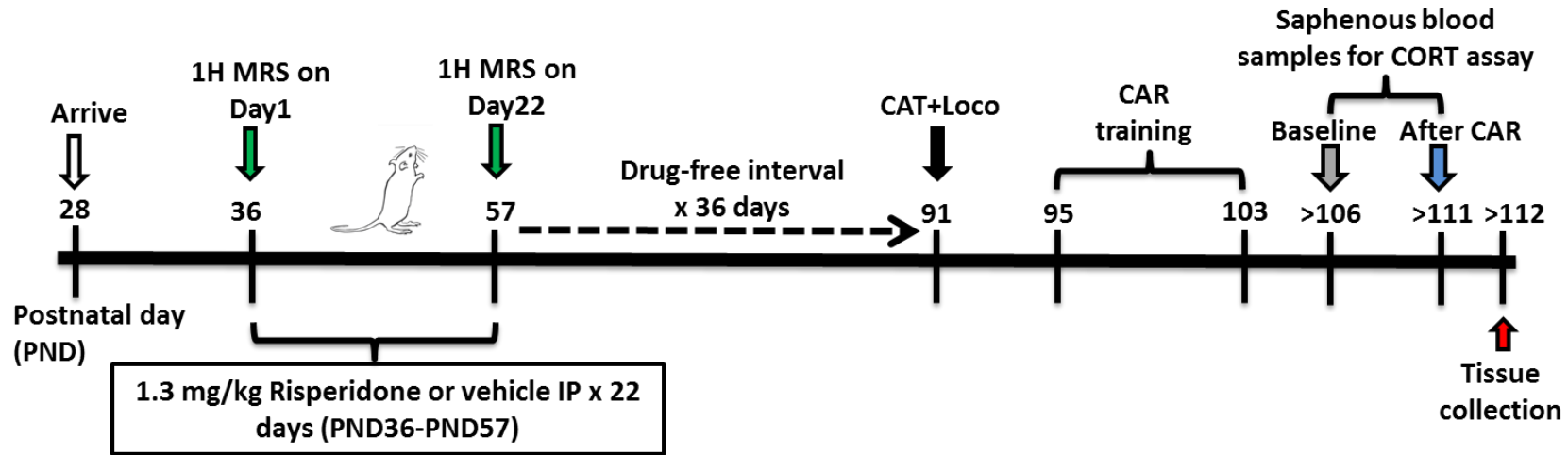
Male SD rats arrived at the animal facility either on PND 28 (n= 24 for adolescent cohort) or PND 70 (for adult cohort, n = 24) (n = 12 per risperidone/vehicle treatment for a given age). Rats from the same drug and age groups were pair-housed in Macrolon cages with Sani chip bedding (Able Scientific) and wire lids in a temperature (21 ± 1 °C) and lighting (lights on at 6:00 h and off at 18:00 h controlled room. All rats were given ad libitum access to food and water throughout the whole experiment. Imaging and behavioural tests were conducted during the light phase of the diurnal cycle.

5.2.2. Experimental design

The timeline of the experiment is shown in Figure 5-1. Rats received once-daily IP injections of 1.3 mg/kg risperidone or vehicle for 22 days either during adolescence (PND36-PND57) or adulthood (PND80-PND100). This is the same risperidone treatment protocol as used in all previous experiments in Chapter 3 and Chapter 4. At Day 1 and Day 22 of chronic treatment, ¹H MRS scans were performed on all rats at both baseline (prior to drug administration) and after administration of risperidone or vehicle. After an equivalent drug-free interval of 36 days (PND91 and PND135 respectively for adolescent and adult cohorts), a challenge dose of 1.3 mg/kg risperidone was administered to all rats (both risperidone and vehicle-treated) from both age groups and their long-term cataleptic responses were examined. Four days after the catalepsy test (PND95 or PND139

respectively for adolescent and adult cohorts), all rats were examined for their ability to *acquire* the CAR over 7 days of training. At least 2 days after CAR training, saphenous blood samples (~100-150 μ l) were obtained from all rats at baseline conditions (under mild restraint only). After an interval of four days, rats were given a CAR challenge session and saphenous blood samples were obtained 5-10 min after the end of this CAR session. One day after blood sample collection with CAR challenge, all rats were culled and their brain tissues collected. One adult control rat died suddenly while resting during the drug-free interval, thus giving $n = 11$ for this particular group.

(a)



(b)

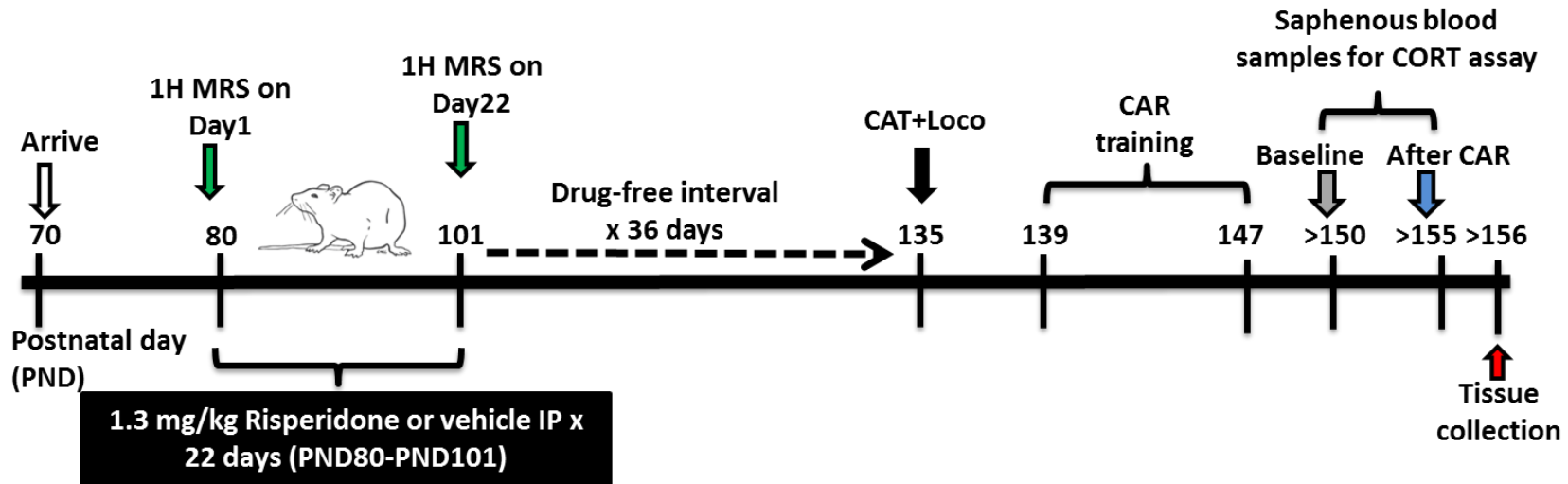


Figure 5-1 Timeline of the final experiment Rats were treated for 22 days with either risperidone or vehicle during (a) adolescence (from PND36 to PND57) or (b) adulthood (from PND80 to PND101). At Day 1 and Day 22 of chronic treatment, ¹H MRS scans were performed at baseline and after administration of 1.3 mg/kg risperidone or vehicle. After a 36-day drug washout period, long-term behavioural outcomes (catalepsy, locomotion and CAR) were assessed along with examination of plasma corticosterone levels and neurochemical assays of regional brain tissues. CAR – conditioned avoidance response; CAT+Loco – horizontal bar test for catalepsy + open field test for locomotor activity; CORT – plasma corticosterone;

5.2.2.1 ¹H MRS scans with risperidone challenge

At Day 1 and Day 22 of chronic risperidone treatment, ¹H MRS spectra were obtained from each rat at both baseline (prior to drug administration) and after administration of 1.3 mg/kg risperidone or vehicle remotely through an IP cannula and attached tubing.

After induction of anaesthesia with 5% isoflurane, each rat received an IP catheterization using 18G cannula and PE50 tubing (Fisher Scientific). After mounting of the rat on the animal bed of 9.4T MRI scanner, anaesthesia was maintained at 1.5-1.7% isoflurane and O₂ flow rate of 1.2 L/min. Axial and sagittal anatomical scans were obtained for localization of the voxel bilaterally over the NAc (6 x 2 x 2 mm³). A smaller voxel size (5.5 x 1.8 x 2 mm³) was used at Day 1 scans of adolescent cohorts to accommodate a smaller NAc size. After minimizing magnetic field inhomogeneity with B₀ map acquisition, first, second and third order shimming was carried out with MAPSHIM. Following acquisition of a reference non-suppressed water spectrum, a baseline water-suppressed ¹H MRS spectrum was obtained from the voxel placed bilaterally on the NAc using PRESS sequence (TE = 9.9 ms; TR = 2500 ms; averages = 356; repetition = 1, time taken = ~ 14.5 min).

Immediately after the baseline scan, fifteen time course ¹H MRS spectra were obtained from the same voxel with either risperidone or vehicle challenge using the same PRESS sequence with the following modifications: averages = 96 (cf. 356 in the baseline scan), time taken for each scan = ~ 4 min (cf. 14.5 min in the baseline scan) and automated local frequency adjustment in between 15 scans. As shown in Figure 5-2, a saline injection was remotely administered IP immediately after completion of the first time course ¹H MRS scan to assess the effect of injection on neuronal metabolism. Next, an injection of risperidone or vehicle was given after the fourth scan and changes in accumbal metabolites were examined for the next 11 scans i.e. approximately for another 44 min.

All ¹H MRS data were processed at TOPSPIN and analysed in LCModel (version 6.3-1J) software (Provencher 1993), using the reference basis sets with the same data acquisition parameter. Metabolites with Cramer-Rao Lower bound (CRLB) or %SD > 20 were rejected from the analysis unless otherwise stated. The concentration of individual metabolites was expressed as a ratio to total creatine (Cr + PCr) following the guidelines in the LCModel manual.

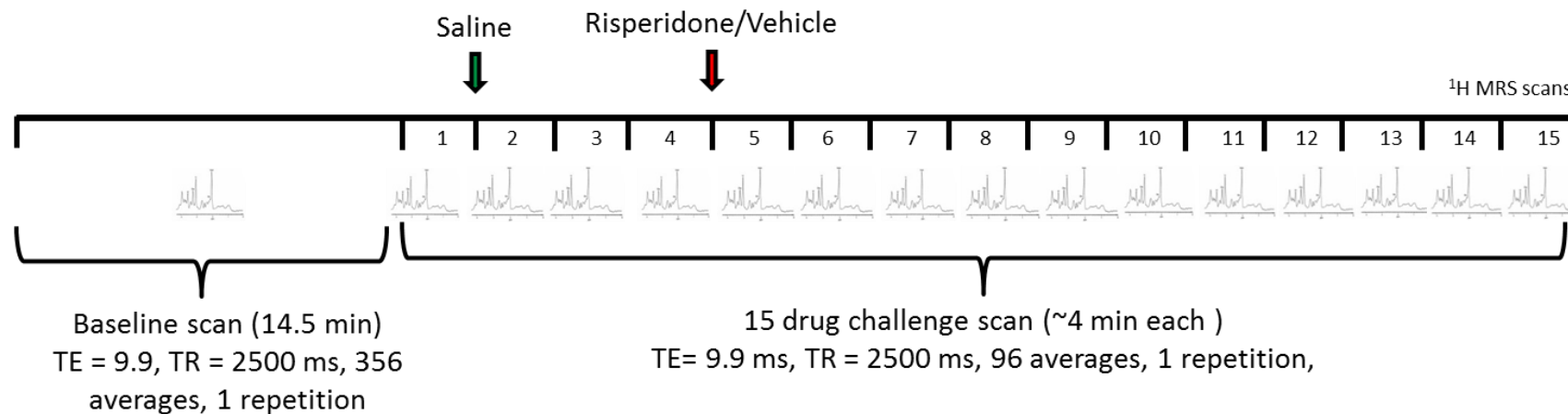


Figure 5-2 Timeline of ^1H MRS data acquisition during chronic risperidone treatment in adolescence or adulthood. At Day 1 and Day 22 of chronic treatment, ^1H MRS spectra were obtained from the voxel bilaterally placed over the nucleus accumbens of vehicle- or risperidone-treated rats from adolescent or adult cohorts. First, a baseline scan was performed to obtain the levels of neural metabolites before drug administration. Next 15 ^1H MRS spectra were obtained, with saline injection immediately after completion of the first scan (Green arrow) and risperidone or vehicle injection after the fourth (Red arrow).

5.2.2.2 Bar test for catalepsy

After a lengthy drug-free interval of 36 days (PND91 for adolescent treatment group and PND135 for adult treatment group), the cataleptic response to a challenge dose of 1.3 mg/kg risperidone (IP) was examined in *both* risperidone- and vehicle-treated rats from both age groups. At 1 h after injection, their cataleptic response was examined with the horizontal bar test (for details, see Chapter 2, Section 2.4). Both forepaws of the rats were placed on the bar and the time rats stayed with both forepaws on the bar was video-recorded. Each rat was examined for a maximum duration of 180 s or a maximum of 12 trials (12 times of placing the forepaws on the bar). Videos were coded, that is, analyst was blind to treatment and analysed in Media Player Classic Home video viewer. The average duration an animal spent with both forepaws on the bar out of the total number of trials in each test was calculated for each animal.

5.2.2.3 Open field test for locomotor activity

Immediately after the catalepsy test i.e. approximately 65 min after injection, long-term locomotor response to the same challenge with risperidone was examined in four black Plexiglas locomotor activity chambers (for details, see Chapter 2, Section 2.5). Rats were individually placed in the locomotor chambers and locomotor activity was examined for a total of 30 min in terms of total distance travelled (cm).

5.2.2.4 CAR training

After a 4-day drug washout period after the catalepsy test, all rats were trained for the CAR with 40 continuous trials per session per day for 7 days. At each training session, after 10-min habituation to the CAR boxes, rats received 40 trials of CS (80 dB white noise) for 10 s, followed by pairing with US (0.6 mA foot-shock) as described previously in Chapter 2 and 3. As before, avoidance (movement of the rat into the other chamber during 10 s CS-only presentation), escape (crossing during the next 10 s of presentation of the CS paired with the US) and escape failure (failure to make a crossing during the entire 20 s period) were recorded. The inter-trial interval varied randomly from 20 to 40 seconds (Natesan et al. 2006b; Wadenberg et al. 2001b). Avoidance, escape and escape failures were calculated as the percentage out of the total trials in the session. Motor activity was recorded as the number of chamber crossings.

5.2.2.5 Plasma corticosterone assay

At a minimum of 2-day interval from the CAR training, two saphenous blood samples were collected from each rat using 23 G needles: one at baseline (under mild restraint) and another after a challenge CAR session. There was an interval of 4 days in between two samples. Plasma samples were stored at -20°C until use. Plasma corticosterone levels were determined by an in-house liquid

chromatography tandem mass spectroscopy (LC-MS/MS). The system consisted of a Shimadzu Nexera[®] UPLC system with a Phenomenex Kinetex[®] 1.7u XB-C₁₈ 100Å (50x2.1mm) column attached to an ABSciex QTrap-5500[®] triple-quadrupole mass spectrometer. Briefly, in 96-well plates, 20 µl of plasma samples and standards were mixed with 10 µl each of internal standards (500 nM corticosterone-[²H₄] in 1:1 acetonitrile : water) and 10 µl of 1M ZnSO₄ and 600 µl of extraction solvents (9:1 ethyl-acetate : acetonitrile). After centrifugation at 500 rpm for 20 min, 500 µl each of sample mixture was transferred to another clean 96-well plate, evaporated to dryness at 55°C for 20 min in the vacuum concentrator and reconstituted in 50 µl of 1:1 methanol : water. 20 µl sample extract was then injected in a 384-well plate and assayed overnight. A gradient elution method at 0.5mL/min was used with the mobile phases A= 0.1% aqueous formic acid and B= 0.1% formic acid in 9:1 acetonitrile : water. The mixture was increased from 50%B to 95%B over 2 min, held at 95%B for 0.5 min and then returned to 50%B for 1 min. This resulted in a retention time of 1.2 min. The mass-spectrometer detection was by way of positive-mode, scheduled multiple reaction monitoring with electrospray ionisation. The mass spectrometer parameters were as follows: for corticosterone: m/z= 247.1 → 329.1, declustering potential (DP) =100V, exit potential (CXP) =12, collision energy (CE) = 23V; for corticosterone-[²H₄]: m/z= 351.1 → 333.0, DP=100, CXP=15, CE=23. Calibration standards over the range 1000 – 10nM and quality controls at three levels were prepared in stripped plasma. Differential quality control samples were prepared by spiking rat serum with 75nM of corticosterone. A ±15% acceptance criterion was applied to all quality controls.

5.2.2.6 HPLC measurements of monoamines and metabolites

At 24 h after saphenous blood sample collection with CAR challenge session, all rats were euthanised and their striatal and the NAc tissues (Paxinos and Watson 2005) collected and stored at -80 °C until use. Briefly, striatal tissues (right hemisphere) and the NAc (bilateral) were quickly weighed (wet weight) and homogenised on ice in a minimum volume of 0.1 ml of 0.1M perchloric acid and 50 mg/ml deoxyepinephrine (internal standard for catecholamines), using ultrasonicator probe (Vibra-Cell, Sonics & Materials, Inc. CT.). After centrifugation at 13,000 rpm for 5 minutes, supernatant from each sample was collected and filtered through 0.2 µm nylon filter. Next, 10 µl of each sample was injected into a HPLC system (See details in Chapter 2) and dopamine, serotonin (5HT), noradrenaline and their metabolites (dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3-MT) and 5-hydroxyindoleacetic acid (5-HIAA)) and measured. Data were processed offline with Chemstation software (Rev B.01.03, Agilent Technologies, Inc. CA). The amount of catecholamines and their metabolites were expressed as pg/mg wet tissue, after correction for the dilution.

5.2.3. Statistical analysis

All data are expressed as mean \pm SEM unless otherwise stated. ^1H MRS, CAR training and plasma corticosterone data were analysed with repeated measures two-way (Age x Drug) ANOVA. Data from catalepsy and locomotor tests, plasma corticosterone assays (baseline and CAR challenge as separate analyses) and HPLC assays were analysed with two-way ANOVA followed by Tukey's post hoc tests. For CAR training data, survival curve analysis was performed using Kaplan-Meier technique and log-rank (Mantel-Cox) test as described previously (Kesby et al. 2015). Pearson's Chi-Square analyses were performed to compare the failure rates of CAR learning in different treatment groups. Correlation between behavioural and neurochemical data was examined using Pearson's correlation analysis. Statistical significance was defined at $p < 0.05$.

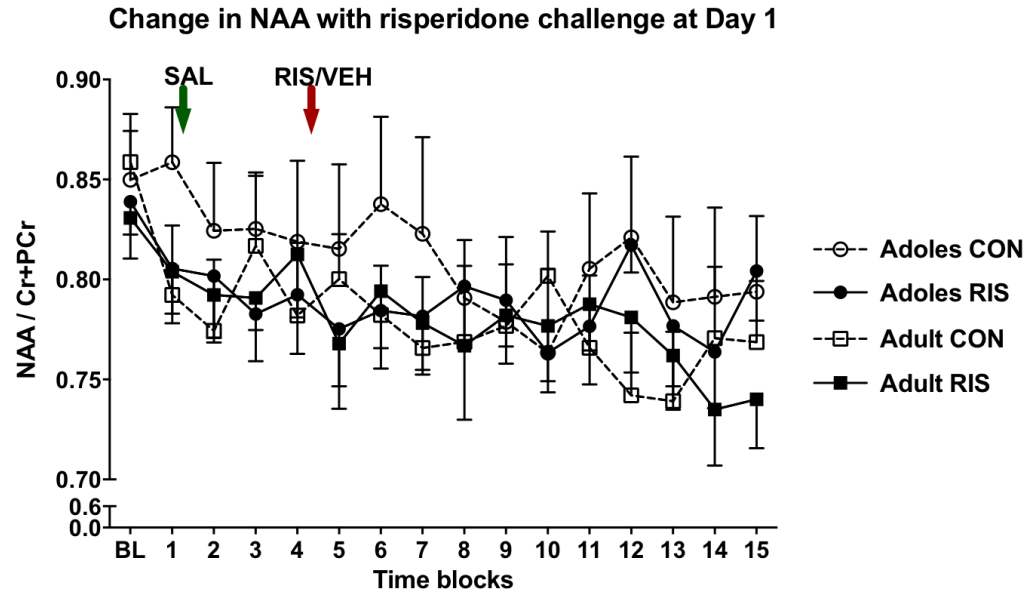
5.3. Results

5.3.1. The effect of chronic risperidone on NAA and other metabolites in the NAc

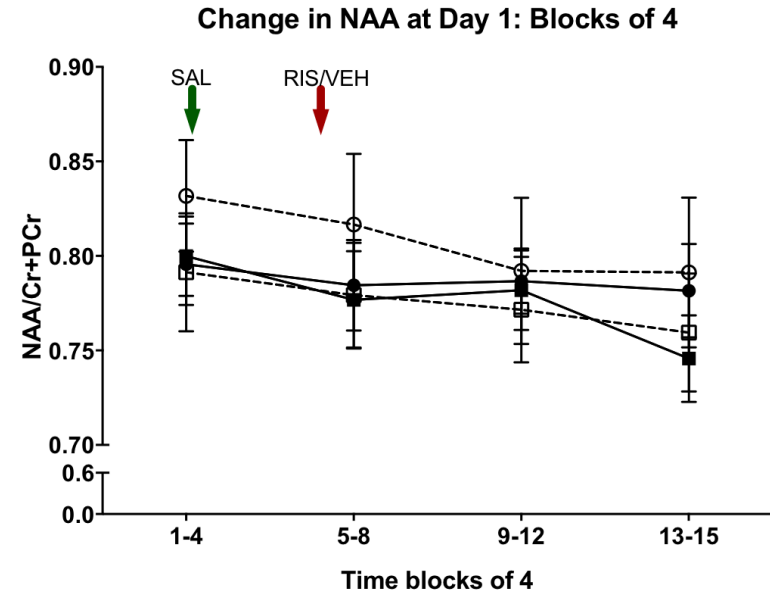
Accumbal NAA could be reliably measured from all rats with ^1H MRS (mean \pm SEM of NAA CRLB – 7.48 ± 0.05). Other metabolites such as glutamate, glutamine, glutathione (GSH), taurine and GABA could also be reliably measured with CRLB of 7.25 ± 0.04 , 12.61 ± 0.07 , 15.28 ± 0.08 , 8.90 ± 0.06 and 18.16 ± 0.11 respectively in the brains of these rats using ^1H MRS.

At both Day 1 and Day 22, chronic risperidone treatment did not appear to alter the levels of NAA or other metabolites in the NAc in both adolescent and adult cohorts. Levels of almost all the detectable metabolites decreased over the course of the study (significant main effect of Time ($p < 0.001$) on repeated measures two-way ANOVA; no significant main effect of Time, Time x Drug, Time x Drug x Age or Age x Drug interaction, all $F < 3$, $p > 0.05$; Details of statistical analysis results reported in two tables in Appendix D). Changes in NAA and glutamate levels with risperidone or vehicle challenge at Day 1 and Day 22 of chronic treatment in adolescence or adulthood are shown in Figure 5-3 and 5-4 respectively. Other metabolites are reported in the two tables in Appendix D. Varying the analysis by examining (1) an average metabolite level from blocks of 4 (time 1-4, 5-8, 9-12 and 13-15) to increase the statistical power, (2) % change from levels at baseline or (3) % change from levels at time 1 or average levels before vehicle or risperidone administration (average of time blocks 1-4) did not show any significant change in the levels of all detectable metabolites in the NAc. Examination of baseline levels of metabolites only also did not reveal any significant difference between risperidone-treated and control rats in both age groups (all $F < 3$, $p > 0.05$). Therefore NAA and other metabolites that were measurable with ^1H MRS were not altered in the NAc by either risperidone treatment regardless of the age of animal.

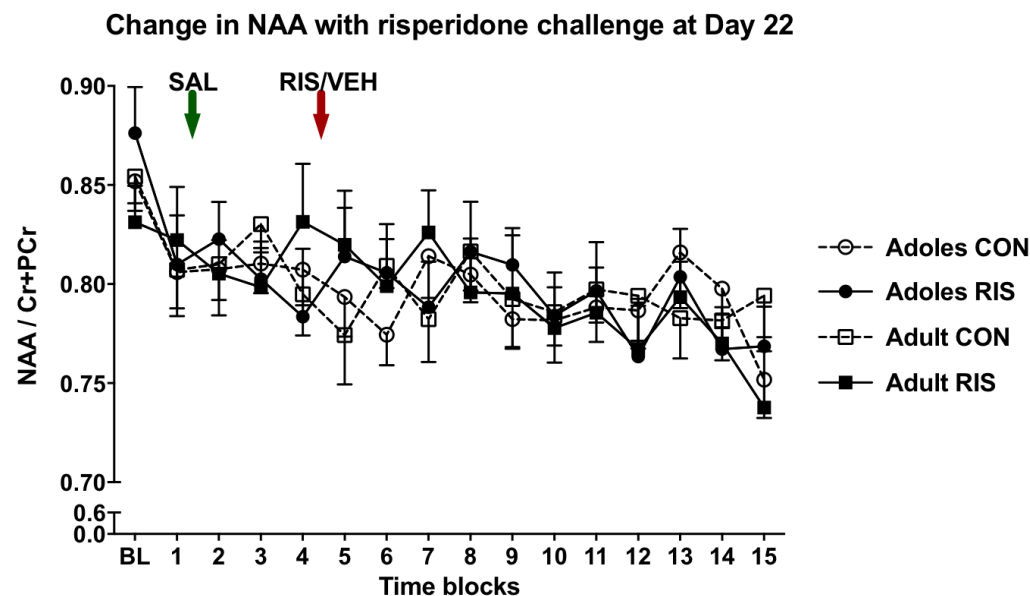
(a) (i)



(a) (ii)



(b) (i)



(b) (ii)

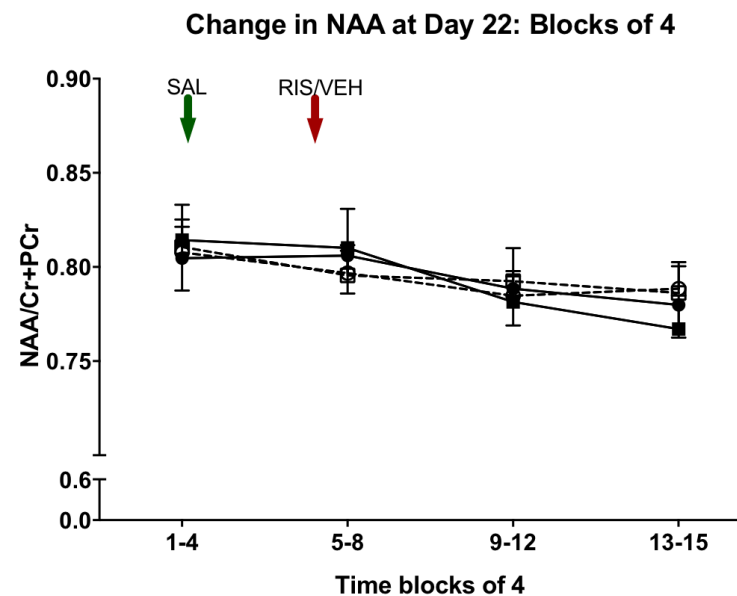
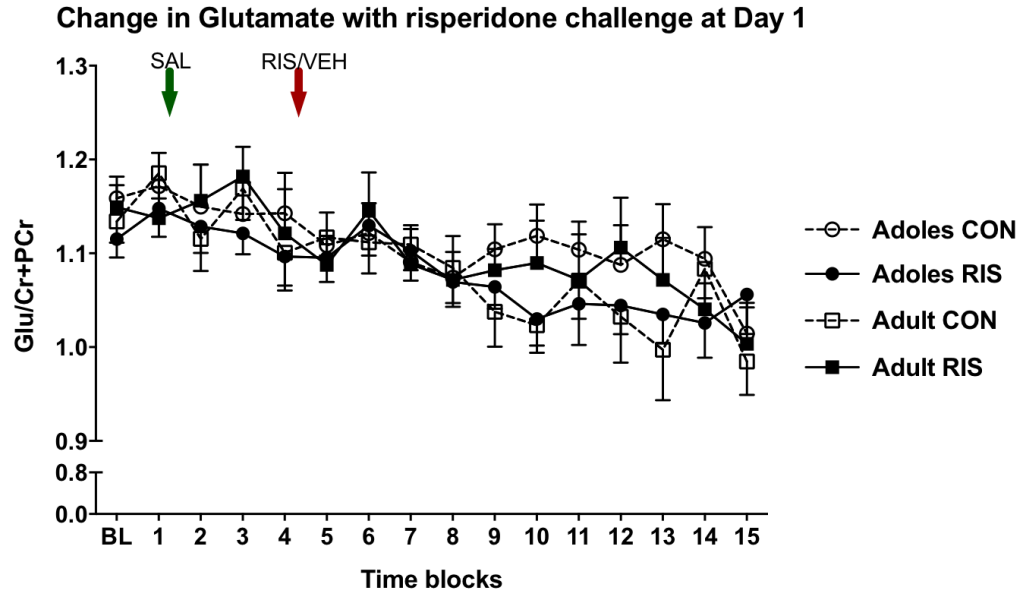
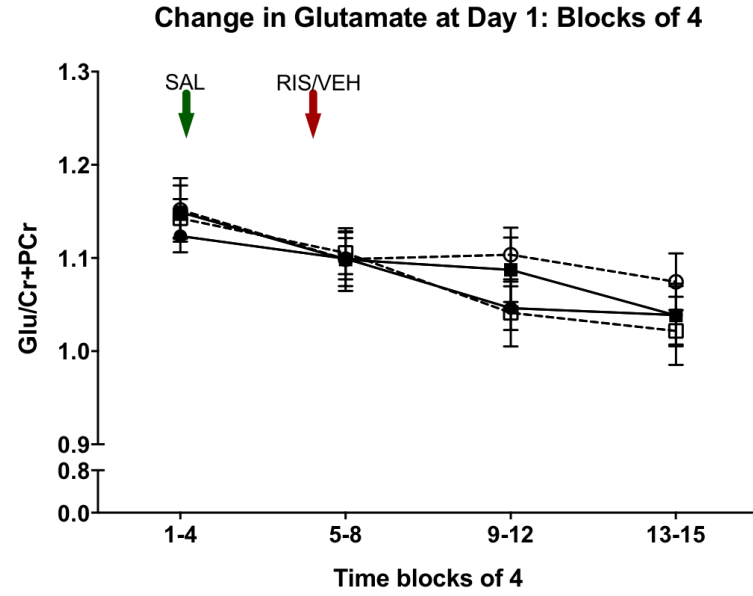


Figure 5-3 Levels of *n*-acetylaspartate (NAA) in the NAc with risperidone treatment in adolescence or adulthood. Changes in NAA in the NAc (at baseline and after risperidone or administration) at (a)(i) Day 1 and (b)(i) Day 22 of chronic treatment are shown. [Analysis as time blocks of 4 is shown in subfigure (a)(ii) and (b)(ii)]. No significant change in NAA was observed at either day in both adolescent and adult cohorts. Green arrow shows the time of saline injection and red arrow risperidone or vehicle injection. Data are expressed as mean \pm SEM. $n = 12$ per drug for a given age; Adoles – adolescent; BL – baseline; CON – vehicle-treated control; RIS – risperidone-treated; VEH – vehicle injection.

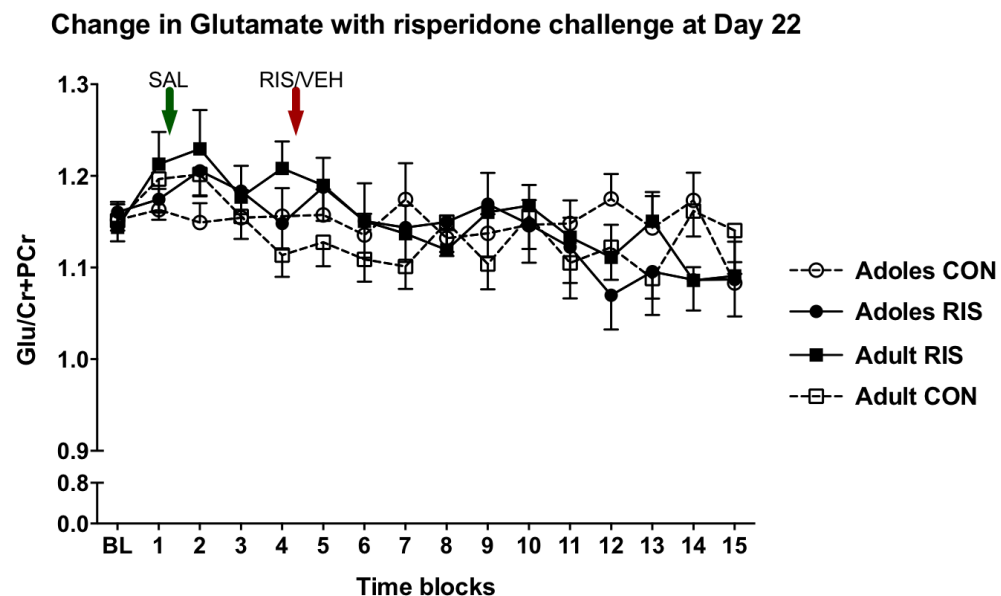
(a)(i)



(a)(ii)



(b)(i)



(b) (ii)

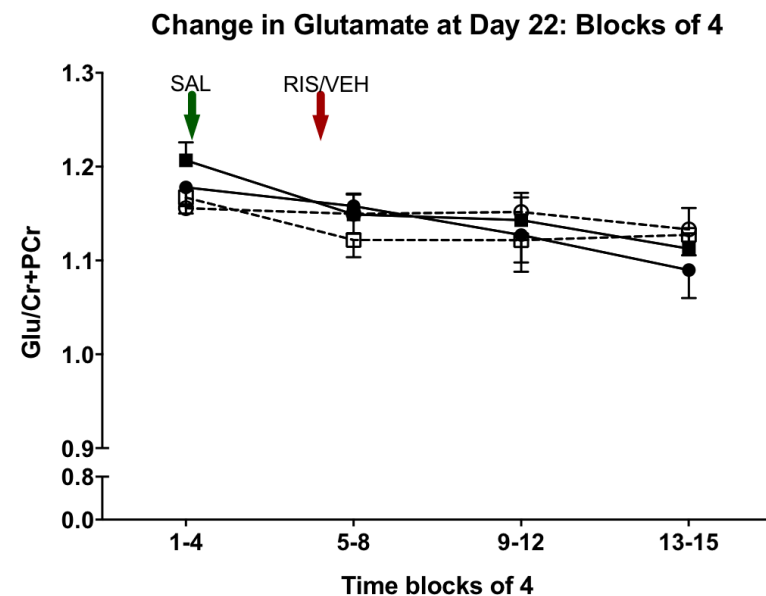


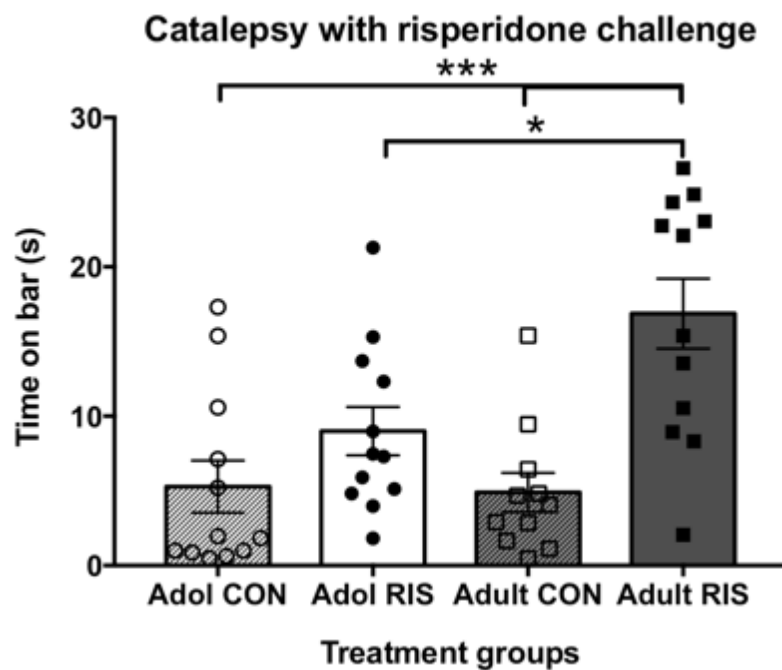
Figure 5-4 Levels of glutamate (Glu) in the NAc with risperidone treatment in adolescence or adulthood. Changes in glutamate in the NAc (at baseline and after risperidone or administration) at (a)(i) Day 1 and (b)(i) Day 22 of chronic are shown. [Analysis as time blocks of 4 is shown in subfigure (a)(ii) and (b)(ii)]. No significant change in glutamate was observed at either day in both adolescent and adult cohorts. Green arrow shows the time of saline injection and red arrow risperidone or vehicle injection. Data are expressed as mean \pm SEM. $n = 12$ per drug for a given age; Adoles – adolescent; BL – baseline; CON – vehicle-treated control; RIS – risperidone-treated; VEH – vehicle injection

5.3.2. Long-term cataleptic response and locomotor activity after a drug-free interval

After a drug-free interval of 36 days, all rats from both age groups received a challenge dose of 1.3 mg/kg risperidone IP and long-term cataleptic response was examined. This dose of risperidone produced a small degree of catalepsy in all animals, with all groups showing an average time-on bar of at least 4-5 s. As shown in Figure 5-5(a), this challenge dose of risperidone induced a significant increase in level of cataleptic response in rats previously treated with this APD in adulthood [two-way ANOVA: significant main effect of Age ($F_{1,43} = 4.254$, $p = 0.045$), Drug ($F_{1,43} = 18.733$, $p < 0.001$), Age x Drug interaction ($F_{1,43} = 5.186$, $p = 0.028$)]. Post hoc tests further confirmed that adult risperidone group stayed longer on the bar than both their vehicle-treated control group and adolescent risperidone- and vehicle-treated groups (all $p < 0.05$). This cataleptic outcome after a lengthy drug-free interval is consistent with the finding *during* chronic treatment in Chapter 4: in that rats treated with risperidone in adolescence, had lower cataleptic response than those treated in adulthood.

Locomotor activity immediately after the catalepsy test is shown in Figure 5-5(b). Two-way ANOVA showed a significant main effect of Age x Drug interaction ($F_{1,43} = 5.509$, $p = 0.024$) without any significant main effect of Age or Drug (both $F < 0.6$, $p > 0.4$). However, post hoc tests did not show any significant difference among the four treatment groups. Therefore, it was concluded that long-term locomotor response to risperidone was not altered by prior treatment in adolescence or adulthood.

(a)



(b)

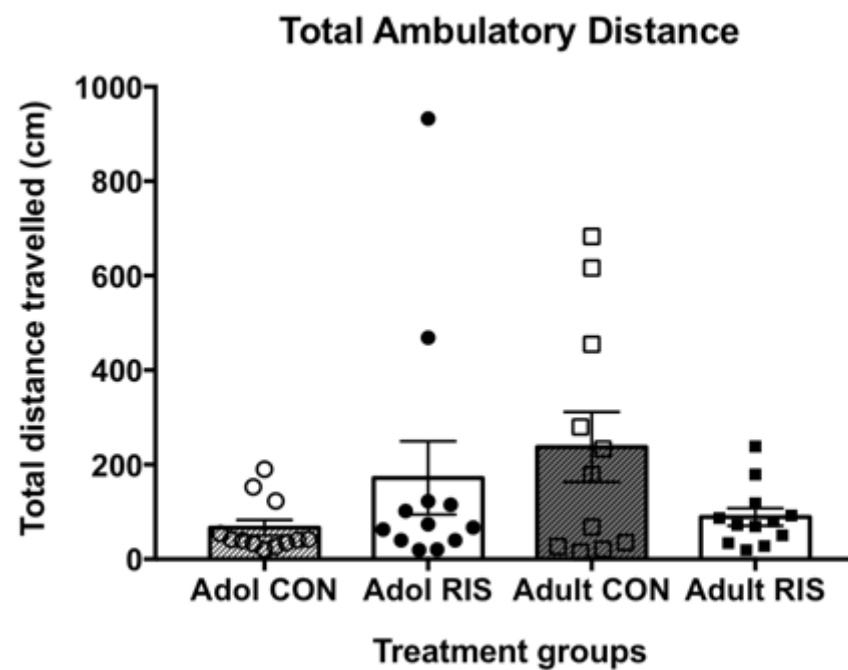


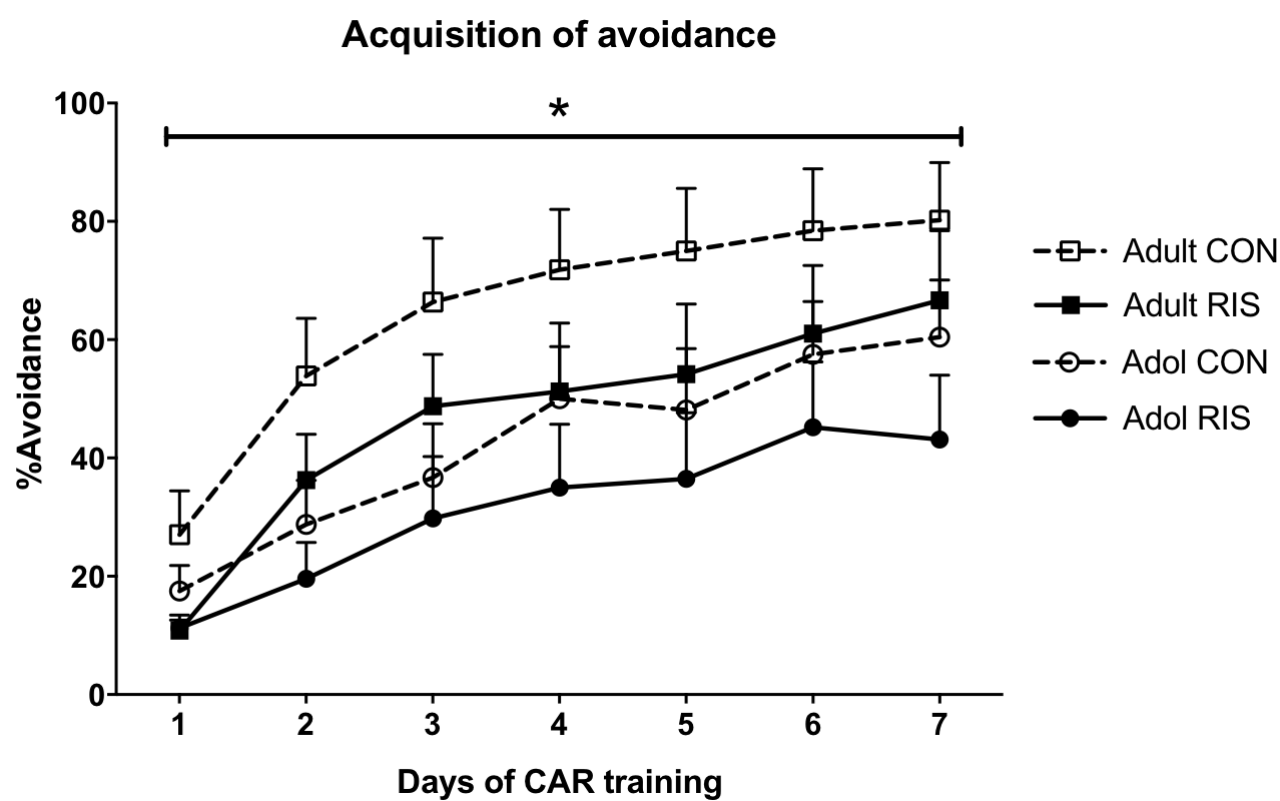
Figure 5-5 Long-term cataleptic response and locomotor suppression induced by a challenge dose of risperidone. (a) Rats treated previously in adulthood showed a significant increase in cataleptic response. Risperidone-induced catalepsy was determined as the mean time (s) the rat stayed with both forepaws on the bar. (b) Long-term locomotor response to a challenge dose of risperidone in rats previously treated in adolescence or adulthood is shown as total distance travelled during 30 min of open field test. Data expressed as mean \pm SEM. $n = 12$ per drug for a given age except $n = 11$ for adult CON; Adol – adolescent; CON – vehicle –treated controls; RIS – risperidone-treated rats; * $p < 0.05$, *** $p < 0.001$

5.3.3. CAR acquisition after a lengthy drug-free interval

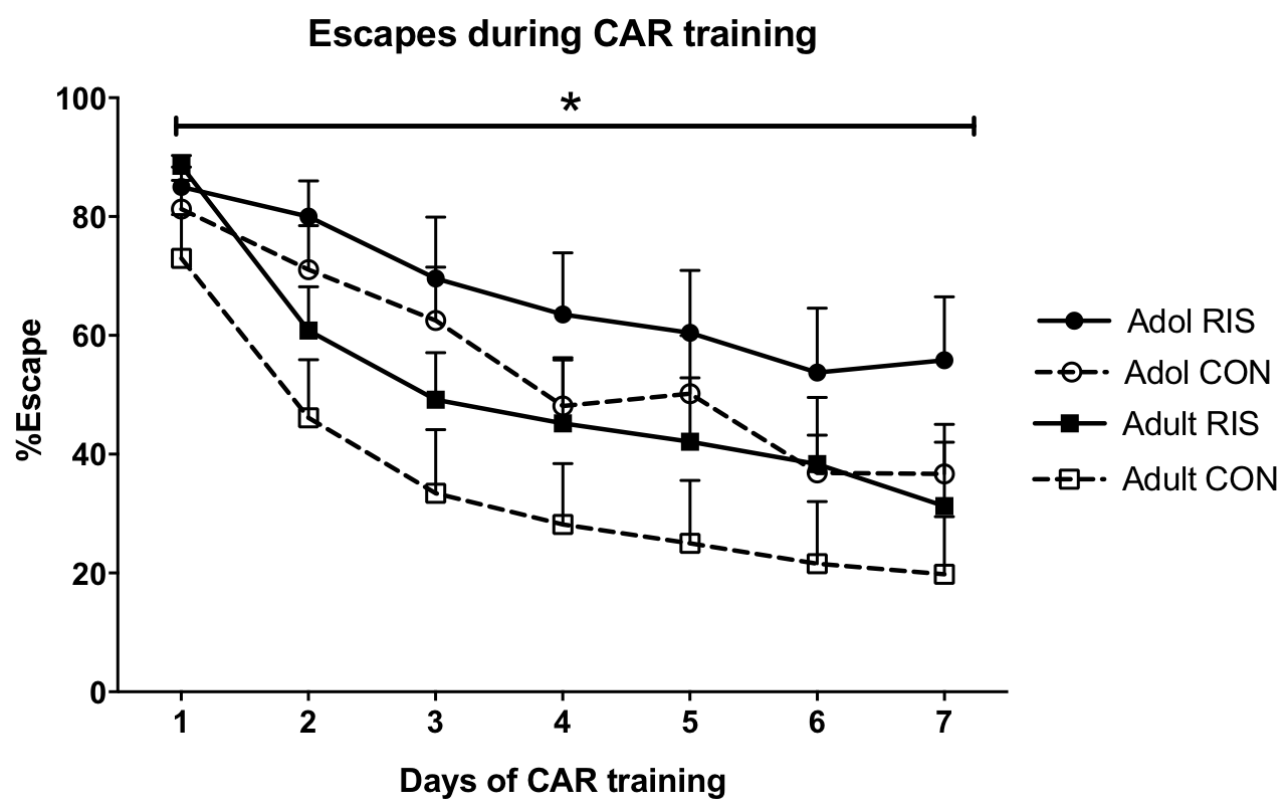
Four days after the catalepsy test, all rats were trained for CAR. The overall avoidance performance of individual groups over 7 days of CAR training is shown in Figure 5-6(a). As expected, the avoidance performance of the majority of rats improved as the training progressed. Notably, the adolescent cohorts showed a slower rate of CAR learning than adult cohorts. This apparent difference seemed to be driven by the adolescent risperidone group that showed the slowest rate of CAR acquisition [repeated measures two-way ANOVA: significant main effects of Day ($F_{6,258} = 47.059, p < 0.001$) and Age ($F_{1,43} = 4.955, p = 0.031$) but no significant main effect of Drug, Age x Drug, Day x Age or Day x Age x Drug (all $F < 3$, all $p > 0.05$)].

Escapes and chamber crossings are shown in Figure 5-6 (b) and (c) respectively. As expected from the avoidance data, adolescent risperidone group showed the highest levels of escapes among the four groups [repeated measures two-way ANOVA: significant main effect of Day ($F_{6,258} = 49.196, p < 0.001$) and Age ($F_{1,43} = 5.134, p = 0.029$), Day x Age ($F_{6,258} = 2.564, p = 0.020$) but no significant main effect of Drug, Age x Drug, or Day x Age x Drug (all $F < 3$, all $p > 0.05$)]. As the avoidance levels improved across training days, chamber crossings also became higher; again adolescent cohorts had lower chamber crossings than adult cohorts [repeated measures two-way ANOVA: significant main effects of Day ($F_{6,258} = 8.985, p < 0.001$) and Age ($F_{1,43} = 4.4482, p = 0.040$) but no significant main effect of Drug, Age x Drug, Day x Age or Day x Age x Drug (all $F < 1.5$, all $p > 0.05$)]. However, none of the four groups showed a significant level of escape failures (Table 5-1).

(a)



(b)



(c)

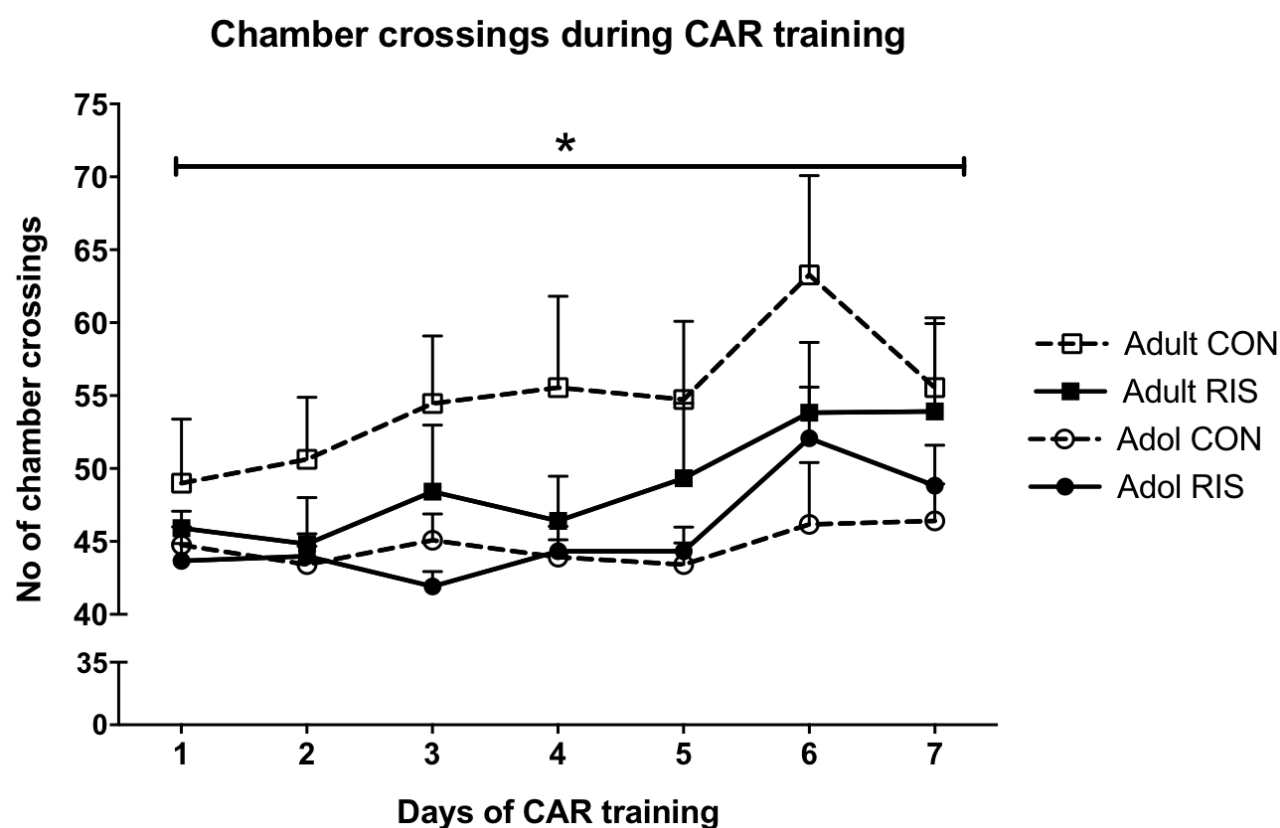


Figure 5-6 Acquisition of the CAR in rats treated with risperidone or vehicle in adolescence or adulthood. (a) % avoidance (b) %escape and (c) number of chamber crossings are shown for rats treated with risperidone or vehicle in adolescence or adulthood. All data expressed as mean \pm SEM. $n = 12$ per drug for a given age except $n = 11$ for adult CON; * $p < 0.05$ Adol cohorts VS adult cohorts. Adol – adolescent; CAR – conditioned avoidance response; CON – control; RIS – risperidone

Table 5-1 Escape failures during 7-day CAR training

Age	Drug	Days of CAR training						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Adolescent	RIS	0 \pm 1.25	0 \pm 0	0 \pm 0.31	0 \pm 0	0 \pm 1.56	0 \pm 0.31	0 \pm 0
	CON	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0.63	0 \pm 0
Adult	RIS	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
	CON	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0

Data are expressed as median \pm semi-interquartile range. $n = 12$ per drug for a given age except $n = 11$ for adult CON; CON – vehicle-treated control rats; RIS – risperidone-treated rats;

Since there was such a disparity in learning between groups, I re-examined CAR acquisition using survival curve analysis. In order to do this, an avoidance criterion needed to be set. A minimum criterion of 40% avoidance was chosen primarily as this allowed the inclusion of all groups (Adolescent risperidone group's performance was ~40% on day 7 (Figure 5-6(a)). As can be observed in survival curves in Figure 5-7, the majority of rats from the adult risperidone and control groups and the adolescent control group reached this criterion by Day 3 or 4 of CAR training. However, only 41.7% of rats from adolescent risperidone group reached this criterion by the last day (Day 7) of CAR training. When the performance was compared across different groups, significantly more rats from the adolescent risperidone group (58.3%) failed to reach the criterion, compared to the adolescent controls (16.7%) ($\chi^2 = 3.929$, $p = 0.047$); adult controls (18.2%) ($\chi^2 = 5.243$, $p = 0.022$). Differences between rats that had prior adolescent risperidone treatment and those with prior adult risperidone treatment did not reach the defined statistical significance ($\chi^2 = 3.379$, $p = 0.066$). Adult risperidone group also did not differ from adult controls ($\chi^2 = 0.791$, $p = 0.374$).

Since such a small (5 out of 12) number of animals from the adolescent risperidone group could successfully acquire the CAR, there was an inadequate sample size to achieve statistical power for further CAR tests. Therefore I chose not to examine their long-term behavioural response to risperidone challenge in the CAR paradigm in order to investigate whether the drug-free interval is a factor in sensitized CAR *suppression* in rats with adolescent risperidone exposure (Chapter 3).

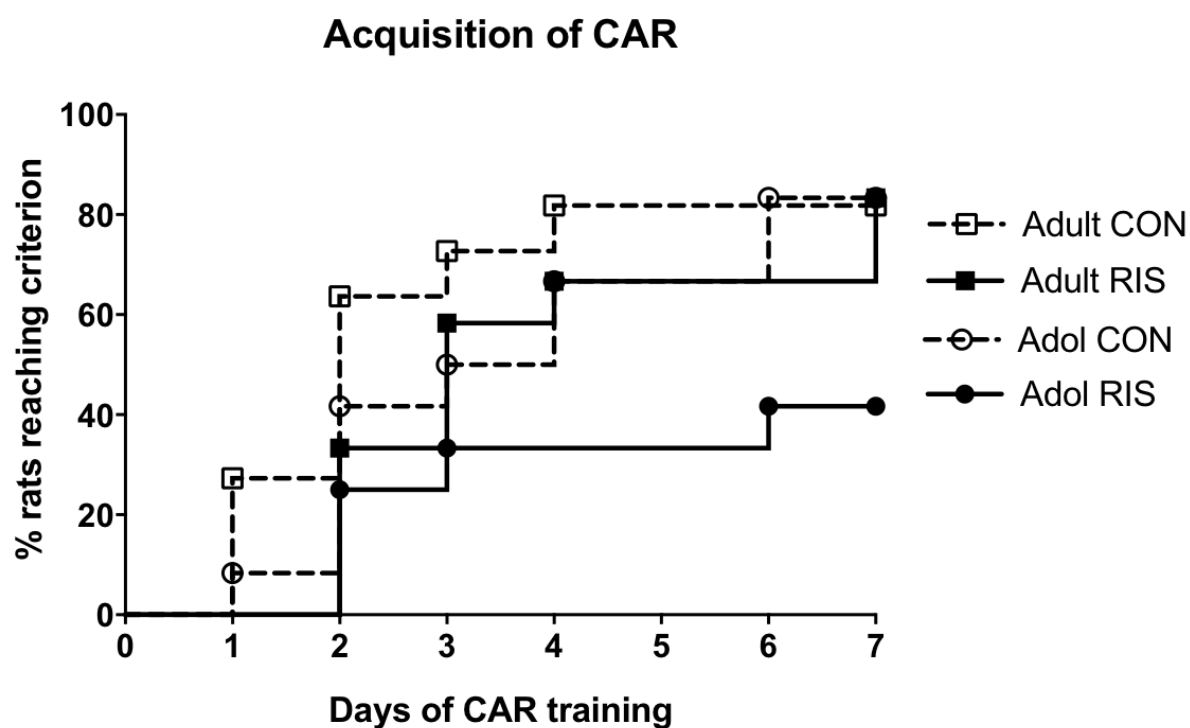


Figure 5-7 Survival curves for rats reaching a criterion of 40% avoidance across 7 days of CAR training. A significantly lower proportion of rats treated with risperidone in adolescence failed to reach the criterion at the end of 7-day training period. Adol – adolescent; CAR – conditioned avoidance response; CON – control; RIS – risperidone

5.3.4. Plasma corticosterone levels at baseline and after a challenge CAR session

The finding of a lower CAR acquisition rate with prior adolescent risperidone exposure raised a question on the role of stress and involvement of hypothalamic-pituitary-adrenal (HPA) axis. Indeed, low avoidance rats, that is, those that show lower ability to acquire the CAR, have been shown to be 'more reactive' or 'hyperemotional' to stressful conditions compared to high avoidance rats, for example, more freezing to white noise stress and higher levels of plasma corticosterone under baseline conditions and stressful events (Steimer and Driscoll 2005; Uvnäs-Moberg et al. 1999). Therefore, plasma corticosterone levels were examined in all rats at baseline (only mild restraint, at a minimum of 2 days after CAR training) and immediately after a challenge CAR session. At baseline, no significant differences in plasma corticosterone levels were observed among four treatment groups (two-way ANOVA: no significant main effect of Age, Drug or Age x Drug interaction, all $F < 2.9$, all $p > 0.05$) (Figure 5-8). As expected, after a challenge CAR session, plasma corticosterone levels became significantly elevated; however, these levels were not different among groups (repeated measures two-way ANOVA: a significant main effect of Sample Type ($F_{1,43} = 34.648$, $p < 0.001$; no significant main effects of Sample Type x age, Sample Type x Drug, Sample Type x Age x Drug, Age, Drug or Age x Drug interaction, all $F < 3.3$, $p > 0.07$). Furthermore, plasma corticosterone levels of CAR learners, i.e. rats that successfully acquired the CAR, did not differ significantly from those of non-learners.

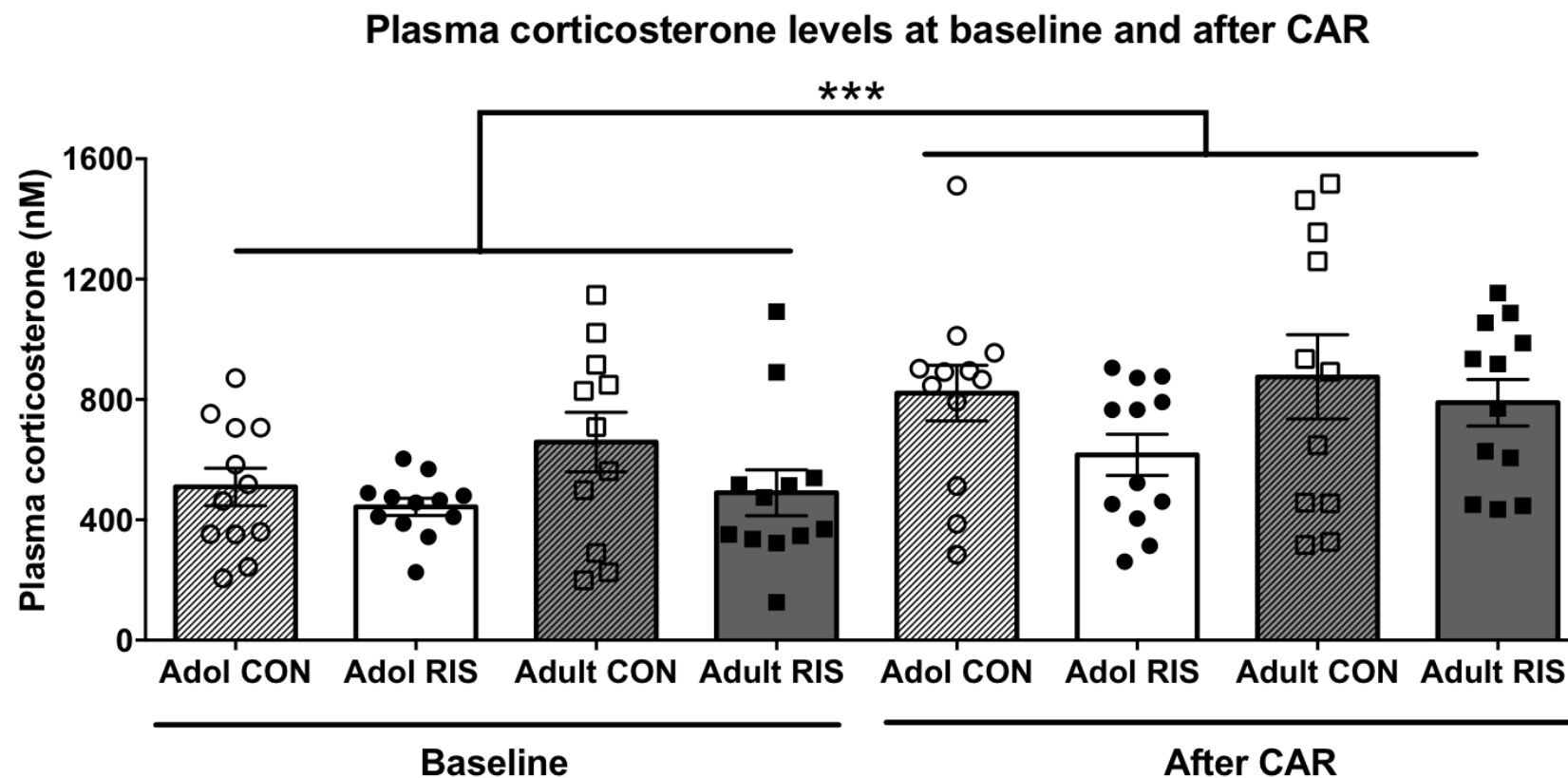


Figure 5-8 Plasma corticosterone levels in rats previously treated with risperidone or vehicle as adolescents or adults. Plasma corticosterone levels were measured from saphenous blood samples collected at baseline and shortly after a challenge CAR session, with an interval of 4 days in between. *** $p < 0.001$ levels at baseline vs levels after CAR. Data are expressed as mean \pm SEM. $n = 12$ per drug group for a given age. Adol – adolescent; CON – vehicle treated controls; RIS – risperidone-treated rats;

5.3.5. Levels of monoamines and their metabolites in the striatum and the NAc

One day after saphenous blood sample collection, all rats were euthanised and regional brain tissues [the striatum and the NAc (Paxinos and Watson 2005)] collected for HPLC assay of catecholamines. Dopamine and its metabolites (DOPAC, HVA and 3MT) in the striatum and the NAc were of particular interest given their roles in catalepsy and CAR performance respectively.

Levels of monoamines (dopamine, 5HT and noradrenaline) and their metabolites in the striatum and the NAc are shown in Table 5-2 and 5-3 respectively. Risperidone treatment in adolescence or adulthood did not induce any significant changes in the levels of monoamines and their metabolites in both striatum and NAc (all $F < 2.35$, $p > 0.05$). Two-way ANOVA of ratios between striatal 5HIAA and 5HT revealed a significant main effect of Age x Drug interaction ($F_{1,43} = 4.89$, $p = 0.032$) with no main effect of Age and Drug (both $F < 1.2$, $p > 0.05$). However, post-hoc tests did not show any significant difference among the four groups. Examination of the ratios between dopamine and its metabolites also did not show any significant difference among four groups. Next correlation analysis was performed to identify any relationship between neurochemical data (catecholamines and their metabolites in the striatum or the NAc) and behavioural data (catalepsy, locomotion and CAR). However, no significant correlation between any measures was observed for any treatment group.

Table 5-2 Levels of catecholamines and their metabolites in the striatum of rats treated with risperidone in adolescence or adulthood

Age group	Drug group	Striatal monoamines and their metabolites (pg/mg tissue) (mean \pm SEM)						
		Dopamine	DOPAC	HVA	3MT	5HT	5HIAA	NA
Adolescent	RIS	5174.30 \pm 218.49	1159.88 \pm 61.76	538.92 \pm 56.01	210.93 \pm 9.71	248.25 \pm 12.16	308.53 \pm 8.76	70.14 \pm 9.86
	CON	4692.98 \pm 482.85	1021.87 \pm 103.35	507.39 \pm 52.52	191.51 \pm 22.14	220.94 \pm 19.97	265.01 \pm 24.80	64.12 \pm 17.02
Adult	RIS	5193.43 \pm 234.55	1120.70 \pm 59.11	492.99 \pm 67.39	203.86 \pm 13.01	270.08 \pm 13.97	292.88 \pm 20.08	56.29 \pm 5.31
	CON	5199.19 \pm 213.01	1184.95 \pm 81.55	451.12 \pm 54.84	203.11 \pm 17.47	245.88 \pm 20.64	303.72 \pm 17.05	59.55 \pm 8.47
F values from Two-way ANOVA ^{\$}	Age	0.71	0.62	0.77	0.02	1.91	0.38	0.68
	Drug	0.58	0.22	0.40	0.39	2.31	0.76	0.02
	Age x Drug	0.61	1.66	0.01	0.33	0.01	2.11	0.17

All data are shown as mean \pm SEM. $n = 12$ per drug for a given age except $n = 11$ for adult CON. ^{\$}All comparisons are $F_{1,43}$, $p > 0.05$; 3MT – 3-Methoxytyramine; 5HIAA – 5-Hydroxyindoleacetic acid; 5HT – 5-hydroxytryptamine or serotonin; Adoles – adolescent; CON – vehicle-treated control rats; DOPAC – 3,4-Dihydroxyphenylacetic acid; HVA – Homovanillic acid; NA – noradrenaline; RIS – risperidone-treated rats;

Table 5-3 Levels of catecholamines and their metabolites in the NAc of rats treated with risperidone in adolescence or adulthood

Age group	Drug group	Accumbal monoamines and their metabolites (pg/mg tissue) (mean \pm SEM)						
		Dopamine	DOPAC	HVA	3MT	5HT	5HIAA	NA
Adolescent	RIS	4219.97 \pm 325.82	1014.85 \pm 70.82	509.89 \pm 56.15	143.06 \pm 15.08	349.22 \pm 32.18	266.60 \pm 28.84	155.79 \pm 32.59
	CON	3746.39 \pm 297.30	856.07 \pm 59.04	394.24 \pm 26.66	143.99 \pm 14.78	350.95 \pm 16.88	291.22 \pm 13.18	142.81 \pm 18.44
Adult	RIS	4217.13 \pm 202.37	970.44 \pm 132.90	429.77 \pm 32.80	148.69 \pm 17.72	402.34 \pm 22.36	309.98 \pm 12.65	138.51 \pm 13.71
	CON	4571.42 \pm 329.10	1065.81 \pm 74.67	430.48 \pm 26.53	170.82 \pm 17.27	374.79 \pm 31.60	271.18 \pm 16.38	103.62 \pm 10.82
F values from Two-way ANOVA ^{\$}	Age	1.98	0.85	0.33	1.00	2.13	0.38	1.93
	Drug	0.04	0.13	2.29	0.50	0.24	0.14	1.39
	Age x Drug	2.010	2.00	2.34	0.43	0.31	2.77	0.29

All data are shown as mean \pm SEM. $n = 12$ per drug for a given age except $n = 11$ for adult CON. $n = 11$ for NA levels of Adoles RIS given one sample did not have a reliably detectable level of NA. ^{\$}All comparisons are $F_{1,43} p > 0.05$; 3MT – 3-Methoxytyramine; 5HIAA – 5-Hydroxyindoleacetic acid; 5HT – 5-hydroxytryptamine or serotonin; Adoles – adolescent; CON – vehicle-treated control rats; DOPAC – 3,4-Dihydroxyphenylacetic acid; HVA – Homovanillic acid; NA – noradrenaline; RIS – risperidone-treated rats;

5.4. Discussion

In this Chapter, I investigated chronic risperidone treatment in adolescents and adults with regards to the short-term effects on NAA and other metabolites in the NAc and long-term behavioural (catalepsy, locomotion and CAR acquisition) and neurochemical changes (in the striatum and the NAc). I showed that, at both Day 1 and Day 22, chronic risperidone treatment did not alter accumbal levels of NAA or other metabolites in both adolescents and adults. More importantly, I showed that after an equivalent drug-free interval, rats treated with risperidone in adolescence had a lower cataleptic response to a challenge dose. These same rats with prior adolescent risperidone exposure were also observed to have a retarded ability to acquire the CAR in adulthood. However, no long-term alterations in levels of plasma corticosterone or monoamines and their metabolites in the striatum and the NAc were observed.

5.4.1. On-drug changes in metabolites of the NAc *during* chronic treatment

To investigate whether chronic risperidone treatment could differentially affect accumbal NAA levels in adolescents and adults, the NAc was examined with ^1H MRS at baseline and after risperidone administration at Day 1 and Day 22. Along with NAA, other metabolites such as glutamate and GSH were also examined. The NAc was chosen given it is the major site for adolescent remodelling changes in dopaminergic and glutamatergic systems (Benoit-Marand and O'Donnell 2008; Huppe-Gourgues and O'Donnell 2012; Mathews et al. 2009; Matthews et al. 2013; Tarazi and Baldessarini 2000; Tarazi et al. 1998) and given the reported long-term changes in GABA and glutamate with adolescent olanzapine treatment (Xu et al. 2015).

In both clinical and preclinical literature, the effects of APDs on NAA have been inconsistent. For instance, preclinical studies in rats have reported a region-dependent increase (Harte et al. 2005; McLoughlin et al. 2009) or no change (Bustillo et al. 2006; Bustillo et al. 2004; Lindquist et al. 2011) in NAA levels following chronic APD treatment. Discrepancy in findings of APDs' effect on NAA may be partly due to heterogeneity in experimental methodologies used in these studies such as route and dosing of APDs (oral vs subcutaneous injections) and method of assessment (HPLC Vs ^1H MRS). Still no study to date has examined on-drug changes in NAA with risperidone challenge. Here, I showed that in the NAc, chronic risperidone treatment did not alter baseline and *on-drug* NAA levels in both adolescents and adults. Lack of any significant effect of risperidone on NAA in my animals without neurobiological pathology could suggest that NAA changes observed in schizophrenic patients are probably due to psychopathological condition per se, with minimal confounds of the effects of chronic APD treatment. Indeed, recent clinical findings indicate that region-specific reductions in NAA occur only in chronic schizophrenia rather than at-risk stage (Brugger et al. 2011; Liemburg et al. 2016), thus suggesting a close relationship between

progression of illness and NAA changes. Another recent preclinical study in rodent model of schizophrenia has also reported a biphasic change in NAA level with MIA (Vernon et al. 2015), thus further supporting the possibility that NAA changes are due to psychopathological changes rather than due to effects of APDs.

Given NAA is regarded as a marker of neuronal viability, function and density (See review by (Rae 2014)) and synthesized almost exclusively in neuronal mitochondria (Niwa et al. 2007; Patel and Clark 1979), the outcome of risperidone treatment on these aspects still needs to be investigated with other techniques. Discrepancies in NAA findings between my previous pilot ^1H MRS experiment and the current experiment may be due to experimental differences in ^1H MRS data acquisition. During a single 1 h long continuous scan in pilot experiment (Appendix C), drifting of the water peak was observed and this affected the quality of spectra obtained and consequently reliability (high CRLB) in quantification of metabolites. In the current experiment, 15 separate ^1H MRS scans (each 4 min long) were performed with automated local frequency adjustment to compensate for drifting of water peak. This technique provided a better spectral quality and a more reliable quantification of metabolites. Difference in pharmacokinetics as a result of different routes of administration in pilot experiment (SC) and the current study (IP) may also play a role in the final outcomes observed. Perhaps, also the small sample size in the pilot experiment suffered from a winner's curse phenomenon (Button et al. 2013).

^1H MRS measures all the available pool of metabolites within the specified voxel, i.e. both extracellular and intracellular levels as well as both neuronal and glial levels. Therefore, given there was no significant alteration in other metabolites such as glutamate, glutamine, GABA and GSH I conclude that risperidone did not alter the metabolism of neurons or glia in the NAc. However, it is still possible that risperidone could differentially affect neurotransmission in GABA-ergic or glutamatergic systems in adolescents and adults, which can be investigated more thoroughly with other techniques such as electrophysiology or microdialysis. In this study, ^1H MRS levels of metabolites were measured only from the NAc. Changes in other brain regions are still to be investigated.

A consistent change in ^1H MRS data observed in all groups was a progressive reduction in the levels of most metabolites, most pronounced with NAA and glutamate, across scan time-points. Perhaps this reduction could be due to suppression of brain metabolism by isoflurane anaesthesia, which can produce potential confounds the neural metabolites examined (Boretius et al. 2013; Herring et al. 2009; Liachenko et al. 1999; Stover et al. 2004). Imaging ^1H MRS on awake fully conscious rats may perhaps provide a better physiological measure of accumbal metabolite levels. Another limitation in interpretation of ^1H MRS findings is the localization of the voxel bilaterally

over the NAc following the published method in the literature (Xu et al. 2015). It is yet to be investigated whether there is any contamination of signal, although expected to be minimal, from anterior commissure and the intervening regions such as medial septal nucleus.

5.4.2. Long-term behavioural outcomes

In the current Chapter, I showed that prior chronic risperidone treatment induced long-term behavioural effects in catalepsy and CAR. These differed depending on whether treatment was administered to rats either as adolescents or adults.

5.4.2.1. Catalepsy and locomotion

When examined after a lengthy drug-free interval, a challenge dose of risperidone induced a robust cataleptic response selectively in rats previously treated with this APD as adults. Rats with prior adolescent risperidone treatment or controls with prior vehicle treatment did not show a significant level of catalepsy. In Chapter 4, a lower level of risperidone-induced cataleptic responses was observed in adolescents, compared to adults *during* ongoing treatment. Furthermore, it was speculated that incomplete wiring status of the adolescent striatum (Matthews et al. 2013; Teicher et al. 1995) would mediate a diminished cataleptic response in adolescents. Again, here in Chapter 5, after a drug-free interval, rats with prior adolescent risperidone treatment were observed to have a lower level of catalepsy to a challenge dose, compared to those with prior adult risperidone treatment. Therefore, it seemed that some aspect of the lower potential for rats with adolescent risperidone treatment to develop catalepsy persisted even after a drug-free interval. The underlying neurobiological basis for this intriguing finding remains unknown. Given that risperidone-induced locomotor suppression did not significantly differ among the four treatment groups, the observed long-term change appeared to be specific to catalepsy only. This finding also suggested differential long-term effects of risperidone on the striatum compared to the NAc since catalepsy is mainly striatum-dependent (Ossowska et al. 1990; Wadenberg et al. 2001b; Yoshida et al. 1994) and locomotion NAc-dependent (Beninger 1983; Kelly et al. 1975; Staton and Solomon 1984). (See chapter 6 for detailed discussion).

Although several investigators have examined *on-drug* cataleptic behaviour with acute or chronic APD treatment, long-term outcomes in catalepsy are less frequently explored. To date, the long-term cataleptic effect of chronic adolescent haloperidol treatment has only been examined in mice (Ushijima et al. 1995). This adolescent haloperidol treatment induced a *decreased* cataleptic response (tolerance) in the short-term (1 and 3 days of drug washout) but an *increased* cataleptic response in the long term (15 and 21 days of drug washout) to a challenge dose of this APD. Together, the findings in the study by Ushijima and colleagues and my current results suggest that

long-term cataleptic outcome of adolescent APD treatment can vary with the type of APDs examined and the species utilized.

5.4.2.2. *Acquisition of the CAR*

When rats were trained to acquire the CAR, differential outcomes were observed depending on the history of prior risperidone treatment. Rats from the adolescent cohorts showed a slower rate of CAR learning. In particular, rats previously treated with risperidone in adolescence were observed to have a retarded ability to acquire the CAR, compared with other treatment cohorts. Only a limited number of rats from the adolescent risperidone cohort successfully acquired the CAR. This low rate of successful CAR acquisition (~41% to reach a criterion of 40% avoidance) following adolescent risperidone treatment was in sharp contrast with the reported rate of CAR acquisition (~75-80% to reach a criterion of $\geq 70\%$ avoidance) in the literature (Choi et al. 2010a) as well as those rates (~75-80% to reach a criterion of $\geq 70\%$ avoidance) observed in Chapter 3. This outcome resulted in an inadequate sample size to study CAR suppression by risperidone challenge; consequently, the fourth aim of this Chapter to determine the role of drug-free interval in sensitized CAR suppression could not be achieved.

The majority of preclinical studies that investigate CAR in chronic APD treated animals examine *suppression* of already acquired CAR by APD treatment in adolescents (Chou et al. 2015; Gao and Li 2014; Qiao et al. 2014a; Qiao et al. 2013; Qiao et al. 2014b; Shu et al. 2014b) or adults (Gao and Li 2013; Natesan et al. 2007; 2008; Natesan et al. 2006a; Samaha et al. 2007; Wadenberg et al. 2000). Similarly, in Chapter 3, I examined *suppression* of the *already acquired* CAR by risperidone treatment in adolescence or adulthood. All rats in Chapter 3 had a prior history of successful CAR acquisition from PND30 to PND34 and therefore, *re-acquired* the CAR in adulthood as rapidly as within 1 day of retraining regardless of age or drug groups. This is consistent with previous reports that CAR is a persistent behaviour (see reviews by (Kapur et al. 2006; Moutoussis et al. 2007)) and that CAR *re-acquisition* is not altered by prior APD treatment (Qiao et al. 2013; Qiao et al. 2014b; Shu et al. 2014b).

Unlike the widely studied *suppression* of previously acquired CAR, there are only a limited number of studies have examined the *acquisition* of CAR in *previously untrained* rats after a drug-free interval from chronic APD treatment. To the best of my knowledge, no study has examined CAR *acquisition* after a lengthy drug-free interval from either adolescent or adult risperidone treatment. In adult rats, CAR *acquisition* has been reported to be unaltered at shortly after termination of chronic risperidone treatment (Drago et al. 1997). Here, I showed that, after a lengthy drug-free interval, adult risperidone treatment did not alter CAR learning. Another study has also investigated CAR acquisition *during* a lengthy risperidone treatment that began in adolescence (Castellano et al.

2009); this study also reported no deficits in CAR acquisition. However, Castellano et al. treated rats with risperidone for an extended duration (>90 days) from adolescence to adulthood, which vastly exceeded duration of adolescence (See Chapter 1). Therefore, the authors' finding may not be specific to adolescent exposure.

Learning of the CAR has been hypothesized to be a two-step process [for example, see (Cain and LeDoux 2008; Choi et al. 2010a)]: (1) Rats have to form an association between the CS and the US (white noise and mild foot-shock respectively in this experiment) through Pavlovian conditioning; in other words, fear is conditioned to the CS (Oleson and Cheer 2013). (2) Rats learn to perform an instrumental avoidance response (crossing into the other chamber) to avoid the foot-shock through the fear aroused by the presence of CS itself. Low avoidance rats have also been reported to be freezing ('fear conditioned') instead of making an instrumental avoidance response (Choi et al. 2010a; Lázaro-Muñoz et al. 2010). Moreover, low avoidance rat strains have also been reported to 'more reactive' to stress, with higher levels of stress hormones of HPA axis such as corticosteroids (Akieda-Asai et al. 2011; Ohta et al. 1999; Steimer and Driscoll 2005; Uvnäs-Moberg et al. 1999). Therefore, I next examined plasma corticosterone levels in all rats at baseline and shortly after a challenge CAR session. CAR challenge increased plasma corticosterone levels significantly from baseline levels, indicating that this CAR challenge was a stressful event. However, plasma corticosterone levels were not different among the four treatment groups at both baseline and after CAR. While examination of plasma adrenocorticotrophic hormones (ACTH) or corticotrophin releasing hormones (CRH) (Akieda-Asai et al. 2011; Ohta et al. 1999) may be complimentary, the current finding with plasma corticosterone levels suggested that impaired CAR learning in rats with adolescent risperidone exposure was less likely due to altered response to stress or stress-related reactions from CAR training. Still, freezing response cannot be ruled out. Indeed, the existing literature has shown that adult rats with prior adolescent olanzapine have heightened freezing to context and impaired fear extinction (Milstein et al. 2013). Further examination of freezing behaviour, for example, in fear-conditioning or during CAR *acquisition* is now required.

5.4.3. Long-term neurochemical outcomes

Next, I continued to examine neurochemical changes in rats with prior adolescent or adult risperidone treatment. Given the striatum is often implicated in the cataleptic response to APDs (Ossowska et al. 1990; Wadenberg et al. 2000; Wadenberg et al. 2001b; Yoshida et al. 1994), this brain region may be responsible for the observed long-term differential cataleptic responses. I also wanted to examine whether short-term increases in striatal dopamine metabolites in risperidone-treated adolescents (Chapter 4) persisted after a lengthy drug-free interval. Mesolimbic dopaminergic system plays a major role in acquisition of the CAR (Fantin and Bottecchia 1984;

Koob et al. 1984; Oleson et al. 2012; Ramirez et al. 2015; Shumake et al. 2010). This mesolimbic dopaminergic activity, in the NAc in particular, is thought to modulate motivational circuits and provide incentive value to neutral stimuli such as white noise or tone (CS) (Oleson and Cheer 2013). Therefore, I hypothesized that differential outcomes in catalepsy and CAR *acquisition* were mediated by neurochemical changes in the striatum and the NAc respectively. To test this hypothesis, I examined monoamines and their metabolites in these two brain regions.

Here, I showed that levels of dopamine, 5HT and their metabolites in both striatum and NAc as examined with *ex vivo* HPLC were not altered by prior risperidone treatment regardless of the age of prior exposure. Also, no significant relationship was observed between catalepsy, locomotion or CAR learning and monoamines or their metabolites. This finding does not rule out dynamic alterations in dopaminergic or serotonergic neurotransmission in the striatum and the NAc, that may be occurring during a behaviour. This obviously would require techniques such as dialysis or voltammetry to address.

Therefore, an outstanding question in this Chapter is which neural mechanism(s) underlie(s) differential long-term behavioural responses in the catalepsy and the CAR *acquisition* in risperidone-treated rats. With regards to catalepsy, neurochemical changes in presynaptic dopaminergic regions such as substantia nigra (SNr) in addition to terminal regions such as the striatum may perhaps underlie differential cataleptic response observed in this study. For instance, chronic haloperidol treatment in adult rats has been reported to induce a long-lasting downregulation (up to 4 weeks) in TH immunoreactivity in the SNr (Levinson et al. 1998). It is still unknown whether TH immunoreactivity in the midbrain can be altered by risperidone treatment selectively in adults or adolescents or both. Moreover, in adult rodents, chronic APD treatment can decrease the number of spontaneously firing neurons in SNr or VTA or both, a condition referred to as ‘depolarization block’ (Chiodo and Bunney 1983; Gill et al. 2014; Grace et al. 1997). These authors have also proposed a relationship between depolarization block of dopaminergic neurons and extrapyramidal side effects (EPS) of APDs. This is relevant because the adolescent midbrain dopaminergic neurons fire faster than their adult counterparts (McCutcheon et al. 2012; McCutcheon and Marinelli 2009) and no study has to date compared midbrain dopaminergic neuron activity following risperidone treatment in adolescence or adulthood.

Successful *acquisition* of the CAR has been related to neural activity in multiple brain regions such as the NAc (Oleson et al. 2012), the VTA (Shumake et al. 2010), the PFC (Stark et al. 1999) and the amygdala (Choi et al. 2010a; Darvas et al. 2011; Lázaro-Muñoz et al. 2010), the striatum (Darvas et al. 2011; Dombrowski et al. 2013). Connections between the NAc and the amygdala (Ramirez et al. 2015) or between the PFC and the amygdala (Moscarello and LeDoux 2013) are also important for

CAR acquisition. Rats that fail to acquire CAR are known to be freezing (Pavlovian ‘fear conditioned’ instead of making an instrumental avoidance). This freezing response is mediated by neural activity in central amygdala nuclei (CeA) (Choi et al. 2010a; Lázaro-Muñoz et al. 2010). Lesioning of CeA (Choi et al. 2010a) or suppression of CeA activity by cortical input from the PFC (Moscarello and LeDoux 2013) suppresses CeA-mediated freezing and significantly improves the CAR. In addition, electrical stimulation of the VTA can improve avoidance acquisition whereas stimulation of lateral habenula can decrease it (Shumake et al. 2010). Therefore, a complex interplay between multiple brain regions is involved in the CAR acquisition. Nonetheless, the NAc appears to have the most central role in CAR acquisition since this brain region modulates motivational circuits and serves as an integrative centre of inputs from the VTA, the amygdala, the PFC and the hippocampus (Humphries and Prescott 2010). For instance, examination of the NAc with voltammetry has shown differential patterns of dopamine release in the NAc during avoidance (increased release) and escape (decreased release) responses during a CAR session or during freezing (decreased release) responses in the fear conditioning (Oleson et al. 2012). The resultant changes in dopamine concentration have been hypothesized to modulate inputs from other brain regions and produce a final behavioural response (Oleson and Cheer 2013). Therefore, examination of dopamine signalling in the NAc in a behaving animal will provide further insight into risperidone-induced neurotransmission changes. Moreover, changes in the brain regions such as the amygdala, the PFC or the VTA that provide inputs to the NAc still need to be examined in rats with prior adolescent risperidone exposure. These aspects will be discussed in more detail in Chapter 6.

A limitation in interpretation of CAR learning deficits in this experiment is that all rats received isoflurane during adolescence. While detrimental neurocognitive outcomes of isoflurane exposure have been reported in neonatal (Jevtovic-Todorovic et al. 2003; Lee et al. 2014), adult (Liu et al. 2014) or aged (Callaway et al. 2015) rodents, no study to date has specifically examined the effects of adolescent isoflurane exposure. However, given that vehicle-treated control rats with the same isoflurane exposure and handling history did not show a similar degree of deficits, this caveat would seem unlikely to be relevant.

To sum up, in Chapter 5, chronic risperidone treatment in adolescence was examined for short-term changes in accumbal NAA and other metabolites and long-term behavioural and neurochemical changes. As in the previous chapters, the outcomes with adolescent risperidone treatment were directly compared with those of adult treatment. The current 22-day risperidone regimen did not alter the levels of NAA or other metabolites in the NAc in both age groups, suggesting no differential age-dependent effect on accumbal metabolism *during* chronic treatment. After a lengthy drug-free interval, this risperidone treatment induced a long-term increase in cataleptic response

only in rats with prior adult risperidone exposure. By contrast, prior risperidone treatment in adolescence impaired the CAR *acquisition* in adulthood again after a lengthy washout period. However, no changes in *ex vivo* levels of monoamines or their metabolites were observed in the striatum and the NAc in both age groups. Further examination of risperidone-induced neurochemical or neurophysiological changes is still required to identify underlying neural mechanism(s) of differential long-term behavioural outcomes in catalepsy and CAR. Taken together, the findings of Chapter 5 provide further support to the hypothesis that the adolescent brain differs from the adult brain in the response to chronic risperidone treatment.

Chapter 6. General discussion

6.1. Introduction

Over the last twenty years, prescription of APDs to children and adolescents has increased dramatically (as much as 5-6 fold) in several different countries (Kalverdijk et al. 2008; Olfson et al. 2006; Olfson et al. 2012; Ronsley et al. 2013; Song and Guo 2013). Consequently, the global increase in prescription of APDs to adolescents in clinical settings has raised concerns about its neurobiological consequences (Ben Amor 2012; Patel et al. 2005; Ronsley et al. 2015; Vitiello et al. 2009). To address whether such concerns are warranted, the effects of APD treatment on adolescent brain neurochemistry and brain function need to be examined. In clinical research, long-term assessment of behavioural and neurochemical changes as a result of adolescent APD prescription can be achieved with certain psychometric tests and imaging modalities such as MRI, ^1H MRS or positron emission tomography (PET). However, such studies are costly, difficult to perform and require time scales outside the remit of most funding bodies.

Neurobehavioural maturation processes are highly conserved between species (Spear 2000; 2007). In addition, a transition period equivalent to human adolescence has been identified in rodents, based on physical, pubertal and neurobehavioural changes (Brenhouse and Andersen 2011; McCutcheon and Marinelli 2009; Schneider 2013; Spear 2000; 2007). This enables us to model adolescent APD treatment in laboratory rodents and investigate the resultant neurobiological changes. Experimental settings, for example, the age at the start of treatment or duration of treatment can be well-controlled in preclinical APD studies. Moreover, invasive neurochemical assays, which are not feasible clinically, can also be achieved in the preclinical APD studies. In particular, studies can be strictly controlled to assess long or short-term outcomes either on or off drug in a timely manner in rodent studies. Furthermore, use of translationally relevant assessments in experimental adolescent rodents, for example, MRI or ^1H MRS, as has been done in the current thesis, can be reverse-translated back to the clinic.

While still limited in number, existing preclinical studies have attempted to identify short-term behavioural and neurochemical effects of adolescent APD treatment [for example, (Choi et al. 2009; Lian et al. 2016; Moran-Gates et al. 2007; Wiley 2008; Wiley and Evans 2008)]. A few preclinical studies over the past five years have also started to examine long-term neurobiological consequences of adolescent treatment with atypical APDs (De Santis et al. 2016; Milstein et al. 2013; Qiao et al. 2013; Vinish et al. 2013). For example, adolescent treatment with olanzapine has been shown to induce long-term deficits in behaviour in adulthood, such as alterations in working memory, fear conditioning, reward behaviour (to amphetamine) and anxiety/depression-related behaviour, as well as changes in neurotransmission in the NAc, and dendritic spine pruning in other major brain regions (Brooks et al. 2016; De Santis et al. 2016; Milstein et al. 2013; Xu et al. 2015).

However, the fact that adolescent APD treatment in these existing studies was not examined together with a comparison age group (Fuhrmann et al. 2015; Spear 2007) raises the question whether or not the reported findings are specific only to the adolescent animals.

To address such issues, the experiments in this thesis have examined chronic 22-day risperidone administration in adolescent rats (PND36-PND56/57) and compared this with outcomes from the same exposure in adulthood (PND80-PND100/101). Risperidone was selected for detailed examination given this atypical APD is most commonly prescribed to adolescents (Hollingworth et al. 2013; Olfson et al. 2006; Olfson et al. 2012). The findings of the current thesis have added to the growing literature on adolescent APD exposure by demonstrating short- and long-term neurobiological consequences either in behaviour or neurochemistry selective to adolescent APD exposure.

In this thesis, behavioural outcomes of adolescent APD treatment were examined with two behavioural tests, namely CAR and horizontal bar test for catalepsy. These two behavioural tests were chosen because they are widely used in the assessment of APDs or compounds with APD potential (Porsolt et al. 2010; Wadenberg 2010). Here, I showed that behaviours in chronic risperidone treated animals were highly dependent on age of exposure:- (1) Long-term alterations in the *acquisition* of the CAR and sensitized *suppression* of CAR were observed in rats treated with risperidone in adolescence compared with adults. (2) Long- and short-term alterations in cataleptic response were observed in rats treated with risperidone in adulthood compared with adolescence. Accompanying alterations in neurochemistry were also observed in brain regions associated with these behaviours. In the following sections, differential behavioural outcomes in CAR and catalepsy are discussed. Given that MRI and ¹H MRS data and possible reasons for negative findings have been discussed in greater details in Chapter 3 and Chapter 5 respectively, these will not be repeated in the current chapter.

6.2. CAR: A further introduction on circuits involved

The CAR is a behavioural paradigm which is widely used not only in studies of APDs (as early as 1950s (Cook and Weidley 1957)) but also in the studies of fear and anxiety (Lovibond et al. 2008). The CAR paradigm in rodents is most often performed in a chamber with two compartments which are separated with a door or a hurdle (See Chapter 2, Section 2.3.1.). Investigators have focused on two aspects of CAR: *acquisition* in untrained rats or *expression* of a previously acquired CAR after some intervention or time period.

Acquisition of the CAR is considered to be a two-step process. First, rats undergo Pavlovian conditioning from repeated pairing of CS (white noise, tone or light) and US (foot-shock), by learning that the CS is followed by the US (formation of CS-US associations). This Pavlovian conditioning leads to a freezing response. Second, rats learn to perform an instrumental avoidance response (crossing into the other chamber) instead of freezing, in order to avoid the foot-shock.

As summarised in Figure 6-1, *acquisition* of CAR is influenced by neural activity in distinct brain regions such as the NAc (Oleson et al. 2012), the VTA (Shumake et al. 2010), the PFC (Stark et al. 1999) and the amygdala (Choi et al. 2010a; Darvas et al. 2011; Lázaro-Muñoz et al. 2010). Connectivity between these regions is critical to perform a CAR, for example, between the NAc and the amygdala (Ramirez et al. 2015) or between the PFC and the amygdala (Moscarello and LeDoux 2013). CS-US associations are formed in the lateral nucleus of the amygdala (LA) (Sah et al. 2003). Lesioning of the LA therefore impairs CAR *acquisition* (Choi et al. 2010a; Lázaro-Muñoz et al. 2010). When CS information is relayed from the basal nucleus of the amygdala (BA) to the NAc, active ‘defensive’ responses (avoidance response in the case of CAR) are produced (Ramirez et al. 2015). If CS information is processed through the central amygdala nucleus (CeA) this leads to Pavlovian responses (freezing) and acts against *acquisition* of CAR (Choi et al. 2010a; Lázaro-Muñoz et al. 2010). Suppression of the CeA activity improves CAR learning and this is thought to be mediated by cortical input from the PFC (infralimbic region) (Moscarello and LeDoux 2013) or recurrent connections between the NAc and the PFC (Ramirez et al. 2015) through the cortico-striato-thalamic loop (Sesack and Grace 2010) (Figure 6-1).

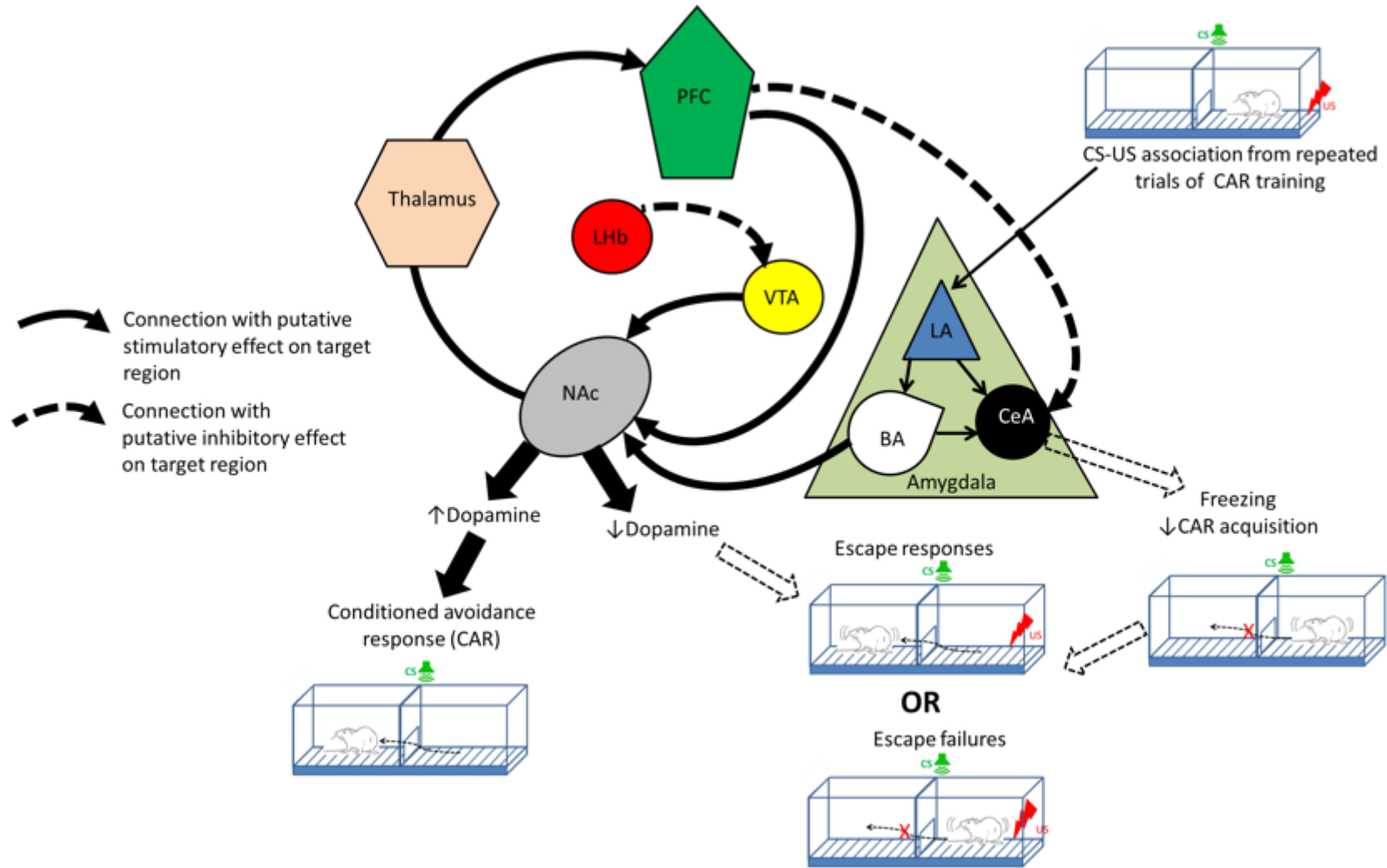


Figure 6-1 Neural pathways and brain regions involved in acquisition of conditioned avoidance response (CAR) Information of CS and US is relayed from the sensory systems to the lateral nucleus (LA) of the amygdala. The LA projects to the basal nucleus (BA) which projects to both central amygdala nucleus (CeA) and the nucleus accumbens (NAc). The NAc is central to mediating an instrumental avoidance response whereas the CeA mediates freezing response. The projections from the prefrontal cortex (PFC, infralimbic region) to the CeA suppress CeA-mediated freezing reactions and promote avoidance response. Stimulation of the ventral tegmental area (VTA), that provides dopaminergic innervation to the NAc, also improves CAR acquisition. CS – conditioned stimulus; DRN – dorsal raphe nuclei; Lhb – lateral habenula; MRN – medial raphe nuclei; SNr – substantia nigra; US – unconditioned stimulus;

Despite an involvement of such a complex interplay between multiple brain regions in the acquisition of the CAR, dopamine signalling within the NAc appears to be the final common pathway in conditioned avoidance behaviour (Oleson and Cheer 2013). The NAc modulates motivational circuits, serves as an integrative centre of inputs from the VTA, the amygdala, the PFC and the hippocampus, and produces a final behavioural output (Humphries and Prescott 2010; Mogenson et al. 1980). Increases in dopamine release in the NAc are accompanied by avoidance responses (McCullough et al. 1993; Oleson et al. 2012) whereas local depletion of dopamine in the NAc (McCullough et al. 1993) or lesioning of the NAc (and the striatum) (Koob et al. 1984) impairs CAR *acquisition*. In addition, electrical stimulation of the VTA, which provides dopaminergic input to the NAc (Elsworth and Roth 2009), can improve CAR *acquisition* (Shumake et al. 2010). By contrast, stimulation of the lateral habenula (LHb), which inhibits 90% of midbrain dopamine neurons (Ji and Shepard 2007), can disrupt CAR *acquisition* (Shumake et al. 2010).

Suppression of avoidance without affecting escape responses is a behavioural effect selective to APDs especially with *acute* administration (Arnt 1982; Wadenberg 2010; Wadenberg and Hicks 1999). It is thought that APDs achieve this selective *suppression* of CAR mainly through the blockade of dopamine neurotransmission in the NAc (Wadenberg 2010; Wadenberg and Hicks 1999). Local administration of dopamine antagonists into the NAc suppresses CAR in a manner analogous to acute systemic administration of APDs (Wadenberg et al. 1990b). At the same time, APD-induced *suppression* of avoidance also correlates tightly with the occupancy of dopamine D2 receptors (Natesan et al. 2007; 2008; Wadenberg et al. 2000; Wadenberg et al. 2001b) (Figure 6-2). *Suppression* of the CAR is therefore considered a robust behavioural readout of APD action (Wadenberg 2010; Wadenberg and Hicks 1999).

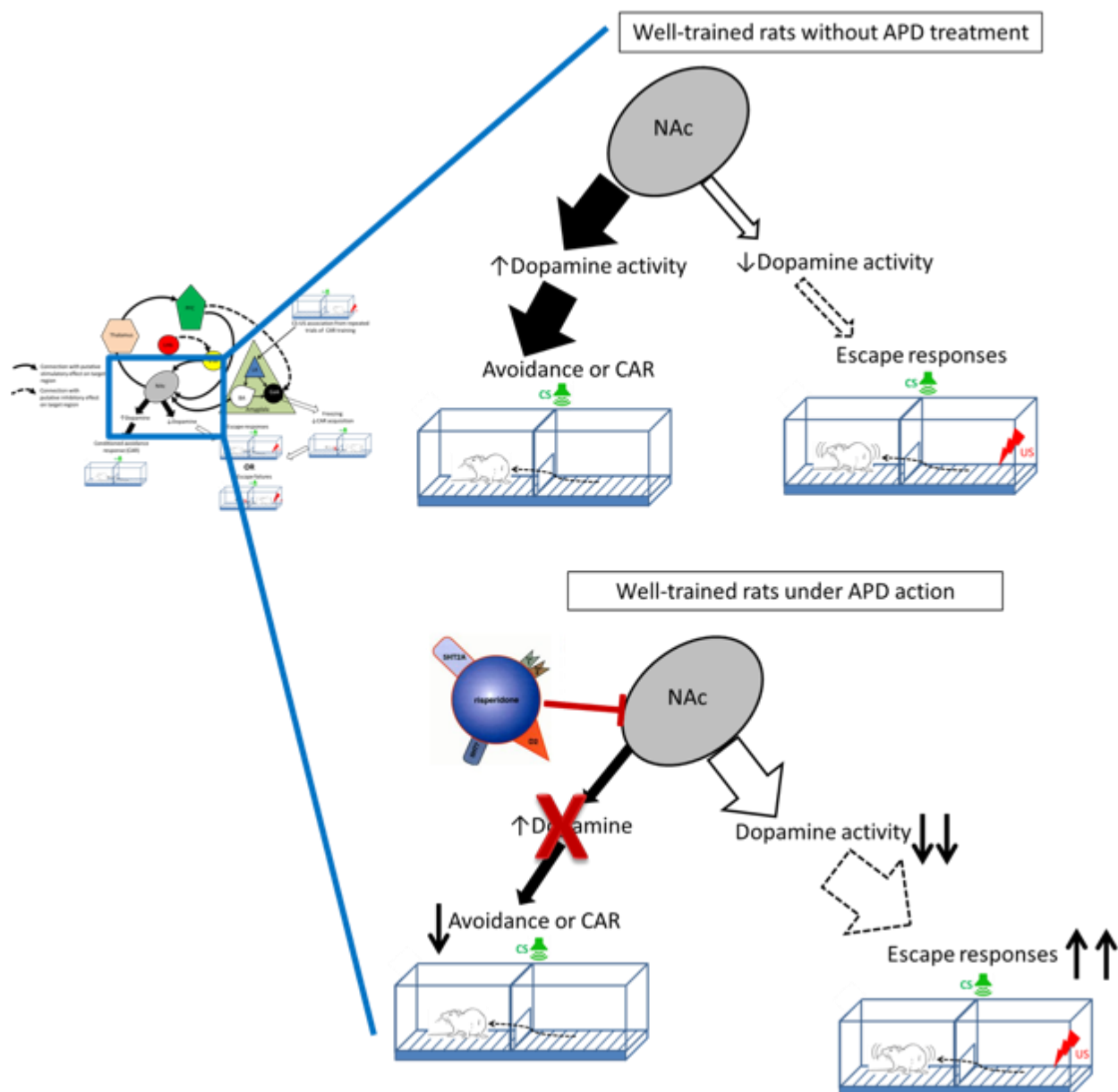


Figure 6-2 Schematic diagram of APD-induced suppression of CAR through blockade of dopamine neurotransmission in the nucleus accumbens (NAc) In normal rats (without APD treatment), increases in the dopamine release are accompanied by avoidance responses (CAR) and decreases by escape responses. In rats treated with APDs, dopamine neurotransmission in the NAc is blocked leading to suppression of CAR without affecting escape responses.

6.3. Risperidone-induced changes in CAR: adolescents vs adults

In this thesis, using CAR, I examined behavioural changes induced by risperidone treatment in adolescents and adults both *during* chronic treatment and after a drug-free interval. Depending on the timing and method (*suppression* or *acquisition*) of assessment, risperidone induced changes in CAR differed between adolescents and adults. *During* ongoing chronic treatment, *suppression* of previously acquired CAR was assessed (Chapter 3). At this time point of assessment, the adolescents experienced fewer escape failures than the adults. After a drug-free interval, both *suppression* of already acquired CAR in previously *trained* rats and *acquisition* of the CAR in *untrained* rats were assessed. At this time point of assessment, rats with prior adolescent risperidone exposure were more sensitive to CAR *suppression* by a challenge dose of this APD, compared to those with prior adult risperidone exposure (Chapter 3). Moreover, after a drug-free interval, rats with prior adolescent risperidone exposure showed a retarded capability to acquire the CAR (Chapter 5).

6.3.1. Risperidone-induced *suppression* of previously acquired CAR

The majority of studies investigating *repeated* APD treatment have examined *suppression* of the CAR in *well-trained* adolescent or adult rats (for example, (Gao and Li 2014; Li et al. 2007; Li et al. 2010; Qiao et al. 2013; Samaha et al. 2007)). Similar to these studies, in Chapter 3, I studied *suppression* of the CAR with chronic APD treatment in adolescents and adults. The experimental features that distinguished my study from the existing reports are: (1) I directly compared adolescent and adult treatment [cf. examination of only one age group in the existing studies, for example, (Gao and Li 2014; Li et al. 2007; Qiao et al. 2013)]; (2) I used CS-US CAR tests unlike the existing studies of *adolescent* APD treatment that utilized CS-only CAR tests [for example, (Qiao et al. 2014a; Shu et al. 2014a; Shu et al. 2014b) but see (Gao and Li 2014)].

In the first part of Chapter 3, I examined three APDs (risperidone, clozapine and haloperidol) whereas in the second part, I continued to examine risperidone only. Rats were given the same handling history including CAR training, the only variable being the age at which APD treatment was administered. I showed in both adolescents and adults that risperidone and haloperidol suppressed the CAR during chronic treatment (Day 17) whereas clozapine no longer blocked the CAR (tolerance). These findings in Chapter 3 are consistent with the reported CAR outcomes of these APDs in adolescents (Qiao et al. 2014a; Qiao et al. 2013) and adults (Li et al. 2007; Li et al. 2010). Importantly, I identified an important age-dependent outcome – adolescents were less susceptible to risperidone-induced escape failures than adults *during* chronic treatment (See the next section for detailed discussion).

After a drug-free interval, when these animals were re-challenged with half of the APD dose they previously received, differential outcomes in CAR were identified depending on the age windows of prior risperidone treatment. Rats treated with risperidone in adolescence were observed to be more sensitive to CAR *suppression* by this APD challenge compared with those treated in adulthood. This finding suggests a sensitization-like CAR *suppression* in rats with adolescent risperidone exposure, as has been reported in the literature (Qiao et al. 2014a). However, no differential effect on escape failure or locomotor responses was observed. This finding selective to the *suppression* of CAR without any effect on escape failures further suggested that the adolescent risperidone treatment preferentially affected the NAc, not the striatum (See the next section for further discussion).

As discussed earlier, the NAc is the major brain region associated with APD-induced suppression of CAR. Therefore, the NAc was the brain region I examined for neurochemical correlates (Chapters 3 and 5). Among the dopaminergic and serotonergic markers examined I observed a significant downregulation of 5HT_{2A} receptor and COMT mRNA in rats previously treated with risperidone in adolescence (Chapter 3). Decreasing 5HT_{2A} receptor activity experimentally by administration of a selective 5HT_{2A} antagonist can potentiate *suppression* of CAR by dopamine antagonists (Wadenberg et al. 2001a). Therefore, I speculate that a decreased 5HT_{2A} receptor activity would potentiate the effect of dopaminergic receptor blockade by risperidone and therefore lead to increased (or 'sensitized') CAR *suppression* by the challenge dose of this APD in these rats. This hypothesis needs to be examined in future studies.

Another aspect of stimulation of the 5HT_{2A} receptor is increased dopamine synthesis and release (Navailles and De Deurwaerdère 2011). Therefore, I speculate that an alternate consequence of decreased 5HT_{2A} receptor levels in rats with adolescent risperidone exposure would be decreased dopamine availability. A reduction in COMT, a major catabolic enzyme for dopamine, in the NAc could represent a compensatory mechanism to decreased dopamine availability. Therefore, I examined the levels of dopamine and its metabolites in another cohort of rats treated with the same adolescent risperidone regimen (Chapter 5). However, no significant alterations in dopamine, 5HT and their metabolites were observed in the NAc. While this finding contradicts this hypothesis, dynamic changes in dopamine availability may still occur for the following reasons: (1) The net functional outcome of 5HT_{2A} receptors are state-dependent; so it is only under activated conditions (e.g. in response to a pharmacological agent) that the activity of 5HT_{2A} receptor may regulate dopamine release (Porrás et al. 2002; Schmidt et al. 1992) or behaviour (e.g. CAR) (Wadenberg et al. 2001a; Wadenberg et al. 1998a). (2) *Ex vivo* HPLC measurement of all the available pools of neurotransmitters and their metabolites may not reflect dynamic changes in synaptic levels as occur

during a behavioural performance. Further examination, for example, with microdialysis under a pharmacological challenge (for example, with risperidone itself or dopamine agonist) may assess functional outcomes of downregulated $5HT_{2A}$ receptor levels on dopamine neurotransmission.

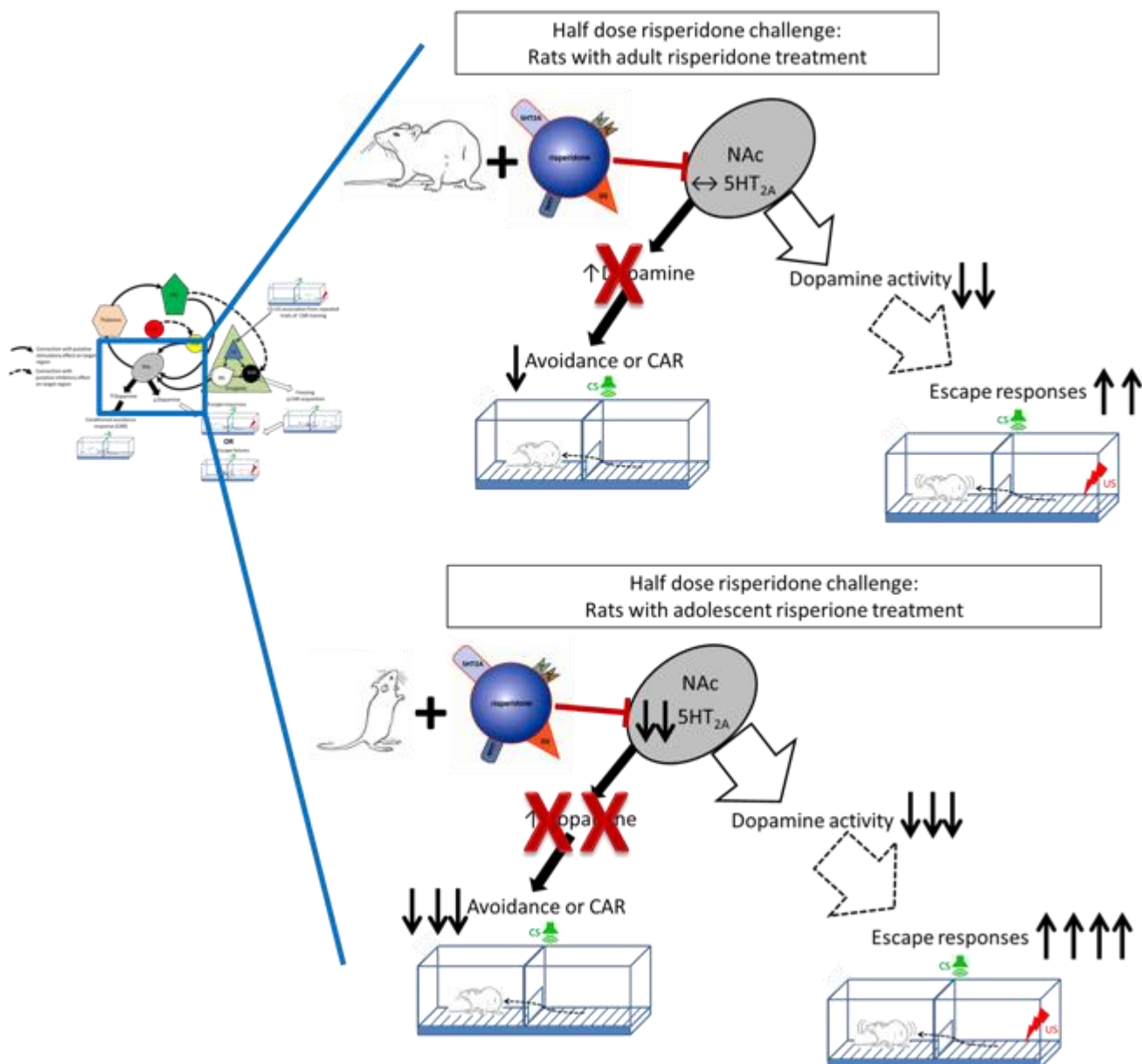


Figure 6-3 Long-term changes in CAR and alterations in neurotransmission in the nucleus accumbens (NAc) of rats with prior risperidone exposure in adolescence or adulthood. Downregulation of $5HT_{2A}$ receptors in the NAc is hypothesized to potentiate CAR suppression by dopamine-blocking effects of half dose risperidone challenge in rats with prior adolescent risperidone treatment. Escape failures induced by risperidone challenge were minimal in both age groups.

6.3.2. Risperidone-induced deficits in *acquisition* of CAR

Following seminal works and influential reviews by Wadenberg and colleagues (for examples, see (Wadenberg et al. 1990b; Wadenberg et al. 1998b; Wadenberg 2010; Wadenberg and Hicks 1999; Wadenberg et al. 2001b)), the majority of preclinical APD studies either in adolescent or adult rats that utilize CAR examined the *suppression* of previously acquired CAR in *well-trained* rats. Wadenberg and colleagues also recommended a selective inclusion of *well-trained* rats in order to avoid the potential confounds of freezing behaviour in the interpretation of the findings (Wadenberg and Hicks 1999). By contrast, only a limited number of studies have investigated the effect of *chronic* APD treatment on the *acquisition* of CAR in *previously untrained* rats. These existing studies (adult animals only) investigated CAR *acquisition* only *during* repeated or chronic treatment [for example, see (Aguilar et al. 1997; Drago et al. 1997; Li et al. 2004)]. To the best of my knowledge, no existing study has examined CAR *acquisition* after a drug-free interval from either adolescent or adult treatment with APDs.

Since the *acquisition* of CAR involves a co-ordinated interplay between multiple brain regions (See Section 6.2.), examination of CAR *acquisition* can provide a mechanistic insight into co-ordinated brain function and behaviour. Moreover, the ability to acquire CAR may also serve as an index of learning and memory or anxiety and fear behaviour. Therefore, in Chapter 5, rats with prior risperidone exposure as adolescents or adults were trained for CAR for 7 days and their ability to acquire CAR was examined. I show rats previously treated with risperidone as adolescents have a retarded ability to acquire the CAR, compared to similarly treated adults. Given that the NAc is the final common pathway to CAR *acquisition*, I hypothesized that neurochemical changes in this brain region would correlate with this observed *acquisition* deficit in animals exposed to risperidone as adolescents. To test this hypothesis, I examined monoamine levels in the NAc at the *conclusion* of the experiment. This *ex vivo* neurochemical examination did not identify any significant alterations in *baseline* pool of monoamines (Dopamine, 5HT and noradrenaline) and their metabolites in the NAc regardless of prior treatment history. Nonetheless, this finding does not exclude alterations in *dynamic* neurotransmission that happens during a behavioural performance in these rats as previously mentioned.

Examination of neural activity in specific region(s), for example, in the NAc with voltammetry or electrophysiology in a behaving animal, could provide insight into the dynamic nature of neurochemical changes in the NAc. Increases in dopamine release in the NAc are observed during avoidance when examined either with voltammetry (Oleson et al. 2012) or microdialysis (McCullough et al. 1993). By contrast, decreases in dopamine release in the NAc are accompanied by escape responses (Oleson et al. 2012). These alterations in the accumbal dopamine release are

thought to be due to different computation and encoding of the CS and the US by dopaminergic neurons which modulates incentive-motivational circuitry (Oleson and Cheer 2013). Therefore, a more detailed analysis of dopaminergic input in the NAc by voltammetry or electrophysiology will provide mechanistic insight into risperidone-induced deficits in CAR learning.

While the NAc serves as the final output pathway to CAR, other brain regions that closely interact with the NAc should also be further examined. As discussed earlier, neural activity in the VTA that provides dopaminergic input to the NAc, strongly influences *CAR acquisition* (Shumake et al. 2010). During adolescence, dopaminergic neurons in the VTA undergo major changes in electrophysiological properties (for example, firing rate) (McCutcheon et al. 2012; McCutcheon and Marinelli 2009) whereas afferent inputs to the VTA from other regions are still incomplete (Yetnikoff et al. 2014). It is still unknown how adolescent risperidone treatment can affect these maturation changes. In adults, 21-day APD treatment can reduce the activity of dopaminergic neurons (a state referred to as ‘depolarization block’) in the VTA or the SNr or both, depending on the drug type (Chiodo and Bunney 1983; Grace et al. 1997). Recently, this finding has been extended to adult offspring of a neurodevelopmental model of schizophrenia (Gill et al. 2014; Valenti et al. 2011). However, VTA neurophysiology has yet to be examined in adolescent risperidone treatment. Investigation of both short- and long-term changes in the VTA and the influence of these changes on the behaviour is therefore required.

Risperidone-induced changes in distinct amygdala nuclei may also play important roles in CAR learning. As introduced previously, amygdala nuclei such as the LA, the BA and the CeA and their connections with the NAc or the PFC are important in *CAR acquisition* (Choi et al. 2010a; Lázaro-Muñoz et al. 2010; Moscarello and LeDoux 2013; Ramirez et al. 2015). Acute administration of APDs in adult rodents is known to induce neuronal activation in the amygdala nuclei (Cohen et al. 2003; Park et al. 2011; Rebec et al. 1983; Takashi et al. 1983; Zhao and Li 2012). Chronic risperidone treatment in adults can also induce neurochemical changes in the amygdala nuclei [for example, see (Terry Jr et al. 2005)]. Yet, the current knowledge of adolescent risperidone treatment on the amygdala is rather limited. In adults, acute administration of APDs increases levels of dopamine and its metabolites in the amygdala but chronic administration induces tolerance to this effect (Essig and Kilpatrick 1991; Kurachi et al. 1995; Kurachi et al. 1994; Takashi et al. 1983). It is still unknown whether similar outcomes can be induced by risperidone treatment in adolescents or which neurotransmitter systems (glutamate, GABA, 5HT or dopamine) are targeted by this treatment.

Serotonergic pathways that have a close interaction with the dopaminergic system are also actively involved in the (associative) learning process (Cools et al. 2008; Harvey 2003; Olvera-Cortes et al.

2008). Downregulation of 5HT_{2A} receptors in the NAc was observed in another cohort of rats with the same adolescent risperidone treatment (Chapter 3). It is still unknown whether this downregulation of 5HT_{2A} receptors has played a role in inducing CAR *acquisition* deficits. Therefore, the role of 5HT systems in the acquisition of the CAR also requires investigation.

To sum up, the *acquisition* of CAR is a complex process involving multiple brain regions. Subsequent investigations of the neural mechanism(s) that underlie CAR learning deficits in rats with adolescent risperidone treatment will require use of techniques to assess neurochemistry and neurophysiology in one or more brain regions such as the NAc in behaving animals. Another outstanding question is whether impaired CAR *acquisition* represents a deficit in general learning ability as a consequence of risperidone exposure in adolescence. Rats treated with olanzapine as adolescents have also been reported to require longer time to reach a defined criterion when examined in delayed non-match to sample task in adulthood (Milstein et al. 2013). Together, the findings in the study of Milstein et al. and in the current thesis raise a hypothesis that APD treatment in adolescence can induce long-term deficits in learning in adulthood. Further investigations of learning and memory are therefore required.

6.4. Risperidone-induced changes in catalepsy: adolescents vs adults

Catalepsy is defined as an inability to correct an unusual posture (Sanberg et al. 1988). In preclinical studies, catalepsy is examined with either a horizontal bar test or grid test (Porsolt et al. 2010; Sanberg et al. 1988). APDs are known to induce catalepsy at high ('supra-therapeutic') doses which occupy >80% striatal dopamine D2 receptors (Wadenberg et al. 2000; Wadenberg et al. 2001b). When given repeatedly in adult rats, low doses of haloperidol which acutely do not induce catalepsy, also induce a progressive increase in this behavioural response (Banasikowski and Beninger 2012a; Schmidt et al. 1999).

At therapeutically relevant doses that occupy 60-80% dopamine D2 receptors, APDs are known to inhibit CAR selectively without affecting escape responses (no escape failures) (Natesan et al. 2008; Wadenberg 2010; Wadenberg and Hicks 1999; Wadenberg et al. 2001b). The dose of risperidone used in this study should achieve a dopamine D2 occupancy of approximately 80% (Wadenberg et al. 2001b). I have shown that the acute administration of this dose of risperidone did not induce a significant number of escape failures in adults (See Pilot Experiment, Chapter 3, Section 3.2). However, with repeated injections *during* chronic treatment, a significant level of escape failures became apparent only in adults (Chapter 3). Although not well-understood, APD-induced escape failures are thought to be due to the development of motor or extrapyramidal side effects from high striatal D2 receptor blockade (Shannon et al. 1999; Wadenberg 2010). Therefore,

I hypothesized the following: Escape failures observed in adult rats would be due to a progressive increase in catalepsy with repeated injections during chronic risperidone treatment. Adolescent rats would experience lower cataleptic responses and therefore lower escape failures during the same treatment. Indeed, supporting my hypothesis, the existing literature shows that the cataleptic responses in adults are higher than those in adolescents *during* 10-day repeated treatment with haloperidol (Wiley and Evans 2008).

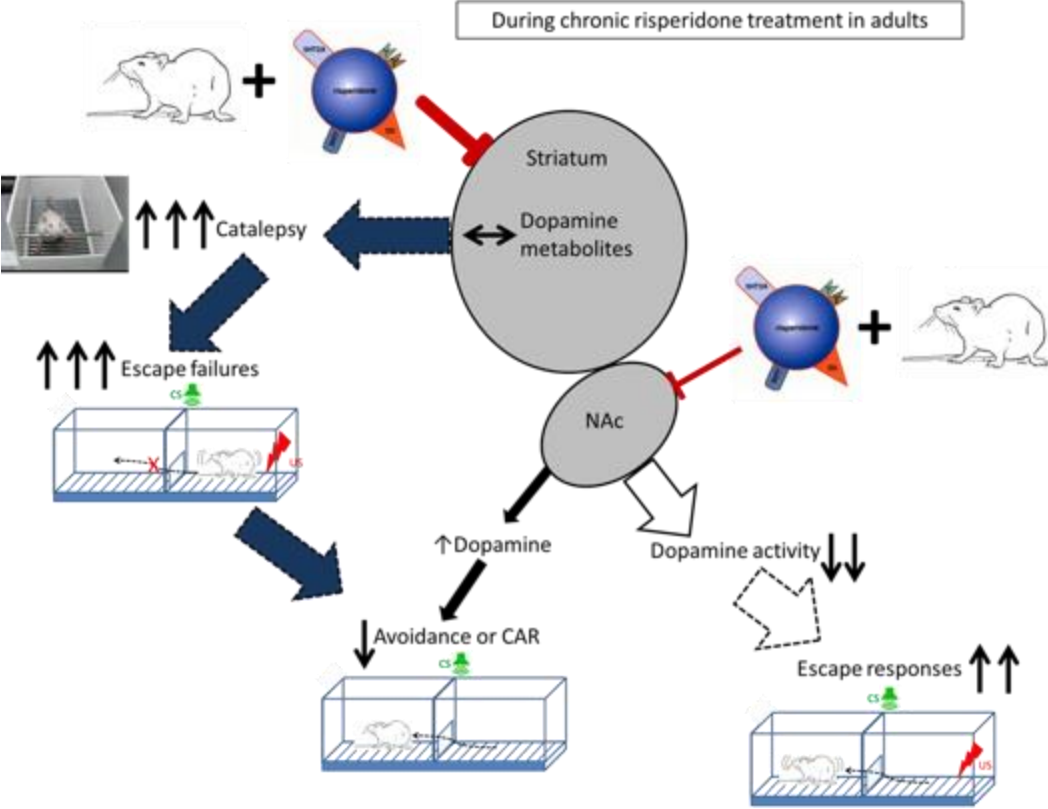
To examine these hypotheses, rats were treated for 22 days with risperidone either as adolescents or adults (Chapter 4). Cataleptic and locomotor responses were examined longitudinally from Day 1 to Day 17 of chronic treatment. Consistent with the reported findings of haloperidol treatment (Wiley and Evans 2008), a lower progression of cataleptic responses was observed in risperidone-treated adolescents, compared to similarly treated adult rats. Moreover, these changes in catalepsy were consistent with the findings in Chapter 3 in which I showed that adolescents developed lower levels of escape failures than adults *during* chronic risperidone treatment. This provides further support to the hypothesis that the observed escape failures in risperidone treated adults are due to cataleptic responses (Wadenberg 2010). This finding also further suggested that cataleptic responses might have partially contributed to *CAR suppression* at least in adults. By contrast, no differential effect on locomotion was observed in adolescents and adults. Given that catalepsy is mainly striatum-dependent (Ossowska et al. 1990; Yoshida et al. 1994) and locomotion mainly NAc-dependent (Beninger 1983; Kelly et al. 1975), this finding further suggested that striatum-dependent behavioural changes (catalepsy and escape failures) became more prominent with repeated risperidone injections in adults whereas the adolescents treated with the same risperidone regime were less sensitive to these behaviour changes (Figure 6-4).

The outstanding question therefore is: ‘Why are adolescent rats less sensitive to striatum-dependent behavioural outcomes than adult rats? The adolescent striatum is still immature with ongoing multiple remodelling processes, for example, in presynaptic dopaminergic innervation (Mathews et al. 2009; Matthews et al. 2013; Naneix et al. 2012; Stamford 1989), and dopaminergic (Andersen et al. 2000; Naneix et al. 2012; Tarazi and Baldessarini 2000; Tarazi et al. 1998; 1999; Teicher et al. 1995) and endocannabinoid receptors (de Fonseca et al. 1993; Klugmann et al. 2011). Therefore, I hypothesized that incomplete wiring of the adolescent striatum would contribute lower striatum-dependent behavioural outcomes in these rats (See the next section for further discussion).

Thus, I elected to examine the neurochemistry of the striatum from rats treated with risperidone in adolescence or adulthood in Chapter 4. Striatal dopamine metabolites were observed to be elevated selectively in risperidone-treated adolescent rats. Interestingly, a *negative* correlation between striatal dopamine metabolites and cataleptic responses were observed in risperidone-treated

adolescent rats. I speculate that increased striatal dopamine metabolites reflect increased dopamine availability in adolescents which in turn is able to counter the hypodopaminergia produced by risperidone. This could act to produce a lower cataleptic response. However, striatal dopamine levels were observed to be normal in these rats, at least at the time of *ex vivo* neurochemical examination. Still this finding does not exclude a possibility of increased dopamine availability in *behaving* rats. Therefore, further investigations are still required to support this hypothesis. Examination of baseline and/or stimulated dopamine release in the striatum with microdialysis, activity of midbrain dopaminergic neurons with electrophysiology [for example, (Grace et al. 1997)] or TH expression in midbrain neurons with immunohistochemistry [for example, (Levinson et al. 1998)] will enable a more accurate assessment of risperidone-induced changes in dopaminergic activity.

(a)



(b)

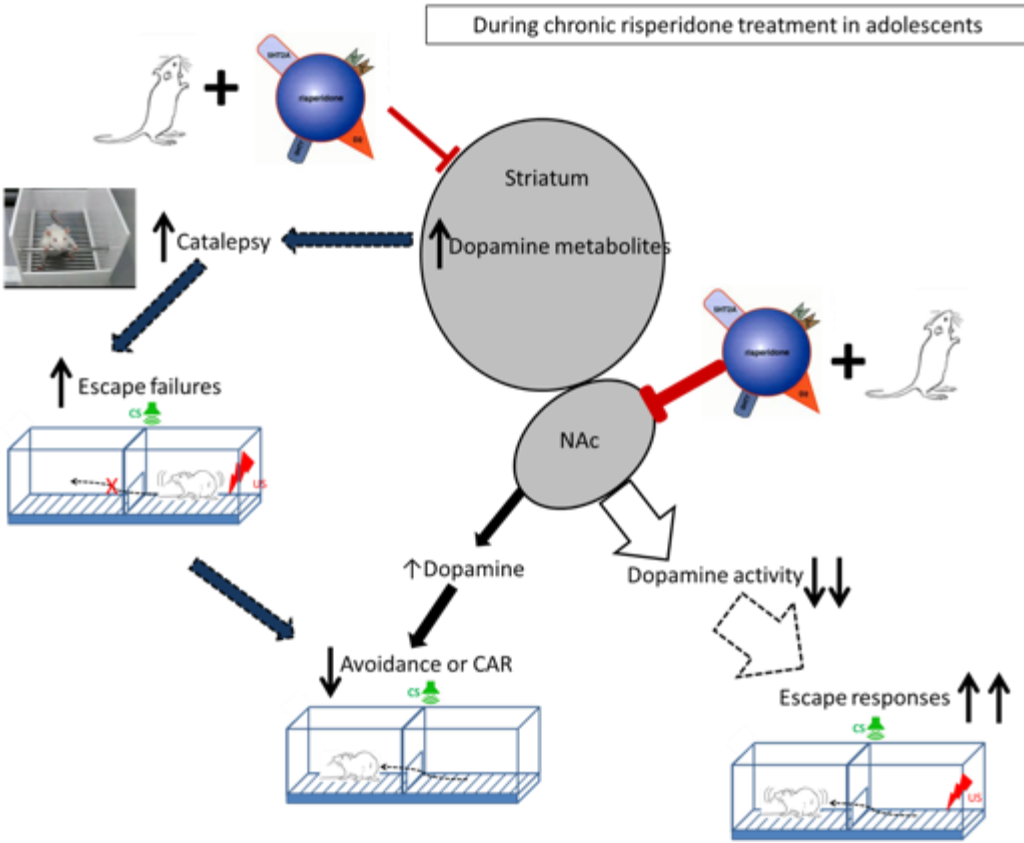


Figure 6-4 Schematic diagram of observed changes in cataleptic behaviour and its contribution to escape failures in the CAR tests during chronic risperidone treatment in adolescent or adult rats. (a) In adult rats, repeated risperidone induced a significant increase in catalepsy and escape failures, striatum-dependent behaviours. These behaviours would contribute to drug-induced CAR suppression observed in these rats. (b) In adolescent rats, given incomplete wiring of the striatum, repeated risperidone treatment did not induce a significant increase in catalepsy and escape failures to a level observed in adults. CAR suppression in adolescents would therefore be due to action mainly on the NAc.

Following up on short-term cataleptic outcomes (Chapter 4), I next examined long-term cataleptic outcomes in another cohort of animals in Chapter 5. Rats treated with risperidone or vehicle for 22 days in either adolescence or adulthood underwent an equivalent drug-free interval. Then all rats received a challenge of risperidone at the same dose they previously received during chronic treatment and their cataleptic behaviour was examined. Remarkably, rats with prior adult risperidone exposure were observed to have a robust sensitization-like cataleptic response. This was not observed in those animals with adolescent risperidone exposure. These findings further suggested that, even after drug-free interval, rats treated with risperidone in adolescence were still less sensitive to striatum-dependent behavioural changes.

However, when the striatal tissues were examined with HPLC, no significant alterations in *baseline* levels of monoamines and their metabolites were observed (Chapter 5). One caveat to the interpretation of this data is that *ex vivo* HPLC measurements of all available monoamines and their metabolites in both extracellular and intracellular pools do not reflect dynamic neurotransmission changes that occur under the influence of an APD or during behavioural performance. Another caveat is that neurotransmitter content was performed after 7 days of CAR training, not immediately after catalepsy testing. Neurochemical examination at a more proximal time point, for example, immediately after bar test, may perhaps provide a better insight into neurotransmission changes responsible for catalepsy while reducing any other possible confounds, for example, from CAR training.

Investigation of changes in the neural activity or neurochemistry in the presynaptic regions, for example, the substantia nigra, may also provide further mechanistic insight into long-term cataleptic response. In adults, APD-induced downregulation of TH levels in the SNr has been observed to last up to 4 weeks and this has been proposed to be one mechanism for extrapyramidal effects like catalepsy (Levinson et al. 1998). This may perhaps be a mechanism for differential catalepsy sensitization in rats with adolescent or adult risperidone exposure. Moreover, in adult rodents, chronic treatment with APDs can decrease the number of spontaneously firing neurons in SNr or VTA or both, a condition referred to as ‘depolarization block’ (Chiodo and Bunney 1983; Gill et al. 2014; Grace et al. 1997). These authors have also proposed a relationship between depolarization block of dopaminergic neurons and cataleptic potential of APDs. Therefore, further examination of midbrain dopaminergic activity is now required.

In addition to dopaminergic systems, changes in other neurotransmitter systems should also be investigated. For example, glutamatergic antagonists have been reported to reduce haloperidol-induced catalepsy sensitization (Riedinger et al. 2011). In particular, glutamatergic neurotransmission in the inferior colliculus appears to have an important role at least in haloperidol-

induced catalepsy (Medeiros et al. 2014; Melo et al. 2010). The neural mechanisms mediating risperidone-induced catalepsy sensitization remain unknown and further investigations are therefore required to better understand the neurobiological underpinnings of differential cataleptic outcomes with risperidone treatment in adolescence or adulthood.

6.5. Risperidone-induced behavioural changes in adolescents vs adults: A synthesis

Together, the findings in the preceding three chapters have provided insights into the neurobiological effects of chronic adolescent risperidone treatment. Risperidone treatment in adolescence was found to preferentially affect CAR – both *suppression* in previously trained rats and *acquisition* in untrained rats. This outcome became evident after a drug-free interval. By contrast, risperidone treatment in adulthood preferentially affected catalepsy both *during* chronic treatment and after a lengthy drug-free interval (See Section 6.7. for discussion on limitations in interpretation of these findings). As discussed in earlier sections, neurotransmission in the NAc is pivotal in both *acquisition* and *suppression* of the CAR. By contrast, catalepsy and escape failures are mainly mediated by the striatum. Therefore, I propose that two distinct brain regions, the NAc and the striatum, are respectively involved in age-dependent outcomes in CAR and catalepsy.

Differences in the effects of risperidone on the NAc and the striatum in adolescents may be related to relative maturation status of these two regions. Here I speculate that due to a less mature wiring state of the striatum, the adolescents are therefore less sensitive to risperidone-induced catalepsy (and escape failures), which are mainly striatum-dependent. In adolescents, presynaptic dopaminergic innervation of the striatum, as identified by TH levels, is still incomplete (Matthews et al. 2013). This may explain why the adolescent striatum has lower levels of dopamine release both at baseline (Naneix et al. 2012) and after stimulation (Matthews et al. 2013; Stamford 1989) compared to adults. Consistent with this, adolescent rats show lower levels of striatum-mediated stereotypic fine movements in response to acute amphetamine (Matthews et al. 2013). The adolescent striatum has also been observed to have higher dopamine turnover than the adult striatum (Naneix et al. 2012). On the other hand, TH innervation of the adolescent NAc is relatively mature (Matthews et al. 2013) (but see (Mathews et al. 2009) and dopamine release in the adolescent NAc was comparable to that in the adult NAc at both baseline and after amphetamine stimulation (Matthews et al. 2013). Dopamine turnover in the NAc is also similar in adolescents and adults (Naneix et al. 2012). Moreover, dopamine receptor changes in the striatum and the NAc also differ. During adolescence, dopamine receptors reach peak levels in the striatum, followed by pruning to adult levels but these changes in dopamine receptors are not seen in the adolescent NAc (Naneix et al. 2012; Teicher et al. 1995) (but see (Tarazi et al. 1998)). Moreover, task-evoked neural activity of the adolescent striatum in operant chambers is different from that of the adult

striatum, suggesting differential processing of stimuli in the striatum of adolescents and adults (Simon and Moghaddam 2015; Sturman and Moghaddam 2012). Therefore, it is plausible that differences in the wiring status of the NAc and striatum in the adolescents lead to differential effects of risperidone on these two brain regions and consequently produce differential effects on CAR and catalepsy.

The outstanding question is: ‘Given a relatively more mature state of the NAc, why are adolescents more sensitive to changes in CAR, which is dependent on this brain region?’ Although presynaptic *dopaminergic* innervation of the adolescent NAc has reached a relatively mature status, the levels of dopamine transporters in the NAc are lower in adolescents than in adults (Matthews et al. 2013). Also, the functional status of NAc medium spiny neurons (MSNs) at the level of integration of glutamatergic and dopaminergic inputs has not reached full maturity (Benoit-Marand and O'Donnell 2008; Huppe-Gourgues and O'Donnell 2012; Huppé-Gourgues and O'Donnell 2012). For example, dopamine D2 receptor-mediated modulation of cortically evoked excitatory postsynaptic potentials (EPSPs) in the accumbal MSNs changes from a suppressive effect in adolescents to a potentiating effect in adults (Benoit-Marand and O'Donnell 2008). Such functional maturation in the NAc can be arrested at the adolescent state by olanzapine treatment in adolescence (Brooks et al. 2016). Therefore, it is plausible that adolescent risperidone treatment has also altered or arrested the functional maturation of MSNs in the NAc and therefore produced changes in the CAR.

In the literature on addiction, *locomotor* sensitization is well-established with exposure to psychoactive or rewarding agents, for example, amphetamine [See review by (Vanderschuren and Kalivas 2000)]. In this thesis, sensitization-like responses in CAR and catalepsy were observed in rats with prior adolescent or adult risperidone exposure respectively. These findings raise the following questions: (1) Does risperidone induce behavioural sensitization like psychostimulants? (2) What mechanisms are common and what are different with regards to risperidone-induced behavioural sensitization (in CAR and catalepsy) and psychostimulant-induced *locomotor* sensitization? (3) How do mechanisms of risperidone-induced sensitization differ in adolescents and adults?

Behavioural sensitization is a progressive increase in a particular behavioural effect with repeated treatment with a drug and an enhanced response in this behaviour on re-exposure to this drug (Vanderschuren and Kalivas 2000). The findings of my thesis, together with those in the existing literature strongly suggest that APDs can produce behavioural sensitization. Here I showed risperidone-induced sensitization in CAR *suppression* and catalepsy, consistent with the reported findings in these two behaviours induced by repeated treatment with risperidone or other APDs such as haloperidol and olanzapine (for example, (Banasikowski and Beninger 2012b; Li et al.

2007; Qiao et al. 2014a; Schmidt et al. 1999)). In addition to CAR and catalepsy, APD-induced sensitization responses have also been demonstrated in other behaviours such as vacuous chewing movements (an index of APD-induced tardive dyskinesia in rodents) (Turrone et al. 2003; Turrone et al. 2005), *suppression* of PCP-induced hyperlocomotion (Shu et al. 2014a; Sun et al. 2009) and *suppression* of lever presses for reward (Trevitt et al. 1998; Varvel et al. 2002). Given the evidence supporting the phenomenon of ‘APD-induced behavioural sensitization’, another question is related to the neural mechanism(s) involved.

In *locomotor* sensitization to psychostimulants, such as amphetamine, two distinct stages are well-recognized: induction (repeated treatment) and expression (rechallenge). Different brain regions (the NAc, the PFC or the VTA) and neurotransmitter systems (dopamine or glutamate) are involved at these different stages (Vanderschuren and Kalivas 2000). It is still unclear whether sensitization to risperidone in either CAR or catalepsy operates according to similar mechanisms as found in *locomotor* sensitization to psychostimulants. The existing studies have shown that induction and expression stages also exist in APD sensitization in CAR (for example, (Qiao et al. 2013)) or catalepsy (for example, (Klein and Schmidt 2003)) [Also see review by (Li 2016)]. However, unlike well-studied *locomotor* sensitization to psychostimulants, the roles of different brain regions or neurotransmitter systems in sensitisation to APDs are far less well understood.

At least in adult APD treatment, an increase in D2 receptor activity, as indicated by heightened locomotor responses to quinpirole, is thought to mediate sensitized responses in CAR *suppression* (which was not observed in this thesis) (Gao and Li 2013; Li 2016). However, the locus of this increased D2 activity (whether in the striatum, the NAc or other brain region) is still unknown. This D2-mediated mechanism appears be less likely in sensitization in adolescent APD treatment. Heightened locomotor responses to quinpirole were not observed in animals that were treated as adolescents with risperidone (1 mg/kg/day for 5 days) (Qiao et al. 2014a) or haloperidol (0.05 or 0.25 mg/kg/day) (Gao and Li 2014) despite sensitized CAR *suppression* responses. Furthermore, a recent study did not find a quantitative change in D2 receptor protein levels in striatum, PFC and hippocampus of adult rats expressing sensitized CAR responses after adolescent asenapine treatment (Shu et al. 2014b). Here I showed that, at least in rats with prior adolescent risperidone exposure, 5HT_{2A} receptors in the NAc may be involved in sensitized CAR *suppression*. Further investigations are still required to better understand neurobiological underpinnings of APD-induced sensitization in CAR.

With regards to haloperidol-induced catalepsy sensitization in *adults*, different roles of dopamine receptors have been identified at different phases. In the induction phase, normal function of D1 receptors is required (Banasikowski and Beninger 2012a) whereas D3 receptors are involved in

expression phase (Banasikowski and Beninger 2012b). Antagonism at NMDA or AMPA receptors has also been shown to lower the rate of catalepsy sensitization during induction phase in adults (Riedinger et al. 2011). However, it is still unknown whether similar mechanisms operate in adolescents and adults with regards to catalepsy sensitization. My findings of differential catalepsy sensitization both *during* chronic treatment and after a drug-free interval strongly suggest that neural mechanisms differ in the two age groups.

At the broader level, how does the adolescent brain handle risperidone-induced neurobiological changes? Do risperidone-induced changes modify normal adolescent remodelling processes? The findings of this thesis, together with the reports in the literature, strongly suggest that normal maturation processes of the adolescent brain can be modified by adolescent APD treatment. For example, adolescent olanzapine treatment has been reported to alter dendritic spine pruning in a region-specific manner (Milstein et al. 2013). The findings of this thesis also suggested modification of adolescent maturation in the striatum. During normal development, dopamine metabolite levels in the striatum reach a peak around PND30 and fall to adult level by PND45 (Naneix et al. 2012). My findings in Chapter 4 indicated a possible alteration of this striatal maturation process by adolescent risperidone treatment as these rats still showed elevated levels of dopamine metabolites even at PND58. This finding raised a possibility of a protracted or arrested maturation of the striatum as a result of adolescent risperidone treatment. A similar ‘protracted’ maturation status has been identified in the activity of MSNs in the NAc after adolescent olanzapine treatment (Brooks et al. 2016). Moreover, neural adaptations during the drug-free interval also seem to play a role due to the following observations: (1) The observed short-term elevations in the striatal dopamine metabolites did not persist after a drug-free interval in rats with adolescent risperidone treatment. (2) Rats treated with risperidone in adolescence became more sensitive to changes in CAR (*suppression* or *acquisition*) after a drug-free interval. Therefore, a more thorough longitudinal assessment is still required to better understand the effects of risperidone on the adolescent brain remodelling.

6.6. Risperidone-induced neurobiological changes in adolescents: Translation back to the clinic?

Through a comparative examination of risperidone treatment in adolescent and adult rats, I showed that the adolescent and the adult brains have significant differences in behavioural and neurochemical responses to this APD regimen (See Section 6.7. for discussion on limitations in interpretation of these findings). This provides supporting evidence that neurobiological consequences of APDs on the adolescent brain cannot be extrapolated directly from the existing

findings in adults. This further suggests that a more thorough understanding of the effects of APDs on the adolescent brain is required.

In this thesis, I used CAR and bar test for catalepsy to probe risperidone-induced alterations in brain function and behaviour in adolescent and adult rats. A *direct* reverse-translation of observed behavioural changes in these two paradigms will not be feasible due to low face validity of these behaviours (Porsolt et al. 2010; Wadenberg and Hicks 1999) and low construct validity given the use of ‘neurodevelopmentally normal’ animals. However, my findings still have important clinical implications. Failure to acquire CAR in rats with adolescent risperidone exposure may perhaps represent an alteration in anxiety/fear status. Or this deficit in CAR *acquisition* may also suggest a deficit in general learning capability or cognitive performance, which needs to be further investigated. Sensitization-like responses in either CAR *suppression* or catalepsy after risperidone also indicate some long-term alteration in brain function. At the same time, these findings raise two questions: (1) Do APD-induced sensitized responses exist in the clinic? (2) What are the implications of APD-induced sensitization?

Psychotic symptoms in schizophrenic patients are thought to be due aberrant assignment of salience to normal stimuli by increased dopaminergic neurotransmission (Kapur 2003). By blocking dopaminergic neurotransmission, APDs are thought to reduce aberrant salience assignment and therefore psychosis (Kapur et al. 2006). In preclinical studies with CAR, the CS provides animals a motivational salience (to make an avoidance response) through dopaminergic neurotransmission in the NAc and APDs block this salience through dopaminergic blockade (Li et al. 2004). CAR suppression by APDs in rodents is therefore thought to be equivalent to APD-induced suppression of psychotic symptoms in schizophrenic patients (Kapur et al. 2006). In schizophrenic patients, APD treatment rapidly induces symptom reduction within 24 h of treatment (Kapur et al. 2005) and this symptom reduction progressively increases (over a duration of several weeks) as this treatment continues (Agid et al. 2003; Leucht et al. 2005) (Figure 6-5(a)). Similarly in preclinical studies in rodents, CAR suppression also progressively increases with repeated APD treatment (over a duration of several days) (Li et al. 2007; Samaha et al. 2008). In the clinic, patients treated with APDs especially with typical APDs can develop a syndrome of EPS called tardive dyskinesia (TD) and APD-induced TD progressively increases over time [See review (Waln and Jankovic 2013)]. Similarly in preclinical studies including the current thesis, cataleptic responses, an index of EPS, progressive increase with repeated APD treatment (Banasikowski and Beninger 2012a; Pezarro Schimmel et al. 2015; Schmidt et al. 1999; Wiley and Evans 2008) (Figure 6-5). Therefore, a progressive increase in symptom reduction or side effects observed in APD-treated patients has

been hypothesized to be analogous to the phenomenon of APD sensitization (CAR or catalepsy) seen in rodents (Li 2016).

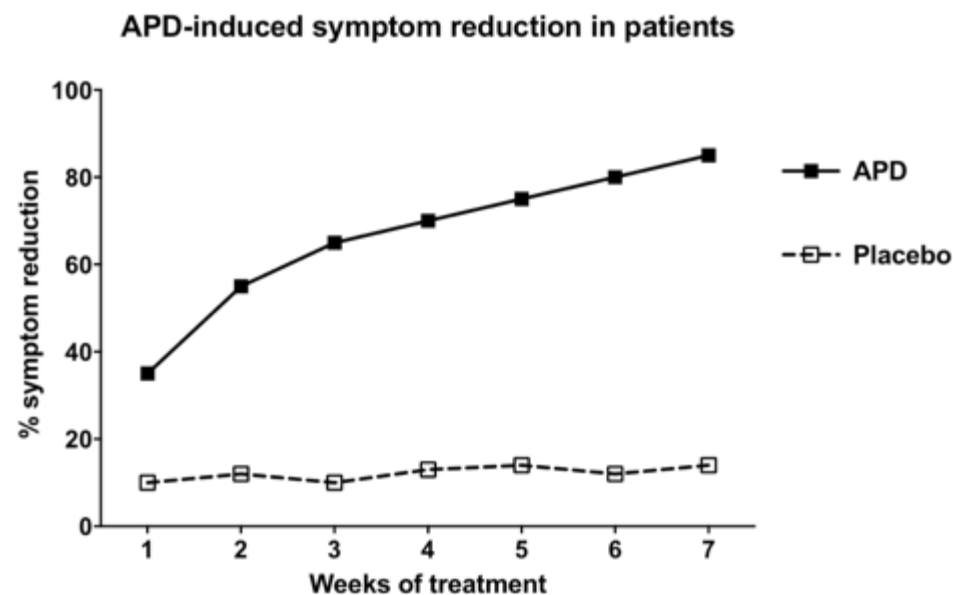
If the phenomenon of ‘APD sensitization’ does exist in the clinic, what are the implications? In sensitization to a drug, a behavioural response becomes increased on subsequent exposure to the same drug (drug challenge as examined here) or to another drug (via ‘cross-sensitization’). In addition, a specific behavioural response can also become decreased on subsequent exposure to another drug (via ‘cross-tolerance’). These outcomes will have important clinical implications, for instance, an adolescent patient who develops a sensitization to risperidone may become ‘cross-tolerant’ to clozapine if he/she needs a switch in APD type. The same patient may also require lower doses if the same APD treatment needs to be repeated for other behavioural symptoms. This same patient may also become ‘cross-sensitized’ to a psychostimulant and therefore more prone to addiction. A recent preclinical study using developmental MAM model has provided evidence for APD-induced cross-tolerance by demonstrating that MAM rats treated previously with haloperidol did not respond to novel agent $\alpha 5$ GABAA positive allosteric modulator (Gill et al. 2014). In the clinical settings, a drug challenge to examine a sensitization response, as has been done here in this thesis or preclinical studies, will not be feasible due ethical issues. Currently, neuroimaging paradigms translated from rodent CAR experiments are available to study the effects of APDs, for example, in healthy volunteers (Bolstad et al. 2015). Perhaps such kinds of neuroimaging studies may enable detection of risperidone-induced sensitization responses in the clinic.

Another potential implication is related to the finding of lower cataleptic sensitization, a striatum-dependent behaviour, in adolescent rats. Although a lower rate of catalepsy sensitization may simply be interpreted as a possibility of lower progression of EPS in patients, I speculate on the possibility of further clinical implications related to striatal function. Here, in this preclinical study, I suggest that chronic risperidone produces less effect on the adolescent striatum than it does on the adult striatum. In patients (often around the age of late-adolescence or young adulthood) at risk for development of schizophrenia, striatal dopamine synthesis capacity is increased and this increase tightly correlates with transition to full-blown psychosis (Howes et al. 2011; Howes et al. 2009). However, the outcomes of clinical trials on early intervention with APDs to prevent this transition in these at-risk individuals have been disappointing (Amos 2014; Preti et al. 2014; Stafford et al. 2013). Lack of effects of APDs in these individuals may be due the fact that APDs are not exerting the required action on the striatum as shown in this study.

In addition to behaviour tests, I also used invasive *ex vivo* neurochemical assessments, which clearly are not feasible in the clinical studies. Using these neurochemical assays, I here showed short-term increases in striatal dopamine metabolites and long-term reduction in accumbal levels of

5HT_{2A} receptor and COMT selectively in rats with adolescent risperidone exposure. Together with my behavioural findings this strongly suggests neurotransmission in the adolescent brain is persistently altered by prior risperidone exposure. How these neurochemical changes can be translated to the clinic, for example, the impact on neurobehavioural or neurocognitive outcomes is still to be investigated.

(a)



(b)

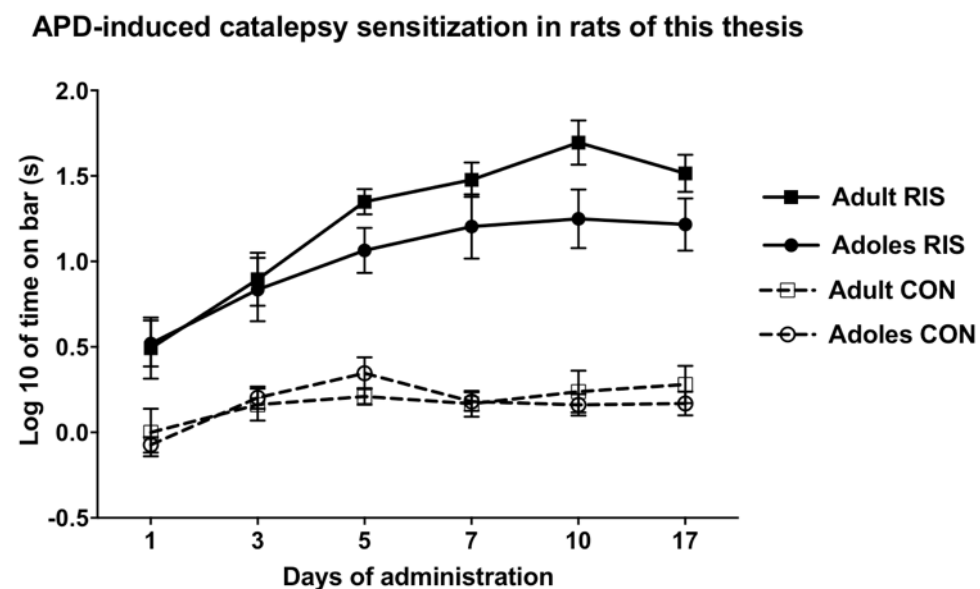


Figure 6-5 Time course of a progressive increase in APD effect (APD-induced sensitization) in both patients and in rodents (a) Schematic diagram shows that in schizophrenic patients, symptom reduction is observed within 24 h of APD administration (Kapur et al. 2005) and this symptom reduction, that is, drug effect, progressively increases over time i.e. sensitization [See details in (Agid et al. 2003; Leucht et al. 2005)]. (b) In male rats studied in this thesis, risperidone induced a small degree of catalepsy with the first dose (Day 1) and this cataleptic response, that is, drug effect, progressively increased over time, i.e. sensitization (Data reported in Chapter 4).

6.7. Limitations and future directions

While the three experiments in this thesis have examined short- and long-term behavioural and neurochemical changes induced by adolescent risperidone treatment, a few areas still need to be addressed. In my experiments, I used daily IP injections as the route of administration. However, as highlighted by (Kapur et al. 2003), this route may not be clinically relevant due to short half-life of APDs in rats which metabolize these drugs faster. Moreover, stress from repeated handling and injections may also have some confounds on the observed findings such as sensitized CAR *suppression*. Supporting this speculation, SC injections of a lower dose of haloperidol in adolescent rats have been reported to induce a more robust sensitized CAR suppression, compared to continuous administration of a higher dose of this APD via osmotic minipumps (Gao and Li 2014). Recent preclinical studies of adolescent APD treatment have attempted to achieve a more clinically comparable pharmacokinetic profiles through the use of oral administration via cookie dough (three times a day) (De Santis et al. 2016; Lian et al. 2015; Lian et al. 2016) or drinking water (Milstein et al. 2013; Vinish et al. 2013; Xu et al. 2015). Yet, only in one of these studies, plasma concentration of APD achieved was examined (Milstein et al. 2013). It is still unknown whether oral administration can induce similar behavioural and neurochemical outcomes as IPD injections. Therefore, a comparative examination of different routes of administration of risperidone in adolescent rats (IP Vs. drinking water Vs. cookie dough Vs osmotic minipumps) will provide an important insight to this phenomenon.

Due to differences in body size and composition between adolescent and adult rats, it is possible that pharmacokinetic factors (Spear 2007), such as relative absorption and distribution of risperidone in different body compartments may be different and any differences in pharmacokinetic factors could contribute to differential outcomes in adolescents and adults rats. In this thesis, I used the same dose of risperidone based on body weight but I did not examine plasma and brain tissue level of risperidone achieved in both age groups to address this issue. Therefore, it is highly recommended to examine the levels achieved in both plasma and brain during chronic treatment as well as after a drug-free interval. For example, in adult rats, haloperidol and risperidone are detectable in whole brain tissues even after a 14-day drug-free interval from chronic treatment although plasma levels were no longer detectable (Terry Jr et al. 2007a). Moreover, it is still to be examined whether this same dose can achieve the same level of dopamine receptor occupancy in adolescents and adults. This is relevant since the adolescent striatum is known to have higher levels of dopamine receptors (Tarazi and Baldessarini 2000; Teicher et al. 1995).

I selected behavioural assessments based on well-validated behavioural tests (CAR and catalepsy) which are specific and sensitive to APDs' action. The effects of APDs on other behavioural

domains, for example, cognition (Llorente-Berzal et al. 2012) or learning are still not well understood. Further examination of risperidone-induced outcomes in behavioural tests of cognition, learning and memory, such as novel object recognition and Morris Water Maze, will complement the current findings of learning deficits in CAR.

The clinical reports are now revealing that APD prescription is increasing not only in adolescents but also in children (Olfson et al. 2006; Olfson et al. 2012; Rettew et al. 2015; Ronsley et al. 2013). In this thesis I focussed on risperidone treatment only in adolescents. Therefore, a comparative examination of atypical APD treatment in juvenile and adolescent rats along with adult cohorts will enable identification of critical windows for specific therapeutic or detrimental outcomes. Adolescence in rats is often considered to have three stages: early (PND23-PND34), mid (PND34-PND46) and late (PND46-PND59) (Burke and Miczek 2014; Tirelli et al. 2003). In this thesis, I examined risperidone treatment starting from mid-adolescence (PND35-PND56). It is still to be investigated which stage of adolescence is the most critical window for inducing a certain behavioural or neurochemical outcome.

In my studies, I only utilized male animals given the scope of the studies. Gender differences in neural outcomes of adolescent APD treatment are still to be investigated. A recent study has elegantly demonstrated the need for inclusion of both males and females, by showing long-term differential behavioural outcomes in male and female rats treated with the same APD regimens in adolescence (De Santis et al. 2016). Future studies should therefore investigate sex-dependent outcomes of adolescent APD treatment.

Moreover, only ‘neurodevelopmentally normal’ adolescent rats were used in this thesis to identify neurobiological consequences specific to risperidone treatment. This use of ‘normal animals’ may not fully reflect the clinical scenario of APD prescription in adolescent patients with ‘neurodevelopmentally altered’ brain. Examination of adolescent risperidone treatment in rodent models of neuropsychiatric disorders [for example, (Piontkewitz et al. 2012; Zhu et al. 2014)] may complement the findings of the current thesis. It is still unknown how APD-induced sensitization can affect behavioural deficits in animal models of neuropsychiatric disorders. At least, sensitized CAR suppression has also been reported with adolescent olanzapine treatment in MIA model (Chou et al. 2015). These authors proposed that although the magnitude and temporal pattern of this sensitized CAR response in MIA animals was similar that seen in ‘normal’ adolescent rats with olanzapine treatment, underlying neural mechanism(s), at least in hippocampal cell proliferation or survival, would be different (Chou et al. 2015). Examination of APD-induced sensitization in rodent models of neuropsychiatric disorders is therefore another possible future direction.

In this thesis, ^1H MRS examination was performed under isoflurane anaesthesia. Therefore potential confounds of the effects of isoflurane anaesthesia on the observed metabolites levels cannot be excluded. Imaging in awake rodents may perhaps provide a better measurement of brain metabolites. In addition, the impact of isoflurane on the adolescent brain maturation is still not known. Deficits in CAR acquisition in rats with adolescent risperidone exposure may perhaps be partially due to the effects of isoflurane and this still need to be investigated. Another limitation of the current thesis is that ^1H MRS examination was focussed on the NAc. Changes in metabolites other brain regions, for example, the PFC, still need to be examined.

An outstanding question that needs to be addressed from Chapter 3 is whether different drug-free intervals in adolescent and adult cohorts lead to differential behavioural responses in CAR (sensitized suppression) and neurochemistry (5HT_{2A} receptor downregulation) selective to adolescents. Although I intended to examine the role of drug-free interval in Chapter 5, this aim was not achieved given a retarded learning of the CAR in adolescent risperidone cohort. Therefore, a more thorough examination of behavioural and neurochemical outcomes after the same drug washout period in adolescent and adult cohorts is still required.

The functional consequences of 5HT_{2A} receptor downregulation in the NAc in rats with adolescent risperidone treatment are still known. Given involvement of 5HT receptors in associative learning (Harvey 2003), investigation of the role of 5HT_{2A} receptor downregulation in the CAR learning is also warranted in rats with adolescent risperidone treatment. As thoroughly reviewed elsewhere (Zhang and Stackman 2015), 5HT_{2A} receptors are strongly involved in behaviours such as novel object recognition and spatial cognition in addition to major neuropsychiatric disorders including depression, schizophrenia and Alzheimer's disease. Therefore, functional significance of 5HT_{2A} receptor downregulation in other behaviours apart from the CAR is also yet to be explored.

Ex vivo neurochemical assessments in this thesis focussed on dopamine-enriched brain regions such as the striatum and the NAc. *In vivo* assessments of neural signalling, for example, with microdialysis, voltammetry or electrophysiology, in behaving animals (for instance, (McCullough et al. 1993; Oleson et al. 2012; Sturman and Moghaddam 2012)) will enable identification of neurotransmission changes relevant to impaired CAR acquisition or catalepsy sensitization. Examination of the PFC, midbrain dopaminergic regions (VTA and SNr) or amygdala will provide further mechanistic insights into these behavioural changes. For instance, the firing rate of dopaminergic neurons in the adolescent VTA is higher, compared to the adult VTA counterparts (McCutcheon et al. 2012; McCutcheon and Marinelli 2009). The effects of adolescent risperidone treatment on these VTA neurons are still unknown. Changes in VTA neurons may perhaps underlie risperidone-induced deficits in CAR acquisition or sensitized CAR suppression. In addition to

changes in dopaminergic and serotonergic systems, APD-induced changes in the endocannabinoid systems should also be investigated. Given a report that short-term endocannabinoid CB1 signalling could be altered by adolescent APD treatment in a drug- and sex-dependent manner (Wiley et al. 2008b), examination of CB1 receptor changes with adolescent risperidone treatment could be another promising direction in the future..

6.8. Conclusion

To conclude, using adolescent male SD rats, this thesis has examined short- and long-term neurobiological outcomes of adolescent risperidone treatment in comparison with the same treatment regime in adults. I used CAR and catalepsy tests, which are sensitive and specific for the actions of APDs on brain function. Along with these behavioural assessments, clinically relevant examination with MRI and ¹H MRS and end-point neurochemical assays of the striatum and the NAc were performed. Through these assessments in this thesis, age-dependent behavioural outcomes were observed to be induced by the same risperidone treatment – In adolescent rats, the CAR was more preferentially affected by risperidone treatment while the outcomes in the catalepsy (and escape failures) are less prominent, compared to adults. Accompanying these behavioural changes, short-term elevations in dopamine metabolites in the striatum and long-term downregulation of 5HT_{2A} receptor and COMT in the NAc were observed selectively with adolescent risperidone treatment. No short-term alteration in NAc metabolites or long-term change in brain structure was observed with the current risperidone regimen in adolescents and adults. My findings provide supporting evidence that the adolescent brain differs markedly from the adult brain in response to risperidone. In short, findings from this thesis plainly indicate that adolescents are not ‘little adults’ and that adolescent APD prescription practices cannot just be extrapolated from adult findings or guidelines. Given risperidone is the most commonly prescribed atypical APD to adolescents, these findings may prove clinically relevant, providing new directions for clinical research on the outcomes of APD treatment in adolescents. These preclinical findings could help shape future clinical trials which will more extensively examine the neurobiological outcomes of adolescent APD prescription.

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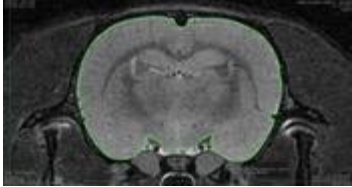

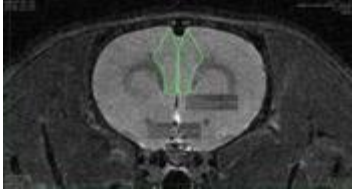
Appendices


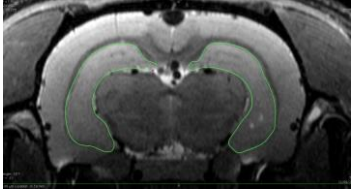
Appendix A – Primer information*Information on the primers used in RT-PCR experiments*

Target Gene	NCBI reference sequence	Forward Primer	Reverse Primer	Product Size (bp)
GAPDH	NM_017008.4	ATCCTGCACCACCAACTGCT	GGGCCATCCACAGTCTTCTG	123
Dopamine D1	NM_012546.2	GGACACCGAGGATGACAACT	TGGCTACGGGGATGTAAAAG	89
Dopamine D2	NM_012547.1	CCCAGAGAGGACCCGGTATAG	CTGGTTTGGCAGGACTGTCA	110
Tyrosine hydroxylase (TH)	NM_012740.3	GCTTCAATGACGCCAAGGAC	CTGGATGGTGTGAGGGCTGT	122
5-hydroxy tryptamine 2A (5HT _{2A})	NM_017254.1	GCGATCTGGATTTACCTGGA	CACACGGCAATGATTTTCAG	155
Catechol-O-methyl-transferase (COMT)	NM_012531.2	ATCTTCACGGGGTTTCAGTG	GAGCTGCTGGGGACAGTAAG	145
Monoamine oxidase-A (MAO-A)	NM_033653.1	GTGTGGAACCCCTTGGCATA	GTCCCATTCCTGAGCGTGTC	117
Monoamine oxidase-B (MAO-B)	NM_013198.1	GATTCCCAGTGATGCTCCAT	ATGGGTCTCCGCAGTTACAC	151
Glutamic acid decarboxylase 65 (GAD65)	NM_012563.1	TGAGGGAAATCATTGGCTGGC	TCTGACGTGAATGCGATGAGC	166

Appendix B – MRI image segmentation

MRI Regions of interest and their identification landmarks

Region of interest	Anatomical boundaries and criteria	Reference to Rat Brain Atlas	No of slices of analyzed	Example ROI
Whole brain (WB)	<p>Rostral start from the base of olfactory bulb (at the demarcation of the olfactory bulb and frontal association cortex)</p> <p>Caudal end at the last slice containing cerebral cortex</p>	Approximately +5.62 mm to -9.36 from bregma)	16-17 (every second slice)	
Striatum	<p>Rostral start at the slice containing caudate putamen surrounded by forceps minor of corpus callosum</p> <p>Caudal end at the slice when the lateral border of hippocampus is lower than the medial</p> <p>Anatomical borders – corpus callosum superiorly, external capsule laterally, lateral ventricles medially</p>	Approximately +2.52 mm to -3.6 mm from bregma	11-13 continuous slices	
Prefrontal Cortex (PFC, Prelimbic and Infralimbic Cortex)	<p>Rostral start at the slice where forceps minor of corpus callosum starts to form clearly</p> <p>Caudal end at the slice immediately before decussation of corpus callosum</p> <p>Anatomical borders – superiorly the line</p>	Approximately from +4.2 mm to +2.52 mm from Bregma	4-5 continuous slices	

	joining the genu of corpus callosum and the most medial ventral point of cortex (at about 45 degree); inferiorly the horizontal line joining the tail of the corpus callosum; laterally the corpus callosum.			
Cerebral Cortex (CCx)	Rostral start from the base of olfactory bulb (at the demarcation of the olfactory bulb and frontal association cortex) Caudal end at the last slice containing cerebral cortex	Approximately +5.62 mm to -9.36 from bregma	16-17 (every alternate slice)	
Hippocampus	Rostral start - when the CA and dentate gyrus coincide with dorsal hippocampal commissure Caudal end at the loss of contrast between the corpus callosum and the subiculum, the absence of the dentate gyrus, and the clear separation of the two cerebral hemispheres	Approximately -1.92 mm to -8.04 mm from bregma	14-15 continuous slices	

Appendix C – Pilot ¹H MRS experiment

Pilot experiment: Examination of accumbal metabolites with acute risperidone administration

Introduction

In this pilot experiment, acute effects of single dose administration of risperidone on accumbal metabolites were examined in adolescents and adults. N-acetylaspartate (NAA), glutamate and GABA were of interest in this experiment, given reported changes in these metabolites with APD treatment.

The aims of this pilot experiment are as follows:

- (1) to establish the feasibility of ¹H MRS examination of time course of risperidone-induced metabolite changes
- (2) to examine whether acute administration of risperidone can differentially affect accumbal metabolites in adolescents and adults

Materials and methods

Subjects

Male adolescent and adult (PND33-35 and PND100-103 respectively at the time of examination) rats were used (n = 8, 4, 7 and 6 respectively for adolescent risperidone, adolescent vehicle, adult risperidone and adult vehicle groups). Rats assigned to risperidone and control groups arrived at different time points with an interval of approximately 1 month.

¹H MRS scans with administration of risperidone

After induction of anaesthesia with 5% isoflurane, each rat received a *subcutaneous* (SC) catheterization using 30G cannula and PE50 tubing (Fisher Scientific). After mounting of the rat on the animal bed of 9.4T MRI scanner, anaesthesia was maintained at 1.5-1.7% isoflurane and O₂ flow rate of 1.2 L/min. Axial and sagittal anatomical scans were obtained for localization of voxel bilaterally over the NAc (6 x 2 x 2 mm³ for adults and 5.5 x 1.8 x 2 mm³ for adolescents). A smaller voxel size was used in adolescent cohorts to accommodate smaller NAc.

After minimizing magnetic field inhomogeneity with B₀ map acquisition, first, second and third order shimming was carried out with MAPSHIM. Following acquisition of a reference non-suppressed water spectrum, a baseline water-suppressed ¹H MRS spectrum was obtained from the

voxel placed bilaterally on the NAc using PRESS sequence (TE = 9.9 ms; TR = 2500 ms; averages = 356; repetition = 1, time taken = ~ 14.5 min). Immediately after the baseline scan, a single time course ^1H MRS scan with either 1.3 mg/kg risperidone or vehicle challenge was performed on the same voxel using the following PRESS sequence: TE = 9.9 ms; TR = 2500 ms; averages = 96; repetition = 15, time taken = 60 min. As shown in Figure 1, a vehicle injection was remotely administered SC immediately after completion of the first repetition and risperidone or another vehicle injection after fourth repetition. Changes in accumbal metabolites were examined for the next 11 repetitions i.e. approximately for another 44 min.

All ^1H MRS data were processed on TOPSPIN and analysed in LCModel (version 6.3-1J) software (Provencher 1993), using the reference basis sets with the same data acquisition parameter. Metabolites with Cramer-Rao Lower bound (CRLB) or %SD > 20 were rejected from the analysis unless otherwise stated. The concentration of individual metabolites was expressed as a ratio to total creatine (Cr + PCr) following the guidelines in the LCModel manual.

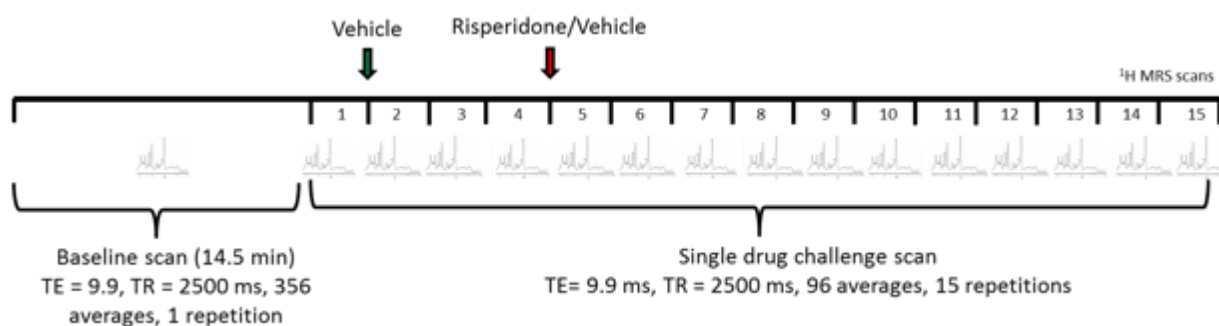


Figure 1 Timeline of ^1H MRS data acquisition with acute administration of 1.3 mg/kg risperidone or vehicle in adolescents and adults.

Statistical analysis

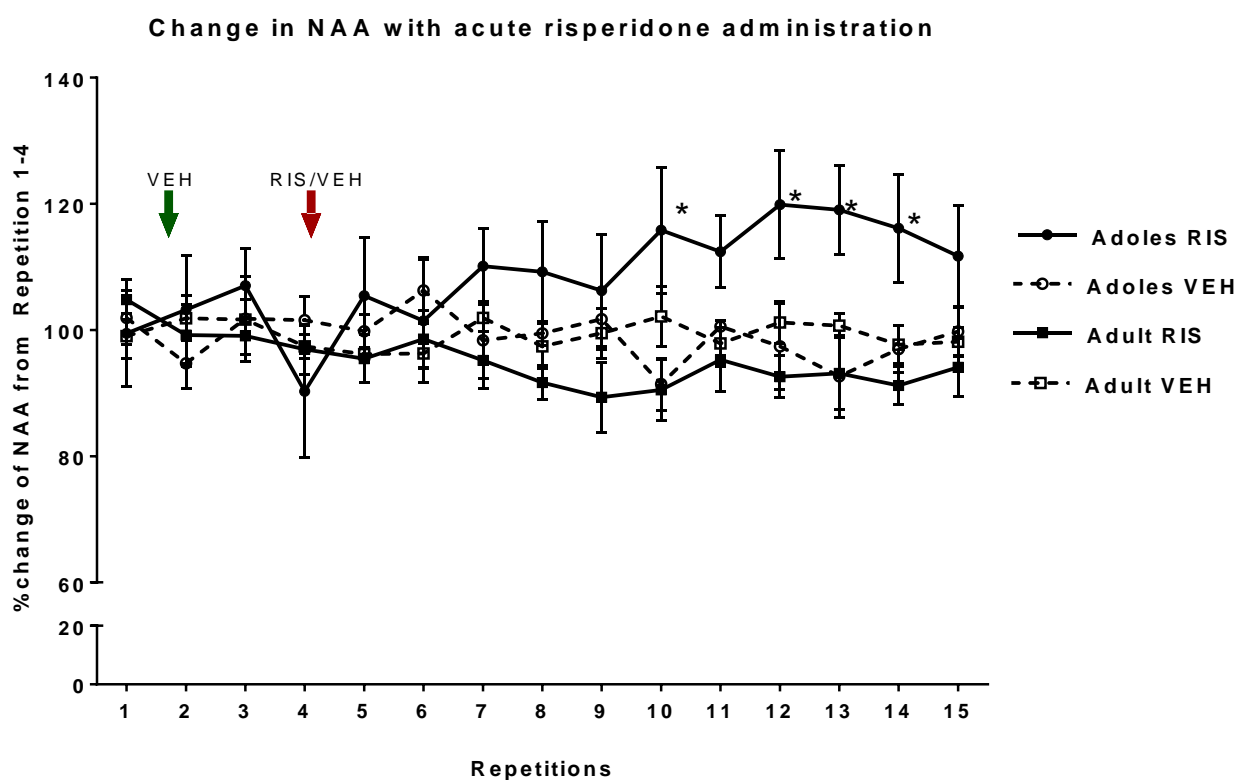
Baseline levels of metabolites were analysed with two-way ANOVA. Levels of metabolites from time course scan were transformed as %change from pre-drug levels (from the average level of repetitions 1-4) and analysed with repeated measures two-way ANOVA, followed by Dunnett's tests. Statistical significance was defined as $p < 0.05$.

Results

Examination of baseline levels showed that control groups (vehicle-vehicle) had significant higher NAA and glutamate levels than risperidone groups: two-way ANOVA for NAA: significant main effect of drug ($F_{1,21} = 15.077$, $p = 0.001$), age ($F_{1,21} = 4.512$, $p = 0.046$) but no age x drug interaction ($F < 1$, $p > 0.8$); two-way ANOVA for glutamate: significant main effect of drug ($F_{1,21}$

= 9.062, $p = 0.007$ but no main effect of age or age x drug interaction (both $F < 2.9$, both $p > 0.1$). Given CRLB was $> 25\%$, GABA data were not further analysed.

Given the observed baseline differences, data from time course 1H MRS scan were analysed as %change from the mean level of repetitions 1 to 4, that is, pre-drug levels. As shown in Figure 2, following the injection of risperidone, a progressive increase in NAA levels was observed and this was selective to adolescent risperidone group (repeated measures two-way ANOVA: a significant main effect of repetition x drug x age ($F_{14,266} = 3.013$, $p < 0.001$) but no main effects of repetition, age, drug, repetition x drug, repetition x age or age x drug (all $F < 3.2$, $p > 0.07$). Further examination at individual repetitions showed that risperidone-treated adolescents had significantly higher NAA levels than risperidone-treated adults at repetitions 10, 12, 13 and 14 (all $p < 0.05$) and vehicle-treated controls at repetition 13 ($p < 0.01$).



*Figure 2 Change in NAA levels in the nucleus accumbens with acute administration of risperidone in adolescents and adults. * $p < 0.05$ for Adoles RIS vs Adoles VEH at repetition 13 and for Adoles RIS vs Adult RIS at repetition 10, 12, 13, 14. Data are expressed as mean \pm SEM. Adoles – adolescent; RIS – risperidone; VEH – vehicle; Data from two risperidone-treated rats were discarded given CRLB $> 20\%$.*

Repeated measures two-way ANOVA of changes in glutamate also showed a significant main effect of repetition ($F_{14,294} = 2.448$, $p = 0.002$) and repetition x age x drug ($F_{14,294} = 2.448$, $p =$

0.003) without a significant main effect of age, drug, age x drug, repetition x age, repetition x drug (all $F < 1.7$, $p > 0.05$). Further examination at individual time points however did not reveal any significant difference among the four groups (Figure 3).

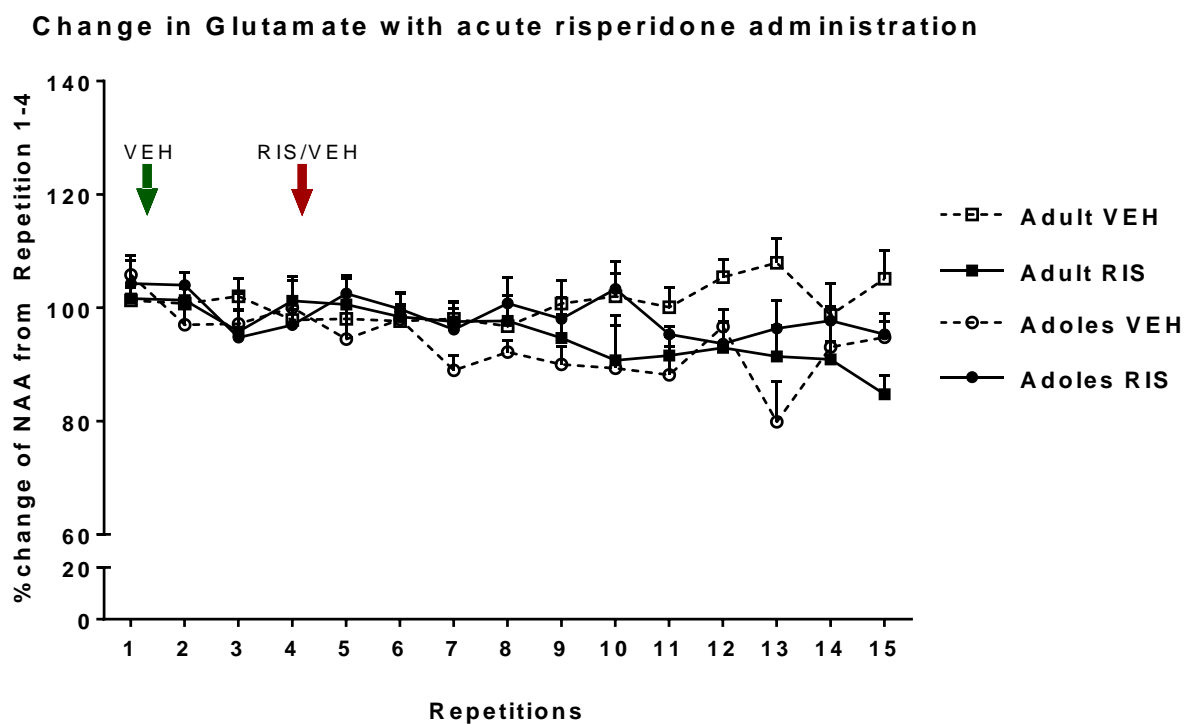


Figure 3 Change in glutamate levels in the nucleus accumbens with acute administration of risperidone in adolescents and adults. Data are expressed as mean \pm SEM. Adoles – adolescent; RIS – risperidone; VEH – vehicle;

Appendix D – ¹H MRS findings of Chapter 5

Levels of neural metabolites in the NAc at baseline and after administration of risperidone/vehicle at Day 1 and Day 22 of chronic treatment in Chapter 5 are shown in the following tables

Table 1 Levels of accumbal metabolites at baseline and after administration of risperidone or vehicle at Day 1 of chronic treatment in adolescence or adulthood

Metabolite	Metabolite concentration (/tCr) (mean \pm SEM) at Day 1																
	Time block	BL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Glutamine	Adoles CON	0.62 \pm 0.02	0.67 \pm 0.02	0.65 \pm 0.02	0.67 \pm 0.02	0.65 \pm 0.03	0.63 \pm 0.02	0.66 \pm 0.02	0.64 \pm 0.03	0.66 \pm 0.02	0.62 \pm 0.02	0.64 \pm 0.03	0.67 \pm 0.02	0.66 \pm 0.02	0.66 \pm 0.03	0.61 \pm 0.02	0.60 \pm 0.02
	Adoles RIS	0.64 \pm 0.02	0.68 \pm 0.02	0.67 \pm 0.02	0.65 \pm 0.02	0.63 \pm 0.03	0.62 \pm 0.02	0.65 \pm 0.02	0.67 \pm 0.03	0.72 \pm 0.02	0.65 \pm 0.02	0.66 \pm 0.03	0.67 \pm 0.02	0.69 \pm 0.02	0.66 \pm 0.03	0.68 \pm 0.02	0.66 \pm 0.02
	Adult CON	0.60 \pm 0.02	0.65 \pm 0.02	0.67 \pm 0.02	0.69 \pm 0.02	0.65 \pm 0.03	0.66 \pm 0.02	0.64 \pm 0.02	0.63 \pm 0.03	0.66 \pm 0.02	0.62 \pm 0.02	0.63 \pm 0.03	0.63 \pm 0.02	0.63 \pm 0.02	0.62 \pm 0.03	0.60 \pm 0.02	0.61 \pm 0.02
	Adult RIS	0.59 \pm 0.02	0.63 \pm 0.02	0.66 0.02	0.66 \pm 0.03	0.67 \pm 0.03	0.64 \pm 0.03	0.66 \pm 0.02	0.65 \pm 0.03	0.68 \pm 0.03	0.63 \pm 0.02	0.65 \pm 0.03	0.61 \pm 0.03	0.66 \pm 0.02	0.67 \pm 0.03	0.63 \pm 0.02	0.61 \pm 0.02
	Stats	Time - $F_{15,660} = 3.396, p < 0.001^{***}$; Time x Age - $F_{15,660} = 1.123, p = 0.331$; Time x Drug - $F_{15,660} = 0.991, p = 0.463$; Time x Age x Drug - $F_{15,660} = 0.624, p = 0.856$; Age - $F_{1,44} = 0.830, p = 0.367$; Drug - $F_{1,44} = 0.810, p = 0.373$; Age x Drug - $F_{1,44} = 0.187, p = 0.668$															
Glutathione	Adoles CON	0.29 \pm 0.01	0.27 \pm 0.01	0.32 \pm 0.01	0.32 \pm 0.01	0.31 \pm 0.02	0.28 \pm 0.02	0.32 \pm 0.02	0.32 \pm 0.01	0.29 \pm 0.01	0.31 \pm 0.02	0.28 \pm 0.01	0.33 \pm 0.02	0.31 \pm 0.02	0.33 \pm 0.02	0.31 \pm 0.02	0.31 \pm 0.01
	Adoles RIS	0.30 \pm 0.01	0.29 \pm 0.01	0.29 \pm 0.01	0.32 \pm 0.01	0.32 \pm 0.02	0.31 \pm 0.02	0.31 \pm 0.02	0.29 \pm 0.01	0.31 \pm 0.01	0.32 \pm 0.02	0.30 \pm 0.01	0.33 \pm 0.02	0.32 \pm 0.02	0.34 \pm 0.02	0.34 \pm 0.02	0.33 \pm 0.01
	Adult CON	0.30 \pm 0.01	0.32 \pm 0.01	0.30 \pm 0.01	0.31 \pm 0.01	0.32 \pm 0.01	0.34 \pm 0.02	0.31 \pm 0.02	0.32 \pm 0.01	0.31 \pm 0.01	0.32 \pm 0.01	0.33 \pm 0.01	0.32 \pm 0.02	0.32 \pm 0.02	0.31 \pm 0.02	0.32 \pm 0.02	0.31 \pm 0.01
	Adult RIS	0.31 \pm 0.01	0.31 \pm 0.01	0.30 \pm 0.01	0.33 \pm 0.01	0.32 \pm 0.01	0.30 \pm 0.02	0.34 \pm 0.02	0.32 \pm 0.01	0.32 \pm 0.01	0.32 \pm 0.01	0.30 \pm 0.01	0.31 \pm 0.02	0.31 \pm 0.02	0.34 \pm 0.02	0.32 \pm 0.02	0.32 \pm 0.01
	Stats	Time - $F_{15,615} = 1.901, p = 0.021^*$; Time x Age - $F_{15,615} = 0.886, p = 0.581$; Time x Drug - $F_{15,615} = 0.691, p = 0.795$; Time x Age x Drug - $F_{15,615} = 1.242, p = 0.235$; Age - $F_{1,41} = 1.009, p = 0.321$; Drug - $F_{1,41} = 0.275, p = 0.603$; Age x Drug - $F_{1,41} = 0.234, p = 0.634$															

Myo-Inositol	Adoles CON	0.67 ± 0.02	0.59 ± 0.03	0.60 ± 0.03	0.59 ± 0.02	0.58 ± 0.02	0.54 ± 0.03	0.57 ± 0.03	0.57 ± 0.03	0.55 ± 0.02	0.57 ± 0.03	0.57 ± 0.03	0.56 ± 0.03	0.56 ± 0.03	0.59 ± 0.03	0.55 ± 0.02	0.58 ± 0.02
	Adoles RIS	0.70 ± 0.02	0.59 ± 0.03	0.62 ± 0.03	0.60 ± 0.02	0.60 ± 0.02	0.61 ± 0.03	0.64 ± 0.03	0.61 ± 0.03	0.62 ± 0.02	0.61 ± 0.03	0.60 ± 0.03	0.61 ± 0.03	0.59 ± 0.03	0.64 ± 0.03	0.64 ± 0.02	0.63 ± 0.02
	Adult CON	0.73 ± 0.02	0.70 ± 0.03	0.70 ± 0.03	0.72 ± 0.02	0.68 ± 0.02	0.68 ± 0.03	0.69 ± 0.03	0.69 ± 0.03	0.72 ± 0.03	0.65 ± 0.03	0.71 ± 0.03	0.69 ± 0.03	0.68 ± 0.03	0.67 ± 0.03	0.69 ± 0.02	0.72 ± 0.02
	Adult RIS	0.77 ± 0.02	0.71 ± 0.03	0.68 ± 0.03	0.73 ± 0.02	0.69 ± 0.02	0.68 ± 0.03	0.71 ± 0.03	0.70 ± 0.03	0.71 ± 0.02	0.68 ± 0.03	0.69 ± 0.03	0.72 ± 0.03	0.69 ± 0.03	0.70 ± 0.03	0.72 ± 0.02	0.69 ± 0.02
	Stats	Time - $F_{15,660} = 5.413, p < 0.001^{***}$; Time x Age - $F_{15,660} = 1.358, p = 0.162$; Time x Drug - $F_{15,660} = 0.962, p = 0.494$; Time x Age x Drug - $F_{15,660} = 0.655, p = 0.829$; Age - $F_{1,44} = 32.863, p < 0.001^{***}$; Drug - $F_{1,44} = 2.125, p = 0.152$; Age x Drug - $F_{1,44} = 0.768, p = 0.385$															
Taurine	Adoles CON	0.84 ± 0.02	0.80 ± 0.03	0.81 ± 0.03	0.79 ± 0.02	0.75 ± 0.03	0.77 ± 0.02	0.807 ± 0.03	0.75 ± 0.03	0.79 ± 0.02	0.78 ± 0.03	0.75 ± 0.02	0.78 ± 0.03	0.73 ± 0.03	0.78 ± 0.03	0.80 ± 0.02	0.73 ± 0.03
	Adoles RIS	0.87 ± 0.03	0.80 ± 0.03	0.78 ± 0.03	0.76 ± 0.02	0.77 ± 0.03	0.77 ± 0.02	0.77 ± 0.03	0.77 ± 0.03	0.76 ± 0.02	0.80 ± 0.03	0.79 ± 0.02	0.77 ± 0.03	0.81 ± 0.03	0.79 ± 0.03	0.81 ± 0.02	0.79 ± 0.03
	Adult CON	0.78 ± 0.02	0.74 ± 0.03	0.73 ± 0.03	0.71 ± 0.02	0.72 ± 0.03	0.74 ± 0.02	0.72 ± 0.03	0.72 ± 0.03	0.72 ± 0.02	0.71 ± 0.03	0.73 ± 0.02	0.74 ± 0.03	0.72 ± 0.03	0.71 ± 0.03	0.74 ± 0.02	0.72 ± 0.03
	Adult RIS	0.82 ± 0.02	0.77 ± 0.03	0.75 ± 0.03	0.75 ± 0.02	0.73 ± 0.03	0.72 ± 0.02	0.74 ± 0.03	0.72 ± 0.03	0.74 ± 0.02	0.73 ± 0.03	0.72 ± 0.02	0.75 ± 0.03	0.74 ± 0.03	0.73 ± 0.03	0.74 ± 0.02	0.75 ± 0.03
	Stats	Time - $F_{15,660} = 4.598, p < 0.001^{***}$; Time x Age - $F_{15,660} = 0.476, p = 0.953$; Time x Drug - $F_{15,660} = 0.912, p = 0.551$; Time x Age x Drug - $F_{15,660} = 0.803, p = 0.675$; Age - $F_{1,44} = 6.444, p = 0.015^*$; Drug - $F_{1,44} = 0.420, p = 0.521$; Age x Drug - $F_{1,44} = 0.025, p = 0.876$															
GABA	Adoles CON	0.35 ± 0.02	0.30 ± 0.02	0.34 ± 0.02	0.29 ± 0.02	0.33 ± 0.02	0.30 ± 0.02	0.33 ± 0.03	0.31 ± 0.03	0.34 ± 0.03	0.30 ± 0.02	0.30 ± 0.02	0.32 ± 0.02	0.31 ± 0.03	0.36 ± 0.02	0.30 ± 0.02	0.26 ± 0.03
	Adoles RIS	0.40 ± 0.02	0.36 ± 0.02	0.35 ± 0.02	0.32 ± 0.02	0.33 ± 0.02	0.3 ± 0.02	0.35 ± 0.03	0.35 ± 0.03	0.34 ± 0.03	0.32 ± 0.02	0.34 ± 0.02	0.35 ± 0.02	0.32 ± 0.03	0.31 ± 0.02	0.36 ± 0.02	0.34 ± 0.03
	Adult CON	0.38 ± 0.02	0.34 ± 0.02	0.30 ± 0.02	0.35 ± 0.02	0.29 ± 0.02	0.30 ± 0.02	0.32 ± 0.02	0.30 ± 0.02	0.33 ± 0.03	0.29 ± 0.02	0.27 ± 0.02	0.33 ± 0.02	0.32 ± 0.03	0.30 ± 0.02	0.33 ± 0.02	0.32 ± 0.03
	Adult RIS	0.36 ± 0.02	0.32 ± 0.02	0.31 ± 0.02	0.33 ± 0.02	0.33 ± 0.02	0.36 ± 0.02	0.33 ± 0.02	0.32 ± 0.02	0.32 ± 0.03	0.34 ± 0.02	0.33 ± 0.02	0.31 ± 0.02	0.32 ± 0.03	0.33 ± 0.02	0.32 ± 0.02	0.29 ± 0.03
	Stats	Time - $F_{15,270} = 3.305, p < 0.001^{***}$; Time x Age - $F_{15,270} = 1.063, p = 0.391$; Time x Drug - $F_{15,270} = 0.823, p = 0.652$; Time x Age x Drug - $F_{15,270} = 2.242, p = 0.006^{**}$; Age - $F_{1,18} = 0.131, p = 0.722$; Drug - $F_{1,18} = 1.248, p = 0.279$; Age x Drug - $F_{1,18} = 0.299, p = 0.591$															

GABA signals from time course ^1H MRS scans of some animals were rejected from analysis given CRLB was $>25\%$. Adoles – adolescent cohort; BL – baseline; CON – vehicle-treated controls; GABA – gamma-aminobutyric acid; RIS – risperidone-treated rats; Stats – statistical analysis results from repeated measure two-way ANOVA

Table 2 Levels of accumbal metabolites at baseline and after administration of risperidone or vehicle at Day 22 of chronic treatment in adolescence or adulthood

Metabolite	Metabolite concentration (/tCr) (mean \pm SEM) at Day 22																
	Time block	BL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Glutamine	Adoles CON	0.59 \pm 0.01	0.63 \pm 0.03	0.57 \pm 0.03	0.60 \pm 0.03	0.60 \pm 0.02	0.63 \pm 0.03	0.62 \pm 0.03	0.64 \pm 0.03	0.59 \pm 0.03	0.61 \pm 0.03	0.63 \pm 0.03	0.59 \pm 0.02	0.59 \pm 0.02	0.62 \pm 0.03	0.60 \pm 0.02	0.58 \pm 0.03
	Adoles RIS	0.60 \pm 0.01	0.60 \pm 0.03	0.62 \pm 0.03	0.62 \pm 0.03	0.63 \pm 0.02	0.63 \pm 0.03	0.61 \pm 0.03	0.61 \pm 0.03	0.57 \pm 0.03	0.56 \pm 0.03	0.58 \pm 0.03	0.60 \pm 0.02	0.60 \pm 0.02	0.61 \pm 0.03	0.60 \pm 0.02	0.59 \pm 0.03
	Adult CON	0.59 \pm 0.01	0.62 \pm 0.03	0.62 \pm 0.03	0.62 \pm 0.03	0.64 \pm 0.02	0.61 \pm 0.03	0.65 \pm 0.03	0.64 \pm 0.03	0.62 \pm 0.03	0.62 \pm 0.03	0.60 \pm 0.03	0.59 \pm 0.02	0.63 \pm 0.02	0.63 \pm 0.03	0.60 \pm 0.02	0.63 \pm 0.03
	Adult RIS	0.62 \pm 0.01	0.63 \pm 0.03	0.68 \pm 0.03	0.65 \pm 0.03	0.67 \pm 0.02	0.63 \pm 0.03	0.64 \pm 0.03	0.65 \pm 0.03	0.66 \pm 0.03	0.67 \pm 0.03	0.64 \pm 0.03	0.64 \pm 0.02	0.65 \pm 0.02	0.63 \pm 0.03	0.61 \pm 0.02	0.64 \pm 0.03
	Stats	Time - $F_{15,660} = 1.099$, $p = 0.353$; Time x Age - $F_{15,660} = 1.068$, $p = 0.383$; Time x Drug - $F_{15,660} = 0.805$, $p = 0.673$; Time x Age x Drug - $F_{15,660} = 0.603$, $p = 0.873$; Age - $F_{1,44} = 3.437$, $p = 0.70$; Drug - $F_{1,44} = 0.453$, $p = 0.505$; Age x Drug - $F_{1,44} = 0.847$, $p = 0.362$															

Glutathione	Adoles CON	0.31 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.31 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.30 ± 0.01	0.32 ± 0.01	0.28 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.30 ± 0.01	0.33 ± 0.01	0.31 ± 0.01	0.32 ± 0.01
	Adoles RIS	0.3 ± 0.01	0.29 ± 0.01	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.30 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	0.32 ± 0.01	0.30 ± 0.01	0.30 ± 0.01	0.30 ± 0.01	0.31 ± 0.01	0.3 ± 0.01	0.33 ± 0.01	0.30 ± 0.01
	Adult CON	0.30 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.29 ± 0.01	0.29 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.29 ± 0.01	0.30 ± 0.02	0.31 ± 0.01	0.33 ± 0.01	0.32 ± 0.01	0.31 ± 0.01	0.27 ± 0.02	0.32 ± 0.01	0.32 ± 0.01
	Adult RIS	0.29 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.27 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.30 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.30 ± 0.01	0.30 ± 0.01	0.30 ± 0.01	0.28 ± 0.01	0.30 ± 0.01	0.30 ± 0.01	0.29 ± 0.01
	Stats	Time - $F_{15,630} = 2.214, p = 0.008^{**}$; Time x Age - $F_{15,630} = 1.539, p = 0.086$; Time x Drug - $F_{15,630} = 0.837, p = 0.636$; Time x Age x Drug - $F_{15,630} = 2.165, p = 0.006^{**}$; Age - $F_{1,42} = 0.09, p = 0.766$; Drug - $F_{1,42} = 1.213, p = 0.277$; Age x Drug - $F_{1,41} = 0.0001, p = 0.997$															
Myo-Inositol	Adoles CON	0.74 ± 0.01	0.66 ± 0.02	0.65 ± 0.02	0.67 ± 0.02	0.66 ± 0.02	0.67 ± 0.02	0.64 ± 0.02	0.66 ± 0.02	0.64 ± 0.02	0.67 ± 0.02	0.67 ± 0.02	0.63 ± 0.02	0.65 ± 0.02	0.68 ± 0.02	0.66 ± 0.02	0.65 ± 0.02
	Adoles RIS	0.73 ± 0.01	0.65 ± 0.02	0.66 ± 0.02	0.66 ± 0.02	0.62 ± 0.02	0.65 ± 0.02	0.62 ± 0.02	0.62 ± 0.02	0.62 ± 0.02	0.63 ± 0.02	0.64 ± 0.02	0.63 ± 0.02	0.65 ± 0.02	0.67 ± 0.02	0.65 ± 0.02	0.64 ± 0.02
	Adult CON	0.72 ± 0.01	0.70 ± 0.02	0.70 ± 0.02	0.72 ± 0.02	0.69 ± 0.02	0.68 ± 0.02	0.67 ± 0.02	0.66 ± 0.02	0.69 ± 0.02	0.68 ± 0.02	0.71 ± 0.02	0.72 ± 0.02	0.67 ± 0.02	0.68 ± 0.02	0.68 ± 0.02	0.68 ± 0.02
	Adult RIS	0.77 ± 0.01	0.71 ± 0.02	0.70 ± 0.02	0.70 ± 0.02	0.68 ± 0.02	0.71 ± 0.02	0.68 ± 0.02	0.69 ± 0.02	0.68 ± 0.02	0.73 ± 0.02	0.69 ± 0.02	0.69 ± 0.02	0.67 ± 0.02	0.71 ± 0.02	0.73 ± 0.02	0.68 ± 0.02
	Stats	Time - $F_{15,660} = 5.513, p < 0.001^{***}$; Time x Age - $F_{15,660} = 0.883, p = 0.583$; Time x Drug - $F_{15,660} = 0.648, p = 0.836$; Time x Age x Drug - $F_{15,660} = 0.815, p = 0.661$; Age - $F_{1,44} = 16.113, p < 0.001^{***}$; Drug - $F_{1,44} = 0.064, p = 0.801$; Age x Drug - $F_{1,44} = 1.586, p = 0.215$															
Taurine	Adoles CON	0.80 ± 0.02	0.75 ± 0.02	0.74 ± 0.03	0.76 ± 0.02	0.75 ± 0.03	0.76 ± 0.03	0.73 ± 0.02	0.75 ± 0.02	0.77 ± 0.03	0.72 ± 0.03	0.76 ± 0.02	0.75 ± 0.02	0.78 ± 0.02	0.74 ± 0.02	0.74 ± 0.03	0.72 ± 0.02
	Adoles RIS	0.84 ± 0.02	0.78 ± 0.02	0.76 ± 0.03	0.74 ± 0.02	0.73 ± 0.03	0.75 ± 0.03	0.76 ± 0.02	0.78 ± 0.02	0.78 ± 0.03	0.76 ± 0.03	0.74 ± 0.02	0.74 ± 0.02	0.77 ± 0.02	0.78 ± 0.02	0.73 ± 0.03	0.75 ± 0.02
	Adult CON	0.83 ± 0.02	0.71 ± 0.02	0.78 ± 0.03	0.78 ± 0.02	0.74 ± 0.03	0.73 ± 0.03	0.76 ± 0.02	0.78 ± 0.02	0.76 ± 0.03	0.76 ± 0.03	0.74 ± 0.02	0.76 ± 0.02	0.74 ± 0.02	0.73 ± 0.02	0.72 ± 0.03	0.74 ± 0.02
	Adult RIS	0.82 ± 0.02	0.75 ± 0.02	0.73 ± 0.03	0.71 ± 0.02	0.72 ± 0.03	0.74 ± 0.03	0.70 ± 0.02	0.72 ± 0.02	0.74 ± 0.03	0.78 ± 0.03	0.71 ± 0.02	0.73 ± 0.02	0.75 ± 0.02	0.72 ± 0.02	0.73 ± 0.03	0.75 ± 0.02
	Stats	Time - $F_{15,660} = 5.491, p < 0.001^{***}$; Time x Age - $F_{15,660} = 0.963, p = 0.493$; Time x Drug - $F_{15,660} = 1.413, p = 0.135$; Time x Age x Drug - $F_{15,660} = 1.070, p = 0.381$; Age - $F_{1,44} = 0.408, p = 0.526$; Drug - $F_{1,44} = 0.031, p = 0.862$; Age x Drug - $F_{1,44} = 0.557, p = 0.460$;															

GABA	Adoles CON	0.38 ± 0.02	0.34 ± 0.04	0.36 ± 0.02	0.33 ± 0.03	0.37 ± 0.02	0.34 ± 0.03	0.31 ± 0.03	0.31 ± 0.03	0.32 ± 0.03	0.33 ± 0.02	0.36 ± 0.03	0.33 ± 0.02	0.36 ± 0.03	0.33 ± 0.03	0.34 ± 0.03	0.30 ± 0.02
	Adoles RIS	0.39 ± 0.01	0.35 ± 0.03	0.33 ± 0.02	0.33 ± 0.02	0.31 ± 0.02	0.33 ± 0.02	0.32 ± 0.02	0.30 ± 0.02	0.32 ± 0.02	0.32 ± 0.01	0.30 ± 0.02	0.32 ± 0.01	0.31 ± 0.02	0.33 ± 0.02	0.31 ± 0.02	0.28 ± 0.02
	Adult CON	0.38 ± 0.02	0.35 ± 0.04	0.31 ± 0.02	0.31 ± 0.03	0.31 ± 0.02	0.34 ± 0.03	0.31 ± 0.03	0.35 ± 0.03	0.34 ± 0.03	0.31 ± 0.02	0.37 0.03	0.32 ± 0.02	0.34 ± 0.03	0.29 ± 0.03	0.37 ± 0.03	0.32 ± 0.02
	Adult RIS	0.35 ± 0.02	0.34 ± 0.05	0.31 ± 0.03	0.31 ± 0.04	0.31 ± 0.03	0.39 ± 0.03	0.35 ± 0.03	0.36 ± 0.04	0.36 ± 0.03	0.44 ± 0.02	0.35 ± 0.04	0.33 ± 0.02	0.30 ± 0.04	0.33 ± 0.04	0.33 ± 0.03	0.36 ± 0.03
	Stats	Time - $F_{15,150} = 1.529, p = 0.102$; Time x Age - $F_{15,150} = 1.525, p = 0.103$; Time x Drug - $F_{15,150} = 1.184, p = 0.290$; Time x Age x Drug - $F_{15,150} = 0.668, p = 0.812$; Age - $F_{1,10} = 0.636, p = 0.444$; Drug - $F_{1,10} = 0.00001, p = 0.997$; Age x Drug - $F_{1,10} = 1.752, p = 0.215$;															

GABA signals from time course ¹H MRS scans of some animals were rejected from analysis given CRLB was >25%. Adoles – adolescent cohort; BL – baseline; CON – vehicle-treated controls; GABA – gamma-aminobutyric acid; RIS – risperidone-treated rats; Stats – statistical analysis results from repeated measure two-way ANOVA;