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

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

## **Abstract**

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

## **Disciplines**

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## **Publication Details**

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**Title: Early Antipsychotic Treatment in Childhood/Adolescent Period has Long-term Effects on Depressive-Like, Anxiety-Like and Locomotor Behaviours in Adult Rats**

**Running title** – *Childhood APD treatment effects on behaviour in adult rats*

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## **Abstract**

Childhood/adolescent antipsychotic drug (APD) use is exponentially increasing worldwide, despite limited knowledge of the long-term effects of early APD treatment. Whilst investigations have found that early treatment has resulted in some alterations to dopamine and serotonin neurotransmission systems (essential to APD efficacy), limited studies into potential long-term behavioural changes have occurred. This study, using an animal model for childhood/adolescent APD treatment, investigated the long-term effects of Aripiprazole, Olanzapine and Risperidone on adult behaviours of male and female rats. Open-field/holeboard, elevated plus maze (EPM), social interaction and forced swim (FS) tests were then conducted in adult rats. Our results indicated that in the male cohort, early Risperidone and Olanzapine treatment elicited long-term hyper-locomotor effects (open-field/holeboard and FS tests), whilst a decrease in depressive-like behaviour (in FS test) was observed in response to Olanzapine treatment. Furthermore, anxiolytic-like behaviours were found following testing in the open-field/holeboard and EPM in response to all 3 drug treatments. Effects in the female cohort however were to a far lesser extent, with behavioural attributes indicative of an increased depressive-like behaviour and hypo-locomotor activity were exhibited in the FS test following early Risperidone and Olanzapine treatment. These results suggest that various APDs have different long-term effects on the behaviours of adult rats.

**Keywords:** Antipsychotic drug, Risperidone, Olanzapine, Motor activity, Anxiety, Depression, Development.

## **Abbreviations**

5-HT, Serotonin

ANOVA, Analysis of Variance

APD, Antipsychotic Drug

DA, Dopamine

EPM, Elevated Plus Maze

FS, Forced Swim

NT, Neurotransmitter

PD, Postnatal Day

SEM, Standard Error of the Mean

## 1. Introduction

The use of antipsychotic drugs (APDs) in children and adolescents is rapidly increasing worldwide, despite serious limitation in the safety and efficacy of APD use in these population groups (Rani et al. 2008; Varley and McClellan 2009; Olfson et al. 2010; Alexander et al. 2011; Olfson et al. 2012; Seida et al. 2012; Hoekstra 2014). Whilst first-generation APDs (e.g. Haloperidol and Chlorpromazine) are used less frequently in children and adolescence due to their serious extrapyramidal side-effects, second-generation APDs including Aripiprazole, Olanzapine and Risperidone are commonly prescribed (mostly off-label) for treating various childhood disorders; from mental illnesses including anxiety, depression and child-onset schizophrenia (Olfson et al. 2010; Memarzia et al. 2014; Schneider et al. 2014), to various behavioural disorders (Haw and Stubbs 2007; Vitiello et al. 2009; Sharma and Shaw 2012). Whilst the therapeutic effects of APDs are predominantly based on their antagonist or partial agonist mechanisms of action on the dopamine (DA) D<sub>2</sub> and serotonin (5-HT) 5-HT<sub>1A</sub> and 5-HT<sub>2A/2C</sub> receptors (Meltzer 2002; Meltzer et al. 2003; Grace et al. 2007; Kegeles et al. 2010; Purves-Tyson et al. 2012; Milstein et al. 2013), the dopaminergic and serotonergic neurotransmitter (NT) systems are also heavily involved in multiple neurodevelopmental processes during the childhood/adolescent period (Levitt et al. 1997; Frost and Cadet 2000; Andersen 2003; Klomp et al. 2012; Milstein et al. 2013). There is therefore the potential for APD use in such a critical neurodevelopmental time period to carry a substantial risk of long-term behavioural pathologies, due to their potent actions on the still developing DA and 5-HT NT systems (Andersen 2003; Andersen and Navalta 2004; Andersen 2005; Vinish et al. 2012; Memarzia et al. 2014).

Although current clinical research into the use of APDs in the adolescent population over both a short term period of between 1-2 months, and longer-term period of up to 6 months has found some benefits in the treatment of some mental illness symptomology (Stigler et al. 2004; Kumra et al. 2008; Zuddas et al. 2011), there is limited data on the potential for long-term alterations to adult behaviours (Milstein et al. 2013; Shu et al. 2014; Varela et al. 2014). Although the effects of childhood/adolescent APD use on adult behaviours is unknown in a clinical setting, current animal studies have shown that early drug treatment for a period of up to 4 weeks can result in various significant alterations in the dopaminergic and serotonergic pathways in the brain long-term (Maciag et al. 2006; Moran-Gates et al. 2006; Vinish et al. 2012; Milstein et al. 2013; Memarzia et al. 2014; Varela et al. 2014), including permanent changes in the distribution of NT receptors and dendritic architecture (Maciag et al. 2006; Moran-Gates et al. 2006; Milstein et al. 2013), with minimal evidence of the direct effects on behaviour. Alterations to the production, transport and binding of DA and 5-HT in the cortical and striatal brain regions have already been directly linked to changes in behavioural attributes, including locomotor activity levels along with depressive-like and anxiety-like behaviours. The present study therefore investigated the long-term effects of APD treatment during the critical neurodevelopmental window of childhood/adolescence, on behavioural attributes in adult rats, including locomotor activity, depressive-like, anxiety-like, exploratory and social behaviours.

## **2. Materials and Methods**

### *2.1. Animals and Housing*

Timed pregnant Sprague-Dawley rats were obtained at gestation day 16 from the Animal Resource Centre (Perth, WA, Australia). They were housed in individual cages under

environmentally controlled conditions (22°C, light cycle from 07:00 to 19:00 and dark cycle from 19:00 to 07:00), and allowed *ad libitum* access to water and standard laboratory chow diet (3.9 kcal/g: 10% fat, 74% carbohydrate, 16% protein). Day of birth was considered postnatal day (PD) 0. Pups were sexed on PD14, and then 96 Sprague-Dawley rats (48 male and 48 female) were weaned on PD20 and housed in individual cages. All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, NSW, Australia (AE 12/20), and complied with Australian Code of Practice for the Care and Use of Animals for Scientific Purpose (2004).

## 2.2. Drug Treatment Groups

Before the drug treatment commenced, the rats were trained for self-administration by feeding them cookie dough (0.3 g) without drugs 2 times per day for PD 18-21. Animals were then assigned randomly to one of four experimental groups per gender on PD21 ( $n = 12/\text{group}$ ): (1) Aripiprazole (Otsuka, Japan), (2) Olanzapine (Eli Lilly, USA), (3) Risperidone (Apotex, Canada), or (4) control (vehicle). The drug treatment period from PD22-50 in the rats was carried out at the equivalent time of the childhood/periadolescent phase in humans (Andersen 2003). A staggered drug treatment pattern, where lower APD dosages are slowly increased to a final dosage amount, was used to mimic a clinical setting (Taylor et al. 2009). The APD doses were initiated on PD22 at 0.2 mg/kg, 3 times per day for Aripiprazole; 0.25 mg/kg, 3 times per day for Olanzapine; and 0.05 mg/kg, 3 times per day for Risperidone, and then increased in 3 steps over the first 7 days of the 4 week treatment period to achieve a final dose on PD28 of 1 mg/kg, 3 times per day for Aripiprazole, 1 mg/kg, 3 times per day for Olanzapine, and 0.3 mg/kg, 3 times per day for Risperidone. The proposed dosages are within the recommended dosage ranges for the psychiatric treatment of paediatric patients, based on the body surface area formula for dosage translation between humans and rats in the FDA

guideline for clinical trials (Food & Drug Administration 2005; Reagan-Shaw et al. 2008; Taylor et al. 2009; Zuddas et al. 2011). It has been previously reported that, at these used dosages, Aripiprazole drug treatment reaches above 90% DA D<sub>2</sub> receptor occupancy rates in the rat brain (Wadenberg 2007), while Olanzapine and Risperidone reach 65-80% DA D<sub>2</sub> receptor occupancy (Kapur et al. 2003; Natesan et al. 2006). These dosages have also been shown to be physiologically and behaviourally effective in our laboratory, with similar dosages seen to induce weight gain and changes in hypothalamic neuropeptide Y expression in adolescent rats (Lian et al. 2015), whilst alterations to both DA and 5-HT receptor binding has been reported in adult rats (Han et al. 2009; Weston-Green et al. 2011). The 0.3 g dry cookie dough pellets were fed to the rats 3 times per day (07:00h, 14:00h and 22:00h; with 8±1 hour intervals) over the 4 week treatment period. Rats were observed throughout the treatment to ensure that they completely consumed the cookie dough pellet. The rats in the control group received an equivalent pellet without the drug.

### *2.3. Behavioural Tests*

All behavioural tests were carried out between 09:00 and 17:00h. All protocols for the behavioural tests used in the present study were adapted from previous studies (Burne et al. 2004; du Bois et al. 2008). Behavioural tests were carried out over a 4 week period from PD72-94 (the equivalent of adulthood) in the following sequence; open-field/holeboard test, elevated plus maze test, social interaction test, and forced swim test, as described below.

#### *2.3.1. Open-Field/Holeboard Test*

The open-field/holeboard test was modified from previous designs and carried out on PD72 to examine exploratory behaviour and locomotor activity (Burne et al. 2004; du Bois et al. 2008). A single rat was placed in a black square arena (60cm x 60cm x 40cm), with a floorboard insert placed 5cm above the base, with four holes, 5cm in diameter, each situated

10cm in from each of the corners. The light intensity was 30lx across the entire arena. Rat behaviour was recorded with a centrally located video camera for 30 minutes. Locomotor activity was later analysed using video-tracking software (Noldus Information Technology, Wageningen, The Netherlands). The frequency of head dips were scored manually.

### *2.3.2. Elevated Plus Maze*

The elevated plus maze test was modified from previous designs and carried out on PD79, to examine anxiety-like behaviours and locomotor activity (Wigger and Neumann 1999; Burne et al. 2004; Mällo et al. 2007; du Bois et al. 2008). It consisted of two open arms (50cm x 7cm) and two closed arms (50cm x 7cm x 30cm) with an open roof, arranged around a central platform (7cm x 7cm). Like-arms opposed each other across the central platform. The maze was a wooden structure, painted with a black acrylic, and elevated 60cm above the floor. The light intensity was set at 100 lx at the open arms and central platform. A single rat was placed on the central platform facing an open arm and rat behaviour was recorded for 7 minutes with a centrally located video camera. We measured the number of open and closed arm entries, and the duration of time spent in the open and closed arms, central platform and ‘open part’ (open arms plus central platform). We also measured the number of arm changes as a measure of general locomotor activity on the maze. Data was scored manually, with criterion for entry into the open/closed arms was whole body and four paws entry; a rat was considered to be in the central platform zone if its head and front paws were in the central platform, with its body positioned in the closed arm.

### *2.3.3. Social Interaction*

The social interaction test was modified from previous designs and was carried out on PD86 to examine social and anxiolytic behaviours (Burne et al. 2004; du Bois et al. 2008). It was performed in a black circular arena (90cm diameter x 30cm high walls) made of a black acrylic, with light intensity at 30 lx across the entire arena. Two unfamiliar rats of a similar

size, of the same sex and from the same treatment group were placed on opposing sides of the arena, allowed to move around freely and behaviour was recorded for 7 minutes with a centrally located video camera. All rats were only tested once. Behaviour was analysed via the use of video-tracking software (Noldus Information Technology, Wageningen, The Netherlands) looking at time spent within a given proximity (set at 20cm – average body length) of one another, and mean distance between each other, and gathered data from each Social Interaction test involving two rats was analysed as a single data point. No aggressive behaviours were observed using this procedure.

#### *2.3.4. Forced Swim Test*

The forced swim test was modified from previous designs and was carried out on PD93 and 94 to examine depressive-like behaviours and locomotor activity (Burne et al. 2004; du Bois et al. 2008). A blue opaque cylinder (30cm x 50cm) was filled to predetermined water level (30°C), dependent upon the body length of each individual rat. Rats were measured from the tip of the snout to the base of the tail, and water depth was then calculated by multiplying this value by 1.25. Rats were tested over 2 days, at a light intensity of 1000 lx (fluorescence strip lighting-stress conditions). On the first day, the rats were placed in the water for a 10 minute duration, then removed and dried with towels and placed in a cage with paper towel for 20 minutes prior to being returned to their home cages. Water was changed between rats. On the second day, the rats were placed in the cylinder for a period of 5 minutes. Locomotor activity was recorded on the second day using a centrally located video camera. We manually evaluated the time spent climbing (defined as vertical movements of the forepaws, usually up against the cylinder), swimming (movement throughout the cylinder, usually in a horizontal direction), and floating (immobile – an absence of movement except for that required to keep its head above water).

## 2.4. Statistical Analysis

All collected data were analysed using SPSS software (Windows version 19.0, SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to examine the distribution of data from all experiments. All normally distributed data from male and female rats were also analysed by two-way analysis of variances (ANOVAs) (Gender x Treatment). Then data from males or females were analysed separately by one-way ANOVA, followed by *post-hoc* Tukey tests for multiple comparisons between the treatment groups. Due to the inter-relationship of activity levels, we also utilized an analysis of covariance (ANCOVA) test with total distance travelled (cm) obtained in Open Field/Hole Board test as the co-variate factor to further analyse data from Elevated Plus Maze and Forced Swim Tests. Data that was not normally distributed was analysed via the non-parametric Mann-Whitney U-test. All data were expressed as mean  $\pm$  standard error of the mean ( $\pm$ SEM), and statistical significance was accepted when  $p \leq 0.05$ .

## 3. Results

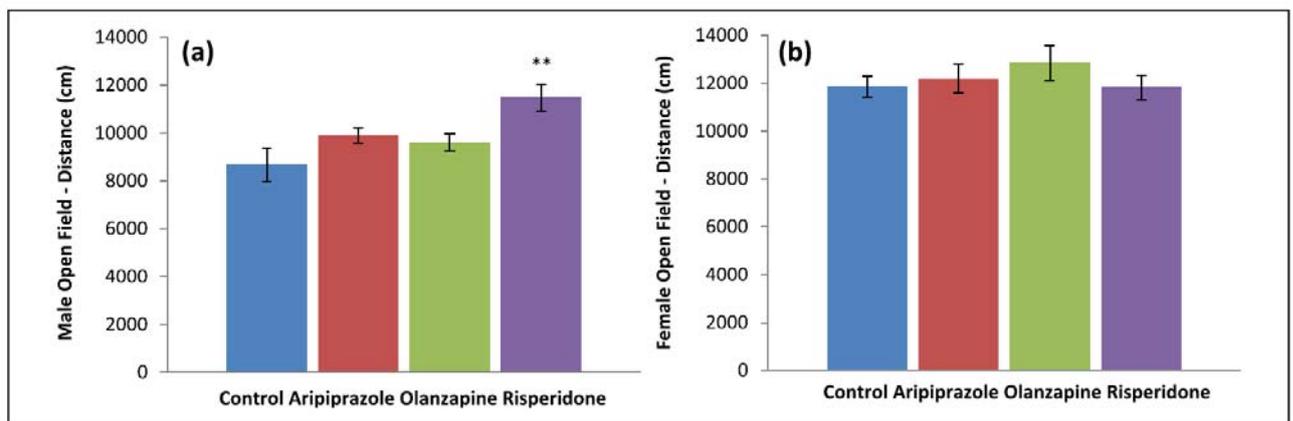
Following a childhood/adolescent treatment of Aripiprazole, Olanzapine, Risperidone or vehicle for 4 weeks (PD22-50), a series of 4 behavioural tests including the Open-Field/Holeboard, Elevated-Plus Maze (EPM) and Social Interaction and Forced Swim (FS) tests, were conducted over a 4 week period (PD72-94).

### 3.1. Open Field/Holeboard

Two-way ANOVAs (Gender x Treatment) revealed significant effects of Gender on average velocity ( $F_{1,90} = 49.780$ ,  $p < 0.001$ ), total distance moved ( $F_{1,90} = 32.838$ ,  $p < 0.001$ ) and rearing ( $F_{1,90} = 17.028$ ,  $p < 0.001$ ), along with a significant interaction between the two factors on total distance moved ( $F_{3,90} = 2.772$ ,  $p < 0.05$ ). Analyses revealed that female rats

had a higher locomotor activity level compared to male rats ( $p < 0.05$ ), and furthermore, male and female rats were also found to respond differently to early APD treatment (Table 1). In male rats, a significant effect of treatment on average velocity ( $F_{3,46} = 4.473, p < 0.01$ ), total distance moved ( $F_{3,46} = 5.115, p < 0.01$ ) and a trend to significance with rearing ( $F_{3,45} = 2.685, p = 0.059$ ) were found. Early Risperidone treatment increased total distance moved (+32%,  $p < 0.01$ ), average velocity (+33%,  $p < 0.01$ ) and rearing (+49%,  $p < 0.05$ ) (Table 1). These increases in average velocity, total distance moved and rearing indicated that male rats treated with Risperidone in the childhood/adolescent period (PD22-50) exhibit hyper-locomotor activity levels following Open-Field/Holeboard behavioural testing in adulthood (PD72)(Figure 1). However, no alterations in locomotor activity were observed in the female treatment groups, indicating that early APD treatment has a stronger long-term effect on the male cohort.

**Figure 1.** Behavioural Effects - Activity Levels (A) Male Open Field/Holeboard Distance Travelled (cm); (B) Female Open Field/Holeboard Distance Travelled (cm) in Sprague-Dawley rats treated chronically with Aripiprazole (1.0 mg/kg, t.i.d), Olanzapine (1.0 mg/kg, t.i.d), Risperidone (0.3 mg/kg, t.i.d) or control (vehicle). Data expressed as mean  $\pm$  SEM. \*\*,  $p < 0.01$  vs. control. Abbreviations: t.i.d.: three times daily.



**Table 1.** Behaviour of Male & Female Sprague-Dawley rats treated with chronic treatment of Aripiprazole (1.0 mg/kg, t.i.d.), Olanzapine (1.0 mg/kg, t.i.d.), Risperidone (0.3 mg/kg, t.i.d.) in the Open-Field/Holeboard tests.

	<b>Control</b>	<b>Aripiprazole</b>	<b>Olanzapine</b>	<b>Risperidone</b>
<b>Male</b>				
<b>Total Distance Travelled (cm)</b>	8672.2 ± 705.8	9902.1 ± 308.6	9607.6 ± 378.3	<b>11461.6 ± 567.8**</b>
<b>Average Velocity (cm/s)</b>	5.2 ± 0.5	6.24 ± 0.3	5.7 ± 0.2	<b>6.8 ± 0.4**</b>
<b>Rearing Frequency</b>	105.2 ± 14.9	145.4 ± 11.9	140.9 ± 10.3	<b>156.5 ± 15.2*</b>
<b>Head Dips</b>	43.6 ± 2.4	48.3 ± 2.4	40.9 ± 1.2	46.85 ± 2.7
<b>Centre Duration (s)</b>	353.1 ± 53.6	445.2 ± 65.8	449.6 ± 84.0	401.6 ± 44.1
<b>Periphery Duration (s)</b>	1384.4 ± 58.8	1303.9 ± 61.6	1298.9 ± 82.7	1248.2 ± 110.4
<b>Centre Frequency</b>	90.5 ± 11.1	112.6 ± 10.8	104.0 ± 10.3	118.8 ± 7.3
<b>Periphery Frequency</b>	115.6 ± 14.2	135.4 ± 8.9	131.5 ± 10.4	147.8 ± 11.0
<b>Female</b>				
<b>Total Distance Travelled (cm)</b>	11863.9 ± 432.7	12194.5 ± 589.2	12848.1 ± 716.7	11835.4 ± 514.7
<b>Average Velocity (cm/s)</b>	7.2 ± 0.2	7.7 ± 0.5	7.9 ± 0.5	8.7 ± 0.4
<b>Rearing Frequency</b>	162.1 ± 9.4	202.2 ± 21.9	209.4 ± 13.0	208.9 ± 16.6
<b>Head Dips</b>	55.4 ± 3.1	59.3 ± 3.3	58.3 ± 3.4	44.4 ± 4.1

<b>Centre Duration</b> (s)	227.2 ± 20.0	357.5 ± 62.4	312.4 ± 28.9	310.7 ± 41.3
<b>Periphery Duration</b> (s)	1465.3 ± 18.9	1333.2 ± 61.8	1383.8 ± 30.4	1396.2 ± 40.0
<b>Centre Frequency</b>	85.8 ± 6.9	110.7 ± 15.6	113.8 ± 11.3	116.9 ± 15.3
<b>Periphery Frequency</b>	152.5 ± 8.5	184.2 ± 20.0	189.1 ± 17.1	178.7 ± 23.1

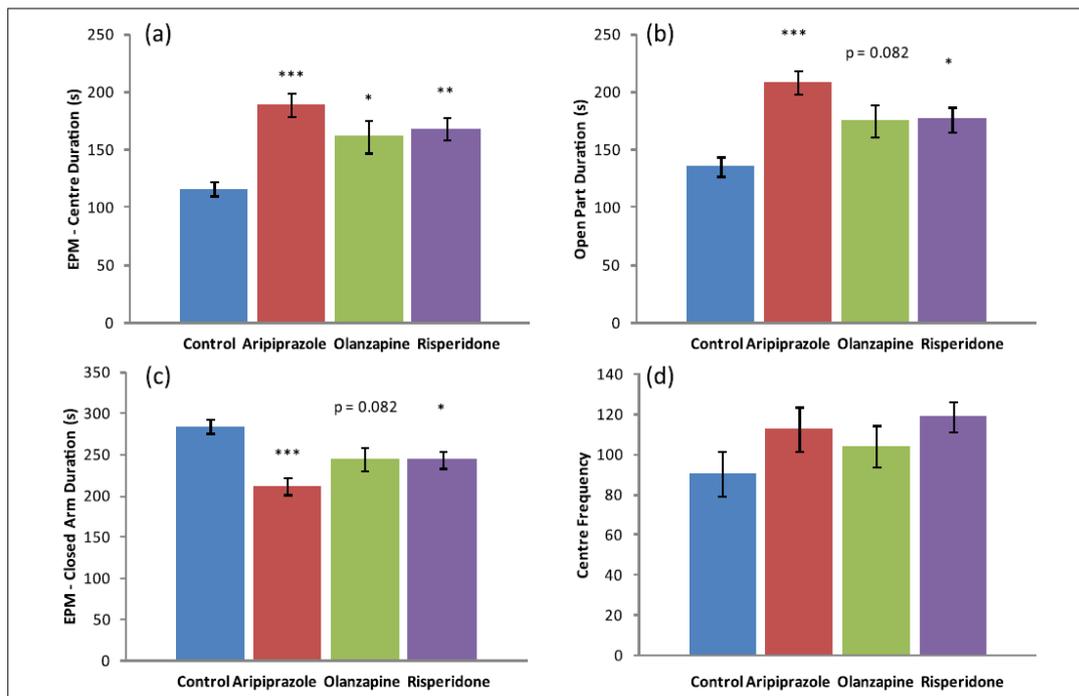
Data are expressed as mean ± SEM. \*  $p \leq 0.05$  vs control; \*\*  $p < 0.01$  vs control; \*\*\*  $p < 0.001$  vs control.

### 3.2. Elevated Plus Maze

Analyses of EPM behavioural data once again revealed that male and female rats were found to respond differently to early APD treatment (Table 2). Two-way ANOVAs (Gender x Treatment) revealed significant interaction between Treatment and Gender on centre duration ( $F_{3,85} = 4.023$ ,  $p < 0.02$ ) and closed arm duration ( $F_{3,85} = 5.149$ ,  $p < 0.01$ ). In male rats, analysis via ANCOVA of closed arm changes data found a significant effect of Total Distance Travelled ( $F_{1,46} = 8.403$ ,  $p < 0.01$ ), whilst one-way ANOVA found that early APD treatment significantly affected centre duration ( $F_{3,45} = 8.924$ ,  $p < 0.001$ ), closed arm duration ( $F_{3,45} = 7.303$ ,  $p < 0.001$ ) and open part duration ( $F_{3,45} = 7.303$ ,  $p < 0.001$ ) (Table 2). Early Aripiprazole (+26%,  $p < 0.001$ ), Olanzapine (+27%,  $p < 0.05$ ) and Risperidone (+14%,  $p < 0.01$ ) treatment also increased centre duration compared to the control group. Furthermore, early Aripiprazole (+53%,  $p < 0.001$ ) and Risperidone (+30%,  $p = 0.05$ ) treatment was found to significantly increase time spent in the open part (open arms + centre platform), with a similar trend also found in the early Olanzapine (+29%,  $p = 0.082$ ) treatment group compared

to the control. There were reduced closed arm durations following the early Aripiprazole (-26%,  $p < 0.001$ ) and Risperidone (-14%,  $p = 0.05$ ) treatments, whilst the early Olanzapine (-14%,  $p = 0.082$ ) treatment resulted in a similar trend (Table 2) (Figure 2). However, there were no significant differences in open arm entries or closed arm entries. In female rats, the early Aripiprazole treatment resulted in a trend to a significant increase in closed arm duration (+57%,  $p = 0.056$ ). These results indicated that early APD treatment with Aripiprazole, Olanzapine and Risperidone in the male rodent model all subsequently led to the expression of anxiolytic-like behaviours in adulthood. In the female model, although no significant effects of early APD treatment on anxiety-like behaviours were found in the female animal model, analysis via ANCOVA found a trend to significant effects of Total Distance Travelled on closed arm changes ( $F_{1,42} = 3.077$ ,  $p = 0.088$ ), indicating again that early APD treatment has stronger long-term effects on the male cohort.

**Figure 2.** Behavioural Effects - Anxiety (A) EPM Centre Duration (s); (B) EPM Open Part Duration (s); (C) EPM Closed Arm Duration (s); (D) Open Field/Holeboard Centre Frequency in male Sprague-Dawley rats treated chronically with Aripiprazole (1.0 mg/kg, t.i.d), Olanzapine (1.0 mg/kg, t.i.d), Risperidone (0.3 mg/kg, t.i.d) or control (vehicle). Data expressed as mean  $\pm$  SEM. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  vs. control. Abbreviations: EPM: Elevated-Plus Maze; t.i.d.: three times daily.



**Table 2.** Behaviour of Male & Female Sprague-Dawley rats treated with chronic treatment of Aripiprazole (1.0 mg/kg, t.i.d.), Olanzapine (1.0 mg/kg, t.i.d.), Risperidone (0.3 mg/kg, t.i.d.) in the Elevated Plus Maze (EPM) tests.

	<b>Control</b>	<b>Aripiprazole</b>	<b>Olanzapine</b>	<b>Risperidone</b>
<b>Male</b>				
<b>Open Arm Entries</b>	1.4 ± 0.4	1.1 ± 0.4	1.0 ± 0.3	0.9 ± 0.3
<b>Closed Arm Entries</b>	1.7 ± 0.3	1.5 ± 0.3	1.1 ± 0.1	1.3 ± 0.2
<b>Closed Arm Changes</b>	8.6 ± 1.1	9.0 ± 1.0	7.4 ± 1.0	10.9 ± 1.1
<b>Open Arm Duration (s)</b>	19.5 ± 6.2	18.8 ± 5.8	13.6 ± 4.5	8.2 ± 3.3
<b>Closed Arm Duration (s)</b>	284.4 ± 8.7	<b>211.8 ± 10.3***</b>	244.5 ± 14.1	<b>243.7 ± 10.7*</b>
<b>Centre Duration (s)</b>	116.1 ± 6.6	<b>189.3 ± 10.3***</b>	<b>161.9 ± 14.2*</b>	<b>168.1 ± 9.5**</b>
<b>Open Part Duration (s)</b>	135.64 ± 8.7	<b>208.17 ± 10.3***</b>	175.50 ± 14.1	<b>176.31 ± 10.7*</b>
<b>Female</b>				
<b>Open Arm Entries</b>	2.0 ± 0.8	1.6 ± 0.6	3.0 ± 0.5	3.0 ± 0.8
<b>Closed Arm Entries</b>	2.3 ± 0.5	2.0 ± 0.4	3.3 ± 0.5	3.2 ± 0.4
<b>Closed Arm Changes</b>	10.1 ± 1.4	10.3 ± 1.0	10.8 ± 0.9	11.9 ± 1.0
<b>Open Arm Duration (s)</b>	23.6 ± 9.0	14.2 ± 6.0	28.8 ± 5.8	30.1 ± 9.5
<b>Closed Arm Duration (s)</b>	159.8 ±	250.3 ± 12.0	214.8 ± 10.8	226.1 ± 7.5

36.3

<b>Centre Duration</b> (s)	151.9 ± 13.7	155.5 ± 9.4	176.4 ± 7.0	163.8 ± 8.3
<b>Open Part Duration</b> (s)	175.55 ± 14.3	169.67 ± 12.0	205.25 ± 10.8	193.91 ± 7.5

Data are expressed as mean ± SEM. \*  $p \leq 0.05$  vs control; \*\*  $p < 0.01$  vs control; \*\*\*  $p < 0.001$  vs control.

### 3.3. Social Interaction

On PD86, all treatment groups and both genders underwent Social Interaction testing to examine behavioural parameters including anxiety and social behaviours. Analysis found no significant differences between the control and drug treatment groups across all of the measured parameters (Table 3). The results indicate that childhood/adolescent APD treatment in both male and female rats had no significant effects on the adult social behaviours examined by the social interaction tests, when compared to the control group.

**Table 3.** Behaviour of Male & Female Sprague-Dawley rats treated with chronic treatment of Aripiprazole (1.0 mg/kg, t.i.d.), Olanzapine (1.0 mg/kg, t.i.d.), Risperidone (0.3 mg/kg, t.i.d.) in the Social Interaction tests.

	Control	Aripiprazole	Olanzapine	Risperidone
<b>Male</b>				
<b>In Proximity Duration (s)</b>	159.0 ± 13.7	140.3 ± 14.4	155.6 ± 17.7	159.5 ± 17.4
<b>In Proximity Duration (%)</b>	38.3 ± 3.4	33.4 ± 3.4	37.1 ± 4.2	38.0 ± 4.1
<b>Mean Distance Between (cm)</b>	12.4 ± 0.3	12.8 ± 0.4	11.1 ± 0.5	11.6 ± 0.4
<b>Female</b>				
<b>In Proximity</b>				

<b>Duration (s)</b>	166.9 ± 17.9	188.5 ± 7.2	212.3 ± 7.9	182.2 ± 12.6
<b>In Proximity Duration (%)</b>	39.7 ± 4.3	44.9 ± 1.7	50.5 ± 1.9	43.4 ± 3.0
<b>Mean Distance Between (cm)</b>	12.0 ± 0.2	11.3 ± 0.2	11.5 ± 0.6	11.6 ± 0.4

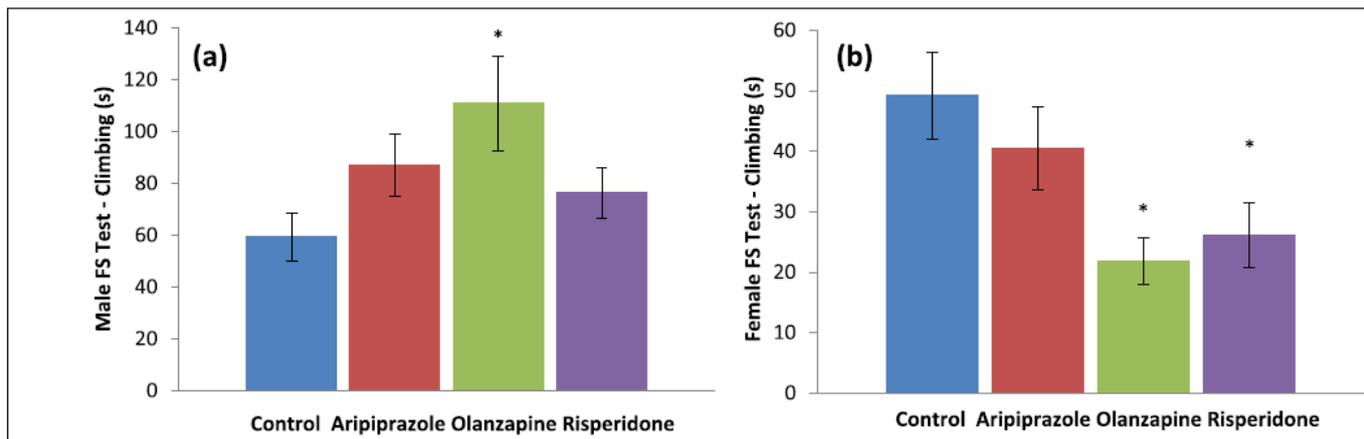
Data are expressed as mean ± SEM. \*  $p \leq 0.05$  vs control; \*\*  $p < 0.01$  vs control; \*\*\*  $p < 0.001$  vs control.

### 3.4. Forced Swim Test

Analyses again demonstrated that male and female rats responded differently to early APD treatment (Table 4). Two-way ANOVAs (Gender x Treatment) revealed significant effects of Gender on climbing duration ( $F_{1,86} = 60.582, p < 0.001$ ), swimming duration ( $F_{1,86} = 67.758, p < 0.001$ ) and floating duration ( $F_{1,86} = 19.833, p < 0.001$ ), along with a significant interaction between the two factors on climbing duration ( $F_{3,86} = 6.929, p < 0.001$ ), swimming duration ( $F_{3,86} = 5.880, p < 0.01$ ) and floating duration ( $F_{3,86} = 7.989, p < 0.001$ ). In male rats, analysis via ANCOVA found a trend to significant effect of Total Distance Travelled on swimming duration ( $F_{1,46} = 3.165, p = 0.083$ ) and a trend to significant effect of Treatment on climbing duration ( $F_{3,46} = 3.012, p < 0.05$ ), whilst analysis via one-way ANOVA found that early APD treatment caused a trend towards significance on floating duration ( $F_{3,45} = 2.720, p = 0.056$ ). Early Olanzapine drug treatment increased climbing duration (+87%,  $p < 0.05$ ) and decreased floating duration (-21%,  $p < 0.05$ ) compared with the control (Table 4). However, similar effects were not observed in any of the other male drug treatment groups (both  $p > 0.05$ ). In female rats, analysis of data via ANCOVA found a significant effect of both Total Distance Travelled ( $F_{1,43} = 4.839, p < 0.05$ ) and Treatment ( $F_{3,43} = 3.477, p < 0.05$ ) on swimming duration, whilst analysis of climbing duration also found a significant effect of Treatment

( $F_{3,43} = 5.019$ ,  $p < 0.01$ ). Furthermore, one-way analysis of floating duration also found a significant effect of early APD treatment ( $F_{3,46} = 4.428$ ,  $p < 0.01$ ) (Table 4). Early Olanzapine drug treatment decreased climbing duration (-55%,  $p < 0.05$ ), and increased floating duration (+17%,  $p < 0.05$ ) when compared to the control group. Furthermore, early Risperidone drug treatment was found to significantly decrease swimming duration (-50%,  $p < 0.05$ ) and climbing duration (-47%,  $p = 0.05$ ), and increase floating duration (+20%,  $p < 0.05$ ) when compared to the control (Table 4). These results indicate that early APD treatment has opposing effects on locomotor activity and depressive-like behaviour in adult male and female rats, indicating again that early APD treatment causes a gender specific response in behavioural activity (Figure 1). Furthermore, the early Aripiprazole treatment was not found to change locomotor activity, nor depressive-like behaviour in either the male or female cohorts.

**Figure 3.** Behavioural Effects – Depressive (A) Male FST Climbing (s); (B) Female FST Climbing (s) in Sprague-Dawley rats treated chronically with Aripiprazole (1.0 mg/kg, t.i.d), Olanzapine (1.0 mg/kg, t.i.d), Risperidone (0.3 mg/kg, t.i.d) or control (vehicle). Data expressed as mean  $\pm$  SEM. \*,  $p < 0.05$  vs. control. Abbreviations: FST: Forced Swim test; t.i.d.: three times daily.



**Table 4.** Behaviour of Male & Female Sprague-Dawley rats treated with chronic treatment of Aripiprazole (1.0 mg/kg, t.i.d.), Olanzapine (1.0 mg/kg, t.i.d.), Risperidone (0.3 mg/kg, t.i.d.) in the Forced Swim (FS) tests.

	Control	Aripiprazole	Olanzapine	Risperidone
<b>Male</b>				
<b>Climbing (s)</b>	59.25 ± 9.2	86.9 ± 12.1	<b>110.8 ± 18.3*</b>	76.3 ± 9.7
<b>Swimming (s)</b>	9.75 ± 0.9	12.75 ± 1.9	7.3 ± 1.4	10.9 ± 2.7
<b>Floating (s)</b>	231.0 ± 9.6	200.3 ± 13.0	<b>181.9 ± 17.9*</b>	212.8 ± 8.6
<b>Female</b>				
<b>Climbing (s)</b>	49.2 ± 7.1	40.5 ± 7.0	<b>21.9 ± 3.8*</b>	<b>26.2 ± 5.3*</b>
<b>Swimming (s)</b>	37.5 ± 4.7	24.5 ± 2.6	28.0 ± 5.1	<b>18.7 ± 2.5*</b>
<b>Floating (s)</b>	213.3 ± 11.1	235.0 ± 8.5	<b>250.1 ± 8.3*</b>	<b>255.1 ± 7.0*</b>

Data are expressed as mean ± SEM. \*  $p \leq 0.05$  vs control; \*\*  $p < 0.01$  vs control; \*\*\*  $p < 0.001$  vs control.

#### 4. Discussion

The present study investigated the effects of childhood/adolescent treatment with Aripiprazole, Olanzapine and Risperidone on long-term (adult) behavioural attributes in both male and female rats. Our results provide evidence that early APD treatment causes long-term changes to locomotor, depressive-like and anxiety-like behaviours, and demonstrate that there are gender differences in both locomotor activity and depressive-like behaviours in response to early APD treatment.

Our study found significant increases in numerous parameters indicative of hyper-locomotor activity in the male rodent model between the drug treatment and control groups, across both the open-field/holeboard and FS behavioural tests (Figure 1). Rats that received early Risperidone treatment showed increased velocity, distance and rearing in the open-field/holeboard test, whilst rats that received early Olanzapine treatment showed increased time spent climbing and decreased time spent floating when compared to the control. In addition, locomotor activity of the male rat early Aripiprazole drug treatment group was unchanged compared to the control. Although increased rearing behaviour has previously been correlated to vertical activity and exploratory/vigilant behavioural attributes (Brenes Sáenz et al. 2006; du Bois et al. 2008), no alterations in the number of head dips, also an indicator of exploratory behaviour was observed. This therefore indicates the differences in rearing activity observed may potentially be due to alterations in activity levels alone.

Thus far, animal studies investigating the effects of early, short-term treatment with APDs on long-term locomotor activity have found differing results (Milstein et al. 2013; Shu et al. 2014; Varela et al. 2014). Milstein and colleagues (Milstein et al. 2013) investigated the effects of early Olanzapine treatment on locomotor activity in adulthood, with no changes to numerous parameters of the open-field test observed. Similarly, the present study found increases in the activity levels of Olanzapine-treated animals in the FS test but not in the Open-Field/Holeboard test, potentially due to the increased stress levels induced by the FS testing procedure. In addition, the investigation by Shu et al (Shu et al. 2014) found that repeated early treatment with Olanzapine and Clozapine induced a persistent inhibition of PCP-induced hyper-locomotion in adulthood, a test commonly used to investigate motor activity levels. However, they reported that on ‘re-habituation’ days in adulthood (when rats

were totally drug free during the long-term drug treatment period), visible increases in activity levels were observed. It is possible that this is due to a drug withdrawal-induced behavioural hypersensitivity, and that the long-term antagonistic actions of drugs, such as the administration of Olanzapine and Risperidone in adolescence, potentially cause the changes in activity levels observed in the present study. Furthermore, concurrent with the findings of our study, numerous studies have also found that Aripiprazole treatment does not cause any impairments to motor activity levels (Leite et al. 2008; Biojone et al. 2011).

Along with increased locomotor activity, the aforementioned changes to behavioural parameters in the FS test was also indicative of changes to depressive-like behaviour in both the male and female rodent models of the present study. In the male cohort, rats that received early Olanzapine treatment showed increased time spent climbing and decreased time spent floating, indicative of a decrease in depressive-like behaviour (Costa et al. 2013). Conversely, in the female cohort, significant decreases in time spent climbing and significant increases in time spent floating were observed in both early Olanzapine and Risperidone drug treatments, indicative of an increase in depressive-like behaviour (Costa et al. 2013).

Previous animal studies looking at the effects of APD treatment on depressive-like behaviour in the FS test has found similar gender-dependent results to those in the present study (Weiner et al. 2003; Sharma et al. 2015). Weiner and colleagues (Weiner et al. 2003) found that acute treatment of adult male rodents with Olanzapine was found to decrease immobility time (time spent floating) in response to the APD treatment, similar to the results found in the present study. Additionally, increases in immobility time was found by Sharma and colleagues (Sharma et al. 2015) following long-term Olanzapine treatment in adult female rodents, observations that were also found in the present study.

The differences in the Olanzapine, Risperidone, and Aripiprazole receptor binding profiles, receptor occupancy levels and mechanisms of action, in particular on the DA and 5-HT receptors, may result in the locomotor activity and depressive-like behavioural differences across these different drug treatment groups. With high-affinity, high receptor occupancy, partial agonist mechanism of action known to have the beneficial effects of normalizing NT activity (Natesan et al. 2006), Aripiprazole (a partial D<sub>2</sub> agonist) may not cause the long-term alterations to the DA NT system seen with adolescent exposure to the potent antagonistic effects of APDs including Olanzapine and Risperidone, and subsequently no changes to locomotor activity. Although changes to locomotor activity-related behavioural parameters were exhibited in the holeboard and FS tests, similar changes were not found in the EPM. One possible explanation for this is the added parameter of height present in the EPM that was not present in the other behavioural tests. The effects of this increased height also potentially affect the number of open-arm entries/duration, and thus the anxiety-like behaviour as examined below. This leads to the possibility that previously documented differences in height and bright-space anxiety (Komada et al. 2008) effect the level of locomotor activity in the EPM test.

Analysis of behavioural data from the male cohort of the present study also found changes to behavioural parameters indicative of anxiolytic behaviour. Compared to the control group, long-term increases in time spent in the open-part (open-arm plus centre duration), and corresponding decreases in time spent in the closed-arms of the EPM were observed in all three APD treatment groups, parameters known to be indicative of anxiolytic behaviour (Mällo et al. 2007; Walf and Frye 2007; Komada et al. 2008; Tsujimura et al. 2008). However, segmenting the open-part data into time spent on the open-arm and centre platform

individually found a significant increase in time spent on the centre platform only, with no differences between drug treatment groups in time spent on the open arms. In addition, a trend to significant increase in centre frequency in the open field/holeboard test following early Risperidone treatment was also found. Thus, in the present study, adolescent APD treatment may potentially cause anxiolytic effects specifically towards bright spaces (due to the differences in light intensity of the open part (100 lx) and closed-arms (darkness)) when compared to the control (Figure 2), with no differences in height-anxiety between the treatment groups and the control were found at the 60cm elevation. Further behavioural testing such as the light/dark transition test is required however to clarify any different aspects in anxiety-like behaviour. However, previous animal studies examining the effects of APD treatment on anxiety-like behaviours have found differing results across treatment groups. Whilst studies in adult rats over a short-term/acute period have found that the partial agonist actions of Aripiprazole induced a similar increase in time spent in the open-arms and a decrease in time spent in the closed-arms of the elevated maze (indicative of anxiolytic effects) (Biojone et al. 2011), studies investigating the effects of treatment with Risperidone and Haloperidol demonstrated anxiogenic effects, opposite to the effects seen in the present study (Karl et al. 2006).

The discrepancy between the results of our study and those previous may be due to both the differences in time between treatment and behavioural testing, and the age of the animals undergoing treatment. Firstly, it is important to consider that whilst previous studies have investigated the immediate effects of short-term/acute or chronic treatment in adult rats on anxiety-related behaviours in adulthood, our study investigated the long-term effects of early (childhood/adolescent) APD treatment on adult behaviour. Hence, changes to NT systems, including the DA and 5-HT systems targeted by APDs, may have occurred following APD

withdrawal in the time period between drug treatment and behavioural testing in the present study, resulting in the differences in both depressive-like and anxiety-like behaviours. Furthermore, the current study used a young animal treatment model compared to the adult model of previous studies. This difference in treatment age may be critical to the different results observed, with the antagonistic actions of Olanzapine treatment during the critical neurodevelopmental period previously found to result in long-term alterations to NT functioning, including the DA and 5-HT NT systems (Vinish et al. 2012; Milstein et al. 2013).

Surprisingly, our study also found gender-specific behavioural effects in response to early APD treatment. Firstly, no anxiety-related changes were observed in adult female rats, whilst the alterations to locomotor activity were found to be both dissimilar and to a far lesser extent to those of the male cohort. Further comparison of activity related changes also identified that the significant gender based difference in total distance moved (~33% greater for females) was almost as strong as the effect of Risperidone drug treatment in the male cohort (~26% greater for Risperidone treatment), reported previously. Also, whilst behavioural observations indicative of changes in depressive-like behaviour were also seen in the adult female rats FS test, early treatment with Olanzapine and Risperidone were found to induce an increase in depressive-like behaviour in adulthood, an opposite effect to that seen in the male cohort. Analysis of the FS test data revealed decreases in time spent climbing (s; Olanzapine treatment) and swimming (s; Risperidone treatment), and subsequent increases in time spent floating (s; Olanzapine and Risperidone treatment). There could be numerous reasons for the differential effects in locomotor, anxiety and depressive-related behaviours observed between the genders. Firstly, gender-based differences in the neurodevelopment of the DA NT system are well-postulated, and may affect the behavioural differences observed in the present study.

During the developmental phase, studies have found both regional and gender-based differences in the development of the DA NT system, including differences in; striatal DA D<sub>1</sub> and D<sub>2</sub> receptor overproduction and elimination, DA D<sub>1</sub> receptor densities in the nucleus accumbens, grey and white matter overproduction and elimination and rates of myelination (Andersen 2003; Sinclair et al. 2014). These gender-based differences in the development of the DA NT system, combined with early APD treatment during this neurodevelopmental window, may explain the observed gender differences in the predominately DA-related locomotor activity changes of the present study.

Furthermore, the sex hormones testosterone and estrogen may also contribute to the gender differences in the behavioural results observed in the present study. During adolescence, testosterone and estrogen influence brain maturation/development in dopaminergic regions including the striatum and prefrontal cortex, and also have a significant role in shaping the dopaminergic signal (Andersen 2003; Purves-Tyson et al. 2012; Sinclair et al. 2014). Previous animal studies have found that augmenting both testosterone and estrogen levels has resulted in altered DA neurotransmission, including DA synthesis, DA receptor mRNA expression and DA transporter levels, as well as changes in DA-related behaviours such as locomotor activity (Purves-Tyson et al. 2012; Sinclair et al. 2014). Additionally, some ‘neuro-protective’ effects of estrogen on DA and 5-HT NT system-related behaviours have been found, with estrogen exhibiting an ability to inhibit the DA D<sub>2</sub> and 5-HT<sub>1A</sub> receptor-induced mediated behavioural changes in sensorimotor gating/information processing, which are deficient in people with mental illnesses (Dunlop and Nemeroff 2007; Gogos et al. 2012). This ability of estrogen to inhibit both DA and 5-HT related changes may account for the differences in the predominately 5-HT-related anxiety, along with the DA and 5-HT related

depressive-like behaviours observed between genders, however further research in this area is needed.

It has been postulated that the physiology behind early insult drug induced hyper-locomotor effects may be due to a drug-treatment induced long-term receptor hypersensitivity (Samaha et al. 2007; Shu et al. 2014). This phenomenon reports that continuous APD treatment (with high affinities for DA receptors) may cause an up-regulation of DA receptors, and thus upon APD withdrawal, enhance dopaminergic signalling in brain regions including the nucleus accumbens, hippocampus and striatum, previously observed in animals exhibiting hyper-locomotor activity (Kelly et al. 1975; Kusljic et al. 2003; Miyakawa et al. 2003; Seo et al. 2008; Beaulieu and Gainetdinov 2011). The potent antagonist actions of chronic Risperidone and Olanzapine APD treatment on the DA D<sub>2</sub> receptors during the critical childhood/adolescent neurodevelopmental period in the present study may have resulted in a long-term DA receptor and consequent DA synthesis up-regulation, enhancing the dopaminergic signal along these brain regions previously heavily implicated in locomotor activity.

Furthermore, the antagonistic actions of Olanzapine and Risperidone on the 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors resulting in decreases in 5-HT projections may also result in similar long-term alterations to dopaminergic signalling and locomotor activity. Previous studies have found strong links between the antagonism of 5-HT<sub>2</sub> receptors and enhanced locomotor activity (Kusljic et al. 2003; Seo et al. 2008), potentially due to the negative correlation between 5-HT and DA functioning. In such cases, a deficiency in 5-HT projections leads to a disinhibition of DA signalling, and thus enhanced DA signal (Kusljic et al. 2003; Seo et al.

2008). Similarly, the antagonistic actions of Olanzapine and Risperidone on the 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub> and DA D<sub>2</sub> receptors, resulting in the aforementioned long-term alterations in signalling, have also been correlated to anxiolytic and decreased depressive-like behaviours (Mora et al. 1997; Andersen and Navalta 2004; Karl et al. 2006; Dunlop and Nemeroff 2007; Seo et al. 2008; de Oliveira et al. 2009; Biojone et al. 2011). Previous studies have demonstrated that repeated antagonism of the 5-HT<sub>2</sub> receptors (both 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub>) have been found to result in a down regulation of both the number and sensitivity of 5-HT<sub>2</sub> receptors (Mora et al. 1997), resulting in a long-term deficiency in serotonergic signalling, correlated with anxiolytic- and decreased depressive-like behavioural effects. Long-term APD use resulting in the up-regulation and hypersensitivity of DA receptors and thus enhanced dopaminergic signalling may also play a role, with enhanced DA signalling in brain regions including the ventral tegmental area, prefrontal cortex and nucleus accumbens correlated with the anxiolytic- and decreased depressive-like behavioural effects exhibited in the present study (Karl et al. 2006; de Oliveira et al. 2009; Biojone et al. 2011).

Finally, despite a high affinity partial agonist mechanism of action on the DA D<sub>2</sub> and 5-HT<sub>1A</sub> receptors (Shapiro et al. 2003), adolescent Aripiprazole APD treatment was also found to elicit anxiolytic-like effects in adult rats. Such effects on anxiety, with no differences in locomotor activity or depressive-like behaviours, it suggests a potential vital role of the 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors in anxiety-like behaviour alone, as Aripiprazole is known to have an antagonistic mechanism of action on the former, and a slightly lower affinity for the latter 5-HT receptor subtypes (Shapiro et al. 2003). Since Olanzapine and Risperidone also have a similar antagonistic and agonistic mechanisms of action on the 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors respectively and exhibit similar effects, this may provide further evidence for the potentially crucial role that mediating these individual receptor subtypes may have in regulating anxiety, although further studies investigating this is necessary.

With clear long-term depressive-like, anxiety behavioural alterations found in the present study, minor alterations to current methods, along with additional investigations will provide further insight into the effects of childhood/adolescent APD use on behavioural attributes. Firstly, altering the social interaction protocol in order to pair naïve age and sex-matched animals may reveal some potential differences in social behaviour that the current method of sex and gender-matched animals may have occluded. Secondly, additional behavioural testing investigating the effects of early APD use on other behavioural parameters including cognitive abilities would be helpful in assessing any potential issues with short and long-term memory in the adult rat, whilst also investigating the effects of other commonly used APDs including Clozapine would also increase the scope of the current investigation. Furthermore, it may also be of benefit for supplementary investigations to be completed on the effects of early APD treatment in disease animal models of mental illness e.g. schizophrenia. Whilst the use of a standard rat strain in the present study is appropriate and clinically relevant due to an inability to investigate in a single animal model for the multiple illnesses (such as schizophrenia, bipolar disorder, autism, ADHD, Tourette's disorder, anxiety disorder, borderline personality disorder and even hemiplegia) APDs are used to treat (Olfson et al. 2010; Memarzia et al. 2014; Schneider et al. 2014), and the reported inadvertent exposure of healthy adolescent brains to APDs due to difficulties in diagnosis at a young age (Andersen and Navalta 2011; Zuddas et al. 2011), increasing the scope of the study to investigate the long-term effects early APD use has on individual illnesses will no doubt be beneficial.

In conclusion, this study indicates that early APD treatment results in long-term changes on locomotor, depressive-like and anxiety-like behaviours in adult rats. In addition, behavioural

differences between genders were also observed in response to early APD treatment. Potential detrimental long-term behavioural changes following early APD treatment may outweigh the initial benefits of childhood/adolescent APD prescription and use. Whilst previous clinical studies have shown gender based differences in APD treatment effects (Ratzoni et al. 2002), our study indicates that these differential effects may not only extend to behavioural attributes but also into adulthood. Hence, careful consideration of both the pharmacology of the APD being prescribed along with the gender of the child may be crucial to decrease any potential long-term effects, including alterations to activity, anxiety or depressive-like behaviours. Further research is required in order to explore the potential physiological reasons behind the behavioural differences found across both treatment groups and genders, which has potentially resulted in the observed effects. Investigations into potential alterations to both the DA and 5-HT NT systems in key brain areas would provide further evidence of these potential detrimental long-term neurotransmitter alterations.

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## **Conflict of Interest Statement**

None Declared.

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