

**The dopamine D<sub>3</sub>-preferring D<sub>2</sub>/D<sub>3</sub> dopamine receptor partial agonist, cariprazine, reverses behavioral changes in a rat neurodevelopmental model for schizophrenia**

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## **Abstract**

Current antipsychotic medication is largely ineffective against the negative and cognitive symptoms of schizophrenia. One promising therapeutic development is to design new molecules that balance actions on dopamine D<sub>2</sub> and D<sub>3</sub> receptors to maximise benefits and limit adverse effects. This study used two rodent paradigms to investigate the action of the dopamine D<sub>3</sub>-preferring D<sub>3</sub>/D<sub>2</sub> receptor partial agonist cariprazine. In adult male rats, cariprazine (0.03-0.3mg/kg i.p.), and the atypical antipsychotic aripiprazole (1-3mg/kg i.p.) caused dose-dependent reversal of a delay-induced impairment in novel object recognition (NOR). Treating neonatal rat pups with phencyclidine (PCP) and subsequent social isolation produced a syndrome of behavioral alterations in adulthood including hyperactivity in a novel arena, deficits in NOR and fear motivated learning and memory, and a reduction and change in pattern of social interaction accompanied by increased ultrasonic vocalisations (USVs). Acute administration of cariprazine (0.1 and 0.3mg/kg) and aripiprazole (3mg/kg) to resultant adult rats reduced neonatal PCP-social isolation induced locomotor hyperactivity and reversed NOR deficits. Cariprazine (0.3mg/kg) caused a limited reversal of the social interaction deficit but neither drug affected the change in USVs or the deficit in fear motivated learning and memory. Results suggest that in the behavioral tests investigated cariprazine is at least as effective as aripiprazole and in some paradigms it showed additional beneficial features further supporting the advantage of combined dopamine D<sub>3</sub>/D<sub>2</sub> receptor targeting. These findings support recent clinical studies demonstrating the efficacy of cariprazine in treatment of negative symptoms and functional impairment in schizophrenia patients.

## 1. Introduction

Schizophrenia is a debilitating, lifelong psychiatric disorder affecting approximately 1% of the population. Current antipsychotics provide therapeutic benefit to the positive symptoms, but have relatively little impact on the negative (social withdrawal and anhedonia) and cognitive deficits (Keefe et al., 2007) which precede psychosis and show a stronger correlation to patient functional outcome (Green et al., 2004; Mintz and Kopelowicz, 2007). All current antipsychotics are dopamine D<sub>2</sub> receptor antagonists, a property thought to contribute to the reduction in positive symptoms but also their high propensity for unwanted extrapyramidal side-effects and tardive dyskinesia limiting therapeutic benefit. Second generation atypical antipsychotics were developed in an attempt to improve therapy but still have limited effect on cognitive deficits or negative symptoms and many cause weight gain and metabolic abnormalities (Miyamoto et al., 2012). Thus new therapeutic agents operating through different pharmacological mechanisms are essential for more effective management of schizophrenia. One potential mechanism, preferential targeting of the dopamine D<sub>3</sub> receptor over the dopamine D<sub>2</sub> receptor, might provide effective relief of positive and negative symptoms combined with improved cognitive function (Gyertyán et al., 2008; Laszy et al., 2005; Millan and Brocco, 2008; Millan et al., 2008).

Current antipsychotics lack selective discrimination of dopamine D<sub>2</sub> and D<sub>3</sub> receptors, however recent positron emission tomography studies (Howes et al., 2015) show these have a differential brain distribution. The D<sub>3</sub> receptor is abundant in the ventral striatal mesolimbic system and frontal cortex but (unlike the D<sub>2</sub> receptor) low in the dorsal striatum, which may account for the paucity of effect of D<sub>3</sub> antagonists on locomotor activity in rodents and provides potential for few extrapyramidal symptom in clinical use in man (see reviews; (Heidbreder and Newman, 2010; Millan and Brocco, 2008). Both gene polymorphisms and increased post-mortem brain D<sub>3</sub> receptor protein levels have also been associated with patients who had schizophrenia. Furthermore converging evidence showing the receptors have contrasting roles on cognition in rodents, encouraged development of drugs with differential D<sub>2</sub>/D<sub>3</sub> receptor pharmacology (Millan and Brocco, 2008; Sokoloff et al., 2006; Watson et al., 2012a). Cariprazine (RGH-188; N'-[trans-4-[2-[4-(2,3-dichlorophenyl)-1-piperazinyl]ethyl]cyclohexyl]-N,N-dimethylurea, Vraylar™) is one such orally active, putative antipsychotic which is described as a dopamine D<sub>3</sub> receptor preferring D<sub>3</sub>/D<sub>2</sub> receptor partial agonist (Ágai-Csongor et al., 2012; Caccia et al., 2013). *In vitro* ligand binding and functional studies show that cariprazine has 6-10 fold higher affinity for the human dopamine D<sub>3</sub> than D<sub>2</sub> receptor (K<sub>i</sub>=0.085 and K<sub>i</sub>=0.49 nM, respectively) and is a moderate affinity partial agonist at 5-HT<sub>1A</sub> receptors (Kiss et al., 2010). Positron emission tomography studies in the monkey confirmed that at the highest dose used

(300µg/kg) cariprazine has >90% D<sub>2</sub>/D<sub>3</sub> receptor occupancy in the striatum with only 30% 5-HT<sub>1A</sub> receptor occupancy in the raphé consistent with the *in vitro* data (Seneca et al., 2011). Unlike other atypical antipsychotics, cariprazine shows a high and balanced occupancy of both D<sub>2</sub> and D<sub>3</sub> receptors in rodents as seen in schizophrenia patients (Kiss et al., 2012; Slifstein et al., 2013). This unique pharmacological profile suggests cariprazine may have antipsychotic effects combined with beneficial effects on cognitive and negative symptoms above that seen with current antipsychotics. Indeed, cariprazine is active in several rodent models predictive of 'antipsychotic-like' activity; including the conditioned avoidance response and inhibition of amphetamine-induced hypermotility, without evidence of induction of 'cataleptic-like' activity (Gyertyán et al., 2011). Furthermore cariprazine has been shown to attenuate the chronic mild stress-induced decrease in sucrose intake in rats which has been suggested by some groups to be a model of anhedonia; a common symptom of schizophrenia as well as depression (Papp et al., 2014). Positive results have been obtained from phase II and III clinical trials in schizophrenia and bipolar mania (Calabrese et al., 2015; Durgam et al., 2014, 2015; Grunder, 2010; Sachs et al., 2015). Recently cariprazine was also shown to produce significant improvement against placebo on Positive and Negative Syndrome Scale total in a 6 week phase III clinical trial (Kane et al., 2015) in patients with an acute exacerbation of schizophrenia and has now received approval by the FDA for the treatment of schizophrenia and acute treatment of manic or mixed episodes associated with bipolar I disorder (McCormack, 2015).

However the preclinical activity of cariprazine against cognitive deficits has so far been limited to reversal of pharmacological impairments in learning and memory. These include scopolamine-induced deficits in a rat water-labyrinth task (where a bell shaped dose-response pattern was observed; Gyertyán et al., 2011) and PCP-induced deficits in rat and mouse tests of visual recognition memory, attentional set shifting/reversal learning and social interaction/social recognition memory (Adham et al., 2012; Zimnisky et al., 2013).

Rearing rats in social isolation from weaning produces long-term neurodevelopmental, behavioral, structural and neurochemical alterations with translational relevance to a spectrum of changes seen schizophrenia (Fone and Porkess, 2008; Jones et al., 2011b; Lapis et al., 2003) including visual learning, social cognition, reversal learning and attentional set shifting deficits (Bianchi et al., 2006; Fone et al., 1996; Fone and Porkess, 2008; King et al., 2009; Marsden et al., 2011; Meffre et al., 2012). Importantly in studies examining new potential therapies for schizophrenia compounds with divergent pharmacology including dopamine D<sub>3</sub> or 5-HT<sub>6</sub> receptor antagonists and mGluR<sub>2/3</sub> or nicotinic receptor agonists (Jones et al., 2011a; King et al., 2013; Marsden et al., 2011; Watson et al., 2012b) reverse components of the isolation-induced syndrome, suggesting this model has useful pharmacological sensitivity.

Neonatal treatment of rats with the non-competitive NMDA receptor antagonist, phencyclidine (PCP), disrupts neuronal development and synaptogenesis and also reproduces in adulthood many of the behavioural features akin to schizophrenia, including hyperactivity to amphetamine, attenuated sensorimotor gating and deficits in cognition (Bubenikova-Valesova et al., 2008; Ingallinesi et al., 2015; Mouri et al., 2007). We recently showed that combining neonatal PCP administration with subsequent isolation rearing, as a novel 'dual-hit' neurodevelopmental model (Lim et al., 2012), causes all the behavioral changes seen with isolation alone (Gaskin et al., 2014), but importantly also attenuated social interaction (McIntosh et al., 2012) which may be a useful predictive index of negative symptoms such as social withdrawal (Sams-Dodd, 1999; Wilson and Koenig, 2014). The current study therefore recorded a battery of behavioral tests in neonatal PCP treated and isolation-reared rats and controls to examine reversal of surrogate markers for positive, negative and cognitive deficits by the novel potential antipsychotic compound, cariprazine.

Initially this study compared the effects of cariprazine and aripiprazole on cognition in a novel object recognition (NOR) test of memory using a model of natural forgetting in adolescent rats to establish suitable doses to be used in the main neurodevelopmental rodent model. This paradigm was selected because of pharmacological sensitivity to drugs which modify dopaminergic activity, reliability, and wide recognition of its translational relevance to visual learning and memory deficits seen in schizophrenia (Lyon et al., 2012; Rajagopal et al., 2014), which are resistant to current medication. Secondly, in the main study, cariprazine and aripiprazole were compared in rats treated with neonatal PCP followed by social isolation to determine their ability to reverse behavioral changes in tests chosen to have relevance to the positive, negative and cognitive deficits seen in patients with schizophrenia.

## **2. Experimental procedures**

### **2.1. Animals**

Male Lister hooded rats were used in all experiments. In the time-delayed NOR studies adolescent rats (Charles River UK weighing between 175-200g) were housed in groups of four and allowed to habituate for a week before testing. For the combined neonatal PCP and social isolation experiment (Gaskin et al., 2014) litters of five male Lister hooded pups arrived with their dam on post-natal day (PND) 3-4 (Charles River UK). Pups from each litter were injected with either sterile saline or PCP HCl (10mg/kg s.c.) on PND 7, 9 and 11. Pups were weaned on PND 24. Those pups receiving saline were housed in conventional cages (50 x 33 x 26cm) in groups of four while rats given PCP were housed in social isolation (41 x

24 x 20cm) such that isolates remained in auditory and olfactory contact with their litter mates but had no physical contact with conspecifics. Rats were isolated for five weeks before undergoing a battery of behavioral tasks, arranged in order of least to most aversive (to minimise any sequential effect on subsequent tasks) at approximately one week intervals. In all experiments food and water was available *ad libitum* and rats were housed under 12h light/dark cycle (lights on 07.00h), temperature  $21 \pm 2$  °C,  $55 \pm 10$  % humidity. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986 and ARRIVE guidelines with approval of University of Nottingham Local Ethical Committee and all efforts were made to minimise animal suffering, reduce the number of animals used, and utilise alternatives to *in vivo* techniques, if available.

## 2.2 Experimental Design

All behavioural studies were performed between 08.00 and 16.00h and following every test the apparatus was thoroughly cleaned with 20%v/v ethanol and dried to remove odor cues. All studies were performed using a blind protocol, so that the observer was unaware of drug treatment. The majority of studies were performed on rats which received both neonatal PCP and were subsequently reared in social isolation following weaning from the dam. The separate impact of exposure to each of these two manipulations has been thoroughly studied before (for reviews see (Bubenikova-Valesova et al., 2008; Fone and Porkess, 2008; Jones et al., 2011b)) and compared with the combined treatment by us (Gaskin et al., 2014). Isolation alone in Lister hooded rats does not attenuate social interaction (a useful index of negative symptoms) while neonatal PCP combined with isolation does, making this dual manipulation the method of choice for use herein. This study was designed to compare the ability of cariprazine and aripiprazole to reverse the resultant behavioral syndrome and not to determine which changes were produced by either neonatal PCP or isolation, so these separate groups were not included. To further reduce unnecessary use of animals, in accordance with the 3Rs principle and ARRIVE guidelines, the effect of cariprazine was compared to that of an antipsychotic in combined PCP/isolation-reared rats rather than to the effect of cariprazine in vehicle group-housed controls which substantially reduced the number of rats utilised. In order to ascertain any potential confounding non-specific 'sedatory-like' motor effect of either drug, indices of locomotor activity were carefully monitored in each behavioral task and this is discussed later.

## 2.3. Drugs and Dosing

For time-delay NOR studies cariprazine (0.03-0.3mg/kg dissolved in 0.154M saline) or aripiprazole (0.3-3.0mg/kg suspended in distilled water with 2% Tween 80) were injected i.p. in a volume of 2ml/kg or 10ml/kg, respectively, 30min prior to the familiarisation trial. Based

on these data (and previous behavioral studies showing lack of sedative or cataleptic effects at these cariprazine doses (Gyertyán et al., 2011; Gyertyán et al., 2008; Papp et al., 2014)) cariprazine (0.1 and 0.3mg/kg) and aripiprazole (3mg/kg) were selected for the combined neonatal PCP and social isolation experiment. Treatments were administered 30min prior to each behavioral test, except for the conditioned freezing associative learning response task where they were given immediately after the third foot shock on the conditioning day and 30min prior to testing on days two and three. Drug administration was given immediately after (and not before) conditioning to prevent any drug effect on perception of and/or response to the foot shock but enable interference with consolidation, and thus ensure detection of any specific effects on learning and memory.

#### **2.4. Spontaneous locomotor activity in a novel arena**

Isolation rearing of rats (both alone and combined with neonatal PCP) causes developmental induction of a reproducible hyperactivity in response to placement in a novel arena, regarded as a surrogate marker for striatal dopamine hyperactivity akin to positive symptoms of schizophrenia, which is attenuated by acute injection of several antipsychotics (Fabricius et al., 2010; Fone and Porkess, 2008; McIntosh et al., 2013). Locomotor activity in a novel arena was therefore measured initially in drug-free rats to confirm development of the isolation syndrome and, one week later, to compare reversal by cariprazine and aripiprazole. Five weeks after weaning, spontaneous locomotor and rearing activity was recorded for 1h in an unfamiliar Perspex arena (39cm x 23.5cm x 24.5cm) using a computerised infra-red activity system (Photobeam Activity System- Home Cage, San Diego Instruments, CA, USA). Horizontal ambulatory activity was measured as the consecutive interruption of two adjacent lower beams, while the number of rears was measured by interruption of a separate layer of upper beams. Cumulative beam-breaks were automatically recorded in 5min time bins for the test duration. Rats received no drug treatment.

Six weeks after weaning, the effects of cariprazine and aripiprazole on spontaneous locomotor and rearing activity were recorded under the same conditions. Rats were assigned to each treatment group in a pseudorandom order using a balanced design to take into account litter, weight and locomotor performance in the previous trial.

#### **2.5. Novel object recognition task**

According to the MATRICS initiative NOR in rodents maps to the visual learning and memory cognitive domain which is attenuated in schizophrenia (Pelletier et al., 2005). NOR is widely regarded as a pertinent cognitive task to assess reversal of recognition memory by antipsychotic drugs because of reliability, knowledge of underlying circuits (Lyon et al., 2012;

Rajagopal et al., 2014) and its differential response to dopamine D<sub>2</sub> (which impair) and D<sub>3</sub> (which restore) receptor antagonists (Watson et al., 2012b). Furthermore time-delay (natural) forgetting of NOR is robustly shorter in rats reared in isolation (both alone and combined with neonatal PCP (Gaskin et al., 2014)) than group-housed littermates and this deficit is reversed by antipsychotics (McIntosh et al., 2013) and drugs under development as adjunctive treatment for cognitive impairment (Jones et al., 2011a; Watson et al., 2012a). The NOR task was adapted from Ennaceur and Delacour (Ennaceur and Delacour, 1988) and is in routine use in our laboratory (Bianchi et al., 2006; King et al., 2004; Watson et al., 2012a; Watson et al., 2012b).

In all studies NOR was assessed 24h after 1h habituation to the apparatus, which also served to collect locomotor activity data in the PCP/social isolation rearing study (see spontaneous locomotor activity above). In studies in group-housed adolescent rats, to select doses of cariprazine and aripiprazole for the isolation study, the effect of drugs was examined on a delay-induced impairment of NOR using a 4h ITI chosen because normal, vehicle treated adolescent group-housed rats can't discriminate the novel from familiar object with this ITI (Watson et al., 2012a). Performance of NOR is highly dependent on the objects used (Li et al., 2014); the close visual resemblance of objects in this task explains why such short ITIs impair performance and justifies selection of this particular trial interval. Furthermore previous validation studies established that rats do not show preferential exploration of the plain or striped objects, so any increase in exploration of the latter is not due to enhanced sensory motivation towards it. A shorter ITI of 2min, where group-housed, vehicle-treated rats readily discriminate the novel object, was also used to examine any possible amnesic drug effects. These dose-response studies used a within-subjects/repeated-measures design such that each rat received every dose of the chosen drug and its vehicle in a pseudorandom order; being tested on four separate occasions, at one week intervals. In studies with PCP isolation rats NOR was conducted in an identical manner but a 2h ITI was selected because these rats show more rapid natural, time delay-induced forgetting and unlike group-housed littermate controls are unable to discriminate after this time. Thus a 2h ITI is ideal to examine drug-induced reversal of PCP/isolate-induced impairment of NOR.

In both studies on the test day rats were briefly reacclimated to the arena for 3min before being returned to the home cage for 1min. During the familiarisation trial two identical objects (plastic bottles, 8cm high and 5cm diameter, wrapped in white masking tape) were placed in opposite corners of the arena (5cm from the side and 10cm from the end wall). The rat was returned to the arena for 3min and exploration of each identical objects recorded with stopwatches. After a 2h inter-trial interval (ITI) in the home cage the rat was returned to the



arena with one familiar and one novel object (identical size and shape bottle wrapped with 4 black stripes 1.2cm in width). During this second, choice, trial exploration of each object was recorded for 3min. Exploratory behavior was defined as sniffing, licking, touching, and direct attention to the object with active vibrissae while the nose was within 1cm of the object. Climbing on or chewing the object was not considered as exploration and not recorded. Object exploration time during the choice trial was used to determine the discrimination ratio (time at novel - time at familiar/ total choice trial object exploration).

## **2.6. Social interaction**

Assessment of the social behavioral repertoire between unfamiliar conspecifics is thought to be ethologically appropriate to monitor social withdrawal, a negative symptom associated with schizophrenia (Sams-Dodd, 1999; Wilson and Koenig, 2014). Social interaction was therefore assessed seven weeks after weaning. Rats were paired with weight-matched conspecifics from a different litter and housing cage of the same developmental condition and acute drug treatment. Rats were lightly coloured on the back with red or blue hair spray (UVGlow, Superdrug, randomly assigned) 30min prior to the test, to aid automated tracking (Ethovision XT ver8.5, Noldus). Each pair was placed in an open arena (75cm diameter, 45cm walls), under dim light (40 lux) for 10min. Movement was automatically tracked while the behavior of each rat (anogenital sniffing, body sniffing, boxing and biting, crawling under and over, following, lying side-by-side and pinning) were scored from the video-recording by a scorer blind to treatment group. As the actual behavior of each rat in the pair is not independent of the action of the partner the mean duration of each individual behavior, total behavior and distance moved was calculated for each pair and the subsequent data analysed such that the *N* value represents the number of pairs. As an additional measure to record communication during social interaction ultrasonic vocalisation was recorded using an electret microphone (Emkay, Avisoft Bioacoustics, Germany) with optimal frequency responses at approximately 20–85kHz (to separately record 22kHz alarm and 50kHz pro-social calls), connected to an ultrasound detection unit (Ultrasound Gate (USG) customised model 112, Avisoft Bioacoustics, Berlin). The resulting signal was digitalised and saved as .wav files. The temporal and frequency characteristic of every call was extracted using associated Avisoft analysis software (SAS-Lab Pro, v 4.38, Avisoft Bioacoustics, Berlin). Once calls had been automatically identified, they were verified by eye before accepting the measurement output for further statistical analysis. A  $\text{Log}_{10}(x+1)$  transformation was used to normalise USV data prior to parametric statistical analysis.

## **2.7. Conditioned freezing response**

Associative learning including memory of preference conditioning is impaired in schizophrenia (Herbener, 2009) and resistant to antipsychotic medication (Kucharska-Pietura et al., 2012). A fear motivated conditioned associated learning task was therefore used to measure amygdala- and/or hippocampal-dependent working-memory based on a protocol previously described by us (Jones et al., 2011a; McIntosh et al., 2013; Woods et al., 2012). The conditioned freezing response (CFR) was examined eight weeks after weaning in a two chamber shuttle box with light and dark sides (27 x 25 x 25cm), separated by an automated door (8 x 8cm), with wire grid floors (PanLab, Slab, Barcelona, Spain) using a three day test protocol previously described (Jones et al., 2011a; McIntosh et al., 2013; Woods et al., 2012). On the first (conditioning) day rats were placed in the light side of the chamber. After 30s the door opened and the latency to cross into the dark side was recorded as an index of anxiety using a floor sensor which also closed the door (ShutAvoid, software v.1.8.2., Panlab S.L, USA). After 30s in the dark side a 5s conditioning stimulus (CS, light and 3kHz, 89dB tone) was immediately followed by delivery of the unconditioned stimulus (US, 1s 0.4mA footshock during the last second of the CS) through the grid floor. A further two consecutive CS/US were delivered separated by 55s. Time spent freezing (completely immobile except for respiration in a hunched posture with inactive vibrissae) between shocks was recorded by stopwatch. After 24 and 48h post-conditioning rats were returned to the dark side for 300s without CS/US and time spent freezing recorded as an index of associative learning and memory (Woods et al., 2012). During the extinction trial (48h post conditioning) the CS alone was presented after 300s and freezing recorded for a further 300s. Behavior was also recorded through a camera in the roof of the CFR chamber using Ethovision (Nodulous) to enable re-assessment if required.

## **2.8. Tissue collection**

One week after the end of the behavioral studies rats were injected with vehicle or drug and 30min later mixed arteriovenous trunk blood (in K<sub>2</sub>EDTA vials which were centrifuged at 4°C for 5min at 1200G) and brain tissue (frontal cortex and hippocampus) was collected and stored at -80°C for subsequent measurement of drug concentrations (in plasma and brain) by using liquid chromatography–mass spectrometry according to the methods described before (Gyertyán et al., 2011).

## **2.9. Statistical analysis**

Locomotor activity time-course data were analysed using repeated measures analysis of variance (ANOVA) with treatment and housing as main factors and time as the repeated measure. The cumulative locomotor activity data were analysed with two-way ANOVA with Bonferroni post-hoc test. Cumulative exploratory times during the familiarisation and choice trials of NOR were compared and the pattern of exploration during the choice trial was analysed using multivariate repeated measures ANOVA (with object as the repeated measure). To take account of any individual difference in object exploration between rats raw NOD data was converted to a discrimination ratio ( $D2 = (\text{novel-familiar times})/(\text{novel+familiar times})$ ) analysed by two-way ANOVA with Bonferroni post-hoc. The individual behavioral components in the social interaction test, latency to enter the dark chamber in the CFR paradigm, and the  $\text{Log}_{10}(x+1)$  transformed (normalised) USV data were analysed using two-way ANOVA with Bonferroni post-hoc while freezing behavior 24 and 48h post-conditioning in the CFR was analysed with two-way repeated measures ANOVA with treatment and housing as variables and time as the repeated measure. Given the unbalanced nature of the groups planned *post-hoc* comparisons were used. These were; vehicle/group-housed/vehicle (Veh/Soc/Veh) vs PCP/isolate/vehicle (PCP/Iso/Veh), PCP/Iso/Veh vs PCP/isolate/cariprazine 0.1mg/kg (PCP/Iso/Car0.1), PCP/Iso/Veh vs PCP/isolate/cariprazine 0.3mg/kg (PCP/Iso/Car0.3), and PCP/Iso/Veh vs PCP/isolate/aripiprazole 3mg/kg (PCP/Iso/Ari). Data was analysed using Prism 6 and SPSS software, with  $P < 0.05$  being considered significant. All data are presented as mean ± SEM.

## **3. Results**

### **3.1. Effect of cariprazine and aripiprazole on natural forgetting in the NOR task**

To assess any pro-cognitive effect in normal rats, the ability of both cariprazine and aripiprazole to reverse a time-delay-induced impairment in a two trial visual recognition

paradigm (NOR) was examined by selecting a 4h ITI (Fig. 1A and C) to produce natural forgetting. In both drug studies vehicle treated control group-housed adolescent rats were unable to discriminate the novel from the familiar object during the choice trial. Both cariprazine (Fig. 1A, object x drug:  $F_{(3,44)} = 6.576$ ,  $p < 0.01$ ) and aripiprazole (Fig. 1C, object x drug;  $F_{(3,44)} = 3.315$ ,  $p < 0.05$ ) improved object recognition, such that rats given the two highest doses of each drug spent significantly longer exploring the novel than the familiar object (cariprazine; 0.1mg/kg  $p < 0.01$ , 0.3mg/kg  $p < 0.001$ , aripiprazole; 1mg/kg  $p < 0.05$ , 3mg/kg  $p < 0.001$ ). This improvement in performance was confirmed by the concomitant significant main effect of each drug on the discrimination ratio (cariprazine; Fig. 1B,  $F_{(3,44)} = 7.670$ ,  $p < 0.001$ , and aripiprazole; Fig. 1D,  $F_{(1,44)} = 3.570$ ,  $p < 0.05$ ) although this only reached significance ( $p < 0.001$ ) from vehicle with the highest dose of cariprazine (0.3mg/kg) and not with aripiprazole. Although the effect of aripiprazole on the discrimination ratio failed to show any *post-hoc* significance even at the highest dose this was probably due to a slightly larger variation in the vehicle results for this study.

During the familiarisation trials neither cariprazine nor aripiprazole produced any significant object place preference (Table 1, cariprazine;  $F_{(1,44)} = 0.095$ ,  $p = 0.780$ , aripiprazole;  $F_{(1,44)} = 0.011$ ,  $p = 0.916$ ). Total object exploration during the choice trial was lower than that in the familiarisation trial in all groups in both drug studies (Table 1, cariprazine; trial  $F_{(1,44)} = 67.081$ ,  $p < 0.001$ , aripiprazole; trial  $F_{(3,44)} = 23.241$ ,  $p < 0.001$ ) but there were no trial x drug treatment interactions (cariprazine;  $F_{(3,44)} = 1.980$ ,  $p = 0.131$ , aripiprazole;  $F_{(3,44)} = 0.845$ ,  $p = 0.477$ ). In addition, drug treatment had no significant main effect on total exploration during the familiarisation (cariprazine;  $F_{(3,44)} = 2.505$ ,  $p = 0.071$ , aripiprazole;  $F_{(3,44)} = 1.123$ ,  $p = 0.350$ ) or choice (cariprazine;  $F_{(3,44)} = 0.941$ ,  $p = 0.429$ , aripiprazole;  $F_{(3,44)} = 0.247$ ,  $p = 0.863$ ) trials. Thus both drugs caused a specific redistribution of exploration towards the novel object during the choice trial without any non-specific change in exploration in either trial consistent with this being due to a specific effect on cognition and not a non-specific 'sedative-like' effect on locomotion or attention.

### 3.2. Effect of cariprazine and aripiprazole on NOR using a short intertrial interval

To ensure that neither drug impaired NOR, in two further groups of rats the effect of cariprazine and aripiprazole was examined on the pattern of NOR using a short ITI of 2min where vehicle treated rats were readily able to discriminate the novel from the familiar object (Fig. 2A and C). Neither cariprazine (Fig. 2A) nor aripiprazole (Fig. 2C) had any significant effect on the pattern of object discrimination (object x drug; cariprazine;  $F_{(3,44)} = 2.335$ ,  $p = 0.087$ , aripiprazole;  $F_{(3,44)} = 0.220$ ,  $p = 0.882$ ) during the choice trial. Thus irrespective of the dose of either drug, rats spent significantly more time exploring the novel than the

familiar object (all  $P < 0.001$ ) during the choice trial. Consistent with this observation from the raw data neither cariprazine (Fig. 2B,  $F_{(3,44)} = 2.217$ ,  $p = 0.100$ ) nor aripiprazole (Fig. 2D,  $F_{(1,44)} = 0.214$ ,  $p = 0.886$ ) had any significant effect on the discrimination ratio which was consistently higher than chance ( $d^2 = 0$ ).

Neither drug treatment induced any preference for object location during the familiarisation trial (Table 1, cariprazine  $F_{(1,44)} = 0.238$ ,  $p = 0.829$ , aripiprazole;  $F_{(1,44)} = 0.604$ ,  $p = 0.441$ ). As expected the total object exploration was reduced in the second choice compared to the familiarisation trial (cariprazine;  $F_{(3,44)} = 0.845$ ,  $p = 0.477$ , aripiprazole;  $F_{(1,44)} = 14.958$ ,  $p < 0.001$ ) but neither cariprazine (trial x drug;  $F_{(3,44)} = 0.939$ ,  $p = 0.430$ ) nor aripiprazole (trial x drug;  $F_{(3,44)} = 0.490$ ,  $p = 0.691$ ) had any effect on the magnitude of this reduction nor did they have a main effect on total exploration during either the familiarisation (cariprazine;  $F_{(3,44)} = 2.127$ ,  $p = 0.110$ , aripiprazole;  $F_{(3,44)} = 2.241$ ,  $p = 0.097$ ) or choice trials (cariprazine;  $F_{(3,44)} = 1.948$ ,  $p = 0.138$ , aripiprazole;  $F_{(3,44)} = 0.462$ ,  $p = 0.710$ ).

### **3.3. Effect of combined neonatal PCP and social isolation on locomotor activity and rearing behavior in a novel arena**

During a one hour, drug naïve, test session neonatal rats that had been treated with PCP and housed in social isolation from weaning for five weeks were hyperactive, showing higher horizontal ambulation (total beam breaks; vehicle/group-housed;  $1737 \pm 132$ , PCP/isolate;  $2012 \pm 44$ ;  $F_{(1,73)} = 6.423$ ,  $p < 0.05$ ) and more rears (total beam breaks; vehicle/group-housed;  $142 \pm 16$ , PCP/isolate;  $180 \pm 6$ ;  $F_{(1,73)} = 0.604$ ,  $p < 0.01$ ) compared to those given neonatal vehicle injections and housed in social groups. This confirmed the expected neurodevelopmental syndrome had been induced by the dual-hit early-life intervention and was used to ascertain there was no marked difference in activity between rats assigned by lots to vehicle, cariprazine or aripiprazole groups prior to drug testing.

### **3.4. Effect of acute cariprazine or aripiprazole on locomotor activity in a novel arena in rats treated with neonatal PCP and housed in social isolation**

One week later the ability of cariprazine and aripiprazole to reverse the PCP/isolation induced hyper-reactivity to the novel environment was examined in the same boxes. Over the entire 1h period PCP/Iso/Veh rats exhibited higher locomotor counts than any other group (Fig. 3A). Although this was not significantly higher than Veh/Soc/Veh controls they displayed significantly more rears than the latter. Thus there was a main effect of acute drug treatment ( $F_{(3,75)} = 21.763$ ,  $p < 0.001$ ) on the total locomotor activity but no main effect of early life/housing ( $F_{(3,75)} = 2.421$ ,  $p = 0.124$ ). PCP-isolate rats treated with either cariprazine (both doses) or aripiprazole made significantly fewer horizontal beam breaks than the

PCP/Iso/Veh group (all  $p < 0.001$ ). Analysis of the time course (data not shown) revealed a significant time x acute treatment interaction ( $F_{(11,770)} = 2.004$ ,  $p < 0.01$ ) but no significant time x housing interaction ( $F_{(33,770)} = 1.359$ ,  $p = 0.188$ ) suggesting that activity declined at the same rate irrespective of early-life housing but was attenuated by acute treatment with either drug.

A similar profile was seen with the total number of rears which was increased by PCP/Iso (main effect  $F_{(3,73)} = 19.582$ ,  $p < 0.001$ ) and reduced by acute drug treatment ( $F_{(3,73)} = 15.452$ ,  $p < 0.001$ ). The PCP/Iso/Veh rats made significantly more rears than the Veh/Soc/Veh group (Fig. 3B,  $p < 0.001$ ) but this was attenuated in cariprazine (at both doses) and aripiprazole treatment (all  $p < 0.001$  compared to PCP/Iso/Veh). Repeated measures ANOVA of the time course data (not shown) revealed a main effect of time ( $F_{(11,748)} = 49.664$ ,  $p < 0.001$ ) but no significant time x housing ( $F_{(11,803)} = 1.461$ ,  $p = 0.141$ ) or time x acute treatment interactions ( $F_{(33,748)} = 1.405$ ,  $p = 0.0067$ ). The rearing data from two rats were excluded due to interruption of the upper beams by paw prints on the walls during recording but locomotor data were unaffected.

### **3.5. Effect of acute cariprazine or aripiprazole on impairment in NOR caused by combined neonatal PCP treatment and social isolation**

Neonatal PCP combined with isolation rearing significantly impaired NOR (object x neonatal treatment/housing;  $F_{(1,70)} = 15.915$ ,  $P < 0.001$ ) which was reversed by cariprazine or aripiprazole (Fig. 4A, object x acute treatment;  $F_{(3,70)} = 5.867$ ,  $p < 0.01$ ). In the choice trial Veh/Soc/Veh rats were readily able to discriminate the novel from the familiar object as confirmed by the discrimination ratio (Fig. 4B). In contrast, neonatal PCP with social isolation significantly reduced the discrimination ratio ( $F_{(1,70)} = 19.875$ ,  $p < 0.001$ ) which was close to chance and this was robustly reversed ( $F_{(3,70)} = 9.789$ ,  $p < 0.001$ ) by either cariprazine (0.1 and 0.3mg/kg) or aripiprazole. Irrespective of early-life treatment/housing or drug treatment none of the groups showed any preference for either the front or back object during the familiarisation trial (Table 2,  $F_{(1,70)} = 0.812$ ,  $p = 0.371$ ) and neither neonatal treatment/housing (object x neonatal treatment/ housing;  $F_{(1,70)} = 0.177$ ,  $p = 0.676$ ) nor drug treatment (object x acute drug treatment;  $F_{(3,70)} = 1.582$ ,  $p = 0.202$ ) affected this. Neither housing nor drug treatment had any significant effect on total object exploration during the familiarisation trial (Table 2,  $F_{(1,70)} = 0.126$ ,  $p = 0.724$  and  $F_{(3,70)} = 2.351$ ,  $p = 0.080$  respectively) but both had significant effects on total object exploration during the choice trial (PCP/housing;  $F_{(1,70)} = 10.524$ ,  $p < 0.01$ ) drug treatment;  $p < 0.01$ ,  $F_{(3,70)} = 8.893$ ,  $p < 0.001$ ). Total object exploration was significantly reduced between the first and second trials (main effect  $F_{(1,70)} = 29.923$ ,  $p < 0.001$ ), but there were no significant trial x housing ( $F_{(1,70)} = 3.962$ ,  $p = 0.050$ ) or trial x drug treatment ( $F_{(3,70)} = 0.144$ ,  $p = 0.144$ ) interactions.

### 3.6. Effect of acute cariprazine or aripiprazole on social interaction in neonatal PCP social isolation rats

As a potential index of change in negative symptoms of schizophrenia, seven weeks after weaning social interaction was recorded in an unfamiliar open field between pairs of weight and treatment matched rats from different litters that had never met before (Fig. 5). Repeated measures ANOVA of individual behaviors revealed significant behavior  $\times$  housing ( $F_{(6,192)} = 34.072$ ,  $p < 0.001$ ) and behavior  $\times$  drug treatment ( $F_{(18,192)} = 2.001$ ,  $p < 0.05$ ) interactions (Fig. 5). Of particular note anogenital and body sniffing were by far the most prevalent behaviors, accounting for over 80% of the total interaction time in most pairs (mean  $\pm$  SEM, 81.4  $\pm$  1.3), so it is particularly pertinent that the major changes were seen in these two behaviors. Anogenital sniffing and following were both significantly reduced in the PCP/Iso/Veh compared to that in Veh/Soc/Veh rats (anogenital sniffing;  $P < 0.001$ , following;  $p < 0.05$ ) but neither cariprazine nor aripiprazole reversed this reduction. Conversely, body sniffing was significantly increased ( $p < 0.001$ ) in PCP/Iso/Veh compared to Veh/Soc/Veh rats and this was only attenuated ( $p < 0.05$ ) by the highest (0.3mg/kg) dose of cariprazine (and not aripiprazole). The small apparent increases in boxing and biting and crawling under and over in PCP/Iso rats did not reach significance from group-housed controls (Veh/Soc/Veh). The total social interaction time between pairs was unaffected by either housing ( $F_{(1,32)} = 0.022$ ,  $p = 0.882$ ) or drug treatment ( $F_{(3,32)} = 1.514$ ,  $p = 0.230$ ). In contrast both housing ( $F_{(1,32)} = 11.404$ ,  $P < 0.01$ ) and drug treatment ( $F_{(3,32)} = 21.336$ ,  $p < 0.001$ ) significantly affected the distance travelled during the task (Fig. 6A), such that Veh/PCP/Veh pairs moved significantly more than Veh/Soc/Veh ( $p < 0.01$ ) rats. This increase in locomotor activity was reversed by aripiprazole (3mg/kg,  $p < 0.001$ ) and, in a dose-related manner, by cariprazine (0.1mg/kg  $P < 0.05$ , 0.3mg/kg  $p < 0.001$ ) analogous to the effect observed in the novel arena. To further examine the impact of housing on social behavior the number of USVs emitted during interaction were also analysed as a potential index of change in communicative interaction, potentially highly relevant to human behavior. The total USVs emitted per pair was significantly increased by PCP/Iso (Fig. 6B,  $F_{(1,9.623)} = 21.336$ ,  $p < 0.01$ ) but unaffected by either cariprazine or aripiprazole ( $F_{(3,32)} = 1.367$ ,  $p = 0.270$ ).

### 3.7. Effect of acute cariprazine or aripiprazole on fear conditioned freezing behavior in PCP and social isolation rats and group-housed controls

During the CFR training process drug injection was not performed to avoid any confounding impact (such as alteration in perception of or aversion to the shock) on acquisition but data was analysed by drug sub-group to determine whether preceding treatment affected behavior during training and acquisition. Neither housing ( $F_{(1,70)} = 0.638$ ,  $p = 0.427$ ) nor drug

treatment ( $F_{(3,70)} = 0.573$ ,  $p=0.634$ ) had any effect on the latency of rats to cross into the dark side of the box (data not shown). As expected freezing duration progressively increased on repeated exposure to the foot shock ( $F_{(1,70)} = 108.886$ ,  $p<0.001$ ) and there was a significant interaction between foot shock x housing ( $F_{(1,70)} = 6.902$ ,  $p<0.05$ ) although no foot shock x drug treatment interaction ( $F_{(3,70)} = 0.657$ ,  $p=0.581$ ).

There was a main effect of housing on freezing upon re-exposure to the context 24h later ( $F_{(1,70)} = 6.687$ ,  $P<0.05$ ) such that the PCP/Iso/Veh froze less than Veh/Soc/Veh rats (Fig. 7,  $P<0.05$ ) but neither drug was able to reverse this effect at this time ( $F_{(3,70)} = 0.370$ ,  $p=0.775$ ). The change was similar at 48h post-conditioning (Fig. 7) such that PCP/Iso/Veh rats froze less than Veh/Soc/Veh controls ( $F_{(1,70)} = 8.092$ ,  $p<0.01$ ) but this was also not altered by cariprazine or aripiprazole ( $F_{(3,70)} = 2.145$ ,  $p=0.102$ ). The presentation of the conditioning cue significantly increased freezing duration ( $F_{(1,70)} = 177.507$ ,  $p<0.001$ ), but this was unaltered by housing condition ( $F_{(1,70)} = 3.612$ ,  $p=0.061$ ) or drug treatment ( $F_{(3,70)} = 1.197$ ,  $p=0.317$ ). After the presentation of the cue the PCP/Iso/Veh rats also froze for significantly less than Veh/Soc/Veh ( $p<0.01$ ) controls and although cariprazine and aripiprazole appeared to increase freezing above that in the PCP/Iso/Veh rats this did not reach significance (all  $p>0.05$ ).

### **3.8. Effect of repeated acute cariprazine or aripiprazole on body weight and plasma and brain drug levels in rats that have received combined neonatal PCP treatment and social isolation from weaning**

Up to the commencement of treatment with cariprazine or aripiprazole there was a significant effect of neonatal treatment on body weight (main effect;  $F_{(1,70)} = 9.617$ ,  $p<0.01$ , time x neonatal treatment/housing interaction;  $F_{(8,584)} = 3.281$ ,  $p<0.01$ ) such that neonatal PCP isolated rats were significantly lighter (from PND 24 up to PND 59) than vehicle group-housed controls. In contrast after commencing of regular drug injection (from PND 66) there were no significant differences in body weight between any of the groups. Blood plasma and brain levels of cariprazine and aripiprazole were determined 30min after the final injection (to coincide with peak behavioral effects, Table 3) in the PCP-isolation study. A previous study (Gyertyán et al., 2011) found that oral cariprazine (1mg/kg) produced a peak plasma concentration of 91ng/ml, which with linear pharmacokinetics would be expected to result in plasma concentrations of ~9 and ~30ng/ml with 0.1 and 0.3 mg/kg i.p., similar to those reported herein (6.6 and 33.4ng/ml, respectively). Furthermore the brain:plasma ratio for 0.1 and 0.3 mg/kg i.p. cariprazine measured at a single time point herein (7.9:1 and 5.1:1 respectively) are very similar to the value (7.6:1) reported by Gyertan et al. (2011) suggesting good CNS penetration with intraperitoneal administration.



#### 4. Discussion

This study demonstrates that the D<sub>3</sub>-preferring D<sub>2</sub>/D<sub>3</sub> dopamine receptor partial agonist, cariprazine, improves learning and memory in two rat models of cognitive impairment. In a delay dependent model of natural forgetting, both cariprazine and aripiprazole attenuated the impairment in NOR in a dose-related manner. In contrast neither drug had any adverse effects on the performance of NOR using a short (2 min) ITI. Cariprazine and aripiprazole are partial agonists at dopamine D<sub>3</sub> receptors however cariprazine displays about 10-times higher affinity and potency in the *in vitro* binding and functional tests, respectively than aripiprazole (Kiss et al., 2010; Tadori et al., 2011). Considerable previous work by ourselves and others suggest that blockade of the dopamine D<sub>3</sub> receptor can improve cognition (Watson et al., 2012a), so this could be an important potential site of action most likely for cariprazine rather than aripiprazole, since the latter shows relatively low occupancy of D<sub>3</sub> receptors *in vivo* at pharmacologically-effective doses in rat studies (Gyertyán et al., 2011). Equally, it is known that blockade of the dopamine D<sub>2</sub> receptor can attenuate visual learning and memory in this paradigm (Watson et al., 2012a). Thus, given that both drugs also have affinity for the dopamine D<sub>2</sub> receptor it can be assumed that, at the doses used, there was either no significant antagonism at the D<sub>2</sub> receptor or that any such effect was surmounted by actions at other receptors. It should also be noted that both drugs are relatively high affinity 5-HT<sub>1A</sub> receptor partial agonists *in vitro* (Kiss et al., 2010) but with low potency in lower lip retraction test *in vivo* (Gyertyán et al., 2011). As selective 5-HT<sub>1A</sub> receptor antagonists reverse impairments in NOR this mechanism could be an alternate or contributory factor to the pro-cognitive action seen in this study (Pitsikas et al., 2003). Both cariprazine and aripiprazole had no effect on total object exploration during either the familiarisation or choice trials. Thus, preferential increases in exploration of the novel object were the result of a redistribution of exploration and not the consequence of altered levels of activity as a result of any 'sedative-like' action of the drugs pointing to the specific nature of these effects on cognition.

In addition, cariprazine and aripiprazole were equally active in reversing the NOR deficit in rats given neonatal PCP and housed in social isolation from weaning. Although PCP/isolate rats were hyperactive in an open field, analysis of this behavior shows they fail to habituate to the arena over time rather than having markedly enhanced locomotion at individual time points which could impair attention of objects in a short 3min NOR trial. Furthermore such hyperactivity would be expected to reduce total object exploration in both familiarisation and choice trials (which did not occur) and not account for a selective redistribution of the pattern of object exploration in the choice trial. Deficits in NOR performance are well documented following social isolation (Bianchi et al., 2006; Jones et al., 2011a; King et al., 2009;

McIntosh et al., 2013; Watson et al., 2012b) and combined neonatal PCP and social isolation but not neonatal PCP alone (Gaskin et al., 2014). Cariprazine has also been reported to have pro-cognitive effects in other rodent paradigms (Gyertyán et al., 2011; Zimnisky et al., 2013) and aripiprazole and cariprazine have been shown to reverse subchronic PCP-induced impairments in NOR in mice and rats (Nagai et al., 2009; Neill et al., 2015; Snigdha et al., 2008). It should be noted that changes in total object exploration were observed during the choice trial (main effects of both neonatal treatment/housing and drug treatment). However, analysis of the discrimination ratio, which takes differences in total object exploration between groups into account, suggests that the cognitive impairment in the PCP/Iso/Veh group was not confounded by any increase in total object exploration. Of particular note, cariprazine produced a dose-related reversal of the PCP-induced impairment of social recognition memory and delayed T-maze alternation in wild type mice but was unable to reverse these deficits in D<sub>3</sub> receptor knockout mice (Zimnisky et al., 2013), consistent with the idea that partial agonist activity at this receptor may play an important contribution to this pro-cognitive effect in social recognition and spatial working memory. It would be valuable to assess the impact of cariprazine on PCP-isolation-induced impairments of other cognitive domains relevant to those impaired in schizophrenia, such as social cognition in future studies.

Combined neonatal PCP and isolation caused the expected hyperactivity in a novel arena and during social interaction which was significantly reduced by cariprazine or aripiprazole. While neonatal PCP alone does not induce hyperlocomotion (Boctor and Ferguson, 2010) this is a consistent trait of social isolation attenuated by a range of antipsychotics, including haloperidol, risperidone and olanzapine (Fabricius et al., 2011; McIntosh et al., 2013). As hyperlocomotion probably results from increased mesolimbic dopamine, thought to contribute to positive symptoms of schizophrenia (Fone and Porkess, 2008), its attenuation herein supports the potential antipsychotic properties of cariprazine.

Several studies have reported that isolation reared rats display reduced motivation for contact with conspecifics in social interaction (Moller et al., 2011; Van Den Berg et al., 1999). However there may be marked strain differences; Wistar rats showing decreased social behavior (such as approach, following, anogenital sniffing (Moller et al., 2011)) while Lister hooded rats show increased aggression without any major change in other behaviors (Fone and Porkess, 2008; Wongwitdecha and Marsden, 1996). Although total social interaction was unaffected by drug treatment or PCP/isolation, the most prevalent pro-social behaviours were significantly altered by the latter; decreased anogenital sniffing and following accompanied by increased body sniffing. Such that social interaction in Veh/Soc/Veh pairs was predominately nose to tail while that in PCP/Iso/Veh pairs was primarily nose to nose.

Only the highest dose of cariprazine (but not aripiprazole) significantly reduced the most prevalent pro-social behavior, body sniffing (by 20% from PCP/Iso/Veh rats), which may reflect a difference in effectiveness of cariprazine and aripiprazole at treating negative symptoms, which is worthy of further investigation. Consistent with the current findings the increase in avoidance and decrease in following behaviour produced by sub-chronic PCP administration to adult rats was also reversed by cariprazine (Neill et al., 2015). Using an identical PCP/isolate paradigm we found that clozapine (at doses which reversed the NOR deficit) was unable to restore the pro-social behavioural deficit (McIntosh et al., 2012), consistent with clozapine's limited efficacy to treat the negative symptoms of schizophrenia and supporting the potential translational value of this test.

The USVs recorded in all groups were in the 50kHz range which occur in social, appetitive situations, during tickling and sexual behaviour thought to reflect positive affective state in rats (Brudzynski, 2013; Seffer et al., 2015). The increase in USVs in the socially isolated rats may reflect an increased motivation for social interaction although this was not accompanied by an increase in total social interaction compared to group-housed rats. At first sight the increase in USVs produced by PCP/Isolate rats would appear to be contradictory to that expected if this paradigm has any correlation to reduced sociability and social withdrawal seen in schizophrenia. However isolation-reared rats don't show the normal approach response to playback of pro-social 50kHz calls (Seffer et al., 2015), consistent with the idea that they fail to mediate a normal response to socio-affective auditory information, which might contribute to the increased calls recorded in this study. Alternatively the increase in USVs may denote an increase in positive affect derived from the social interaction. USVs in the 50kHz range are associated with dopaminergic activity, being elicited by apomorphine, amphetamine and cocaine (Brudzynski et al., 2012; Mu et al., 2009; Wintink and Brudzynski, 2001) but not by other psychostimulants, such as MDMA, caffeine or nicotine (Sadananda et al., 2012; Simola et al., 2012; Simola et al., 2010). Furthermore, direct stimulation of USVs by injection of quinpirole into the shell of the nucleus accumbens is mediated in a complex triphasic way by both dopamine D<sub>2</sub> and D<sub>3</sub> receptors (Brudzynski et al., 2012). Social isolation (without neonatal PCP) has been shown to increase basal dopamine release in the nucleus accumbens and enhance release in response to amphetamine (Hall et al., 1998), cocaine (Howes et al., 2000) and emotional stimuli (Fulford and Marsden, 1998). Thus the increase in USVs seen in PCP/isolated rats might represent an enhanced response resulting from the priming of the dopaminergic mesolimbic system.

Although freezing following the first shock in CFR was significantly lower in PCP/isolates than controls both groups froze for an equivalent time after the second shock, indicating that both successfully responded to conditioning. Therefore, reductions in freezing following re-

exposure to the context or cue probably result from a deficit in cognition rather than alteration in pain perception or emotional motivated associative learning. Attenuation of freezing in isolates in response to the context and cue are consistent with previous findings (Gaskin et al., 2014; Jones et al., 2011a; McIntosh et al., 2013) and may result from altered hippocampal and amygdala function (Goosens and Maren, 2001; Richmond et al., 1999). At no point did acute drug treatment with either cariprazine or aripiprazole significantly reverse the CFR deficit in the PCP/Iso/Veh group. Our previous studies have shown that neither risperidone nor the mGluR<sub>2/3</sub> agonist, LY379268, could reverse the isolation-induced CFR deficit (Jones et al., 2011a; McIntosh et al., 2013), but that CFR is enhanced in control rats by drugs which modify central glutamatergic or cholinergic function such as 5-HT<sub>6</sub> receptor antagonists (Woods et al., 2012). Indeed, isolation-induced deficits in CFR appear to involve impaired consolidation mediated by altered cholinergic, and not dopaminergic, regulation of the CREB cascade in the hippocampus (Okada et al., 2015). Impaired associative learning is a common symptom of schizophrenia showing limited response to existing antipsychotics (Herbener, 2009; Shohamy et al., 2010), so development of novel compounds which reverse this cognitive impairment in a rodent model may be a valuable addition to drug discovery.

In summary, neonatal PCP combined with social isolation from weaning caused significant behavioral changes in locomotor activity, NOR, social interaction and CFR tasks. Acute cariprazine and aripiprazole reversed the hyperlocomotor activity and impairment in NOR. While, neither drug significantly affected the deficits seen in CFR, cariprazine, but not aripiprazole, modestly altered the changes seen in the most abundant prosocial behaviour during social interaction. Thus, cariprazine was as effective as aripiprazole in reversing the neurodevelopmental deficits thought to model positive symptoms and cognitive impairments seen in schizophrenia but, unlike aripiprazole, was also modestly effective in attenuating a model of the negative symptoms. In considering the translational relevance of this work it is important to note that the changes reported resulted from acute (albeit repeated) drug injection while chronic administration is essential before any clinical benefit from antipsychotics is observed. Furthermore, whilst many drugs operating through diverse pharmacological targets demonstrate pro-cognitive activity in preclinical tasks (e.g. NOR) and reverse indices of negative symptoms (e.g. social interaction), none have translated to proven efficacy in the clinic (Millan et al., 2014; Young and Geyer, 2015). Therefore further studies are essential to determine if multiple-hit developmental models such as this one provide more reliable predictive test beds for these vital unmet medical needs.

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### **Contributors**

DJG Watson, MV King and KCF Fone proposed the study design, conducted the experiments, performed the statistical analysis and interpretation of the data. B. Kiss, I. Gyertyán, and N. Adham participated in the study design and interpretation of data. DJG Watson, MV King and KCF Fone wrote the first draft of the manuscript and all authors contributed to and approved the final manuscript.

### **Conflict of Interest**

MV King and KCF Fone are employed by The University of Nottingham and DJG Watson was at the time of the study. N. Adham is an employee of Forest Research Institute, an affiliate of Actavis, Inc. B. Kiss is an employee of Gedeon Richter Plc. I. Gyertyán was an employee of Gedeon Richter Plc at the time of the study.

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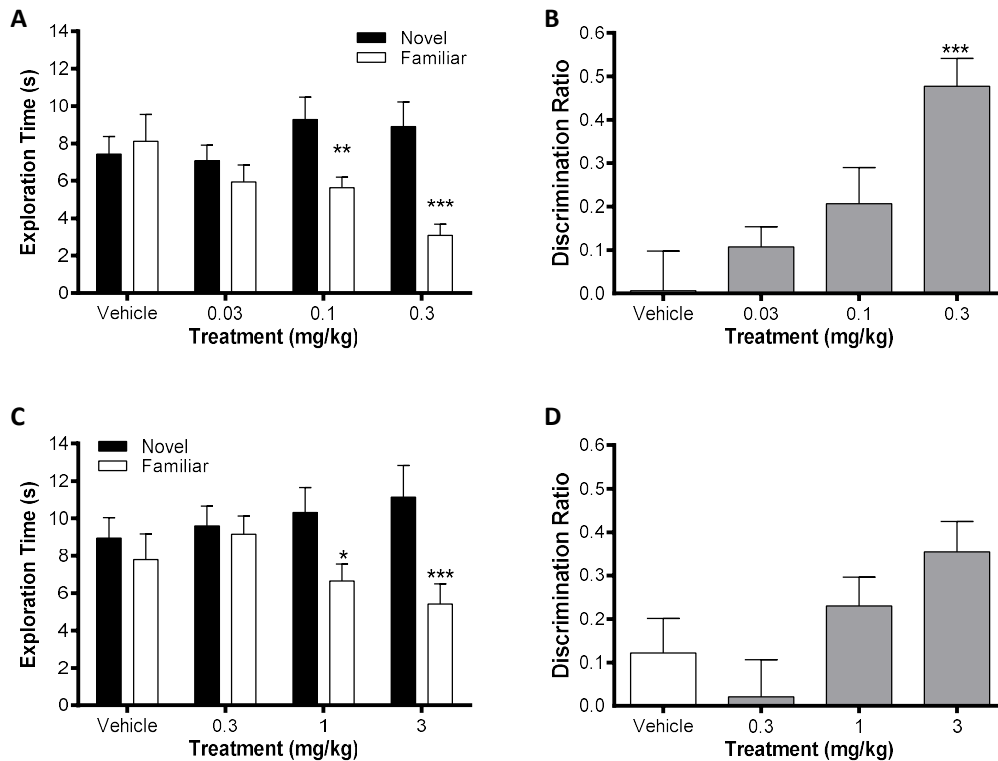
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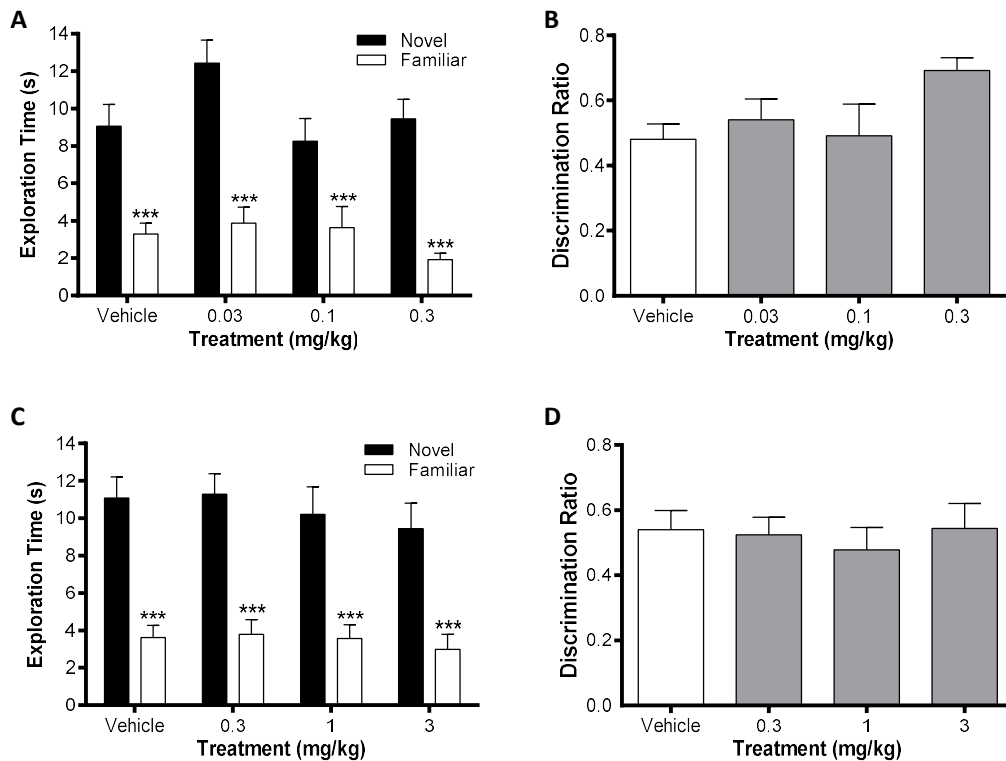
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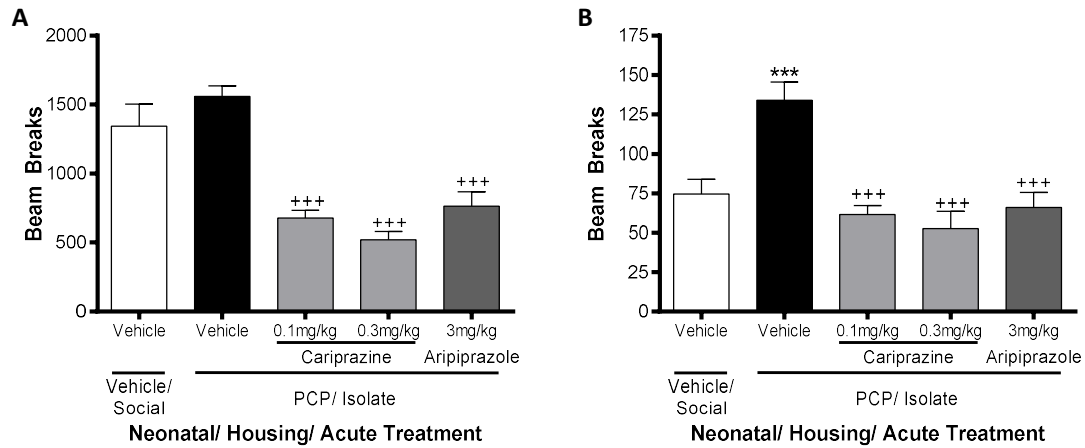
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**Figure 1** Both cariprazine and aripiprazole delay natural forgetting in a 4h ITI NOR paradigm. Comparison of the effect of cariprazine (**A** and **B**, 0.03-0.3mg/kg, 2ml/kg) and aripiprazole (**C** and **D**, 0.3-3mg/kg, 10ml/kg) injected i.p. 30min prior to the familiarisation trial in group-housed male Lister hooded rats (N=12, data shown as mean±SEM). **A** and **C**: Object exploration (s) during the second choice trial. **B** and **D**: Discrimination ratio (d2, novel object exploration – familiar object exploration/ total object exploration). In **A**: Repeated measures ANOVA showed an object x cariprazine treatment interaction ( $p<0.01$ ), \*\*  $p<0.01$ , \*\*\*  $p<0.001$  from the novel object in same treatment group, Bonferroni *post-hoc* test. In **B**: ANOVA showed a main effect of cariprazine treatment ( $P<0.001$ ), \*\*\*  $p<0.01$  from the vehicle group Dunnett's *post-hoc* test. In **C**: Repeated measures ANOVA showed an object x aripiprazole treatment interaction ( $p<0.05$ ), \*  $p<0.05$ , \*\*\*  $p<0.001$  from novel object in same treatment group, Bonferroni *post-hoc* test. In **D**: ANOVA showed a main effect of aripiprazole treatment ( $p<0.05$ ),  $p=0.091$  3mg/kg vs. vehicle group, Dunnett's *post-hoc* test.

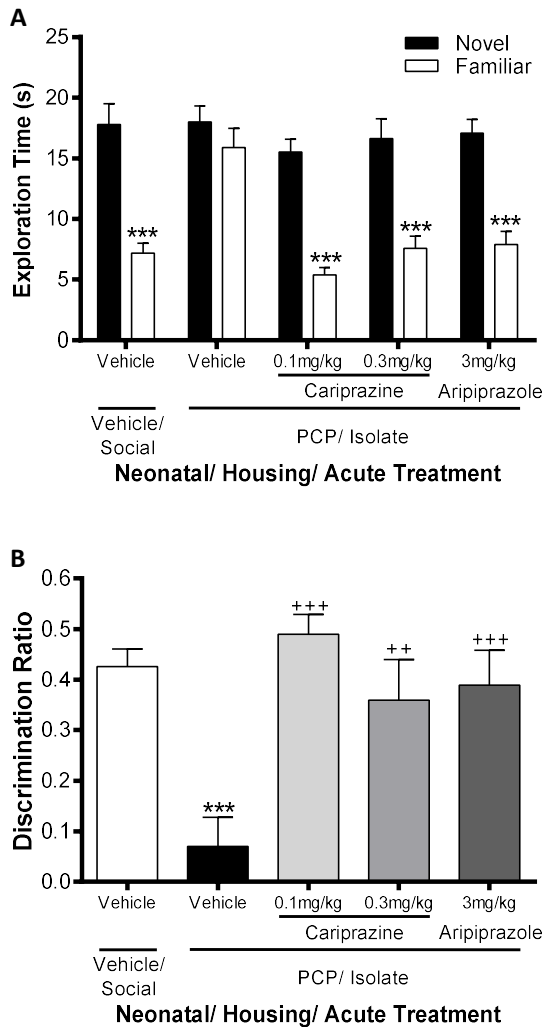


**Figure 2** Both cariprazine and aripiprazole had no effect on NOR with a short (2min) ITI in which normal rats readily discriminate the novel from familiar object. Comparison of the effect of cariprazine (**A** and **B**, 0.03-0.3mg/kg, 2ml/kg) and aripiprazole (**C** and **D**, 0.3-3mg/kg, 10ml/kg) injected i.p. 30min prior to the familiarisation trial in group-housed male Lister hooded rats ( $N=12$ , data shown as mean $\pm$  SEM). **A** and **C**: Object exploration (s) during the second choice trial. **B** and **D**: Discrimination ratio (d2, novel object exploration – familiar object exploration/ total object exploration). In **A**: Repeated measures ANOVA confirmed there was no object x cariprazine treatment interaction ( $p=0.087$ ), \*\*\*  $p<0.001$  from novel object in same treatment group, Bonferroni *post-hoc*. In **B**: ANOVA confirmed no main effect of cariprazine treatment ( $p=0.100$ ). In **C**: Repeated measures ANOVA confirmed there was no object x aripiprazole treatment interaction ( $p=0.882$ ), \*\*\*  $p<0.001$  from novel object in same treatment group, Bonferroni *post-hoc*. In **D**: ANOVA confirmed there was no significant effect of aripiprazole ( $p=0.886$ ). Note as every rat received every dose and vehicle over four weeks the data also demonstrate the behaviour is reproducible and consistent in rats of comparable age.

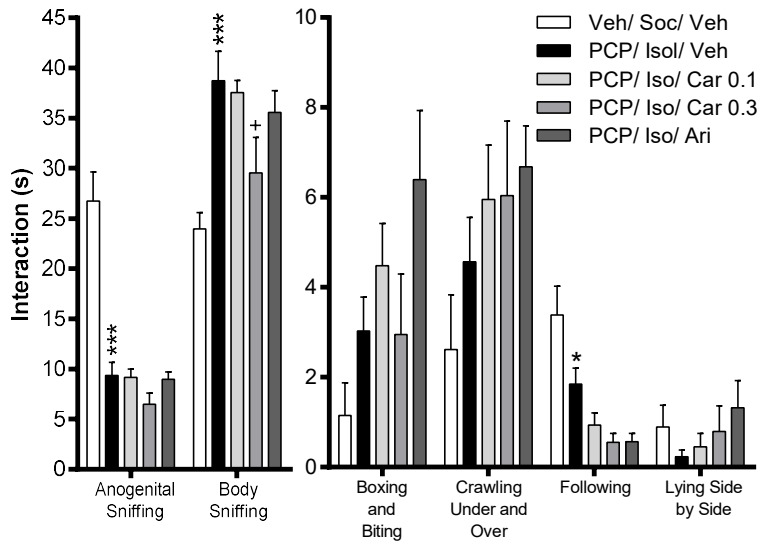


**Figure 3** Cariprazine and aripiprazole attenuated the hyperactivity in a novel arena produced by combined neonatal PCP and social isolation rearing from weaning of male Lister hooded rats. **(A)** Total locomotor activity and **(B)** number of rears (mean±SEM, N=14-16) measured by infra-red beam breaks in a novel arena over 1h. Behavior was recorded 6 weeks after weaning, following i.p. injection of either vehicle (2ml/kg), cariprazine (0.1 or 0.3mg/kg) or aripiprazole (3mg/kg in 10ml/kg) 30min prior to being placed in the arena. In **A**: two-way ANOVA showed a main effect of acute drug treatment ( $p<0.001$ ) only,  $+++ P<0.001$  from PCP/isolate/vehicle group, Bonferroni planned *post-hoc* comparisons. In **B**: two-way ANOVA showed a main effect of acute drug treatment ( $p<0.001$ ) and of neonatal treatment/ housing ( $p<0.001$ ),  $*** p<0.001$  from vehicle/ social/ vehicle group;  $+++ p<0.001$  from PCP/isolate/vehicle group, Bonferroni planned *post-hoc* comparisons.

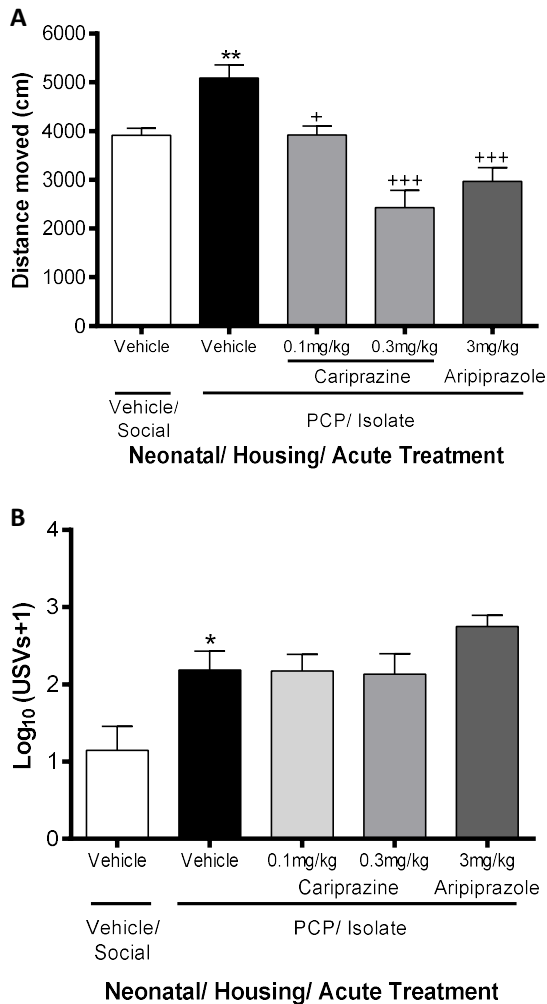




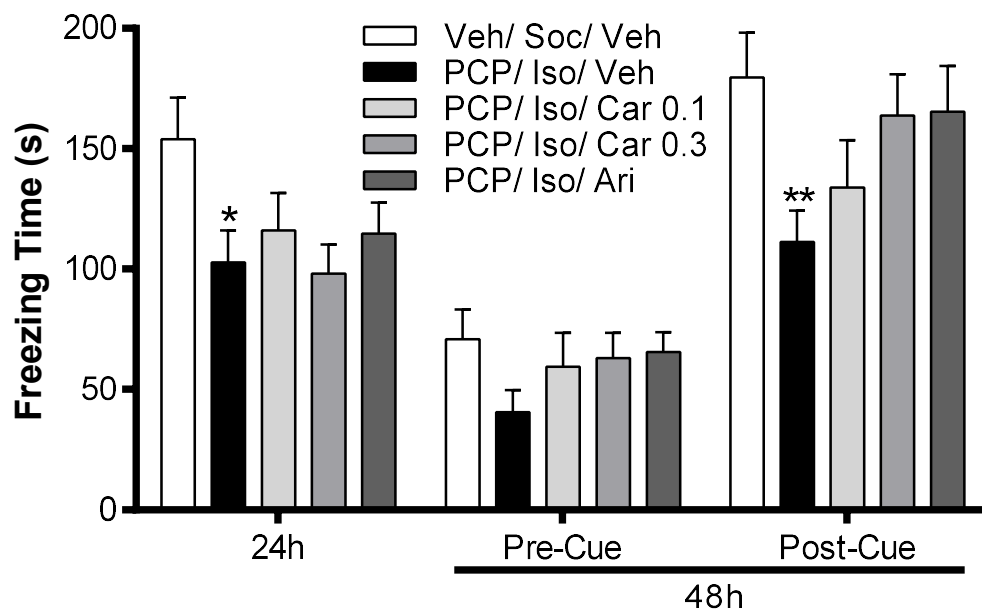
**Figure 4** Cariprazine and aripiprazole reversed the impairment in NOR in male Lister hooded rats produced by neonatal treatment with PCP and social isolation from weaning. Rats were injected i.p. with either vehicle (2ml/kg), cariprazine (0.1 or 0.3mg/kg) or aripiprazole (3mg/kg in 10ml/kg) 30min prior to familiarisation trial and data shown is exploration recorded from the choice trial 2h later, mean±SEM, N=14-16. **A:** Novel and familiar object exploration time (s). **B:** Discrimination ratio (d2, novel object exploration – familiar object exploration/ total object exploration). In **A:** RM-ANOVA showed a significant object x neonatal treatment/ housing interaction ( $p<0.001$ ) and a significant object x acute drug treatment interaction ( $p<0.01$ ), \*\*\*  $P<0.001$  from the novel object in same treatment group, Bonferroni *post-hoc*. In **B:** two-way ANOVA showed significant main effects of neonatal drug treatment/ housing ( $p<0.001$ ) and acute drug treatment ( $p<0.001$ ), \*\*\*  $p<0.001$  from vehicle/social/vehicle group; ++  $p<0.01$ , +++  $p<0.001$ , from PCP/isolate/vehicle group, Bonferroni planned *post-hoc* comparisons.



**Figure 5** Comparison of the effect of cariprazine (Car at the dose indicated in the legend) and aripiprazole (Ari) on social interaction in male Lister hooded rats treated with neonatal PCP and socially isolated from weaning. Social interaction (s, mean $\pm$ SEM, of the mean time for each pair) between two rats of similar weight from different litters but the same developmental condition and drug treatment over a 10min trial,  $N=7-8$  pairs. Rats were injected i.p. with either vehicle (2ml/kg), cariprazine (0.1 or 0.3mg/kg) or aripiprazole (3mg/kg in 10ml/kg) 30min prior to the trial. Repeated-measures ANOVA revealed a significant behavior  $\times$  neonatal treatment/housing interaction ( $p<0.001$ ) and a significant behavior  $\times$  acute drug treatment interaction ( $P<0.05$ ), \*  $p<0.05$ , \*\*\*  $p<0.001$  from vehicle/social/vehicle group for that behavioral component; +  $p<0.05$  from PCP/isolate/vehicle group in that behavior, Bonferroni planned *post-hoc* comparisons. Note the different time scale used to report anogenital and body sniffing (which account for over 80% of the total interaction time in most pairs) and the other much less frequent behaviors.



**Figure 6** Effect of cariprazine and aripiprazole on social interaction in male Lister hooded rats treated with neonatal PCP and socially isolated from weaning. Social interaction between two rats of similar weight from different litters but the same developmental condition and drug treatment over a 10min trial, n=7-8 pairs. The average time spent in each behavioral component for each pair is presented as mean±SEM and rats received i.p. injection with either vehicle (2ml/kg), cariprazine (0.1 or 0.3mg/kg) or aripiprazole (3mg/kg in 10ml/kg) 30min prior to trial. Figures show the total; **A**: locomotor distance (cm) covered and **B**: number of ultrasonic vocalisations (USV) per pair during the 10min trial, following a log<sub>10</sub> transformation to normalise USV data. In **A**: Two-way ANOVA showed a main effect of neonatal treatment/ housing ( $p<0.01$ ) and acute drug treatment ( $p<0.001$ ), \*\*  $p<0.01$  from vehicle/ social/vehicle group; +  $p<0.05$ , +++  $P<0.001$  from PCP/ isolate/vehicle group, Bonferroni planned *post-hoc* comparisons. In **B**: Two-way ANOVA showed a main effect of neonatal treatment/ housing ( $p<0.01$ ) but no effect of acute drug treatment ( $p=0.270$ ). \*  $p<0.05$  from vehicle/social/vehicle group, Bonferroni planned *post-hoc* test.



**Figure 7** Effect of cariprazine (Car at the dose indicated) or aripiprazole (Ari) on the retention and extinction of memory in a fear motivated conditioned freezing response in controls or male Lister hooded rats treated with neonatal PCP and socially isolated from weaning. Rats received i.p. injection of either vehicle (2ml/kg), cariprazine (0.1 or 0.3mg/kg) or aripiprazole (3mg/kg in 10ml/kg) immediately after fear conditioning (three 1s 0.4mA foot shocks accompanied with light and tone cue delivered with a 55s delay between each cue/foot shock) and 30min prior to assessment of memory at 24h and 48h post-conditioning. Data shown is freezing time. Total time (s, mean $\pm$ SEM, n=14-16) spent freezing was recorded following reintroduction (for 5min) into the conditioning chamber at 24h and 48h later and (at 48h only) with presentation of the light and tone cue. At 24h, two-way ANOVA showed a significant effect of neonatal treatment/ housing ( $p < 0.05$ ) but no effect of acute drug treatment ( $p = 0.775$ ). At 48h repeated-measures ANOVA showed a significant effect of cue ( $p < 0.001$ ) and a main effect of neonatal treatment/housing ( $P < 0.01$ ) but no main effect of acute drug treatment or any interactions (all  $p > 0.05$ ), \*  $p < 0.05$ , \*\*\* $p < 0.01$  from vehicle/ social/ vehicle group at that time point, Bonferroni planned *post-hoc* comparisons.

**Table 1** Object exploration during the familiarisation and choice trials.

<i>Intertrial Interval</i>	<i>Drug</i>	<i>Dose</i>	<i>Familiarisation Trial</i>			<i>Choice Trial</i>
			<i>Front Object (s)</i>	<i>Back Object (s)</i>	<i>Total Exploration (s)</i>	<i>Total Exploration (s)</i>
4h	Cariprazine	Vehicle	11.2 (1.2)	10.9 (1.3)	22.1 (2.4)	15.6 (2.0)
		0.03 mg/kg	14.6 (1.3)	12.4 (1.1)	27.0 (2.2)	13.0 (1.7)
		0.1 mg/kg	13.4 (1.7)	13.5 (1.2)	26.9 (2.3)	14.9 (1.4)
		0.3 mg/kg	9.2 (1.4)	10.7 (1.4)	20.0 (2.1)	12.0 (1.7)
4h	Aripiprazole	Vehicle	10.7 (1.2)	10.3 (1.0)	21.0 (2.0)	16.7 (2.3)
		0.3 mg/kg	10.9 (1.2)	13.3 (2.0)	24.3 (3.0)	18.8 (1.2)
		1 mg/kg	13.9 (1.5)	13.3 (1.3)	27.2 (2.5)	17.0 (2.0)
		3 mg/kg	13.1 (1.5)	11.3 (0.8)	24.4 (2.0)	16.6 (2.5)
2min	Cariprazine	Vehicle	10.7 (0.8)	8.9 (1.2)	19.7 (1.8)	12.3 (1.7)
		0.03 mg/kg	10.3 (1.0)	9.5 (1.1)	19.9 (2.0)	16.3 (1.6)
		0.1 mg/kg	8.6 (1.2)	9.7 (0.9)	18.3 (2.0)	11.9 (1.9)
		0.3 mg/kg	6.4 (1.4)	7.1 (1.1)	13.5 (2.3)	11.4 (1.4)
2min	Aripiprazole	Vehicle	9.5 (0.9)	9.0 (0.9)	18.5 (1.7)	14.7 (1.6)
		0.3 mg/kg	10.7 (0.9)	10.8 (1.3)	21.5 (2.1)	15.1 (1.7)
		1 mg/kg	9.3 (0.9)	10.3 (1.0)	19.6 (1.6)	13.8 (1.8)
		3 mg/kg	7.0 (1.1)	8.1 (1.2)	15.2 (1.7)	12.4 (1.8)

Neither cariprazine (0.03-0.3mg/kg) nor aripiprazole (0.3-3mg/kg i.p.) given 30min prior to the familiarisation trial in group-housed male Lister hooded rats had any effect on the distribution of object exploration time (mean (SEM),  $N=12$  each drug group) during any of the familiarisation trials (all  $p>0.05$ ). There was a significant reduction in object exploration during the choice compared to the familiarisation trial in all experiments (all  $p<0.001$ ) but drug treatment did not significantly alter the total object exploration time during any familiarisation or choice trial (all  $p>0.05$ ). Thus the both drugs altered the pattern of object exploration during the choice trial without affecting the total choice trail object exploration time, suggesting there was no confounding non-specific effect of the drugs on attention of the objects and no major 'sedative-like' effect of either drug at the doses used.

**Table 2** Object exploration during the familiarisation and choice trials.

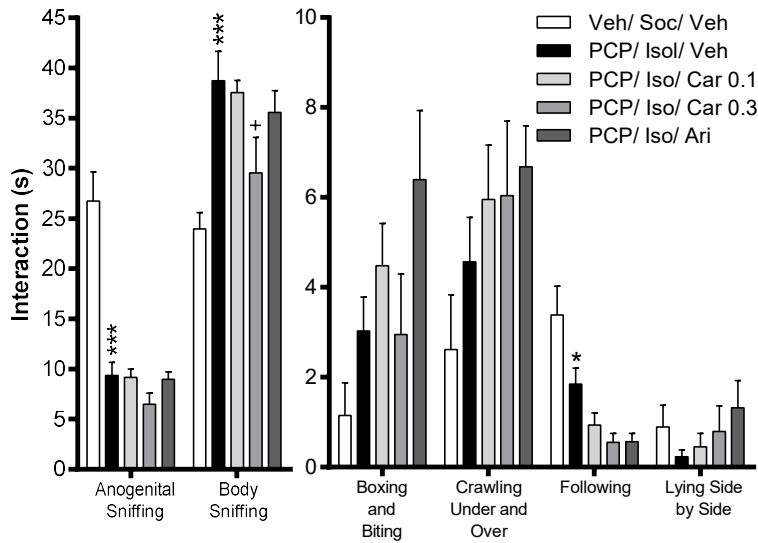
<i>Intertrial Interval</i>	<i>Neonatal</i>		<i>Familiarisation Trial</i>			<i>Choice Trial</i>
	<i>Treatment/ Housing</i>	<i>Drug Treatment</i>	<i>Front Object (s)</i>	<i>Back Object (s)</i>	<i>Total Exploration (s)</i>	<i>Total Exploration (s)</i>
2h ITI	Vehicle/ Group	Vehicle	17.7 (1.4)	17.2 (1.3)	34.9 (2.1)	25.0 (2.3)
	PCP/ Isolate	Vehicle	17.8 (1.1)	18.2 (1.2)	36.0 (1.7)	33.9 (2.3)
	PCP/ Isolate	Cariprazine (0.1mg/kg)	17.5 (1.5)	14.7 (1.2)	32.1 (1.8)	20.9 (1.4)
	PCP/ Isolate	Cariprazine (0.3mg/kg)	15.0 (1.4)	13.0 (1.9)	28.0 (3.0)	24.2 (1.9)
	PCP/ Isolate	Aripiprazole (3mg/kg)	14.4 (1.4)	16.4 (1.3)	30.8 (2.1)	25.0 (1.7)

Neither cariprazine (0.1 or 0.3mg/kg) nor aripiprazole (3mg.kg) injected i.p. 30min prior to the novel object discrimination task had any effect on the pattern of object exploration (s) during the familiarisation trial either in controls or rats treated with neonatal PCP and social isolation from weaning (all  $p>0.05$ ). There was a significant reduction in exploration in the choice compared to the familiarisation ( $p<0.001$ ) trial which was accompanied by a main effect of neonatal drug treatment/ housing ( $p<0.001$ ) and acute drug treatment during the choice trial ( $p<0.01$ ). Data shown as mean (SEM),  $N=14-16$ .

**Table 3** Drug concentrations in blood plasma and brain tissue.

<b><i>Drug Treatment</i></b>	<b><i>Blood Plasma (ng/ml)</i></b>	<b><i>Brain (ng/g)</i></b>
Cariprazine (0.1mg/kg)	6.6 (0.8)	52.0 (3.0)
Cariprazine (0.3mg/kg)	33.4 (3.7)	171.8 (23.0)
Aripiprazole (3mg/kg)	93.5 (9.2)	127.3 (19.6)

Blood plasma (ng/ml) and brain tissue (combined right frontal cortex and right hippocampus, ng/g) concentrations were determined 30min after injection of cariprazine (0.1 or 0.3mg/kg) or aripiprazole (3mg/kg) i.p. in male Lister hooded rats treated with neonatal PCP and then housed in social isolation from weaning. Drug concentrations were determined using liquid chromatography–mass spectrometry. Data shown as mean (SEM),  $N=14-15$ .



**Figure 5** Comparison of the effect of cariprazine (Car at the dose indicated in the legend) and aripiprazole (Ari) on social interaction in male Lister hooded rats treated with neonatal PCP and socially isolated from weaning. Social interaction (s, mean±SEM, of the mean time for each pair) between two rats of similar weight from different litters but the same developmental condition and drug treatment over a 10min trial,  $N=7-8$  pairs. Rats were injected i.p. with either vehicle (2ml/kg), cariprazine (0.1 or 0.3mg/kg) or aripiprazole (3mg/kg in 10ml/kg) 30min prior to the trial. Repeated-measures ANOVA revealed a significant behavior x neonatal treatment/housing interaction ( $p<0.001$ ) and a significant behavior x acute drug treatment interaction ( $P<0.05$ ), \*  $p<0.05$ , \*\*\*  $p<0.001$  from vehicle/social/vehicle group for that behavioral component; +  $p<0.05$  from PCP/isolate/vehicle group in that behavior, Bonferroni planned *post-hoc* comparisons. Note the different time scale used to report anogenital and body sniffing (which account for over 80% of the total interaction time in most pairs) and the other much less frequent behaviors.



