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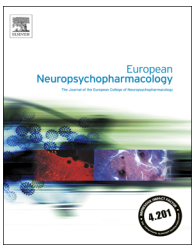

Putative therapeutic targets for symptom subtypes of adult ADHD: D4 receptor agonism and COMT inhibition improve attention and response inhibition in a novel translational animal model

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Abstract
Prefrontal cortical dopamine plays an important role in cognitive control, specifically in attention and response inhibition; the core deficits in ADHD. We have previously shown that methylphenidate and atomoxetine differentially improve these deficits dependent on baseline performance. The present study extends this work to investigate the effects of putative therapeutic targets in our model. A selective dopamine D4 receptor agonist (A-412997) and the catechol-*O*-methyl-transferase (COMT) inhibitor; tolcapone, were investigated in the combined subtype of adult ADHD (ADHD-C). Adult female rats were trained to criterion in the 5C-CPT (5-Choice Continuous Performance Task) and then separated into subgroups according to baseline levels of sustained attention, vigilance, and response disinhibition. The subgroups included: high-attentive (HA) and low-attentive with high response disinhibition (ADHD-C). The ADHD-C subgroup was selected to represent the combined subtype of adult ADHD. Effects of tolcapone (3.0, 10.0, 15.0 mg/kg) and A-412997 (0.1, 0.3, 1.0 μmol/kg) were tested by increasing the variable inter-trial-interval (ITI) duration in the 5C-CPT. Tolcapone (15 mg/kg) significantly increased sustained attention, vigilance and response inhibition in ADHD-C animals, and impaired attention in HA animals. A-412997 (1.0 μmol/kg) significantly increased vigilance and response inhibition in ADHD-C animals only, with no effect in HA animals. This is the first study to use the translational 5C-CPT to model the adult ADHD-C subtype in rats and to study new targets in this model. Both tolcapone and A-412997 increased vigilance and response

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inhibition in the ADHD-C subgroup. D4 and COMT are emerging as important potential therapeutic targets in adult ADHD that warrant further investigation.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is typically characterised by high levels of age-inappropriate attention deficits, impulsivity and hyperactivity. These behaviours are associated with dysfunctions in the neuronal networks involved in controlling and regulating response inhibition and attention (Costellanos et al., 2006). The dopaminergic system remains the main target for currently used psychostimulant ADHD medication, drugs that increase extrasynaptic dopamine levels in a non-selective manner (Volkow et al., 2002, 2003). Conforming to the dopamine theory of ADHD, COMT; the main enzyme responsible for dopamine metabolism, and the dopamine D4 receptor (DRD4) have been widely associated with ADHD.

DRD4 are highly expressed in brain regions known to be affected in ADHD including frontal cortex (Ariano et al., 1997; Tarazi and Baldessarini, 1999) with low expression in the cerebellum (Barili et al., 2000) in humans and rodents. The most replicated genetic association with ADHD is a variable number of tandem repeats in exon 3 of the DRD4 (Faraone et al., 2005; Gizer et al., 2009; Li et al., 2006). These meta-analyses confirm that individuals with these genetic alterations have an increased risk of developing ADHD. Functional imaging studies have shown that the DRD4 7-repeat allele can alter dopaminergic function in brain regions involved in inhibitory control, including the right anterior prefrontal cortex and left middle frontal gyrus (Mulligan et al., 2014), and the left middle and the left inferior frontal gryri (Gilsbach et al., 2012). In support of these findings, Young et al. (2011) have elegantly demonstrated that D4 knockout (KO) mice have attenuated response inhibition, which in turn produces abnormal attentional performance. Based on the clinical literature, the DRD4 clearly presents an interesting target for improving deficits in response inhibition, and to a lesser extent inattention. To date the procognitive effects of DRD4 agonists have been demonstrated only in unimpaired rats (Woolley et al., 2008) and in the spontaneously hypertensive rat (SHR) (Browman et al., 2005). The SHR is the result of genetic manipulations, and has been widely used as a model of ADHD (Sagvolden, 2000). However, this approach utilizes a manipulation with little aetiological relevance (induced hypertension) and therefore lacks translational validity for ADHD. The present experiments assess the effects of A-412997 in our rat model of ADHD in order to investigate the role of DRD4 in ADHD symptoms of inattention, response disinhibition and impaired vigilance. This compound was chosen due to its selectivity, and full agonism at the DRD4 in rats (Moreland et al., 2005) and procognitive effects observed in rats (Woolley et al., 2008).

The second agent we investigate here is tolcapone currently used as an adjunct treatment in Parkinson's disease (Ceravolo et al., 2002). Tolcapone was chosen as it readily crosses the blood brain barrier and is a selective COMT inhibitor (Budygin et al., 1999). The effect of COMT in the PFC on dopamine metabolism, has led to interest in the putative role of COMT in the aetiology of polygenic ADHD (Beiderman et al., 2004; Salatino-Oliveira et al., 2011; Tekin and Cummings, 2002). COMT has been examined as a candidate gene for ADHD, and in contributing to possible neuropsychological phenotypes, however these studies have yielded conflicting results (Bellgrove et al., 2005; Boonstra et al., 2008; Matthews et al., 2012; Taerk et al., 2004). Some groups have found no effect of the COMT genotype on executive function in children (Boonstra et al., 2008; Mills et al., 2004; Taerk et al., 2004) and adults (Boonstra et al., 2008) with ADHD. Bellgrove et al. (2005) have shown in children with ADHD, a negative association between the met allele and sustained attention. Biehl et al. (2015) have demonstrated that adults with the met/met COMT genotype were at a disadvantage in a working memory task, however others have found no effect in ADHD. In a recent imaging study, children and adolescents with ADHD and the met genotype have reduced white matter connectivity between various brains regions compared with COMT-met carriers. In the same sample, white matter integrity was correlated with impaired performance in the CPT, specifically for attentional outcomes (Hong et al., 2014).

Reduced COMT activity either via genetic (Papeleo et al., 2008) or pharmacological (Lapish et al., 2009; Turnbridge et al., 2004) manipulation improves cognitive function, in domains of working memory, attention, executive function and emotional processing in animal studies. In an interesting study using the widely used rodent test of attention and impulsivity—the 5-choice serial reaction time task (5-CSRTT), Papeleo et al. (2012) showed numerous sex and COMT dependent effects using COMT KO mice. Specifically they showed that COMT KO mice were more impulsive; as measured by increased premature responding. The COMT KO mice (males only) were also shown to respond differently to the removal of food restriction (a potential stressor inducing reward seeking/motivation), their performance improved compared to the control wildtype mice (Papeleo et al., 2012). In support of these findings, tolcapone has also been shown to improve attentional set-shifting in adult rats (Turnbridge et al., 2004). To our knowledge only one study has examined the effects of tolcapone on attention and impulsivity in rats, finding no effects in the 5-CSRTT (Paterson et al., 2011). The 5-CSRRT is broadly based on human continuous performance tasks (CPTs), which are one of the most widely used tasks in the study and assessment of ADHD. The 5-CSRTT includes target trials, like the human CPTs but lacks non-target trials; this limits the ability to measure vigilance and impulsivity in a manner consistent with human CPTs. Therefore the lack of effect reported by Paterson et al. (2011) could be due to inability of the 5-CSRTT to robustly detect changes in vigilance and

response inhibition. To overcome the limitations of the 5-CSRTT, including the reduced transferability of results from animal studies to human studies using (CPTs), here we present studies using the recently validated 5C-CPT.

The rodent 5C-CPT has been specifically developed to include both target and non-target trials, enabling the additional measurements of vigilance and response inhibition (Young et al., 2009). In the 5C-CPT the rodent must attend and respond to target light stimuli (one aperture is illuminated) and inhibit their response to non-target stimuli (all apertures illuminated). The form of impulsivity (premature responding) measured in the 5-CSRTT is distinctly different from the additional type of impulsivity measured in the 5C-CPT—response inhibition; where the animal must inhibit from responding to a stimulus. A growing literature suggests that impulsivity (a hallmark of ADHD) is not a unitary construct but instead a phenomenon involving complex cognitive, neural and emotional processes (Evenden, 1999). There are two forms of impulsivity widely recognised in behavioural studies, these include impulsive choice and impulsive action (Broos et al., 2012). Impulsive choice (not measured in these studies) is regarded as an impulsive decision made from a distorted evaluation of delayed consequences of behaviour and a preference for more immediate, smaller rewards instead of beneficial delayed rewards. Whereas impulsive action assessed by human CPTs, is regarded as a failure to inhibit an inappropriate response to prepotent stimuli (Evenden, 1999; Reynolds et al., 2006; Winstanley et al., 2006). Whilst the 5-CSRTT does assess impulsive action as premature responding, it is not analogous to the human CPT as there is no prepotent stimulus to inhibit responding to. However, the 5C-CPT does include a prepotent stimulus (non-target trial) that must be ignored and the behaviour inhibited (response inhibition) just like in the human CPT.

Recently using the 5C-CPT, we have shown that subgroups of animals can be selected with high levels of impulsivity (HI subgroup) and with impairments in sustained attention and reduced vigilance (LA subgroup). Our work with this model has demonstrated that the effects of standard ADHD medication, (methylphenidate and atomoxetine) varies according to the subtype selected (Tomlinson et al. 2014). Therefore, this study aims, first, to extend our previous work by selecting a subgroup of animals using the 5C-CPT, with deficits in all key aspects assessed in the human CPT—response inhibition, vigilance and sustained attention (ADHD-C subgroup). Premature responding was not used as part of the selection criteria as in our experience; it is less consistent with impulsive responding in the human CPT, therefore reducing the translational relevance of the results. Furthermore, groups have shown that levels of attention can directly influence premature responding (Cole and Robbins, 1987; Puumala et al., 1996); we wanted to ensure that the subgroup selected had specific deficits in attention (not secondary to premature response changes). Our previous work separated attention and impulsivity to form subgroups, the current work aims to extend this and combine them, without having the effect of premature responding distorting attention. Therefore, premature responding was *not* included and response inhibition (false alarm responding) *was* included in the selection criteria as the parameter of impulsivity for this model of ADHD.

2. Experimental procedures

2.1. Subjects and housing conditions

Subjects were 40 adult female Lister-hooded rats (Charles River, UK; weighing 240 ± 10 g at the start of training) housed in groups of five on a reversed 12 h light: dark cycle (lights on at 19:00 h). All animals were approximately 8–10 weeks old at the beginning of training. Female rats were used for the present study as the 5C-CPT has been thoroughly validated for female rats in our laboratory (Barnes et al. 2012a; 2012b; Tomlinson et al., 2014), female rats show enhanced cognitive function in certain tests in our laboratory (Sutcliffe et al., 2007), female rats do not gain weight rapidly which allows maintenance of group housing for the lengthy periods involved with 5C-CPT training and testing, thus avoiding the stress of single housing for rats. Both genders suffer from mental illness, whilst there are gender differences in the prevalence of ADHD, the estimated male versus female ratio is reported to be 1.6:1 in the DSM-V therefore, although higher in males, females are still greatly affected by the disorder (Ramtekkar et al., 2011). It is also argued that the reason for the higher prevalence in males may be due to males having more noticeable symptoms including disruptive hyperactive-impulsive symptoms. Therefore it is at least as scientifically valid to use female rodents for research. Indeed, a recent meta-analysis demonstrates that female mice show *less* variability than males in a large array of traits (not just behavioural) when oestrous cycle was not controlled for (Prendergast et al., 2014). All animals were housed in a temperature (21 ± 2 °C) and humidity (55 ± 5%) controlled environment. Animals had free access to food (Special Diet Services, UK) and water until one week prior to training when food restriction was initiated. Thereafter rats were maintained at approx. 90% of their free-feeding body weight (10 g rat chow/rat/day). Water was available for the duration of this study ad libitum. All experiments took place in the dark phase of the light: dark cycle under red light, between 0900 h and 1600 h, illumination of the light in the chamber was a punishment following an omission, incorrect or premature response. All experiments were conducted in accordance with the UK Animals (Scientific Procedures) 1986 Act and University ethical guidelines.

2.2. Apparatus

The 5C-CPT apparatus consisted of eight 25 cm × 25 cm aluminium chambers, each enclosed within a wooden sound attenuating box. Within each box there was a low-level fan to provide ventilation and mask extraneous background noise. The rear wall of the testing chamber contained nine individual apertures, four of which were occluded, leaving apertures 1, 3, 5, 7 and 9 free for presentation of light stimuli. All eight chambers were connected to a PC and data collection and initial analysis was controlled by K-limbic software (Conclusive Solutions), which generated an Excel spreadsheet (Microsoft) containing the raw data for statistical analysis.

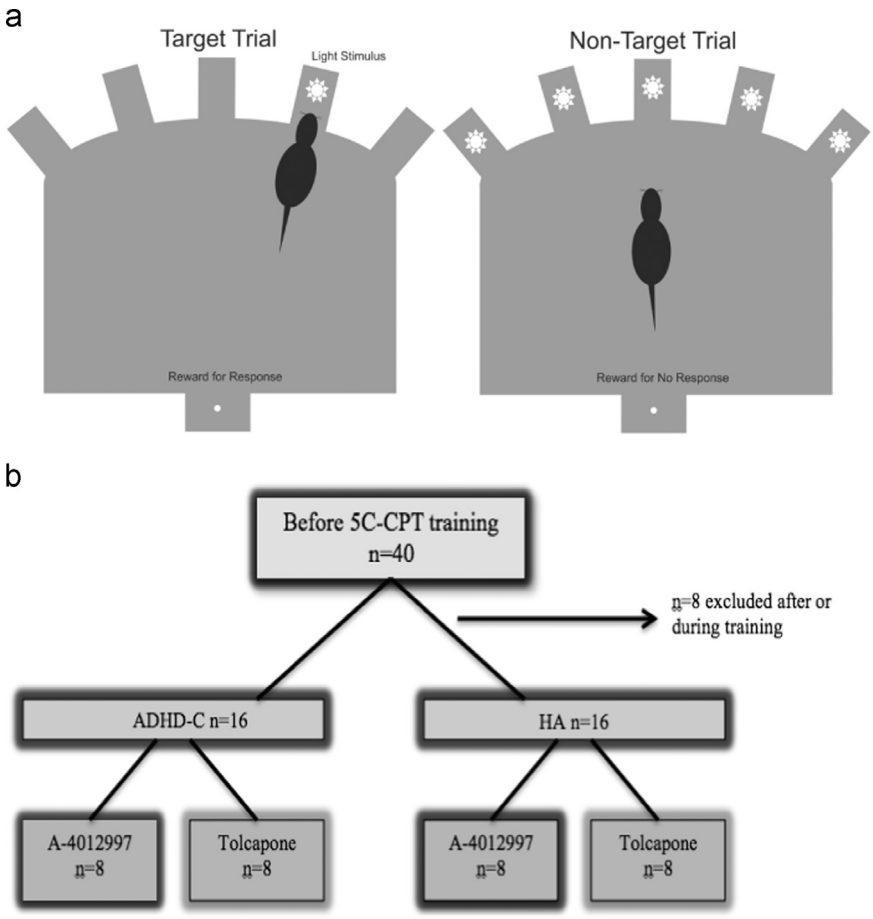

Fig. 1 (a) Example of the two trial types in the 5C-CPT. During target trials the rat must respond to the stimulus by nose-poking beyond the infra-red (IR) beam in the location of the cue stimulus (aperture). Cue stimuli can appear in any one of the five locations. Non-target trials occur when all five cue lights come on at once, and the rat must inhibit from responding in any of the five locations (apertures) adapted from Young et al (2013). (b) A schematic showing how the cohort of animals were separated into groups following training. Also showing the number of animals receiving each drug treatment.

2.3. 5C-CPT training procedure

The 5C-CPT procedure is similar to the standard 5C-SRTT procedure; both methods include target (go) trials in which the animal must respond to a light stimulus by nose-poking in the illuminated aperture. However, the 5C-CPT procedure also includes non-target (no-go) trials, when the animal must withhold from responding to a five-light (all apertures are illuminated) stimulus in order to receive a food reward (Fig. 1a). For a detailed description of the standard 5C-SRTT training and testing procedure see Eagle and Robbins (2003).

The training procedure used was based on the mouse 5C-CPT procedure (Young et al., 2009), with one adaptation—initially placing greater emphasis on non-target trials by adjusting the proportion of target and non-target trials. The training procedure is well established in our laboratory and has been described in detail previously see Barnes et al. 2012a; 2012b and most recently in Tomlinson et al. (2014). The measures used during the selection criteria included (1) accuracy (a measure of selective attention); (2) False alarm rate (a measure of response inhibition, calculated by measuring the number of non-target trials wrongly responded to); (3) Sensitivity index (a measure of vigilance calculated using the signal detection theory, SI corresponds with d'—commonly used in human CPTs as a measure of vigilance). Responsivity index was not used as a measure in the selection criteria, because it is a measure of the response bias of the animal. However RI is used in the analysis, because a change in SI accompanied by a change in RI may not be due to alterations in vigilance but due to response bias. See Table 1 for a full description of 5C-CPT measures.

Training sessions consisted of 120 trials or lasted 30 min. Training began with the parameters set at: stimulus duration (SD) and limited hold duration (LH) set at 10 s. The proportion of target and non-target trials per session was 77 target trials and 43 non-target trials, later increased to 84 target trials and reduced to 36 non-target trials. The inter-trial interval (ITI) and time out (TO) remained constant throughout training (TO—5 s and ITI—variable mean 5 s). However, as animals' performance improved, the SD was reduced to 2 s, and the LH reduced to 2 s. The SD was reduced for individual rats in stages (10, 8, 4 and 2 s). Animals had to satisfy set criteria to progress to the next stage of training until reaching the target criterion of $>70\%$ accuracy, $<25\%$ omissions, $>65\%$ correct rejections for two consecutive days, previously described in Tomlinson et al. (2014). Animals were fully trained when they reached the

Table 1 Description of the measurements from target and non-target trials in the 5C-CPT.

Trial type	Measurement	Definition	Correlated Behaviour
Target trial measures	Accuracy (%)	The percentage of correct responses not including omissions	Selective attention
	Correct (%)	Similar to accuracy, but including omitted trials	General task performance
	Omissions (%)	The percentage of go trials not responded to	Sustained attention/ motivation
	Correct latency (s)	Time from trial presentation to correct response	
	Incorrect latency (s)	Time from trial presentation to incorrect response	
	Correct rejections (%)	Percentage of non-target trials not responded to	Response inhibition
Non-target measures	Premature Response	Response during inter-trial interval	Motor impulsivity
Non trial measures	Magazine latency (s)	Time from reward dispense to collection	Motivational state
	Total trials	Number of target and non-target trials completed	
	Hite rate, p[HR]	Percent correct expressed as a proportion	General task performance
Signal detection theory calculation	False alarm rate, p[FA]	Proportion of non-target trials responded to	Response Disinhibition
	Sensitivity index, SI	A non-parametric calculation based on signal detection theory $SI = \frac{p[HR] - p[FA]}{2(p[HR] + [FA]) - (p[HR] + p[FA])^2}$	Vigilance
	Responsivity Index, RI	A non-parametric calculation based on signal detection theory $RI = \frac{p[HR] - p[FA] - 1}{1 - (p[HR] - p[FA])^2}$	Response strategy

target parameters under the standard training conditions (2 s SD, 5 s ITI, 2 s LH) for two consecutive days; this took approximately 24 weeks. After this, all animals were then trained once-three times per week Monday to Friday. Two animals were excluded at this point, as they failed to reach the set criteria.

2.4. 5C-CPT testing procedure—Selection of ADHD-C and HA subgroups

The procedure used for subgroup selection is described in detail in Tomlinson et al. (2014). Briefly, following acquisition of the 5C-CPT all rats were screened for 5 consecutive days under standard training (baseline) conditions. The means of the attentional measures (accuracy and SI) and response inhibition (p[FA]) were calculated for the 5 days for individual animals. Using the calculated attentional means and response inhibition scores, the rats were then assigned to either a high-attentive (HA) or low attentive with response inhibition deficits (ADHD-C) subgroup, according to the following parameters: HA (accuracy $>90\%$, SI >0.3, p[FA] <0.5) and ADHD-C ($<90\%$ accuracy, SI <0.3, p[FA] >0.5). The 6 animals that showed significant fluctuations in attentional performance over the 5 days (i.e. means meeting highest and lowest quartiles) were excluded, leaving 16 rats per subgroup (ADHD-C and HA) remaining.

2.5. Drugs

A-412997 dihydrochloride (2-(30,40,50,60-tetrahydro-20*H*-[2,40]bipyridinyl-10-yl)-*N*-*m*-tolyl-acetamide) a highly selective DRD4 receptor agonist with high affinity for the rat and human dopamine D4 receptor (Moreland et al., 2005), (Tocris Cookson, Bristol, UK) was dissolved in acetic acid; pH=6.0 with 1 M NaOH, and made-up to a final volume with 0.9% NaCl. A-412997 was administered intraperitoneally (IP) in a volume of 1.0 ml/kg 30 min before behavioural testing, doses are in μmol/kg. Tolcapone (Kemprotec Limited, Middlesbrough, UK) was dissolved in 0.9% NaCl with a few drops of tween (Tunbridge et al., 2004) and administered IP in a volume of 1 ml/kg 30 min prior to testing. Each test day was separated by a two or three-day washout period (no drug administration) (see Table 2 for schematic of testing schedule).

2.5.1. 5C-CPT drug testing in ADHD-C and HA

A-412997 and tolcapone were tested in HA and ADHD-C rats using the 5C-CPT. Drugs were administered according to a

Table 2 Schedule during testing weeks.

Day	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Activity	Rest	**Test day**	Training	Rest	**Test Day**	Rest	Rest

Table 3 Schedule during testing weeks.

Drug	Vehicle	A-412977 0.1 μmol/mg	A-412977 0.3 μmol/mg	A-412977 1.0 μmol/mg	Tolcapone 3.0 mg/kg	Tolcapone 10 mg/kg	Tolcapone 15 mg/kg
Day 1	ADHD-C $n=4$	ADHD-C $n=2$	ADHD-C $n=2$	ADHD-C $n=2$	ADHD-C $n=2$	ADHD-C $n=2$	ADHD-C $n=2$
	HA $n=4$	HA $n=2$	HA $n=2$	HA $n=2$	HA $n=2$	HA $n=2$	HA $n=2$

Dose schedule showing an example test day, conforming to a latin square design. This schedule was repeated for each test day (four in total), ensuring each animal received each dose in a randomized manner, ADHD-C ($n=16$), HA ($n=16$).

fully randomized Latin-square within subjects design with a minimum of 72 h between drug challenge sessions. Experiments were separated by a 1-week washout period. Animals were tested on a Tuesday and Friday and trained under standard conditions in between test days for one day (Wednesday). On the remaining days (Monday and Thursday) the animals were not trained or tested (Table 2).

Following separation into subgroups—HA ($n=16$) and ADHD-C ($n=16$) animals were then randomized to either receive A-412997 or tolcapone (half of each subgroup received each drug, i.e. $n=8$) see Fig. 1b for schematic. A-412997 (0.1, 0.3, 1.0 μmol/kg) or vehicle (0.9% NaCl) was administered to HA ($n=8$) and ADHD-C ($n=8$) subgroups (Table 3). Tolcapone (3.0, 10, 15 mg/kg) or vehicle (0.9% NaCl) was administered to HA ($n=8$) and ADHD-C ($n=8$) subgroups (Table 3). Doses of both compounds were selected based on previous studies in male rats assessing attention, impulsivity and hyperactivity in similar behavioural paradigms (Browman et al., 2005; Paterson et al., 2011). Animals were tested in the 5C-CPT at an increased variable ITI (10 s), 2 s SD and 2 s LH, as previously described (Tomlinson et al. 2014).

2.6. Data and statistical analysis

Data are expressed as mean ± SEM and analysed using SPSS version 16.0. The data were checked for normality and where appropriate (normally distributed) were analysed using parametric measures. Overall subgroup performance using individual measures (e.g. accuracy, premature responses etc.) were normally distributed and analysed by one-way between-subjects ANOVA. Where appropriate, when the overall ANOVA was significant, the LSD *post-hoc* Fisher's Least Significant Difference test was applied.

Analysis of performance (individual performance measures) following drug treatment during challenge sessions was carried out by using a repeated measure two-way ANOVA analysis with treatment as the repeated measure and the subgroup as the between-subjects measure followed by LSD planned comparisons analysis. This allowed reduction of variability by utilising the within-subjects analysis to compare each animal to itself in each treatment group.

3. Results

3.1. ADHD-C and HA subgroup comparison

One-way ANOVA revealed that ADHD-C animals performed significantly worse compared with HA animals under standard conditions. The attentional measures of accuracy (sustained attention) [$F_{(1,38)}=6.47$, $p<0.001$; Fig. 2a] and SI (vigilance) [$F_{(1,38)}=6.47$, $p<0.001$; Fig. 2b] were significantly reduced in ADHD-C compared with HA animals. Response inhibition was significantly reduced in ADHD-C animals, shown as significantly increased p[FA] [$F_{(1,38)}=6.47$, $p<0.001$; Fig. 2c] (Table 4).

3.2. Effects of A-412997 in ADHD-C and HA animals

Treatment of the ADHD-C subgroup with A-412997 significantly improved vigilance in the 5C-CPT compared with vehicle. Repeated measures two-way ANOVA showed a significant Dose X Group interaction for vigilance as measured by SI [$F_{(3,42)}=3.54$, $p<0.05$; Fig. 3b]. Planned comparisons analysis showed a significant increase in the HA vehicle group compared with vehicle treated ADHD-C animals ($p<0.05$). In the ADHD-C subgroup, SI was significantly increased at the highest doses; 0.3 and 1.0 μmol/kg ($p<0.05$; $p<0.01$ respectively; Fig. 3b), and increased to a level that closely approached significance at the lowest dose; 0.1 μmol/kg ($p=0.06$; NS) compared with vehicle. No effects of A-412997 were observed in HA animals (Table 5). The measure of RI showed a significant Dose X Group interaction following repeated measures two-way ANOVA [$F_{(3,42)}=5.19$, $p<0.01$]. RI is the nonparametric measure used to assess the response bias of the animal; the 'tendency to respond', and therefore changes in RI suggest a change in response bias. Post-hoc analysis revealed a difference between the two vehicle groups that did not

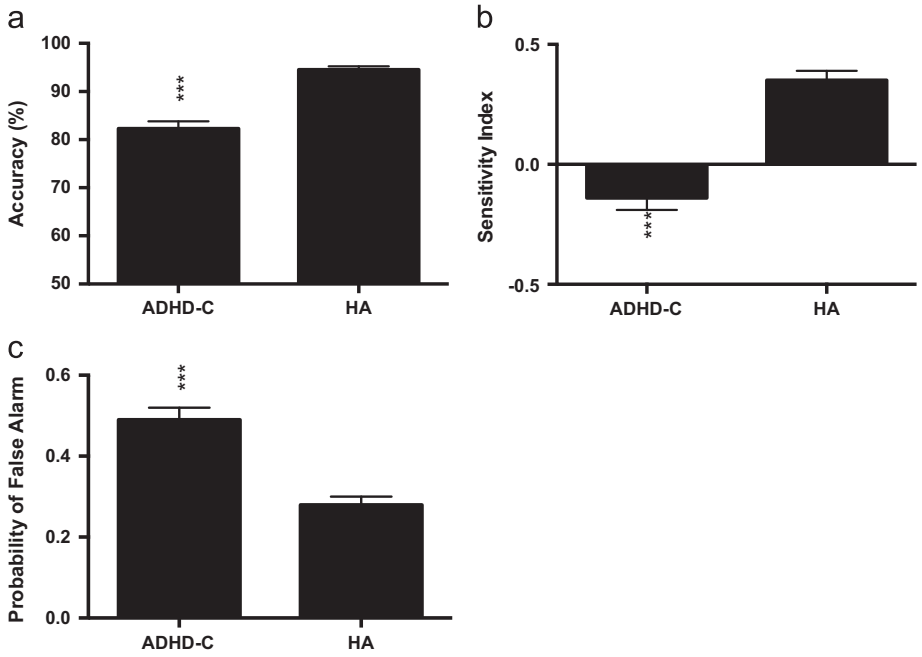

Fig. 2 Subgroup differences in attention. (A) Subgroup differences in target go-trial accuracy in the 5C-CPT under standard conditions, (B) subgroup differences in vigilance (SI). (C) Subgroups differences in false alarm responding (p[FA]). The subgroups are HA and ADHD-C. Data are expressed as the mean ± SEM over 5 days, $n=16$ per sub-group. One-way ANOVA followed by post-hoc analysis showed a significant reduction in accuracy, SI and p[FA] (***$p<0.001$) ADHD-C compared with HA.

Table 4 5C-CPT measures in the HA and ADHD-C subgroups.

5C-CPT measures	HA	ADHD-C
Processed trials	117.00 ± 2.45	116.89 ± 2.58
Accuracy (%)	94.50 ± 0.77	**82.30 ± 1.52****
% Omission	13.00 ± 3.14	**28.00 ± 4.60****
Correct latency (go/no-go)	0.73 ± 0.05	0.77 ± 0.06
Incorrect latency (go)	0.74 ± 0.20	0.89 ± 0.14
Magazine latency (go)	1.12 ± 0.15	1.62 ± 0.31
Premature responses	15.13 ± 4.49	23.44 ± 3.46
p[HR]	0.59 ± 0.08	**0.31 ± 0.09****
p[FA]	0.29 ± 0.02	**0.49 ± 0.03****
Incorrect latency (no-go)	0.63 ± 0.03	0.69 ± 0.07
Magazine latency (no-go)	1.38 ± 0.06	1.43 ± 0.12
SI	0.32 ± 0.04	**−0.14 ± 0.07****
RI	−0.14 ± 0.09	−0.18 ± 0.07

Measures are shown as observed mean ± SEM. Training parameters consisted of 2 s SD, 5 s TO, 2 s LH, a variable ITI averaging 5 s. Target trials are represented as (go) and non-target trials are represented as (no-go).
***$p<0.001$.

reach statistical significance ($p=0.09$; NS). However in ADHD-C animals, following planned analysis, 1.0 μmol/kg significantly reduced RI when compared to the vehicle group ($p<0.05$; Fig. 3d) (Table 6). Unexpectedly, RI was reduced to a level even lower than that in HA rats although this effect was not significant ($p=0.19$; NS).

A significant Dose X Group interaction was observed for response inhibition (false alarm responding) as revealed by repeated measures two-way ANOVA [$F_{(3,42)}=6.95$, $p<0.01$]. HA vehicle animals made significantly less false alarm responses to non-target stimuli compared to the ADHD-C vehicle group ($p<0.01$, Fig. 3c). Planned comparisons analysis also showed that, in ADHD-C animals, A-412997 at 0.3 and 1.0 μmol/kg significantly reduced false alarm responding ($p<0.01$; $p<0.001$ respectively compared with the vehicle control; Fig. 3c) to a level equivalent to that of HA animals.

3.3. Effects of tolcapone in ADHD-C and HA animals

Treatment with tolcapone increased attention in ADHD-C animals only. Repeated measures two-way ANOVA revealed a significant Dose X Group interaction for the total number of trials [$F_{(3,42)}=4.19$, $p<0.01$]. Planned comparisons revealed that ADHD-C and HA vehicle animals completed a similar number of trials ($p=0.144$; NS). Tolcapone at 15 mg/kg significantly ($p<0.05$) decreased the total number of trials completed in HA (Table 7), but not ADHD-C animals (Table 8). Repeated measures two-way ANOVA also showed a significant Dose X Group interaction for accuracy in the go-trials [$F_{(3,42)}=3.81$, $p<0.05$]. Planned comparisons showed a significant increase in accuracy in HA compared with ADHD-C vehicle treated animals ($p<0.001$; Fig. 4a). In the ADHD-C group, accuracy was significantly increased at the highest doses; 10 and 15 mg/kg ($p<0.01$; $p<0.05$ respectively), to a level

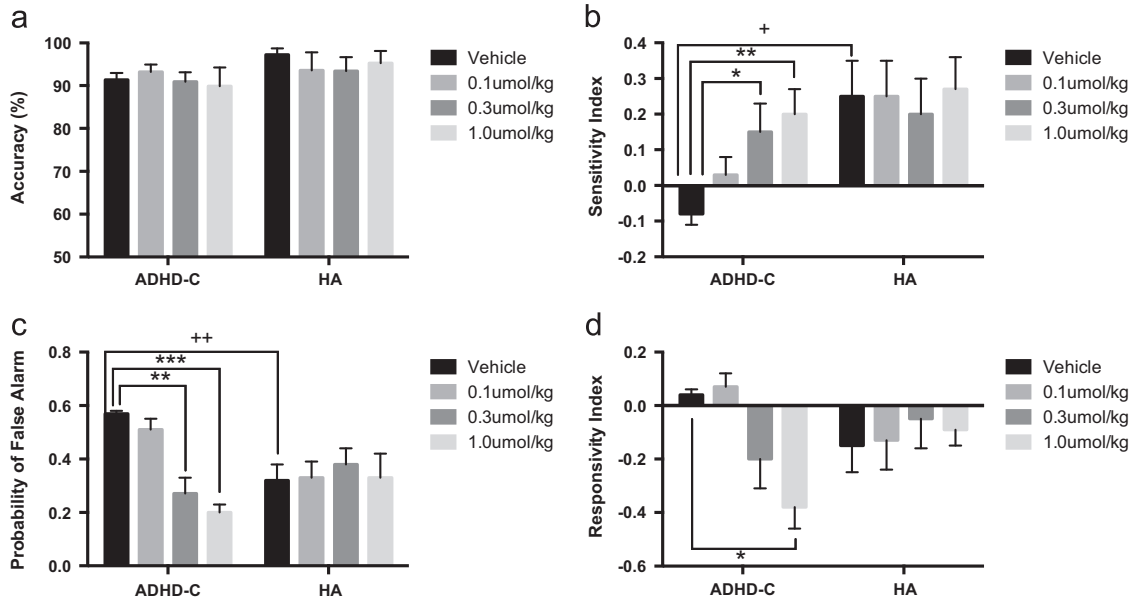

Fig. 3 Effects of A-412997 (0.1, 0.3 and 1.0 mg/kg, i.p. 30 min prior to testing) on overall performance in the 5C-CPT with an enhanced attentional load (variable ITI 10 s) in HA and ADHD-C subgroups. (A) Attentional measures in target-trials (percent accuracy). (B) Vigilance in the two subgroups; SI denotes sensitivity index; the non-parametric measure of vigilance and the ability to discriminate between go and non-target trials. (C) Response inhibition (false alarm responding). (D) Response bias of the animals, RI denotes responsivity index, in the two subgroups. All data are expressed as mean ± SEM, asterisks ($^{**}p<0.05$, $p<0.01$, $p<0.001$) indicate significant differences compared to vehicle, crosses ($^{+}p<0.05$, $^{++}p<0.01$) indicate significant differences between vehicle HA and vehicle ADHD-C animals. All data were analysed using repeated measures two-way ANOVA followed by planned post-hoc comparisons.

Table 5 Effect of A-412997 in the HA subgroup (0.1, 0.3, 1.0 μmol/kg) following a reduced event-rate challenge in the 5C-CPT.

5C-CPT measures	A-412997			
	Vehicle	0.1 μmol/kg	0.3 μmol/kg	1.0 μmol/kg
Processed trials	92.00 ± 8.00	94.13 ± 8.63	90.38 ± 10.70	88.88 ± 5.92
Accuracy (%)	97.21 ± 1.50	93.62 ± 4.20	93.42 ± 3.21	95.29 ± 2.80
% Omission	18.45 ± 3.14	17.06 ± 3.00	16.72 ± 3.20	16.24 ± 2.04
Correct latency (go/no-go)	0.69 ± 0.04	0.65 ± 0.02	0.70 ± 0.03	0.70 ± 0.03
Incorrect latency (go)	0.74 ± 0.20	0.89 ± 0.14	0.90 ± 0.19	0.95 ± 0.19
Magazine latency (go)	1.41 ± 0.15	3.86 ± 1.66	9.80 ± 8.28	10.02 ± 8.46
Premature responses	14.13 ± 5.26	10.88 ± 3.46	11.00 ± 5.63	16.00 ± 5.55
p[HR]	0.55 ± 0.08	0.57 ± 0.07	0.57 ± 0.08	0.59 ± 0.05
p[FA]	0.25 ± 0.10	0.25 ± 0.10	0.20 ± 0.10	0.27 ± 0.09
Incorrect latency (no-go)	0.69 ± 0.04	0.67 ± 0.08	0.66 ± 0.08	0.81 ± 0.05
Magazine latency (no-go)	4.09 ± 1.91	3.34 ± 1.40	5.87 ± 3.51	5.69 ± 2.37
SI	0.25 ± 0.10	0.25 ± 0.10	0.20 ± 0.10	0.27 ± 0.09
RI	−0.15 ± 0.10	−0.13 ± 0.11	−0.05 ± 0.11	−0.09 ± 0.06

Measures are shown as observed mean ± SEM. Reduced event-rate challenge parameters consisted of 2 s SD, 5 s TO, 2 s LH, a variable ITI averaging 10 s. Target trials are represented as (go) and non-target trials are represented as (no-go).

comparable to the vehicle HA group. A significant Dose X Group interaction was observed for hit rate [$F_{(3,42)}=3.47$, $p<0.05$]. At 15 mg/kg, tolcapone significantly increased hit rate ($p<0.01$) in ADHD-C animals, but decreased hit rate in HA animals at all 3 doses ($p<0.05$; $p<0.01$; $p<0.01$). A significant Dose X Group interaction was also observed for premature responses [$F_{(3,42)}=3.78$, $p<0.05$]. At 15 mg/kg, tolcapone significantly decreased hit rate ($p<0.01$) in ADHD-C animals and in HA animals ($p<0.05$). Repeated measures two-way ANOVA also showed a significant Dose X Group interaction for vigilance as measured by SI [$F_{(3,42)}=5.57$, $p<0.01$; Fig. 4b]. Planned comparisons showed a significant increase in HA

Table 6 Effect of A-412997 in the ADHD-C subgroup (0.1, 0.3, 1.0 μmol/kg) following a reduced event-rate challenge in the 5C-CPT.

5C-CPT measures	A-412997			
	Vehicle	0.1 μmol/kg	0.3 μmol/kg	1.0 μmol/kg
Processed trials	83.75±3.70	86.13±5.15	78.50±7.30	76.63±3.49
Accuracy (%)	91.41±1.55	93.22±1.70	90.94±2.21	89.86±4.37
% Omission	19.61±1.11	17.32±1.40	20.75±2.65	23.62±2.45
Correct latency	0.76±0.02	0.79±0.04	0.75±0.02	0.82±0.04
Incorrect latency	0.92±0.07	0.76±0.10	1.04±0.17	0.80±0.11
Magazine latency	1.23±0.05	1.25±0.08	1.50±0.12	1.33±0.09
Premature responses	23.88±4.02	15.38±2.58	18.25±6.25	20.25±5.54
p[HR]	0.48±0.02	0.55±0.03	0.46±0.06	0.40±0.06
p[FA]	0.57±0.01	0.51±0.04	**0.27±0.06****	**0.20±0.03*****
Incorrect latency	0.69±0.02	0.67±0.03	0.61±0.09	0.78±0.04
Magazine latency	2.17±0.56	2.55±1.06	1.64±0.31	2.12±0.54
SI	−0.08±0.03	0.03±0.05	**0.15±0.04***	**0.20±0.03****
RI	0.04±0.02	0.07±0.05	−0.20±0.11	**0.38±0.08***

Measures are shown as observed mean±SEM. Significant values are in bold, compared with vehicle. Reduced event-rate challenge parameters consisted of 1.0 s stimulus duration, 5 s Time Out Period, 2 s Limited Hold, a variable inter-trial interval (ITI) averaging 10 s.

*$p<0.05$.
**$p<0.01$.
***$p<0.001$.

Table 7 Effect of tolcapone in the HA subgroup (3.0, 10.0, 15.0 mg/kg) following a reduced event-rate challenge in the 5C-CPT measures.

5C-CPT measures	Tolcapone			
	Vehicle	3.0 mg/kg	10.0 mg/kg	15.0 mg/kg
Processed trials	94.63±4.78	83.00±8.61	81.63±7.90	**80.38±5.96***
Accuracy (%)	96.36±1.68	92.57±2.28	91.99±1.82	**86.49±3.98***
% Omission	13.59±1.64	19.99±2.88	20.26±1.35	19.63±3.65
Correct latency (go/no-go)	0.70±0.03	0.71±0.04	0.72±0.03	0.72±0.03
Incorrect latency (go)	1.05±0.23	0.98±0.18	0.94±0.16	0.66±0.19
Magazine latency (go)	1.55±0.16	1.50±0.15	1.28±0.08	1.35±0.07
Premature responses	27.13±1.17	29.38±3.86	23.38±3.20	**14.88±2.45***
p[HR]	0.65±0.04	**0.48±0.06***	**0.48±0.03****	**0.47±0.08****
p[FA]	0.34±0.04	0.27±0.03	0.29±0.05	0.40±0.07
Incorrect latency (no-go)	0.69±0.04	0.78±0.04	0.79±0.05	0.65±0.03
Magazine latency (no-go)	2.12±0.38	3.21±1.75	4.90±2.57	3.30±1.78
SI	0.33±0.04	0.22±0.07	0.21±0.09	**0.08±0.08****
RI	−0.01±0.08	−0.26±0.06	−0.25±0.05	−0.13±0.13

Measures are shown as observed mean±SEM. Reduced event-rate challenge parameters consisted of 2 s SD, 5 s TO, 2 s LH, a variable ITI averaging 10 s. Target trials are represented as (go) and non-target trials are represented as (no-go).

*$p<0.05$.
**$p<0.01$.

vehicle animals compared with ADHD-C animals for this measure ($p<0.001$, Fig. 4b). SI was significantly increased at the highest dose of 15 mg/kg ($p<0.05$) in ADHD-C animals and significantly *reduced* in HA animals compared with the respective vehicle groups ($p<0.01$, Fig. 4b). The effect of 10 mg/kg in the ADHD-C group to increase SI closely approached statistical significance ($p=0.06$; NS). There were no significant effects of tolcapone on RI [$F_{(3,42)}=2.19$, $p=0.13$; NS] Fig. 4d.

Repeated measures two-way ANOVA revealed that impulsivity measures were also affected by tolcapone; false alarm

Table 8 Effect of tolcapone in the ADHD-C subgroup (3.0, 10.0, 15.0 mg/kg) following a reduced event-rate challenge in the 5C-CPT measures.

5C-CPT measures	Tolcapone			
	Vehicle	3.0 mg/kg	10.0 mg/kg	15.0 mg/kg
Processed trials	79.75±5.12	79.13±3.75	76.50±5.75	80.13±3.87
Accuracy (%)	85.21±2.49	90.48±3.67	**94.17±1.69****	**91.46±2.56***
% Omission	13.58±1.06	17.36±2.00	12.91±1.70	16.88±1.95
Correct latency	0.71±0.03	0.72±0.03	0.71±0.03	0.72±0.04
Incorrect latency	0.90±0.15	0.89±0.17	0.87±0.12	0.94±0.09
Magazine latency	1.28±0.13	1.49±0.23	1.28±0.14	1.74±0.29
Premature responses	46.50±10.08	26.88±3.19	23.63±7.92	**20.75±2.33****
p[HR]	0.37±0.07	0.42±0.05	0.42±0.06	0.39±0.07
p[FA]	0.66±0.04	0.72±0.08	0.71±0.04	0.76±0.08
Incorrect latency	0.72±0.06	0.72±0.06	0.77±0.07	0.71±0.06
Magazine latency	1.59±0.26	1.66±0.42	1.39±0.18	1.62±0.27
SI	−0.14±0.06	−0.05±0.06	0.08±0.07	**0.07±0.04***
RI	0.30±0.08	0.13±0.10	0.25±0.09	0.05±0.08

Measures are shown as observed mean±SEM. Reduced event-rate challenge parameters consisted of 1.0 s stimulus duration, 5 s Time Out Period, 2 s Limited Hold, a variable inter-trial interval (ITI) averaging 10 s.
*Represents $p<0.05$.
**$p<0.01$.

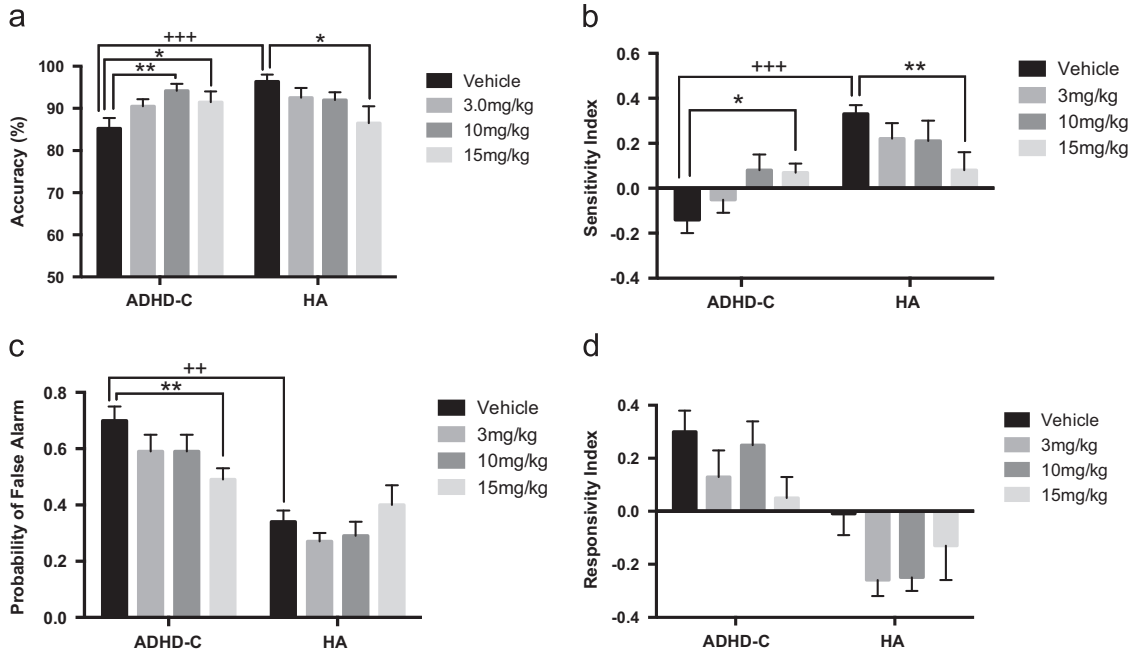

Fig. 4 Effects of tolcapone (3.0, 10 and 15 mg/kg, i.p. 30 min prior to testing) on overall performance in the 5C-CPT with an enhanced attentional load (variable ITI 10 s) in HA and ADHD-C subgroups. (A) Attentional measures in go-trials (percent accuracy). (B) Vigilance in the two subgroups; SI denotes sensitivity index; the non-parametric measure of vigilance and the ability to discriminate between target and non-target trials. (C) Response inhibition (false alarm responding). (D) Response bias of the animals, RI denotes responsivity index, in the two subgroups. All data are expressed as mean±SEM, asterisks (*$p<0.05$, **$p<0.01$) indicate significant differences compared to vehicle, crosses ($^{++}p<0.1$; $^{+++}p<0.001$) indicate significant differences between vehicle HA and vehicle ADHD-C animals. All data were analysed using repeated measures two-way ANOVA followed by planned post-hoc comparisons.

responding showed a significant Dose X Group interaction [$F_{(3,42)}=3.40$, $p<0.05$; Fig. 4c]. Planned comparisons showed that vehicle treated ADHD-C animals had significantly increased p[FA] compared with HA vehicle treated animals ($p<0.01$), and at the highest dose (15 mg/kg) p[FA] was significantly reduced in ADHD-C animals only ($p<0.01$, Fig. 4c).

4. Discussion

The present study demonstrates that rats selected with deficits in sustained attention, vigilance and response inhibition in the 5C-CPT, represent a translational animal model of symptoms in the combined subtype of adult ADHD. These deficits in attention and vigilance are shown by decreased accuracy in responding to target stimuli and decreased SI (a non-parametric measure of vigilance taking into account target and non-target stimulus responses). The increased false alarm responding of ADHD-C rats to non-target stimuli is indicative of a deficit in response inhibition. Taken together; deficits in response inhibition and attention in the 5C-CPT mimic the deficits in clinical adult ADHD, as enhanced false alarm responding often accompanies impaired CPT performance (Groman et al., 2009).

These findings extend our earlier work in rats modeling the inattentive subtype (ADHD-I) of adult ADHD, and the impulsive traits in the combined (ADHD-C) and predominantly hyperactive-impulsive (ADHD-HI) subtypes of adult ADHD (Tomlinson et al., 2014). The first aim of the present study was to combine symptoms of inattention and deficits in response inhibition to form a model of the symptoms of ADHD-C. We have expanded our previous work as we have now characterized a combined model of inattention and impulsivity. This model can be utilised to investigate the mechanisms underlying attention and impulsivity (specifically response inhibition) and how they are interconnected. Impulsivity is suggested to be a multifaceted construct with motor impulsivity mediated by the serotonergic system, and response inhibition via the dopaminergic system (Paterson et al., 2012; Worbe et al., 2014). Future work should aim to assess compounds such as WAY-100635 which acts as a 5HT1A antagonist as well as a DRD4 full agonist (Pattij and Schoffelmeer, 2014). We have further demonstrated the value of the 5C-CPT in dissociating between the two forms of impulsivity, motor impulsivity and response inhibition.

The second aim of the present study was to investigate the effects of putative therapeutic targets, to enhance attention and response inhibition in ADHD-C animals. Our findings extend previous studies and demonstrate the cognitive enhancing properties of tolcapone and A-412997. In the present study, both compounds increased vigilance and reduced false alarm responding at the highest doses in ADHD-C animals. A-412997 did not enhance sustained attention but increased the SI at the two highest doses in ADHD-C, indicative of an increase in vigilance in this subgroup. However, at the highest dose there was a reduction in responsivity index (RI), suggesting that the change in SI may not be due to increased vigilance but may be due to a change in strategy or response bias of the animal. Thus, we cannot conclude that the change in SI at 1.0 μmol/kg is due to an increase in vigilance. In contrast, however at the lower dose of 0.3 μmol/kg there was no accompanying change in RI indicating that, at this dose, there was indeed an increase in vigilance in ADHD-C animals produced by D4 receptor stimulation.

For the first time we have shown that A-412997 at 0.3 and 1.0 μmol/kg reduced false alarm responding in ADHD-C adult rats. This reduction was to a level comparable to that of vehicle HA animals, normalizing the response inhibition deficits. Importantly, the decrease in false alarm responding was not due to an overall decrease in responding, as the hit rate did not change. However, at 1.0 μmol/kg there was a change in RI indicating a possible change in strategy or response bias of the animals. These findings support the role of dopamine and specifically the role of DRD4 in response inhibition. Our findings are consistent with previous findings in DRD4 knock-out mice that show response inhibition deficits in the 5C-CPT (Young et al., 2011). To date previous studies investigating the role of the D4 receptor in ADHD have focused on hyperactivity, using tests of the locomotor hyperactivity to mimic that observed in childhood ADHD. Administration of CP-293019, u-101958, L-745870 and S-18126 (DRD4 antagonists) attenuate the hyperactivity present in the neurotoxin 6-hydroxydopamine neonatal lesion model of ADHD in rats (Zhang et al., 2002, 2001) and mice (Avale et al., 2004), suggesting a role of the DRD4 in hyperactivity symptoms of childhood ADHD. We did not measure hyperactivity between subgroups in adult rats and this is a limitation. Also as our results suggest, we would predict different levels of activity between the subtypes. A-412997 has also been shown to restore delay dependent deficits in the novel object recognition task in rats (Woolley et al., 2008). Others have also shown A-412997 to improve cognitive performance in SHR rat pups (proposed as a model of childhood ADHD) in the 5-trial inhibitory avoidance test (Browman et al., 2005). We have extended these studies by using selection of adult rats from within a normal population, based on extremes of normal behaviours and a translational task; the 5C-CPT. Our findings show that the DRD4 mediates response inhibition and vigilance in adult ADHD with no effects on motor impulsivity or sustained attention in the ADHD-C subtype. These data suggest that the DRD4 may denote a new target for treatment of improving symptoms in the adult ADHD-C subtype. Woolley et al. (2008) have also shown that A-412997 does not have the potential drug abuse liability associated with psychostimulant treatment making DRD4 a desirable target in ADHD.

The second agent used; tolcapone exerts its effects by COMT inhibition, it increases extracellular levels of dopamine in the rat PFC (Tunbridge et al. 2004) and has little or no effect on extracellular dopamine levels in the striatum (Budygin et al., 1999). The PFC plays an important role in cognitive function, including attentional and inhibitory control processes. Here, we have shown that tolcapone at the two highest doses (10 and 15 mg/kg) increased sustained attention while 15 mg/kg also increased vigilance in ADHD-C animals only. The increase in SI in ADHD-C at 15 mg/kg was not accompanied by a significant effect on RI suggesting that the SI increase was due to a real increase in vigilance in ADHD-C animals. However, despite the increase in SI in ADHD-C animals, they did fail to reach the vigilance level of the HA vehicle animals, suggesting that the level of increase was not sufficient to completely overcome the deficit. Tolcapone at the highest dose (15 mg/kg) also reduced false alarm responding in ADHD-C animals, indicative of an increase in response inhibition.

Interestingly, 15 mg/kg of tolcapone decreased vigilance and sustained attention in HA animals to the same level as that observed following 15 mg/kg in ADHD-C animals. However, at 15 mg/kg in HA animals, the number of trials

completed decreased. This suggests a reduction in motivational state, not due to a change in motor activity as response latencies did not differ across groups. Another possibility could be genetic differences between the groups as COMT activity is genetically influenced, with the highest level of variance resulting from the Val158Met polymorphism. The difference between homozygotes Val-COMT and homozygotes Met-COMT is estimated to be 35% COMT enzyme activity in the human brain (Chen et al., 2004). Given that dopamine in the PFC and cognitive performance have an inverted U-relationship, i.e. with too much or too little dopamine leading to impairments in cognition e.g. in working memory (Cools and D'Esposito, 2011; Robbins and Arnsten, 2009; Tunbridge, 2010; Williams and Goldman-Rakic, 1995), genetic differences in COMT could affect this shift. Farrell et al. (2012) have elegantly shown how these genetic differences can effect the direction of cognitive consequences produced by tolcapone. In humans pharmacological COMT inhibition has been shown to impair performance in Met-COMT subjects, and improve performance in Val-COMT (higher COMT activity) subjects (Farrell et al., 2012; Giakoumaki et al., 2008). While not suggesting that the ADHD-C rats had this polymorphism, given the strong genetic influence on COMT, these animals may have discrete genetic or epigenetic differences. This genetic difference may be leading to a differential response to tolcapone in both rats and humans. This possibility requires further exploration.

Using the translational 5C-CPT we have selected subgroups of rats with opposite extremes of behaviour (Tomlinson et al., 2014). By utilizing the ADHD-C rat subgroup displaying severe impairments in attention and response inhibition we have now demonstrated an animal model of the symptoms of the ADHD-C subtype. The majority of animal models of ADHD focus on the hyperactivity component in ADHD. Hyperactivity diminishes in adult ADHD; by using our rat model of the symptoms of ADHD-C (inattention and response disinhibition) we have been able to examine the precognitive effects of two compounds targeting the dopaminergic system. For the first time we have shown that tolcapone improves attention and response inhibition in a rat model of the symptoms of the ADHD-C subtype. We have also shown that A-412997 improves response inhibition and vigilance in ADHD-C animals. These findings highlight the role of D4 agonism, and COMT inhibition, in the processes of attention and response inhibition. Our findings emphasize the importance of focusing on the endophenotypes of adult ADHD and selecting appropriate treatments dependent on symptom expression. We have also highlighted important novel therapeutic targets for treating inattention and response inhibition.

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Conflict of interest

JCN has received expenses to attend conferences and fees for lecturing and consultancy work (including attending advisory boards) from the manufacturers of various neuropsychiatric drugs.

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