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Selective and interactive effects of D_2 receptor antagonism and positive allosteric mGluR4 modulation on waiting impulsivity

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ABSTRACT

Background: Metabotropic glutamate receptor 4 (mGluR4) and dopamine D_2 receptors are specifically expressed within the indirect pathway neurons of the striato-pallidal-subthalamic pathway. This unique expression profile suggests that mGluR4 and D_2 receptors may play a cooperative role in the regulation and inhibitory control of behaviour. We investigated this possibility by testing the effects of a functionally-characterised positive allosteric mGluR4 modulator, 4-((*E*)-styryl)-pyrimidin-2-ylamine (Cpd11), both alone and in combination with the D_2 receptor antagonist eticlopride, on two distinct forms of impulsivity.

Methods: Rats were trained on the five-choice serial reaction time task (5-CSRTT) of sustained visual attention and segregated according to low, mid, and high levels of motor impulsivity (LI, MI and HI, respectively), with unscreened rats used as an additional control group. A separate group of rats was trained on a delay discounting task (DDT) to assess choice impulsivity.

Results: Systemic administration of Cpd11 dose-dependently increased motor impulsivity and impaired attentional accuracy on the 5-CSRTT in all groups tested. Eticlopride selectively attenuated the increase in impulsivity induced by Cpd11, but not the accompanying attentional impairment, at doses that had no significant effect on behavioural performance when administered alone. Cpd11 also decreased choice impulsivity on the DDT (i.e. increased preference for the large, delayed reward) and decreased locomotor activity.

Conclusions: These findings demonstrate that mGluR4s, in conjunction with D_2 receptors, affect motor- and choice-based measures of impulsivity, and therefore may be novel targets to modulate impulsive behaviour associated with a number of neuropsychiatric syndromes.

1. Introduction

Maladaptive impulsivity, the tendency to act prematurely without foresight, is a multi-faceted behavioural trait associated with impaired response inhibition and a preference for instant gratification (Robbins and Dalley, 2016). Impulsive behaviour is a core feature of attention-deficit/hyperactivity disorder (Castellanos et al., 2006) and drug addiction (de Wit, 2009; Hester and Garavan, 2004; Lee et al., 2009; Moeller et al., 2001), and is thought to manifest from abnormalities in a distributed network of brain regions centred on the prefrontal cortex (PFC), hippocampus, and basal ganglia (Baunez and Robbins, 1997; Dalley et al., 2011; Jentsch and Taylor, 1999; Rieger et al., 2003; Winstanley et al., 2006). Motor and choice impulsivity represent two neurobiologically-dissociable, yet potentially overlapping forms of 'waiting impulsivity' – defined as an intolerance for delayed rewards and an

Abbreviations: cAMP, cyclic adenosine monophosphate; CSF, cerebrospinal fluid; DDT, delay discounting task; EC_{30}, effective concentration at 30%; EC_{50}, effective concentration at 50%; EPSC, excitatory post-synaptic current; 5-CSRTT, five-choice serial reaction time task; GABA, gamma-aminobutyric acid; GP, globus pallidus; HI, high-impulsive; ITI, inter-trial interval; IPSC, inhibitory post-synaptic current; LH, limited hold; LI, low-impulsive; mGluR, metabotropic glutamate receptor; MI, mid-impulsive; MSN, medium spiny neuron; SD, stimulus duration; STN, subthalamic nucleus; TO, timeout.

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inability to refrain from responding during delays signalling future reward (Dalley et al., 2011; Robinson et al., 2009).

Glutamate is the principal excitatory neurotransmitter within the mammalian central nervous system and acts *via* two distinct receptor sub-types; ionotropic (iGluR) and metabotropic (mGluR) glutamate receptors (Conn and Pin, 1997; Schoepp, 2001). Based on distinct neuroanatomical distributions and functional dissociations, as well as the increasing availability of selective allosteric modulators, mGluRs may provide novel targets for therapeutic intervention in a number of neuropsychiatric disorders (Conn and Pin, 1997; Nakanishi, 1992; Schoepp and Conn, 2002). Metabotropic glutamate receptor 4 (mGluR4) is a group III, inhibitory mGluR expressed pre-synaptically within both the ventral and dorsal divisions of the striatum and pallidum (Bradley et al., 1999; Corti et al., 2002), specifically at cortico-striatal glutamatergic and striato-pallidal GABA-ergic synapses (Beurrier et al., 2009; Bradley et al., 1999; Cuomo et al., 2009; Gubellini et al., 2014). Thus mGluR4 is ideally located to modulate the D_2 receptor-expressing indirect pathway (Bradley et al., 1999) projecting from the striatum to the pallidum and subsequently the subthalamic nucleus (STN; Albin et al., 1989; DeLong, 1990; Missale et al., 1998; Smith et al., 1998). Functionally, mGluR4 activation suppresses glutamatergic and GABA-ergic neurotransmission in the striatum and globus pallidus (GP), respectively (Beurrier et al., 2009; Cuomo et al., 2009; Gubellini et al., 2014; Pisani et al., 1997; Valenti et al., 2003).

In the present study, we investigated the effects of positive allosteric mGluR4 modulation on motor and choice impulsivity. Following functional characterisation *in vitro*, we assessed the effects of a selective positive allosteric mGluR4 modulator 4-((*E*)-styryl)-pyrimidin-2-ylamine (Cpd11; East et al., 2010), on premature responding on the 5-CSRTT (Robbins, 2002). To reveal a putative involvement of the D_2 receptors, we investigated the effects of administering the D_2 receptor antagonist eticlopride alone and in combination with Cpd11 on 5-CSRTT performance. We subsequently assessed the effects of sub-chronically administered Cpd11 to reveal possible compensatory effects on different aspects of performance in the 5-CSRTT. For comparative purposes, we also assessed the effects of Cpd11 on choice impulsivity using the delay discounting task (DDT).

2. Material and methods

2.1. Subjects

Male Lister-hooded rats (Charles River, Germany), weighing 250–280 g, were trained and assessed for performance on the 5-CSRTT and DDT. A separate group of Lister-hooded rats, weighing 250–300 g (Charles River, Germany), were used for the assessment of locomotor activity. All rats were housed in groups of four under a 12 h light/dark cycle with food and water initially available *ad libitum*. Food restriction was initiated in the trained rats when body weights were at least 300 g. Animals were provided with environmental enrichment, consisting of red Perspex tunnels and wooden gnawing blocks. Body weight was then maintained at approximately 85% of free feeding weight. All training and testing commenced between the hours of 07:00 and 15:00, five days a week. All experimental procedures were authorised by the Local Animal Care and Use Committee in accordance with local animal care guidelines, AAALAC regulations and the USDA Animal Welfare Act.

2.2. Drugs

Cpd11 was synthesised at Boehringer Ingelheim, Germany. For the functional characterisation experiments conducted *in vitro*, Cpd11 was dissolved in 100% DMSO at a stock concentration of 10 mM and stored at −20 °C. For the *in vivo* studies, all drugs were administered according to a Latin square design unless otherwise stated. Cpd11 was dissolved in 10% Tween80 (0.1% v/v) and 90% Natrosol (0.5%) and administered orally (p.o.) at 2 ml/kg, 30 min before testing. Eticlopride hydrochloride was purchased from Sigma Aldrich (Germany), dissolved in saline (0.9%) and administered subcutaneously (s.c.), at 1 ml/kg, 20 min before testing. *D*-amphetamine was purchased from Sigma Aldrich (Germany), dissolved in saline (0.9%) and administered intraperitoneally (i.p.), 2 ml/kg, 15 min before testing. Drugs that were administered i.p. or s.c. were adjusted to pH 7.4.

The selected dose ranges and pre-treatment times for Cpd11 and eticlopride were based on initial pharmacokinetic studies and preliminary behavioural experiments conducted *in house* (East et al., 2010). For example, 30 min following administration of a 30 mg/kg dose of Cpd11, plasma and CSF concentrations of 11.6 μM and 0.7 μM were measured (CSF:plasma ~0.06). *In vitro,* an EC_{50} of ~1 μM was calculated for Cpd11. Based on these findings, it was necessary to select a dose range sufficient to produce CSF exposures in line with this value. In preliminary behavioural experiments, 30 mg/kg Cpd11 failed to modulate 5-CSRTT performance, indicating a minimum effective dose of approximately 40 mg/kg (based on the estimated CSF:plasma ratio and *in vitro* data). A maximal dose of 80 mg/kg was chosen to ensure high selectivity towards mGluR4; Cpd11 has shown to exert mGluR5 modulation activity at high concentrations (IC_{50} ~ 10 μM) (East et al., 2010). To confirm suitable drug exposures were attained in the behavioural studies, plasma exposures for Cpd11 and eticlopride were assessed using satellite rats (Table 1). CSF exposures for Cpd11 were also assessed and compared to the EC_{50} values calculated *in vitro*.

2.3. Compound characterisation – cAMP assay

The functional and allosteric properties of Cpd11 were assessed *in vitro* using the LANCE® *Ultra* cAMP assay kit (Perkin Elmer, USA) for the determination of changes in intracellular cAMP *via* G_i-coupled receptor modulation. The protocol was based on that provided by Perkin Elmer and is described in detail in the supplementary material (S1).

2.4. Behavioural measures

2.4.1. Five-choice serial reaction time task training

Thirty-two operant chambers (Med Associates Inc, St. Albans, Vermont) were used, as described previously (Bari et al., 2008; Carli et al., 1983). Each chamber consisted of five evenly-spaced apertures containing an LED light, set into a curved wall at the rear of the chamber. A centrally-located food magazine was located on the opposite wall, into which 45 mg reward pellets could be delivered

Table 1
Mean plasma and CSF concentrations of Cpd11 measured 45 min after drug administration (±SEM). Mean plasma concentrations of eticlopride measured 35 min after drug administration (±SEM).

	Mean Plasma Concentration (nM)	Mean CSF Concentration (nM)
Cpd11 (mg/kg; p.o.; n = 4)		
60	17950 ± 2.96	1562.3 ± 299.71
80	20000 ± 1.42	1715 ± 312.89
Eticlopride (mg/kg; s.c.; n = 4)		
0.005	0.78 ± 0.23	–
0.01	1.57 ± 0.83	–
0.02	3.51 ± 0.69	–

(Sandown Scientific, UK). Infrared beams located at the entrance of each aperture and the food magazine allowed detection of nose pokes.

The 5-CSRTT training protocol has been described previously (Isherwood et al., 2015). Briefly, 96 rats were habituated to the behavioural apparatus for three days, with gradual food restriction being initiated three days prior. Each training session consisted of 100 trials and lasted no longer than 30 min. Training sessions started with the illumination of the house and magazine light, and by the delivery of a reward pellet. Reward collection initiated the first trial. A single trial consisted of an inter-trial interval (ITI), followed by the pseudo-random illumination of one of the five apertures for a fixed duration (stimulus duration; SD). Following stimulus onset, a nose-poke to the corresponding aperture, within a fixed time interval (limited hold; LH) was required for reward delivery. Premature responses made during the ITI, incorrect responses and responses made outside the LH (an omission) resulted in a timeout (TO), during which time no food was delivered and the house light was extinguished for 5 s. Animals were trained for at least 1 week before starting each drug study; performance was deemed stable when they consistently completed 100 trials with $\geq$70% accuracy and $\leq$25% omissions (SD 0.7 s; ITI 5 s; LH 5 s). At this stage, perseverative responses resulted in a 5 s TO and loss of food reward. A SD of 0.7 s was used rather than 0.5 s (e.g. Carli et al., 1983) to compensate for the less-bright light emitting diodes (LEDs) present in Med Associates boxes. On the day before each drug challenge, a baseline session was run to ensure that performance did not drift during the course of the experiment.

Premature responding was calculated as a percentage of completed trials (correct + incorrect + omissions). A premature response was deemed an incomplete trial and re-set the current trial. Percentage accuracy was defined as the number of correct responses divided by the sum of correct and incorrect responses. Perseveration was calculated as the number of additional responses made in the same aperture, following a correct response. Omissions were calculated in terms of the percentage of completed trials.

2.4.2. Impulsivity screening

Based on neurobiological differences that exist between trait high- and low-impulsive rats assessed on the 5-CSRTT (e.g. Caprioli et al., 2014; Dalley et al., 2007; Jupp et al., 2013; Zeeb et al., 2016), it was hypothesised that modulating impulsivity *via* pharmacological mechanisms that target mGluR4 may produce differential effects on motor impulsivity that depend on dopaminergic mechanisms (Besson et al., 2010; Fernando et al., 2012; Moreno et al., 2013). Thus, the motor impulsivity studies were conducted in rats selected for high-, mid- and low-levels of trait impulsivity. Due to the limited effect of positive allosteric mGluR4 modulation on DDT performance, no such screening took place for this behavioural task.

Screening for impulsivity consisted of four 'challenge' training sessions where the ITI was extended to 7 s to increase the occurrence of premature responses (Dalley et al., 2007). Each challenge session was separated by four baseline training sessions, where task parameters were restored to the training configuration. The mean percentage of premature responses made by each rat across the challenge sessions was calculated. Rats were excluded from the study if they exhibited poor or unstable performance or failed to complete 100 trials on the majority of challenge sessions. Of the 96 rats trained, 16 rats were excluded in this way. All rats were ranked, based on the mean % premature responses, from highly-impulsive to low-impulsive. The upper and lower 15th centiles of premature responders were termed high-impulsive (HI) and low-impulsive (LI) rats, respectively. The remaining rats were categorised as mid-impulsive (MI).

2.4.3. Delay discounting task

Thirty-two operant chambers were used (Med Associates Inc, St. Albans, Vermont), as described previously (Mar and Robbins, 2007; Winstanley et al., 2003). Each chamber consisted of two retractable levers located on either side of a centrally-located food magazine into which reward pellets could be delivered (Sandown Scientific, UK). A stimulus light was located above each lever and an infrared beam at the entrance of the food magazine detected reward collection. Rats were habituated to the behavioural apparatus for three days under a fixed ratio-1 schedule of reinforcement before starting pre-training. See the supplementary material (S2) for details on habituation and pre-training.

During DDT training, each session consisted of 6 blocks of 10 trials, with each trial lasting 72 s. Each block began with four forced-choice trials whereby the left and right levers were each presented twice in a random order. Responding on the right lever resulted in the immediate delivery of a single reward pellet. Responding on the left lever resulted in the delayed delivery of three reward pellets, with increasing delay across blocks from 0 s (block 1), 2 s (block 2), 4 s (block 3), 8 s (block 4), 16 s (block 5) and 32 s (block 6). Following the completion of four forced trials, six free-choice trials were introduced. As in the pre-training protocol, each trial was initiated by the illumination of the house and magazine lights. Rats were required to nose poke the food magazine within 10 s to trigger the presentation of both levers and lever lights. A failure to respond on either lever within 10 s (omission) resulted in the retraction of both levers with all lights extinguished and an ITI initiated before the next trial. Responding on one of the levers within 10 s resulted in the retraction of both levers and all lights being extinguished. Reward delivery was preceded by the illumination of the magazine light either immediately or after the chosen delay. The length of the ITI was dependent on the choice of the immediate or delayed lever, and followed reward delivery to ensure each trial was exactly 72 s in duration. Task parameters and data collection for the 5-CSRTT and DDT were controlled by Med Associates Inc. software (St. Albans, Vermont).

2.4.4. Locomotor activity

Locomotor activity was assessed using eight Tru Scan arena chambers measuring 39 × 41 × 41 cm, equipped with two sensing rings (Couldbourn Instruments, USA) to detect activity along three orthogonal planes; mean distance travelled (cm), total rearing events, and rearing time (sec). All rats were habituated in an annexe to the experimental room before testing.

2.5. Experimental design

2.5.1. Experiment 1: effect of mGluR4 modulation and D_2 receptor antagonism on 5-CSRTT performance

Rats which had either not undergone impulsivity screening (unscreened rats; n = 10) or those selected for high and low levels of impulsivity (n = 12 per group) were used to assess the effect of systemic Cpd11 administration on 5-CSRTT performance. Rats received vehicle, 40, 60 and 80 mg/kg Cpd11 and were tested 30 min later.

Mid-impulsive rats were used to assess the interactive effect of D_2 receptor antagonism and positive allosteric mGluR4 modulation on 5-CSRTT performance. Initially, 12 rats were used to assess the systemic effects of eticlopride when administered alone; rats received vehicle, 0.005, 0.01 and 0.02 mg/kg eticlopride and were tested 20 min later. Subsequently, 16 rats were used to investigate the interactive effects of 60 mg/kg Cpd11 and eticlopride treatment (0.05 and 0.01 mg/kg). Cpd11 was administered 30 min before behavioural testing, as before, followed by the administration of eticlopride 10 min later.

2.5.2. Experiment 2: effect of sub-chronic Cpd11 treatment on 5-CSRTT performance in MI rats

Nineteen MI rats were used to investigate the effects of sub-chronic Cpd11 treatment on 5-CSRTT performance. Following two pre-treatment baseline test sessions, rats were divided into two groups matched for levels of premature responding. For five subsequent days, rats were administered Cpd11 (80 mg/kg), or vehicle, and tested on the 5-CSRTT 30 min later. To investigate whether sub-chronic Cpd11 treatment resulted in longer-lasting effects on 5-CSRTT performance, behaviour was assessed for an additional two days immediately following treatment termination (post-treatment baseline).

2.5.3. Experiment 3: effect of positive allosteric mGluR4 modulation on DDT performance

Rats that were trained on the DDT were used to assess the effects of systemic Cpd11 treatment on choice performance (n = 15). Cpd11 was administered at 40, 60 and 80 mg/kg, 30 min before the task. As a positive control, all rats also received *d*-amphetamine (0.5 mg/kg) 15 min before the task to ensure the DDT was sufficiently sensitive to detect changes in choice performance.

2.5.4. Experiment 4: effect of positive allosteric mGluR4 modulation on locomotor activity

Thirty-two untrained rats were assessed for locomotor activity following systemic Cpd11 administration. Using a between-subjects design, rats received 40, 60 or 80 mg/kg Cpd11, or vehicle, during the habituation period. Thirty minutes later, rats were placed into the locomotor activity chambers and permitted free exploration for 30 min.

A summary of experimental groups and group sizes for each experiment conducted in this study is shown in Table 2. Group sizes of 12 for the 5-CSRTT and 8 for the locomotor studies were chosen based on previous experiments (Isherwood et al., 2015). As HI and LI rats were selected from the extremes of the distribution, a group of MI rats (n = 16) was also available for the pharmacological interaction study. Given the relatively high individual variability in DDT performance, a larger group size (n = 15) was used to investigate the effects of Cpd11 on delay discounting impulsivity.

2.6. Statistical analysis

All analyses were conducted using SPSS for Windows (version 21) and GraphPad Prism 6. Behavioural data were analysed by analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$. In the case of a violation of sphericity, as shown by a significant main effect in Mauchly's test of sphericity, the Greenhouse-Geisser correction (GG ε) was used to adjust the degrees of freedom for correction of *p* values. Behavioural data on the 5-CSRTT were analysed using repeated measures ANOVA. In the study involving HI, LI and unscreened rats, impulsivity group and drug dose served as between- and within-subject factors, respectively. In studies involving MI rats, drug dose served as a within-subjects factor. Where significant main effects or interactions were observed, further analysis using Dunnett's or Bonferroni *post hoc* tests was performed. In the study involving a sub-chronic treating regime, drug and treatment day served as between- and within-subject factors, respectively. Planned comparisons, using the Bonferroni correction, were performed here. DDT data were analysed using repeated measures ANOVA with both delay and drug dose serving as within-subject factors, followed by Fisher's least significant difference (LSD) *post hoc* test where indicated by a significant delay × dose interaction. Locomotor activity data were analysed using one-way ANOVA followed by Dunnett's *post hoc* test where indicated by a significant main effect of drug dose (between-subjects factor).

3. Results

3.1. Compound characterisation – cAMP assay

Cells over-expressing mGluR4 exhibited concentration-dependent increases in intracellular cAMP production following forskolin stimulation (Fig. 1A). A concentration of 1.2 μM forskolin was selected to stimulate the cells in all further experiments. Glutamate attenuated the effect of forskolin stimulation on cAMP production with an EC_{50} of 54.08 ± 0.04 μM (Fig. 1B). The concentration of glutamate necessary to evoke approximately 30% of the maximal glutamate response (EC_{30}) was determined (30 μM) and subsequently used in the allosteric modulation experiments.

Positive allosteric mGluR4 modulation, by Cpd11, concentration-dependently potentiated the effects of 30 μM glutamate on intracellular cAMP production with an EC_{50} of 1.46 ± 0.05 μM. However, Cpd11 failed to elicit a significant effect on cAMP production in the absence of glutamate (Fig. 1C). Increasing concentrations of Cpd11 evoked parallel, leftward shifts of the glutamate concentration-response curve (Fig. 1D); shifting the glutamate EC_{50} approximately 20 fold (maximal shift factor = 20.02), thus indicating enhanced potency of glutamate at mGluR4 by Cpd11 application. These results provide strong evidence for allosteric modulation of mGluR4 by Cpd11. The subtle effect observed in the absence of glutamate and the slight downward shift of the glutamate concentration-curve may imply some agonistic properties of Cpd11. However, this effect was minimal and was only seen at the highest concentrations (approximately 10 fold higher than the EC_{50}). A similar pharmacological profile has been reported previously (East et al., 2010).

Consistent with these data, Cpd11 administered at 60 and 80 mg/kg reached plasma concentrations of 17.9 and 20 μM and CSF concentrations (i.e. free-fraction) of 1.56 and 1.72 μM, respectively, 45 min after drug administration (Table 1). These values are

Table 2
Experimental groups, **impulsivity groups** and group sizes.

Study	Drug administration	Experimental groups	Impulsivity groups	Group size
5-CSRTT	Cpd11	3 experimental groups + control	HI, LI and US	n = 10/12 per group
	Eticlopride	3 experimental groups + control	MI	n = 12
	Cpd11 + Eticlopride	3 experimental groups + control	MI	n = 16
	Sub-chronic Cpd11	1 experimental group + control	MI	n = 9/10 per group
DDT	Cpd11	3 experimental groups + control + positive control	US	n = 15
Locomotor	Cpd11	3 experimental groups + control	UT	n = 8 per group

HI, high-impulsive; MI, mid-impulsive; LI, low-impulsive; US, unscreened; UT, untrained.

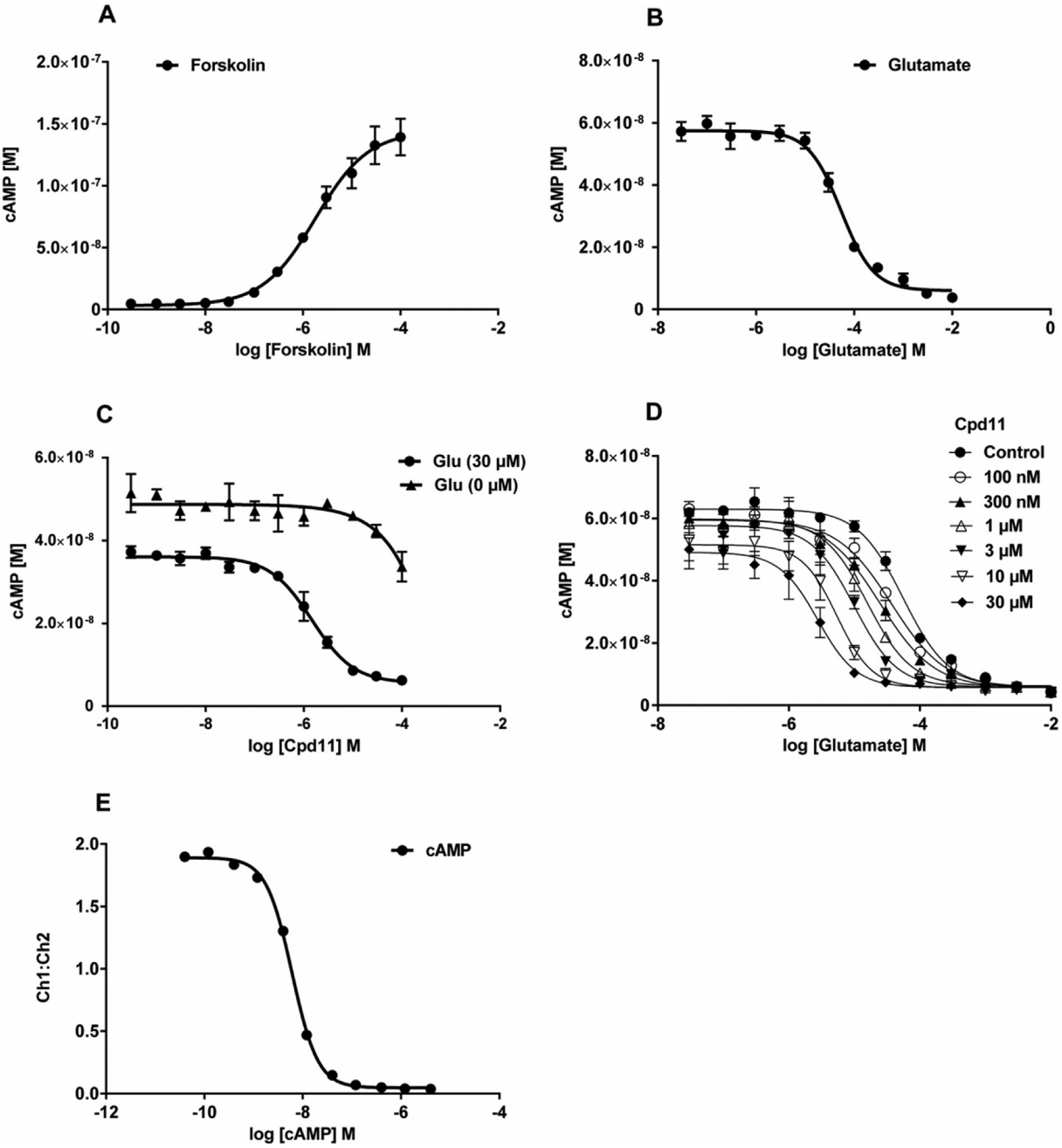

Fig. 1. Functional response of mGluR4-expressing cells to varying concentrations of forskolin, glutamate and Cpd11. **(A)** Forskolin concentration-response curve. **(B)** Glutamate concentration-response curve in the presence of 1.2 μM forskolin. **(C)** Cpd11 concentration-response curve in the presence of 1.2 μM forskolin and 0 or 30 μM glutamate. **(D)** Concentration-dependent leftward shift of the glutamate concentration-response curve by Cpd11. Compound concentration-response curves were generated from triplicates of a single experiment and are expressed as mean ± SD. Raw data were normalised to a cAMP standard **(E).**

perfectly in line with those reported previously (East et al., 2010), and with the EC_{50} value measured for Cpd11 *in vitro*. This is important because, although Cpd11 shows high selectivity towards mGluR4 (limited activity on a panel of 68 other targets), it has shown to exhibit some negative allosteric modulation activity at mGluR5. However, the window between mGluR5 and mGluR4 activity is approximately 10-fold (East et al., 2010); exposures which are not achieved with the given systemic doses. Together, these findings confirm that Cpd11 exerts positive allosteric activity over mGluR4 *in vitro* and that the selected dose ranges are sufficient to exert a pharmacological effect *in vivo.*

3.2. Experiment 1: effect of mGluR4 modulation and D_2 receptor antagonism on 5-CSRTT performance

Fig. 2 shows the effects of systemic Cpd11 administration on 5-CSRTT performance in HI and LI rats and those unscreened for impulsivity. Cpd11 dose-dependently increased premature responding (Fig. 2A; main effect of dose, $F_{3,31} = 9.25$, $p < 0.001$) and decreased attentional accuracy (Fig. 2B; main effect of dose, $F_{3,23} = 23.7$, $p < 0.001$). These effects were independent of impulsivity group and were present for all doses of Cpd11 tested (premature: 40 mg/kg, $p < 0.01$; 60–80 mg/kg, $p < 0.001$ and accuracy:

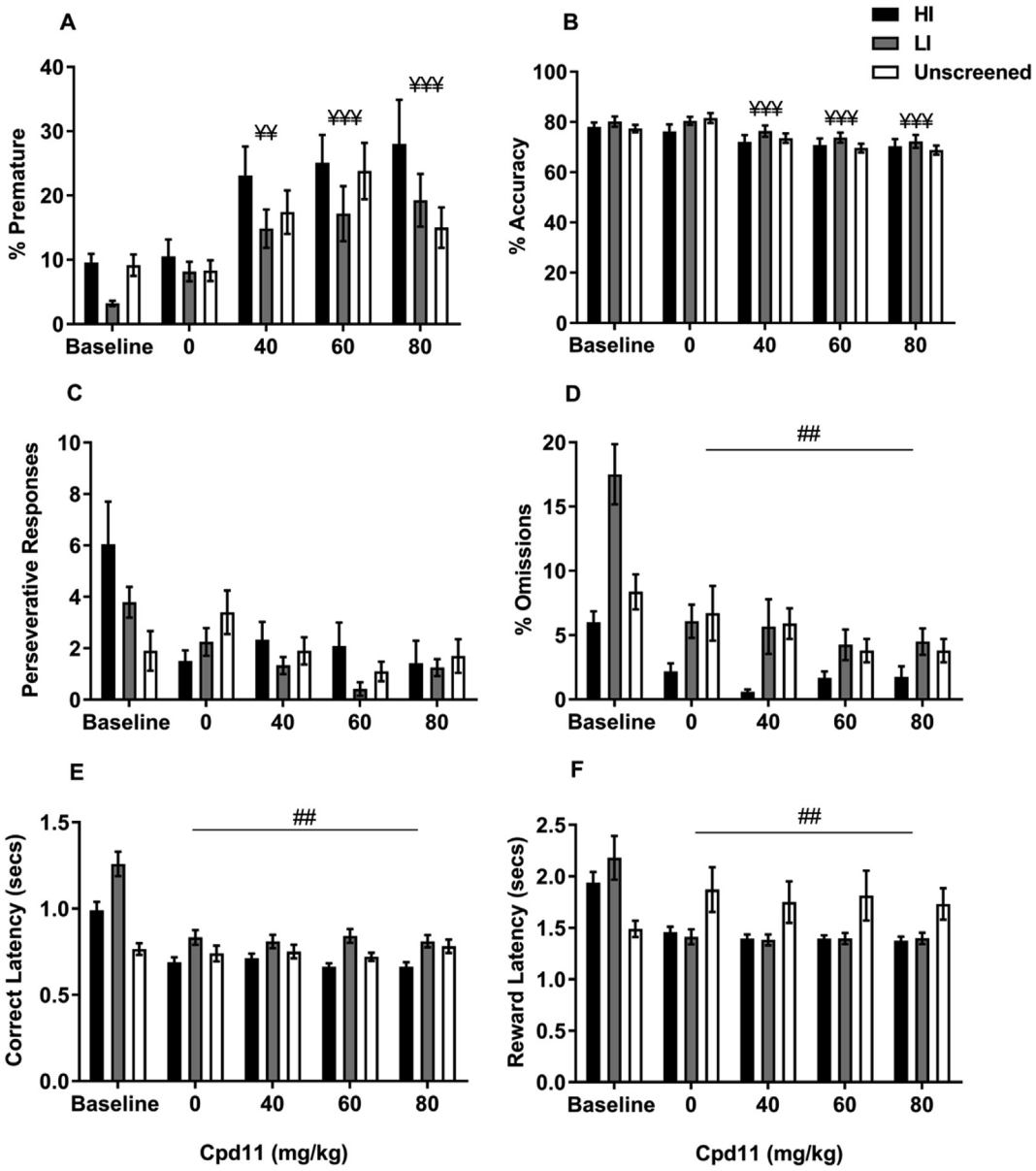

Fig. 2. Effect of Cpd11 on 5-CSRTT performance in HI (n = 12), LI (n = 12) and unscreened (n = 10) rats: (**A**) percent premature responses, (**B**) percent accuracy, (**C**) perseverative responses, (**D**) percent omissions, (**E**) correct response latency and (**F**) reward latency. Bars represent mean ± SEM. Repeated measures ANOVA (mixed design), $^{\#\#}p < 0.01$ main effect of group; Dunnett's *post hoc* test, $^{¥¥}p < 0.01$, $^{¥¥¥}p < 0.001$ HI, LI and unscreened (combined) verses vehicle control.

40–80 mg/kg, $p < 0.001$). Cpd11 treatment also had a significant effect on perseverative responding (main effect of dose, $F_{2,71} = 4.08$, $p < 0.05$, $X^2 = 12.94$, GG $\varepsilon = 0.76$), however this failed to reach statistical significance for any single dose (Fig. 2C). Although omissions and correct response latencies were unaffected by Cpd11 treatment (Fig. 2D and E), these measures were consistently reduced in HI rats (omissions and correct latency: main effect of group, $F_{2,31} = 5.51$, $p < 0.01$ and $F_{2,31} = 6.49$, $p < 0.001$, respectively). *Post hoc* analysis revealed that whereas omissions were reduced in HI rats compared with both LI and unscreened rats ($p < 0.01$ and $p < 0.05$, respectively) correct response latencies were reduced in HI rats compared with LI rats ($p < 0.01$). Reward latencies were also unaffected by Cpd11 treatment (Fig. 2F), however these were consistently increased in unscreened rats (main effect of group, $F_{2,31} = 5.75$, $p < 0.01$, $X^2 = 14.32$, GG $\varepsilon = 0.82$) compared with HI ($p < 0.01$) and LI ($p < 0.01$) rats.

The effect of D_2 receptor antagonism on 5-CSRTT performance is shown in Fig. 3A–D. Task performance was largely unaffected by eticlopride treatment; repeated measures ANOVA indicated that premature responding (Fig. 3A), attentional accuracy (Fig. 3B) and correct response latencies (Fig. 3D) were no different compared with vehicle treated controls. This is unlikely due to insufficient drug exposure given the significant increase in omissions (main effect of dose, $F_{3,33} = 5.0$, $p < 0.01$) observed at the highest dose tested (0.02 mg/kg, $p < 0.05$) (Fig. 3C).

Although eticlopride treatment was without effect when administered alone, interactive effects with Cpd11 were observed following co-administration, as shown in Fig. 3E–H. One rat was excluded from this study due to an error in oral drug administration (i.e. did not receive full dose). Nevertheless, repeated measures ANOVA revealed a significant effect of drug treatment on premature responding (main effect of drug, $F_{2,31} = 5.9$, $p < 0.01$, $X^2 = 12.4$,

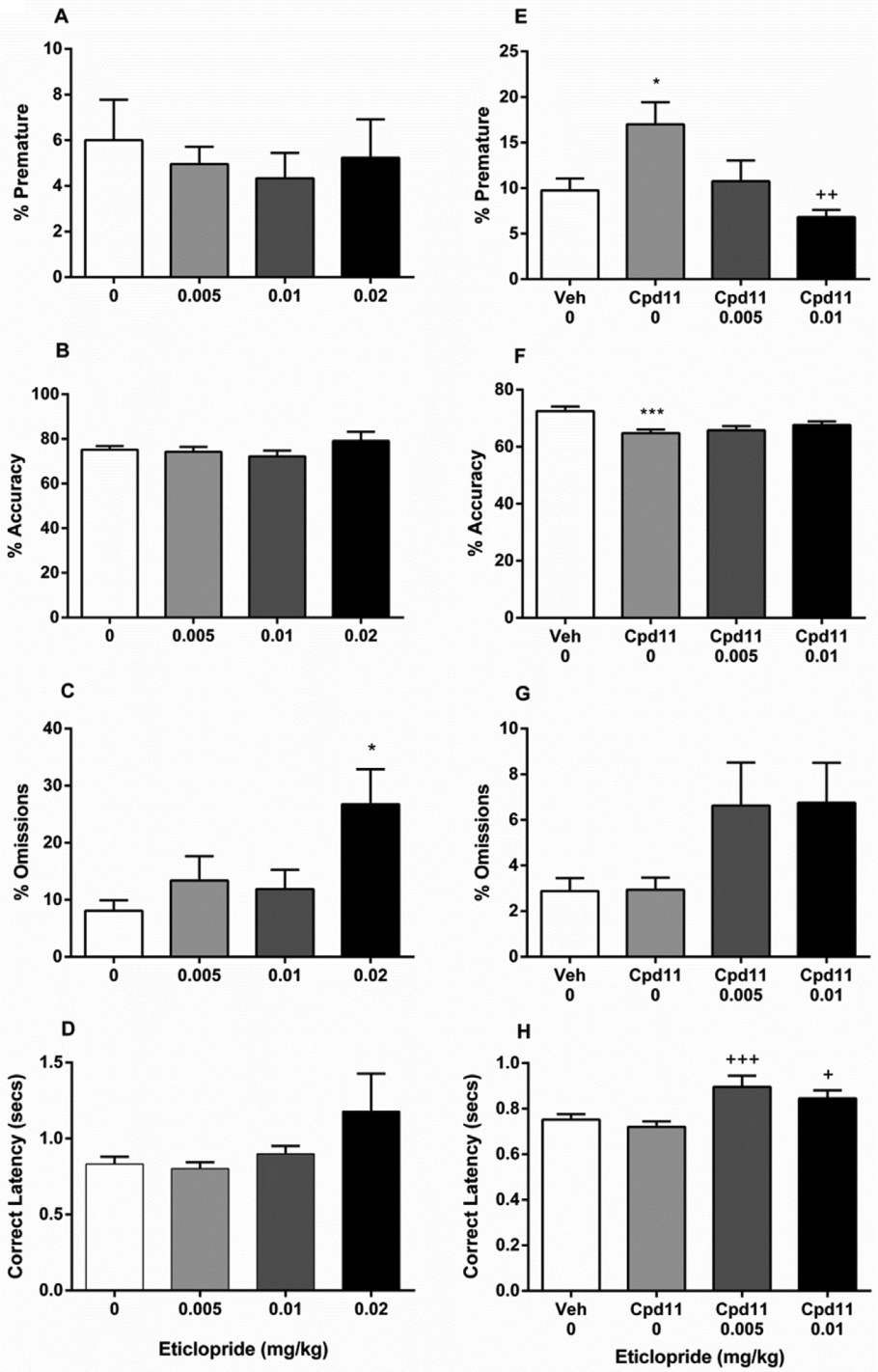

Fig. 3. Effects of eticlopride alone (n = 12; **A-D**) and combined with Cpd11 (n = 16; **E-H**) on 5-CSRTT performance in MI rats: (**A** and **E**) percent premature responses, (**B** and **F**) percent accuracy, (**C** and **G**) percent omissions and (**D** and **H**) correct response latency. Bars represent mean ± SEM. Repeated measures ANOVA; A-D, Dunnett's *post hoc* test, $^{*}p < 0.05$ verses vehicle control; E-H, Bonferroni *post hoc* test, $^{*}p < 0.05$, $^{***}p < 0.001$ versus vehicle (labelled 'Veh-0'), $^{++}p < 0.01$ versus Cpd11 alone (labelled 'Cpd11-0').

GG $\varepsilon = 0.70$). *Post hoc* analysis revealed that Cpd11 evoked an incremental effect on premature responding in the absence of eticlopride ($p < 0.05$), increasing this measure from an average of 9.75 ± 1.3% under the vehicle condition to a maximum of 17 ± 2.5% following 60 mg/kg Cpd11 administration (Fig. 3E). This behavioural response was dose-dependently attenuated with eticlopride treatment (0.01 mg/kg, $p < 0.01$). Furthermore, repeated measures ANOVA also revealed a significant effect of drug treatment on attentional accuracy (Fig. 3F) (main effect of drug, $F_{3,45} = 6.7$, $p < 0.01$), but had no effect on omissions (Fig. 3G). Consistent with the behavioural response to Cpd11 described previously for the unscreened and HI/LI rats, *post hoc* analysis revealed that Cpd11 treatment significantly decreased attentional accuracy ($p < 0.01$). However, unlike as in the case of premature responding, eticlopride treatment had no effect on impaired attentional accuracy. Finally, drug treatment evoked a significant effect on the time taken to

respond correctly (Fig. 3H) (main effect of drug, $F_{3,45} = 8.1$, $p < 0.001$) and the time required for reward collection (main effect of drug, $F_{3,45} = 3.6$, $p < 0.05$; data not shown). *Post hoc* analysis revealed that whilst alone, Cpd11 treatment had no effect on latencies; co-administration with 0.005 and 0.01 mg/kg eticlopride treatment significantly increased the latency to respond correctly compared with the Cpd11-vehicle treated group ($p < 0.001$ and $p < 0.05$, respectively).

3.3. Experiment 2: effect of sub-chronic Cpd11 treatment on 5-CSRTT performance in MI rats

The performance of MI rats on the 5-CSRTT during sub-chronic Cpd11 treatment (80 mg/kg) is shown in Fig. 4. The rat groups were selected based on pre-treatment baseline performance, and matched for levels of premature responding. There was no significant difference between groups in any task parameter during the pre-treatment baseline sessions apart from attentional accuracy. Although the group to be treated with Cpd11 exhibited significantly higher accuracy ($F_{1,17} = 5.7$, $p < 0.05$), this behavioural profile was reversed during chronic treatment and lost in the post-treatment sessions (see below). The pre/post-treatment baseline data were not included in the statistical analysis of 5-CSRTT performance during Cpd11 treatment. Overall, the effect of Cpd11 treatment on 5-CSRTT performance was comparable to that described previously for the unscreened and HI/LI rats; an increase in premature responding and decrease in attentional accuracy. Repeated measures ANOVA failed to reveal a significant effect of Cpd11 treatment

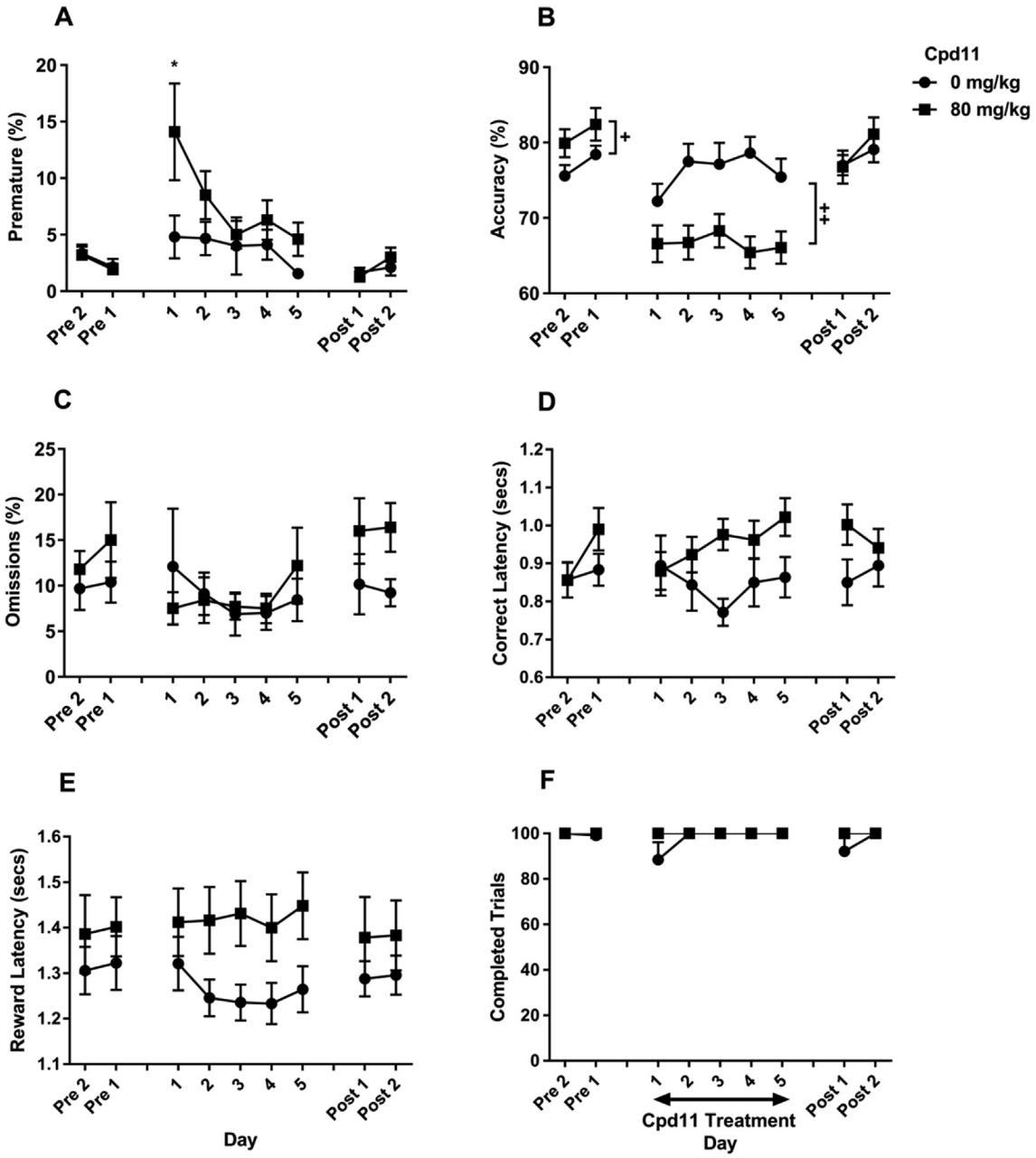

Fig. 4. Effect of sub-chronic Cpd11 treatment on 5-CSRTT performance in MI rats (n = 9/or 10 per group): (**A**) percent premature responses, (**B**) percent accuracy, (**C**) percent omissions, (**D**) correct response latency, (**E**) reward latency and (**F**) completed trials. Data represent mean ± SEM. Repeated measures ANOVA, $^{+}p < 0.05$, $^{++}p < 0.01$ (main effect of drug); *a priori* Bonferroni test, $^{*}p < 0.05$ versus relative vehicle control.

on premature responding ($F_{1,17} = 3.17$, $p = 0.093$), nor a significant drug × day interaction ($F_{4,68} = 1.87$, $p = 0.13$) (Fig. 4A). However, a planned comparison between vehicle and Cpd11 treated groups indicated that whilst acute treatment significantly increased premature responding approximately 3-fold (treatment day 1; $p < 0.05$), this behavioural response was lost with subsequent drug administrations and testing sessions (treatment days 2–5). In contrast, Cpd11 treatment significantly decreased attentional accuracy in the 5-CSRTT (main effect of drug, $F_{1,17} = 12.8$, $p < 0.01$). This effect was maintained throughout the five days of Cpd11 treatment, as shown by a lack of drug × day interaction, decreasing accuracy by approximately 10% on each test session (Fig. 4B). To investigate whether chronic Cpd11 treatment resulted in long-lasting effects on attentional accuracy, data from two post-treatment test sessions were analysed. No significant difference in attentional performance was observed between groups in either post-treatment test session. Consistent with the behavioural profile evoked by acute Cpd11 administration observed earlier, Cpd11 had no effect on any other task parameter (Fig. 4C–F).

3.4. Experiment 3: effect of positive allosteric mGluR4 modulation on DDT performance

The effect of Cpd11 treatment on DDT performance is shown in Fig. 5. As the delay to the large-magnitude reward increased, the choice for that reward decreased (main effect of delay, $F_{2,29} = 89.2$, $p < 0.001$, $X^2 = 112.5$, GG $\varepsilon = 0.41$). This effect was dependent on the drug dose, as demonstrated by a significant delay × dose interaction ($F_{15,210} = 1.7$, $p < 0.05$). *Post hoc* analysis revealed that Cpd11 increased preference for the delayed reward under the 2 s delay (40 mg/kg, $p < 0.01$; 60 mg/kg, $p < 0.001$; 80 mg/kg, $p < 0.01$). However, it should be noted that under the vehicle condition, preference for the delayed reward dropped to as low as 25% at the 2 s delay. Used as a positive control, and analysed separately, *d*-amphetamine administration (0.5 mg/kg) significantly increased the preference for the larger delayed reward. This effect occurred in a delay dependent manner, as indicated by a significant drug × delay interaction ($F_{3,38} = 12.3$, $p < 0.001$, $X^2 = 33.8$, GG $\varepsilon = 0.54$). Thus, *d*-amphetamine increased choice for the large delayed reward when the delay to the reward was 2 and 4 s long ($p < 0.001$), but decreased preference for the large reward, when there was no delay ($p < 0.01$).

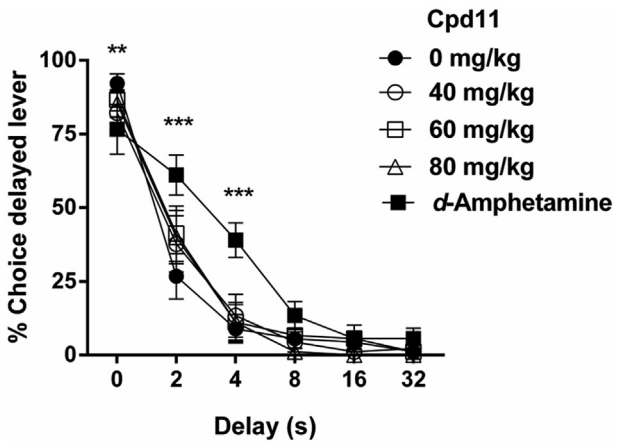

Fig. 5. Effect of Cpd11 on delay discounting performance (80 mg/kg; n = 15). Data represent mean ± SEM percent choice for the delayed reward. Repeated measures ANOVA, Fisher's LSD *post hoc* test, Cpd11 40 mg/kg, $p < 0.01$; 60 mg/kg, $p < 0.001$; 80 mg/kg, $p < 0.01$ verses vehicle control (significance levels not shown), $^{**}p < 0.01$, $^{***}p < 0.001$ *d*-amphetamine versus vehicle control.

3.5. Experiment 4: effect of positive allosteric mGluR4 modulation on locomotor activity

The effect of positive allosteric mGluR4 modulation, by systemic Cpd11 administration, on locomotor activity is shown in Table 3. Cpd11 treatment produced significant effects on all behavioural measures of motor activity, including rearing time ($F_{3,28} = 37.0$, $p < 0.001$), rearing sum ($F_{3,28} = 14.4$, $p < 0.001$) and distance travelled ($F_{3,28} = 3.0$, $p < 0.05$). Whereas total rearing and the time spent rearing were significantly reduced by all doses of Cpd11 (sum: 40 and 60 mg/kg, $p < 0.01$, 80 mg/kg, $p < 0.001$; time: 40–80 mg/kg, $p < 0.001$), the distance travelled was unaffected by the lower doses (40 and 60 mg/kg). In contrast Cpd11, administered at a dose of 80 mg/kg, significantly decreased total distance travelled ($p < 0.05$).

4. Discussion

This study investigated the role of mGluR4 in modulating two distinct forms of waiting impulsivity and its putative interaction with D_2 receptors in modulating motor impulsivity on the 5-CSRTT. The main findings indicate a prominent role of mGluR4 in modulating motor, and to some extent choice, impulsivity as well as aspects of visual attention. Specifically, Cpd11 increased premature responding and decreased attentional accuracy on the 5-CSRTT, an effect that was independent of impulsivity sub-group. Furthermore, the data provide support for an involvement of D_2 receptor-expressing indirect pathway in mediating this behavioural response. Thus D_2 receptor antagonism by eticlopride significantly attenuated the effect of Cpd11 on premature responding, but was without effect when administered alone. By contrast, Cpd11 decreased choice impulsivity, increasing preference for the large-magnitude, delayed reward.

Although the neural mechanism responsible for the observed increase in motor impulsivity is unclear, it may involve the indirect pathway within the basal ganglia. In rodents the striatum is the primary input nucleus of the basal ganglia, receiving glutamatergic input from the cortex, and extending GABA-ergic projections directly to the output nuclei and indirectly *via* the pallidum and STN (Albin et al., 1989; DeLong, 1990). It is well established that mGluR4s are discretely expressed pre-synaptically within the striatum and pallidum, specifically within cortico-striatal and striato-pallidal glutamatergic and GABA-ergic projections, respectively (Bennouar et al., 2013; Beurrier et al., 2009; Bradley et al., 1999; Corti et al., 2002; Cuomo et al., 2009; Gubellini et al., 2014). Thus mGluR4s are ideally located to modulate neurotransmission within this circuitry, primarily by suppressing neurotransmitter release. By contrast, mGluR4 is only weakly expressed within the direct pathway (Bradley et al., 1999). Based on these findings, it was hypothesised that positive allosteric mGluR4 modulation evoked the observed behavioural effects by suppressing cortico-striatal or striato-pallidal neurotransmission. In either case, this would result in increased activity of the pallidum through

Table 3
Effect of Cpd11 (40–80 mg/kg, p.o.) on locomotor activity: rearing time (sec), total rearing (sum) and distance travelled (cm).

Cpd11 (mg/kg)	Rearing time (sec)	Rearing (sum)	Distance (cm)
0	375.9 ± 21.55	125.0 ± 5.42	4141 ± 323.2
40	174.8 ± 22.37 ***	84.25 ± 10.65 **	3424 ± 333.0
60	175.4 ± 19.59 ***	86.25 ± 7.20 **	3453 ± 208.5
80	91.19 ± 15.32 ***	50.75 ± 7.83 ***	2883 ± 309.6 *

Values represent mean ± SEM (n = 8 per group). One-way ANOVA, Dunnett's *post hoc* test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ verses vehicle control.

disinhibition (see Fig. 6 for summary). Studies in brain slices appear to support this hypothesis; electrical stimulation of striatal or GP afferents evokes glutamate-mediated EPSCs and GABA-mediated IPSCs within the respective brain regions. Such evoked potentials are dose-dependently attenuated by the application of mGluR4 agonists (Beurrier et al., 2009; Cuomo et al., 2009; Gubellini et al., 2014; Marino et al., 2003; Pisani et al., 1997; Valenti et al., 2003). It is also noteworthy that the effect of Cpd11 on motor impulsivity was paralleled by a decrease in locomotor activity, confirming that the increase in premature responding was not simply a consequence of drug-induced hyperactivity.

We also investigated the cooperative role of mGluR4 and D_2 receptor modulation on 5-CSRTT performance. It was hypothesised that pharmacological blockade of D_2 receptors would counteract the effect of mGluR4 activation (see Fig. 6 for summary). We found that D_2 receptor antagonism by systemic eticlopride administration dose-dependently attenuated the effects of Cpd11 on premature responding but failed to improve disruptive effects of Cpd11 on attention, indicating that the origin of premature responding is dissociable from attentional deficits. Importantly, systemic eticlopride treatment, up to a dose of 0.03 mg/kg (although i.p.), has no effect on spontaneous locomotor activity (Schindler and Carmona, 2002) thus indicating a selective reduction in impulsivity by D_2 receptor antagonism. Moreover, eticlopride treatment had no effect on premature responding when administered alone nor did it modulate any other task parameter, apart from omissions at the highest dose tested (0.02 mg/kg) (Pattij et al., 2007; van Gaalen et al., 2006). Such a behavioural profile reflects the suitability of the selected dose range when administered subcutaneously; the increase in omitted trials is likely to reflect non-selective motoric effects of the drug at this dose. Together, these findings suggest that the effect of Cpd11 on motor impulsivity is subject to modulation by D_2 receptor antagonism. Further experiments are needed, however, to identify the neural loci responsible for mediating this interaction.

Several lines of evidence suggest that Cpd11 may exert its behavioural effect on 5-CSRTT impulsivity by suppressing GABAergic neurotransmission. Local GP infusion of the mGluR4 agonist LSP1-2111 has been shown to increase premature responding on a reaction time task (Beurrier et al., 2009). Moreover, blocking GABAergic neurotransmission post-synaptically produced a similar effect (Lopez et al., 2007). Whether mGluR4 activation within the ventral division of this circuitry produces a similar effect has not yet been investigated. However, it should be noted that the action of allosteric modulators, such as Cpd11, is restricted to endogenously active receptors. This is important since the striato-pallidal synapse is GABA-ergic where the basal levels of glutamate are likely low and may be insufficient to sustain mGluR4 activation for allosteric modulation. Indeed, positive allosteric mGluR4 modulation fails to inhibit striato-pallidal neurotransmission *in vitro* (Gubellini et al., 2014; Marino et al., 2003). Increasing glutamate concentration by applying TBAO, a broad-spectrum glutamate transporter blocker, enabled positive allosteric mGluR4 modulation to show efficacy (Gubellini et al., 2014). Since the GP receives glutamatergic input from the STN (Parent and Hazrati, 1995), it is predicted that this may provide sufficient levels of glutamate within the striato-pallidal synapse for allosteric efficacy *in vivo* (Marino et al., 2003). By contrast, the cortico-striatal synapse is glutamatergic, where sufficient levels of glutamate are likely present. Additional studies are required to determine which neural mechanism prevails.

Neurally, it is currently unknown how suppression of cortico-striatal or striato-pallidal neurotransmission might mediate the observed increase in motor impulsivity. Recent evidence suggests that the STN may play a significant role (Baunez et al., 2001; Florio et al., 2001; Phillips and Brown, 2000; Phillips and Brown, 1999). Indeed, bilateral lesions to the STN as well as pharmacological inactivation increases premature responding and decreases attentional accuracy on the 5-CSRTT (Baunez and Robbins, 1997, 1995). Thus, positive allosteric mGluR4 modulation is hypothesised to increase pallidal activity and subsequently reduce STN activity (Beurrier et al., 2009; Cuomo et al., 2009; Gubellini et al., 2014). Consistent with this hypothesis, the dopamine receptor antagonist α-flupenthixol has been shown to selectively attenuate the effect of STN lesions on premature responding (Baunez and Robbins, 1997).

Sub-chronic administration of Cpd11 acutely increased premature responding on the first challenge day but failed to evoke this

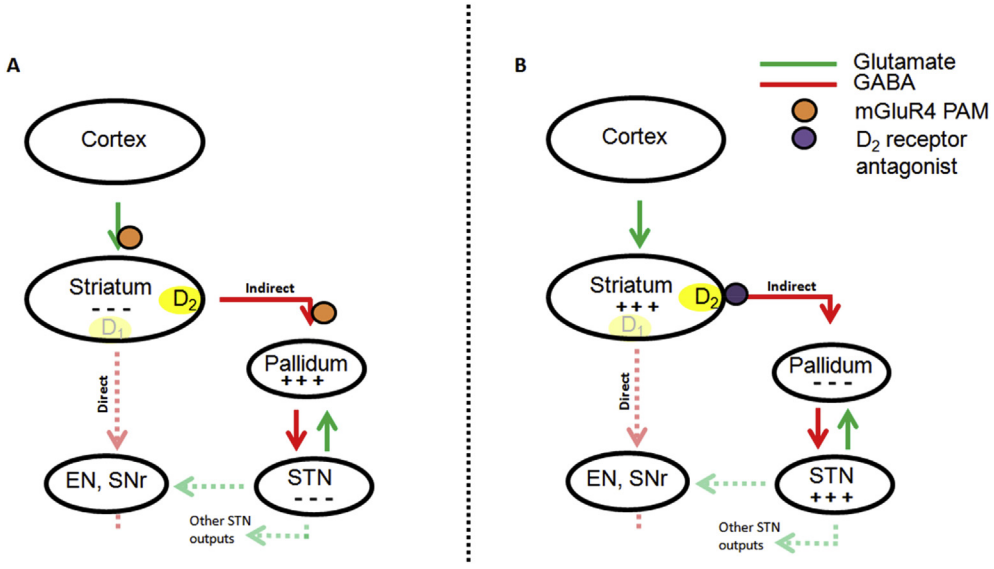

Fig. 6. Schematic diagram summarising the hypothesised involvement of the direct and indirect pathways and the effects of positive allosteric mGluR4 modulation and D_2 receptor antagonism on striatal, pallidal and subthalamic activity. The diagram shows that positive allosteric mGluR4 modulation suppresses cortico-striatal glutamatergic and/or striato-pallidal GABAergic neurotransmission, leading to pallidal disinhibition and subsequently STN inhibition (**A**). By contrast, D_2 receptor antagonism increases striato-pallidal GABA-ergic neurotransmission, leading to pallidal inhibition and subsequently STN disinhibition (**B**).

effect on subsequent treatment days (days 2–5). Indeed the magnitude of increase in premature responding on day 1 was comparable to that observed in the earlier experiments. In contrast, the effect of Cpd11 administration on attentional accuracy was consistently maintained throughout drug treatment and recovered to pre-drug levels following drug discontinuation. This dissociation highlights a separation in neural mechanisms mediating impulse control and attentional accuracy in the 5-CSRTT (Robbins, 2002) and tends to rule out the possibility that the decline in effect of Cpd11 on premature responding was due to mGluR4 desensitisation.

Whilst systemic Cpd11 administration increased motor impulsivity, the same compound decreased choice impulsivity in the DDT. Although the task was sufficiently sensitive to detect the expected impulsivity-reducing effects of d-amphetamine, the conclusion that Cpd11 reduces choice impulsivity requires replication as control animals appeared to show an unexpectedly steep rate of discounting at 2 s. Indeed in our recent study preference for the delayed reward was higher in control rats at this delay (Isherwood et al., 2015). Nevertheless, the apparently opposing effect of Cpd11 on motor and choice impulsivity is consistent with studies demonstrating that lesions of the STN produce similarly opposing effects on motor and choice impulsivity (Baunez and Robbins, 1997; Uslaner and Robinson, 2006; Winstanley et al., 2005). Furthermore, the profile of effects of Cpd11 on motor and choice impulsivity is consistent with a common effect on waiting impulsivity, which may involve impairments in response inhibition and/or temporal perception (Cope et al., 2016; Evenden, 1999). Indeed, we cannot fully exclude the possibility that increased premature responding resulting from Cpd11 administration might reflect an independent deficit in the perception of wait time, and lost opportunity to respond for reward, rather than an effect on response inhibition.

In summary, we have demonstrated that mGluR4 and D_2 receptors play a cooperative role in the regulation and inhibitory control of behaviour. In particular, Cpd11 increased premature responding and decreased attentional accuracy whilst apparently also reducing choice impulsivity. D_2 receptor antagonism attenuated the effect of Cpd11 on premature responding, but had no effect on the attentional deficits incurred. These findings implicate an involvement of the D_2 receptor-expressing indirect striato-pallidal-sub-thalamic pathway in mediating the effects of positive allosteric mGluR4 modulation on impulsivity. Together, these findings enhance our understanding of the neurobiological substrates underpinning impulsivity and suggest that mGluR4 may be a novel target to treat specific aspects of maladaptive impulsivity.

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Statement of interest

Sarah Isherwood, Janet Nicholson and Anton Pekcec are employees of Boehringer Ingelheim Pharma GmbH & Co. KG. Jeffrey Dalley holds research grants from Boehringer Ingelheim Pharma GmbH & Co. KG, and editorial honoraria from Wiley and Sage. Trevor Robbins consults for Cambridge Cognition, Lundbeck, Teva, Shire Pharmaceuticals, Otsuka, and holds research grants from Lundbeck and GSK, royalties from Cambridge Cognition (CANTAB), and editorial honoraria from Springer and Elsevier.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2017.05.006.

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