A preclinical assessment of D,L-govadine as a potential antipsychotic and cognitive enhancer

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Abstract

Tetrahydroprotoberberines (THPBs) are compounds derived from traditional Chinese medicine and increasing preclinical evidence suggests efficacy in treatment of a wide range of symptoms observed in schizophrenia. A receptor-binding profile of the THPB, D,L-govadine (D,L-Gov), reveals high affinity for dopamine and noradrenaline receptors, efficacy as a D2 receptor antagonist, brain penetrance in the 10–300 ng/g range, and thus motivated an assessment of the antipsychotic and pro-cognitive properties of this compound in the rat. Increased dopamine efflux in the prefrontal cortex and nucleus accumbens, measured by microdialysis, is observed following subcutaneous injection of the drug. D,L-Gov inhibits both conditioned avoidance responding (CAR) and amphetamine-induced locomotion (AIL) at lower doses than clozapine (CAR ED50: D,L-Gov 0.72 vs. clozapine 7.70 mg/kg; AIL ED50: D,L-Gov 1.70 vs. clozapine 4.27 mg/kg). Catalepsy is not detectable at low biologically relevant doses, but is observed at higher doses. Consistent with previous reports, acute d-amphetamine disrupts latent inhibition (LI) while a novel finding of enhanced LI is observed in sensitized animals. Treatment with D,L-Gov prior to conditioned stimulus (CS) pre-exposure restores LI to levels observed in controls in both sensitized animals and those treated acutely with d-amphetamine. Finally, possible pro-cognitive properties of D,L-Gov are assessed with the spatial delayed win-shift task. Subcutaneous injection of 1.0 mg/kg D,L-Gov failed to affect errors at a 30-min delay, but decreased errors observed at a 12-h delay. Collectively, these data provide the first evidence that D,L-Gov may have antipsychotic properties in conjunction with pro-cognitive effects, lending further support to the hypothesis that THPBs are a class of compounds which merit serious consideration as novel treatments for schizophrenia.

Key words: Antipsychotic, cognitive enhancer, dopamine, latent inhibition, schizophrenia.

Introduction

Cardinal features of schizophrenia include positive psychotic symptoms arising from abnormal perceptual processes, severe disturbance in cognition, and other negative symptoms linked to poor social interaction and apathy (Andreasen, 2000; Freedman, 2003). Pharmacotherapies that act as dopamine D2 (DA D2) receptor antagonists are consistently effective in treating positive symptoms of schizophrenia (Carlsson, 2006; van Os & Kapur, 2009). Unfortunately, many antipsychotic drugs often evoke unwanted side-effects and fail to address negative/cognitive symptoms. Over the past decade, a compelling case has been made for pharmacological agents that target the fronto-cortical DA system for the treatment of negative and especially cognitive symptoms of schizophrenia (Goldman-Rakic et al. 2004; Weinberger et al. 2001).

In preclinical studies, the disruption of conditioned avoidance responding (CAR) and suppression of psychostimulant-induced locomotion are considered hallmarks of potential antipsychotic efficacy (Svensson, 2000). Disrupted latent inhibition (LI) is observed in individuals with schizophrenia and in a number of preclinical rodent models, including those involving treatment with either acute or repeated d-amphetamine.
(d-Amph) (Gray & Snowden, 2005; Lubow, 2005; Weiner, 2003; Weiner & Arad, 2009). LI is manipulated similarly in rodents and humans by a variety of different pharmacological treatments, suggesting that the neural systems that subserve this phenomenon are largely conserved across species (Lubow, 2005; Weiner & Arad, 2009). A number of experimental protocols that abolish LI have also been used to explore brain regions and neurotransmitter systems that may generate positive symptoms. Moreover, there is increasing interest in experimental protocols that enhance LI as a means of examining the neurobiology of negative and cognitive symptoms (Weiner & Arad, 2009). As such, a treatment that performs well on classical measures of antipsychotic efficacy [CAR, amphetamine-induced locomotion (AIL)], while also restoring LI, when abolished or enhanced, may indicate greater efficacy in addressing the wide range of pathologies observed in schizophrenia.

Impairments in cognitive function mediated by the prefrontal cortex (PFC), along with hypoactivity of this brain region are commonly observed in schizophrenic individuals (Goldman-Rakic, 1999; Seamans & Yang, 2004; Weinberger et al. 2001). Clinical data suggest a beneficial relationship between improved cognition and positive social and clinical outcomes (Andreasen, 2000), but the development of effective treatment strategies that address cognitive deficits has been challenging. Preclinical studies provide strong support for the hypothesis that optimal DA concentrations in the frontal lobes facilitate efficient cognitive function (Goldman-Rakic, 1995; Phillips et al. 2004). Furthermore, increasing the bioavailability of DA or local stimulation of DA receptors in the PFC has proven to be an effective preclinical strategy to improve cognition in circumstances where cognitive performance is suboptimal (Fletcher et al. 1996; Floresco & Phillips, 2001; Hotte et al. 2005; Lapish et al. 2009; Tunbridge et al. 2004). Accordingly, pharmacotherapies that restore optimal PFC DA levels or activity at PFC DA receptors may yield an effective pro-cognitive treatment strategy.

The tetrahydroprotoberberine (THPB) L-stepholidine (L-SPD) has been the subject of extensive preclinical research primarily motivated by its ability to dynamically target DA D_1 and DA D_2 receptors (Mo et al. 2007; Natesan et al. 2008). Preclinical measures of antipsychotic efficacy such as attenuated psychostimulant-induced locomotion, disrupted CAR, and the induction of catalepsy, provide evidence of L-SPD’s DA D_1 antagonism (Natesan et al. 2008). It has also been hypothesized that L-SPD may stimulate PFC DA receptors via a full/full partial D_1 agonist mechanism (Natesan et al. 2008) or by increasing the firing rate of ventral tegmental DA neurons in a 5-HT_1A-dependent manner (Gao et al. 2010). Data supporting this hypothesis include increased cyclic-AMP in DA D_1-expressing cells lines, preferential elevation of c-FOS in the ventral vs. the dorsal striatum (Mo et al. 2007; Natesan et al. 2008), induction of ipsiversive turning in unilateral 6-OHDA-lesioned rodents (Zhang et al. 1999), and increased spontaneous excitatory postsynaptic currents in PFC pyramidal neurons (Gao et al. 2007). Moreover, a pro-drug derivative of L-SPD mitigated deficits in social interaction and novel object recognition in PCP-treated mice, while also suppressing CAR (Guo et al. 2009), thus providing compelling behavioural evidence that L-SPD, and possibly other THPBs, may improve cognition while retaining classical antipsychotic properties.

Amongst other THPBs, L-tetrahydropalmatine exhibits efficacy as an antinociceptive agent, acting as a selective DA D_2 receptor antagonist (Chu et al. 2008) and has been shown to inhibit ATP-sensitive potassium channels (Wu et al. 2010). D,L-Govadine (D,L-Gov) is a THPB with a similar structure to L-SPD and has been characterized as a noradrenaline a_2 antagonist in peripheral cardiovascular tissue, but to date no assessment of this compound has been performed in the central nervous system (Guh et al. 1999; Ko et al. 1996). We commissioned the synthesis of this compound, and assessed its receptor-binding and neurochemical profile, as well as antipsychotic and pro-cognitive properties in preclinical rodent models.

Materials and methods

Animals

All experiments were performed on male Long-Evans rats (Charles River, Quebec) weighing 250–400 g except in the pharmacology experiments where male Sprague-Dawley (SD) rats were used. All animals were maintained on a reversed light/dark cycle (lights on 20:00 hours) Animals were pair-housed, except in the delayed win-shift experiments where they were singly housed to control feeding in order to maintain rats at 85% free-feeding weight. All protocols were approved by the University of British Columbia Animal Care Committee and conducted in accordance with policies outlined by the Canadian Council on Animal Care.

Drug preparation

D,L-Gov (Fig. 1) was provided by Panora Pharmaceuticals (USA) and NMR spectral analysis confirmed
chemical purity (Alphora Research, USA). dl-Gov was dissolved in saline with 30% dimethylformamide (Sigma-Aldrich, USA) and 1% acetic acid (Sigma-Aldrich, USA). Clozapine (Clz; Sigma-Aldrich, USA) was dissolved in saline containing 1% acetic acid. d-Amph (US Pharmacopeia, USA) was dissolved in saline.

**Pharmacology**

The affinity of dl-Gov for DA, serotonin, and norepinephrine receptors was assessed via standard methods (see Dearly *et al.* 1990; Zhou *et al.* 1990) by MDS Pharma Services Inc. (USA). Briefly, human clones were used for each receptor subtype except α₁A, α₁B,
and 5-HT₁ where rat clones were employed. All receptors were expressed in CHO cells and IC₅₀ was determined by a nonlinear, least squares regression analysis. Kᵢ was calculated from the observed IC₅₀ of the tested compound, the concentration of radioligand employed in the assay, and the historical values for the Kᵢ of the ligand (obtained experimentally at MDS Pharma Services). Functional binding efficacy was assessed (Chempartners, USA) via LANCE cAMP kit (PerkinElmer, USA) for each isomer as well as positive and negative controls. The fluorescence intensity measured at 665 nm is decreased in the presence of cAMP from test samples, thus, for ease of interpretation, all data are presented as 1/655 nM signal (Fig. 1c–f). Assays were performed in HEK293 cells, expressing the human D₁ or D₂ receptor and all values were determined in duplicate. For D₂ receptor experiments, cells were stimulated by forskolin (5 μM) and 30 nM DA simultaneously. Brain concentration was assessed after subcutaneous (s.c.) injection of each individual isomer in 230–290 g male SD rats that had free access to food and water. At various time-points after injection (Fig. 1), rats were euthanized via pure CO₂ inhalation and brains were collected and stored at −80 °C until analysis. For analysis whole brain tissue was homogenized for 2 min with 3 volumes (v/w) of homogenizing solution (PBS, pH 7.4). Thirty μl of brain sample was partitioned via liquid-liquid extraction using 1 ml MTBE as an organic solvent. The mixture was vortexed and then centrifuged at 7000 g for 5 min and an 850 μl aliquot of supernatant was removed and evaporated under N₂ until dry. The extract was then reconstituted in 100 μl methanol, vortexed, and 20 μl of the solution was assessed via LC-MS/MS.

**Behavioural assays**

**Catalepsy**

Catalepsy testing occurred in a Plexiglas box (28 cm × 45 cm × 20 cm) containing a 8 cm high horizontal steel bar. After acclimation (1 h) animals were injected s.c. with vehicle (n = 8), or d,l-Gov at 6 mg/kg (n = 5), 3 mg/kg (n = 5), 1.5 mg/kg (n = 5), or 1.0 mg/kg (n = 6). Catalepsy testing occurred at 1, 15, 30, and 45 min after injection. Testing at each time-point lasted 2 min. Behaviour was recorded with a video camera (Cohu, USA) and scored offline. The cumulative time spent immobilized with both forelimbs on the horizontal bar was scored for each test. Videos selected at random were scored by a second observer and a high inter-rater reliability was observed (R² = 0.93, F = 393.4, p < 0.05). The ED₅₀ was calculated from the number of animals that exhibited 60 s of contact time across each dose at the 45-min time-point.

**AIL**

Animals were habituated to locomotor arenas (40 cm × 40 cm × 45 cm Plexiglas box) for 1 h on four consecutive days prior to testing. Activity was assessed on day 1 of habituation and treatment groups were counterbalanced by distance travelled. Animals received intraperitoneal (i.p.) injections of d,l-Gov at 0.3 mg/kg (n = 7), 1.0 mg/kg (n = 6), 3.0 mg/kg (n = 6), and 5.0 mg/kg Clz, or vehicle (Veh) (n = 7) 15 min before 1.5 mg/kg d-Amph or saline on day 5. Distance travelled was assessed for 1 h post-d-Amph/Sal injection. A separate group of animals received d,l-Gov (n = 8) or Veh (n = 7) on days 3 and 4 prior to d-Amph testing on day 5 to assess if previous experience with d,l-Gov affects AIL. Distance travelled was recorded with Ethovision XT (Noldus, USA) and analysed offline. ED₅₀ was calculated as the dose necessary to inhibit d-Amph induced locomotion by 50% compared to animals that received Veh.

**CAR**

Conditioned avoidance responding (CAR) was assessed by a two-way avoidance procedure (Arnt, 1982; Wadenberg et al. 2000). Each avoidance box (Med Associates, USA) consisted of two compartments separated by an open door. Each compartment contained a speaker, house-light, and four photocells. All behavioural protocols were programmed in MED-PC. Before training, each animal received two acclimation sessions lasting 30 min with only the house-light illuminated. Pairings of the conditioned stimulus (CS; white noise, ~60 dB) and unconditioned stimulus (US; foot-shock, 0.75 mA) were presented at a variable interval with a mean of 60 s. The CS was presented for 10 s prior to foot-shock. If the animal crossed to the opposite compartment during this time, the white noise was terminated, no foot-shock delivered, and an avoidance was scored. If the animal did not cross to the opposite compartment during the first 10 s, a foot-shock was administered for 2 s. Movement to the opposite compartment during the 2-s foot-shock resulted in termination of both the CS and US and an escape was scored. Failure to avoid or escape was scored as a failure. If an animal reached a criterion of 90% avoidance for a block of 20 trials within 120 CS–US pairings, it advanced to the drug-testing phase. On the drug test day, animals were injected s.c. with either vehicle (n = 8), or d,l-Gov (0.3 mg/kg, n = 7; 1.0 mg/kg, n = 8; 3.0 mg/kg, n = 7) and placed in the avoidance
box with house-lights illuminated. After 15 min, the presentation of the 100 CS–US pairings began and were scored as during training.

Sensitization

Animals were removed from the colony and taken to a holding room where they received either i.p. d-Amph or saline every Monday, Wednesday, and Friday. Doses started at 1.0 mg/kg and increased 1.0 mg/kg each week over 5 wk to 5.0 mg/kg. After 25 d, behavioral sensitization was assessed over 3 d in locomotor chambers (Plexiglas box, 40 cm × 40 cm × 40 cm) where speed and distance travelled were recorded. The first day consisted of a 2-h acclimation session, and prior to counterbalanced injections of either 0.5 mg/kg d-Amph or saline on days 2 and 3 animals were acclimated for 1 h.

LI

LI tests were performed in rats treated with 1.0 mg/kg acute d-Amph, sensitized rats, and drug-naive rats. Sensitized animals were tested 5 d post-d-Amph challenge. Animals in both conditions (chronic saline or sensitized) were randomly assigned to either non-pre-exposed (NPE) or pre-exposed (PE) groups. Sensitized or saline animals were randomly assigned to Veh or 1.0 mg/kg D,L-Gov groups and received s.c. injections 15 min before each pre-exposure trial. Animals in the PE group received 50 presentations of the CS (10 s duration) per day at a variable interval 60 s schedule for the first 2 d. Conditioning consisted of 100 CS–US pairings. Latency to cross into the other compartment and the number of total beam breaks were recorded. Drug-naive animals receiving acute 1.0 mg/kg Amph were injected i.p. 45 min before the conditioning trial.

Delayed spatial win-shift task

The delayed spatial win-shift task was performed in sensitized and drug-naive animals. For a detailed description of the maze and test protocol see Lapish et al. (2008, 2009). Briefly, animals received a single trial each day that consisted of three phases; training, delay, and test. During the training phase, animals were placed on the maze with four of eight arms open. After the fourth food pellet (Noyes, USA) was consumed, the animal was removed from the maze, returned to its home cage, and sequestered in a quiet dark environment for the duration of the delay. After the delay, access to all eight arms was granted, but food was only available in the four arms blocked during the training phase. Re-entry into an arm constituted an error, and a criterion of one error or less for two consecutive days was required to advance to the next delay or the test day. Animals were trained to criterion at a 1-, 5-, and 30-min delay. Prior to each test, rats received counterbalanced s.c. injections of either Veh or 1.0 mg/kg D,L-Gov 30 min before the test phase. After the first injection, criterion performance was re-established and the second counter-balanced injection was delivered. Separate groups of animals were trained for testing at the 30-min or 12-h delay.

Microdialysis

Rats (275–300 g) were anaesthetized with 4% isoflurane (Baxter, Canada) in oxygen, and then maintained with 1.5–2.5% isoflurane for the remainder of the surgery. Animals were secured in a stereotaxic frame for bilateral implantation of stainless-steel guide cannulae (19-gauge × 15 mm) in the nucleus accumbens (NAc) (from bregma +1.7 mm anterior and ±1.1 mm lateral; from dura −1.0 mm ventral) or PFC (+3.0 mm anterior, ±0.6 mm lateral; −1.0 mm ventral) and secured via stainless-steel screws and dental cement.

Microdialysis probes were constructed from Filtran 12 AN69HF semi-permeable hollow fibres (2 mm long, 340 μm OD × 4 μm, 65 kDa molecular weight cut-off; Hospal, Germany) and silica inlet-outlet lines (75/150 μm ID/OD). The day prior to microdialysis experiments, probes were flushed with artificial cerebrospinal fluid (aCSF) (10.0 mM sodium phosphate buffer with 147.0 mM NaCl, 3.0 mM KCl, 1.0 mM MgCl₂, and 1.2 mM CaCl₂; pH 7.4) and inserted via guide cannulae (dialysis membrane spanned −4.8 to −6.8 mm ventrally for NAc; −1.6 to −3.6 mm for PFC. Rats remained in the testing chamber overnight (14–16 h) with continuous perfusion of aCSF at 1 μl/min. In the morning, dialysates were collected at 10-min intervals and assayed for DA, DOPAC, HVA and 5-HIAA. Once a stable baseline was established (<10% fluctuation over four consecutive samples), the drug treatment phase was initiated with a 1.0 mg/kg s.c. injection of D,L-Gov or Veh.

Samples were analysed via high-pressure liquid chromatography (HPLC) with electrochemical detection. HPLC systems were composed of the following; an ESA 582 pump (Bedford, USA), a pulse damper (Scientific Systems, USA), an inert manual injector (Rheodyne, USA), a Super ODS TSK column (Tosoh Bioscience, USA) and an Intro Electrochemical detector (Antec Leyden, The Netherlands). The mobile phase [70 mM sodium acetate buffer, 40 mg/l EDTA...
and 5 mg/l sodium dodecyl sulfate (adjustable); pH 4.0, 10% methanol] flowed through the system at 0.17 ml/min. EZChrome Elite software (Scientific Software, USA) was used to acquire and analyse chromatographic data. Probe placements were histologically verified following each experiment.

Data analysis

Data were imported into Matlab (Mathworks, USA) or R (R Development Core Team, 2005) for statistical analysis. All data were subjected to analysis of variance (ANOVA) testing. ANOVA testing of delayed spatial win shift (DSWSH) data employed a between-subjects design: treatment was as a within-subjects variable. Microdialysis data was assessed via two-way ANOVA, with repeated variable. Tukey’s post-hoc testing was applied for multiple comparisons with a = 0.05. A general linear model using a probit kernel was applied to the data to determine the ED₅₀ of D,L-Gov on catalepsy, CAR, and AIL.

Results

D,L-Gov exhibits a high affinity for DA receptors and D₂ antagonist properties

D,L-Gov exhibited high affinity for DA receptors, moderate affinity for noradrenaline receptors, and weak affinity for serotonin receptors (Table 1). Higher affinity for D₁ receptors compared to D₂ was observed for D,L-Gov, which is reflected as a low D₁/D₂ ratio (Table 2). Penetration of the blood–brain barrier was confirmed in whole brain homogenate isolated 5, 15, 30, 60, and 120 min (n=4/time-point) after s.c. injection of D,L-Gov (Fig. 1b). Each isomer was assessed independently along with positive and negative controls in a functional binding assay to determine the ability of Gov to directly affect cAMP formation. l-Gov exhibited antagonist properties at D₁ (EC₅₀ = 5.6 × 10⁻⁵) and D₂ (EC₅₀ = 3.9 × 10⁻⁵) receptors (Fig. 1d,f). D-Gov exhibited weak antagonist effects and no agonist effects at the D₁ receptor (EC₅₀ > 1.0 × 10⁻⁴) while conferring weak D₂ antagonist properties (EC₅₀ = 1.4 × 10⁻⁴).

D,L-Gov increases DA efflux in the PFC and the striatum

Basal DA levels, uncorrected for probe efficiency, were detected as 0.18 ± 0.03 and 0.17 ± 0.02 nm in the PFC; 1.59 ± 0.20 nm and 1.97 ± 0.34 nm in the NAc for Veh and D,L-Gov-treated animals, respectively. A single injection of 1.0 mg/kg s.c. D,L-Gov increased DA in both the PFC (two-way ANOVA, main effect of treatment: F₁,₂₄₅ = 9.07, p = 0.0028) and NAc (two-way ANOVA, main effect of treatment: F₁,₂₄₅ = 18.64, p < 0.0001) relative to Veh injection. In the PFC, DA levels peaked during the first 10 min after injection whereas the peak in the NAc was observed 20–30 min post-injection (Fig. 3a1,b1). Increases in both DA metabolites were observed in the PFC (DOPAC, two-way ANOVA, main effect of treatment: F₁,₂₄₅ = 6.01, p = 0.0148; HVA, two-way ANOVA, main effect of treatment: F₁,₂₄₅ = 5.34, p = 0.0215) and the NAc (DOPAC, two-way ANOVA, main effect of treatment: F₁,₂₄₅ = 16.92, p = 0.001; HVA, two-way ANOVA, treatment × time interaction: F₁,₂₄₅ = 18.48, p < 0.0001). A transient increase in DA, caused by the injection procedure, was also observed in the PFC in response to Veh injection that was not observed in the NAc (Fig. 3a1). No increase in the serotonin metabolite, 5-HIAA, was observed in either brain region (Fig. 3a4,b4). All probe placements were confirmed via histology (Fig. 2).

Catalepsy is observed with high doses of D,L-Gov

A dose-dependent increase (R² = 0.91, F = 28.41, p < 0.05) in immobilized contact time was observed with increasing doses of D,L-Gov. Strong and extended catalepsy was observed at the 6.0 mg/kg dose (Fig. 4a,b) whereas mild motor suppression was observed at 1.0 mg/kg (Fig. 4a), without any sign of overt catalepsy. Furthermore, this dose did not

Table 1. D,L-Gov affinity for various receptors subtypes

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Affinity (Kᵢ) (µM)</th>
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<tr>
<td>D₁</td>
<td>0.0064</td>
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<tr>
<td>D₂</td>
<td>0.28</td>
</tr>
<tr>
<td>D₃</td>
<td>0.56</td>
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<tr>
<td>D₄</td>
<td>0.58</td>
</tr>
<tr>
<td>D₅</td>
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</tr>
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<td>5-HT₁</td>
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<td>0.28</td>
</tr>
<tr>
<td>α₂₃D</td>
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induce a transient increase in cataleptic-like behaviour at 15 min post-injection, unlike the 1.5 mg/kg and 3.0 mg/kg doses (multiple comparison, \( p < 0.05 \), Fig. 4b). The ED_{50} value for catalepsy calculated at 45 min from the four doses of D,L-Gov used (Fig. 4a) yielded a value of 4.7 mg/kg.

**D,L-Gov impairs CAR**

D,L-Gov dose-dependently suppressed avoidances \( (R^2 = -0.5, F = 29.02, p < 0.0001) \) in animals proficient with CAR (Fig. 4c). Decreased avoidance behaviour was accompanied by an increase in escape responses in D,L-Gov- and Clz-treated animals (dose \times measure interaction: \( F_{15,6} = 16.56, p < 0.001 \)), providing strong evidence that the disruption of avoidance behaviour was not due to motor impairment. The ED_{50} value for disruption of CAR was calculated at 20 min post-injection from the three doses of D,L-Gov used (Fig. 4d) and yielded a value of 0.723 mg/kg.

**D,L-Gov dose dependently inhibits d-amphetamine induced locomotion**

1.5 mg/kg i.p. d-Amph increased locomotor activity relative to saline injection, and this was dose-dependently suppressed \( (R^2 = -0.4, F = 18.87, p = 0.00022) \) with increasing doses of D,L-Gov prior to d-Amph (Fig. 4e). An ED_{50} of 1.7 mg/kg was calculated for D,L-Gov-mediated suppression of AIL. The effect of D,L-Gov (1.0 mg/kg s.c.) alone on locomotion was assessed for 1 h following injection. A modest non-significant increase in locomotion \( (p < 0.05) \) was observed between D,L-Gov-treated and Veh-treated animals (Fig. 4e, f).

**D,L-Gov treatment at CS pre-exposure reverses the bidirectional effects of acute d-Amph and sensitization on LI**

Injections of Veh or D,L-Gov immediately before each CS pre-exposure session had no subsequent effect on LI in non-sensitized animals as both treatments produced a similar number of avoidances in PE animals (Fig. 5a). Acute d-Amph has been repeatedly shown to abolish LI (Moser et al. 2000; Weiner, 2003), and this effect was replicated in the current study (Fig. 5b, left). Administration of D,L-Gov prior to each CS pre-exposure session ameliorated this effect and restored LI in acute d-Amph-treated animals (Fig. 5b, middle). To address the possibility that pretreatment with D,L-Gov blunts the motor-stimulant effect of d-Amph, thereby mediating the decreased number of avoidances observed, a separate group of animals was

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**Table 2. Pharmacological efficacy of D,L-Gov compared to clozapine and l-stepholidine**

<table>
<thead>
<tr>
<th></th>
<th>( \frac{D_1}{D_2} )</th>
<th>( D_1 (K_i) )</th>
<th>( D_{11} (K_i) )</th>
<th>CAR (ED_{50})</th>
<th>AIL (ED_{50})</th>
<th>Catelepsy (ED_{50})</th>
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<tr>
<td><strong>D,L-Gov</strong></td>
<td>0.023&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0064 ( \mu M &lt;sup&gt;b&lt;/sup&gt; )</td>
<td>0.283 ( \mu M &lt;sup&gt;cd&lt;/sup&gt; )</td>
<td>0.720 mg/kg&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.70 mg/kg&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.70 mg/kg&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><strong>Clozapine</strong></td>
<td>0.438&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.189 ( \mu M &lt;sup&gt;bd&lt;/sup&gt; )</td>
<td>0.431 ( \mu M &lt;sup&gt;ed&lt;/sup&gt; )</td>
<td>7.70 mg/kg&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.27 mg/kg&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.a.</td>
</tr>
<tr>
<td><strong>L-Stepholidine</strong></td>
<td>0.150&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.013 ( \mu M &lt;sup&gt;bl&lt;/sup&gt; )</td>
<td>0.085 ( \mu M &lt;sup&gt;ef&lt;/sup&gt; )</td>
<td>0.270 mg/kg&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.36 mg/kg&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.60 mg/kg&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

<sup>a</sup> Present data.  
<sup>b</sup> Comparison ligand = 0.14 nM SCH-23390.  
<sup>c</sup> Comparison ligand = 0.16 spiperone.  
<sup>d</sup> NIMH PDSP database certified data.  
<sup>e</sup> Natesan et al. (2008).  
<sup>f</sup> Xu et al. (1989).

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**Fig. 2.** Histology. The anatomical location of microdialysis probes were confirmed as being in the prefrontal cortex (a) or the nucleus accumbens (b).
pretreated with either D,L-Gov or Veh on two consecutive days and placed in an open field. On day 3 animals were injected with 1.5 mg/kg d-Amph, returned to the open field, and speed and distance travelled were recorded. No effect of D,L-Gov pretreatment was observed on this measure of AIL (d-Amph + prior D,L-Gov, 12 798 ± 1504 cm vs. d-Amph + prior Veh, 12 158 ± 1971 cm (mean ± S.E.M.; Student’s t test, p > 0.05), suggesting that D,L-Gov pretreatment did not suppress the motor-stimulant effect of d-Amph but rather suppressed the ability of d-Amph to facilitate the association between CS and US. We next assessed if pretreatment with 5.0 mg/kg s.c. Clz restored LI in the same manner as D,L-Gov. We observed that Clz pretreatment decreased the number of avoidances in PE animals, however, NPE animals also showed a similar decrease in the number of avoidances relative to other NPE animals (Fig. 5b, right). No difference (p > 0.05) was found between NPE or PE animals receiving Clz indicating a lack of LI, thus Clz pretreatment does not offset acute d-Amph-induced deficits in LI in the same manner as D,L-Gov.

Fig. 3. Microdialysis. Data from 1.0 mg/kg s.c. D,L-Gov-treated (grey squares) and vehicle-treated (open circles) animals are shown in each graph and expressed as the mean ± S.E.M. Injection is indicated by the arrow in each graph. Dialysis was performed in the PFC (a1–a4) and the NAc (b1–b4) for each treatment group and significant increases in dopamine (a1, b1) as well as metabolites (a1–a2, b1–b2) were observed in response to D,L-Gov. No change in the serotonin metabolite 5-HIAA was observed (a4, b4). (Vehicle vs. D,L-Gov, * Tukey’s post hoc test, p < 0.05; † Tukey’s post-hoc test, p < 0.05.)
Fig. 4. Antipsychotic properties of d,l-Govadine. An increase in catalepsy is observed with increasing doses of d,l-Govadine. Cumulative catalepsy scores for all test points are shown (a). A time-series plot of immobilized contact time for each of the 2-min testing epochs reveals a transient increase at 15 min for the 1.5 and 3.0 mg/kg dose (b). All data are shown as the mean ± S.E.M. immobilized contact time with the catalepsy bar for each 2-min epoch. (n=29, one-way ANOVA: F<sub>4,24</sub> = 66.12, p < 0.00001; comparison with vehicle: * Tukey’s post-hoc test, p < 0.05; ** Tukey’s post-hoc test, p < 0.001). A significant decrease in mean number of total avoidances (c) is observed in the 1.0 mg/kg d,l-Govadine, 3.0 mg/kg d,l-Govadine, and 5.0 mg/kg clozapine groups compared to vehicle controls. A time-series plot for each treatment group is shown (d) and each point represents the mean number of avoided trials ± S.E.M. for a block of 10 trials. d,l-Govadine doses of 1.0 and 3.0 mg/kg increase avoidance behaviour for 60 trials compared to vehicle controls (main effect of treatment: F<sub>4,37</sub> = 8.46, p < 0.0001; comparison with Veh: * Tukey’s post-hoc test, p < 0.05; ** Tukey’s post-hoc test, p < 0.01). d,l-Govadine inhibited amphetamine-induced locomotion in a dose-dependent manner (e, f). 1.5 mg/kg d-Amph increased locomotion relative to saline injection which was suppressed by each dose of d,l-Govadine and clozapine (one-way ANOVA: F<sub>6,30</sub> = 16.382, p = 0.00051; comparison with Veh+Sal: * Tukey’s post-hoc test, p < 0.05; ** Tukey’s post-hoc test, p < 0.01; *** Tukey’s post-hoc test p < 0.001). Time-series plot showing locomotor activity after i.p. injection of 1.5 mg/kg d-Amph at time = 0 (arrow) is presented in (f). Veh+d-Amph (open circles), Veh+Sal (closed circles), Veh+1.0 d,l-Govadine (open squares), d-Amph+0.3 d,l-Govadine (light-grey circles), d-Amph+1.0 d,l-Govadine (mid-grey square), d-Amph+3.0 d,l-Govadine (dark-grey diamond), d-Amph+5.0 mg/kg clozapine (black triangles) are shown as the mean ± S.E.M. in 5-min bins.
Finally, we explored the consequences of the 5-wk escalating dose of the d-Amph sensitization regimen on the expression of LI (Fig. 5c). Sensitization was confirmed in animals receiving repeated d-Amph as an increase in locomotion to a 0.5 mg/kg i.p. d-Amph challenge relative to those who received repeated saline [saline 178 ± 16 % vs. sensitized, 247 ± 22 % (mean ± s.e.m.); Student’s t test, p < 0.05]. The 5-wk escalating dose regimen of d-Amph had the unanticipated effect of significantly decreasing the number of avoidances selectively in the PE group, indicative of increased LI (Fig. 5c, left). Thus a bidirectional effect on LI was observed when comparing all Veh-treated animals (background × exposure interaction: $F_{3.44} = 3.96$, $p < 0.05$) and this interaction was not observed in d,L-Gov-treated animals ($p > 0.05$). This was further substantiated by a highly significant interaction when only the PE animals were considered in the acute d-Amph and sensitized groups (background × treatment interaction: $F_{3.45} = 15.65$, $p < 0.001$). Post-hoc testing revealed a significant difference (Tukey’s post-hoc test, $p < 0.05$) between d-Amph acute Veh PE and d,L-Gov PE animals and a trend (Tukey’s post-hoc test, $p < 0.08$) between d-Amph-sensitized Veh PE and d,L-Gov PE animals. However, post-hoc testing found no difference between the d,L-Gov-treated PE animals given acute d-Amph or sensitized. It should be noted that in each of the groups tested, an inverse relationship was observed between avoidances and escapes (exposure × measure interaction: $F_{1.188} = 28.4$, $p < 0.001$), thus providing further evidence of intact motor performance in rats treated with either d,L-Gov or Clz.

**d,L-Gov improves performance of the delayed spatial win-shift task**

A significant main effect of treatment (three-way ANOVA, main effect of treatment: $F_{1.48} = 4.40$, $p < 0.04$) was observed, confirming that d,L-Gov improved the ability of rodents to use a memory-based foraging strategy. This effect was largely attributable to a significant increase in errors at the 12-h delay in non-sensitized animals that was blocked by treatment with d,L-Gov 30 min prior to the test phase (Fig. 6, left). However, d,L-Gov had no effect at the 30-min delay in non-sensitized animals. In the current study, sensitized animals made significantly fewer errors at a 12-h delay (three-way ANOVA, background × delay interaction: $F_{1.48} = 4.74$, $p < 0.05$; Fig. 6, right) than their non-sensitized counterparts indicating that sensitization improved cognitive
of atypical antipsychotics such as Clz, which evokes DA release in both regions, whereas the typical antipsychotic haloperidol more selectively evokes DA release in the NAc than the PFC (Moghaddam & Bunney, 1990). This compelling neuropharmacological profile motivated a detailed analysis of D_{1,L}-Gov’s effects on a battery of behavioural tests routinely used to screen for antipsychotic efficacy.

Although DA D_{2} antagonists are effective in treating positive symptoms of schizophrenia, their use is limited by side-effects such as tardive dyskinesia and catalepsy. D_{1,L}-Gov at a dose of 6 mg/kg induced strong catalepsy, whereas only transient bouts of inactivity are observed briefly with the lower doses tested (Fig. 4a, b). Accordingly, these lower, non-cataleptic doses are used for all subsequent experiments. Inhibition of AIL and CAR by all antipsychotics currently approved for clinical use justifies the utilization of these behavioural tests as preclinical screening tools (Arnt, 1982; Arnt & Skarsfeldt, 1998; Natesan et al. 2006; Wadenberg et al. 2000). Importantly, non-cataleptic doses of D_{1,L}-Gov suppress AIL, consistent with an ability to mitigate behavioural abnormalities arising from hyperdopaminergia, including the positive symptoms of schizophrenia. The CAR paradigm is a well-established screen for potential typical and atypical neuroleptic drugs, based on the selective inhibition of avoidance but not escape responses (Ader & Clink, 1957; Janssen & Niemegeers, 1961; Niemegeers & Janssen, 1965; Niemegeers et al. 1969; Wadenberg et al. 2000). D_{1,L}-Gov, at doses of 1.0 and 3.0 mg/kg, but not 0.3 mg/kg, suppress avoidance behaviour without affecting escape responses. The efficacy of D_{1,L}-Gov on measures of catalepsy and CAR is probably comparable to l-SPD evidenced by their similar ED_{50} values (Table 2, Natesan et al. 2008). Compared to Clz, both D_{1,L}-Gov and l-SPD exhibit a lower ED_{50} to suppress AIL and CAR, suggesting that both these compounds may provide an effective treatment option for the positive symptoms of schizophrenia.

LI refers to a phenomenon readily observed in both humans and animals where pre-exposure to a CS in the absence of a US impairs the subsequent ability to form an association between these two stimuli. Disrupted LI is commonly observed in schizotypal individuals (Braunstein-Bercovitz & Lubow, 1998; Della Casa et al. 1999) and subsets of schizophrenia patients (Baruch et al. 1988; Gray et al. 1992, 1995; Rascle et al. 2001; Vaitl & Lipp 1997). An inability to ignore irrelevant information or classify stimuli as insignificant is thought to underlie this disruption and is hypothesized to reflect attention deficits that are often
observed in schizophrenia (Gray & Snowden, 2005; Lubow, 2005). Similarly, disrupted LI is observed in animals following d-Amph treatment, as animals pre-exposed (PE) to the CS form an association between a CS and US as readily as animals non-pre-exposed (NPE) to the CS (Moser et al. 2000; Fig. 5b).

The neurobiological mechanisms that mediate LI in humans and rodents are thought to be similar (Dunn et al. 1993), therefore recommending its use in screening potential antipsychotic compounds and for examination of the neurobiology of antipsychotic action. In contrast to animals treated acutely with a d-Amph, we observed enhanced LI in d-Amph-sensitized animals (Fig. 5c). There is growing consensus that enhanced LI may model negative symptoms and cognitive deficits that are characteristic of schizophrenia (Bay-Richter et al. 2009; Bitanihirwe et al. 2011; Knapman et al. 2010; Murphy et al. 2001; Nelson et al. 2010; Weiner & Arad, 2009). Markedly, D1-L-Gov treatment reverses both the disruption and enhancement of LI. When given during CS pre-exposure, D1-L-Gov does not affect the expression of LI in saline-treated animals, indicating that neither the motivational salience of the CS nor the ability to form an association between the CS and US are affected by this drug (Li et al. 2009; Mead & Li, 2009). Moreover, the effect of D1-L-Gov on avoidance behaviour is restricted to rats in the PE group. This further suggests that D1-L-Gov does not affect conditioning, but rather has a selective effect on the neurocognitive processes necessary for the reclassification of previously insignificant stimuli that subsequently serves as a CS predictive of a motivationally relevant US.

A number of atypical and typical antipsychotics are differentiated by an ability to alter LI either at conditioning or pre-exposure (Shadach et al. 2000). In the present study, systemic administration of Clz or D1-L-Gov during CS pre-exposure blocked the increase in avoidance responses normally produced by acute d-Amph in animals pre-exposed to CS (Fig. 5b, black bars). Importantly, Clz also disrupted avoidance behaviour in NPE rats given acute d-Amph (Fig. 5b, white bars), suggesting a more general effect on the formation of a CS–US association. This general impairment was not observed with D1-L-Gov treatment suggesting that, relative to Clz, it may more selectively target the compromised attention processes that disrupt LI.

d-Amph-induced behavioural sensitization is purported to be a valid animal model of schizophrenia (Featherstone et al. 2007; Robinson & Becker, 1986). In the current study, enhanced LI was observed in sensitized animals. This is unlikely to reflect an inability to initiate appropriate motor responses as sensitized animals in the NPE condition acquired CAR at a rate comparable to control animals. An alternative explanation is to attribute enhanced LI to an inability to reclassify stimuli previously deemed to be inconsequential or to problems in forming an association with such stimuli. Enhanced LI is also observed in mouse lines bred to be hyper-reactive to stress and these same mice show reduced D1 receptor mRNA in the cingulate cortex plus reduced D2 mRNA in the ventral tegmental area (Knapman et al. 2010). The possibility remains that increased sensitivity to foot-shock-induced stress in sensitized animals (Barr & Phillips, 2002) may have impaired the CS–US association resulting in enhanced LI. Although further research is needed to clarify the underlying processes by which D1-L-Gov restores LI in this preclinical model of negative symptoms, this finding is particularly marked as this compound also restored LI following acute d-Amph treatment. Together, these data indicate that D1-L-Gov may have the unique ability to influence both positive and negative symptom poles of schizophrenia.

Cognitive deficits are a core feature of schizophrenia, and there is an urgent and unmet medical need for an effective pharmacotherapy to address this abnormality. Neuropsychological and brain-imaging studies of clinical populations diagnosed with schizophrenia reveal deficits in cognitive functions mediated by the frontal lobes, including executive function and working memory (Andreasen, 2000; Featherstone et al. 2007; Floresco et al. 2009; Goldman-Rakic, 1999). The delayed spatial win-shift task performed on an 8-arm radial maze employed here is a well-studied preclinical test of PFC-based executive memory processes (Packard & White, 1990; Seamans et al. 1998). Contrary to our initial hypothesis, d-Amph-sensitized rats are not impaired on this task, which is in agreement with recent studies demonstrating that sensitized animals may actually be more proficient at certain aspects of cognition (Ito & Canseliet, 2010). Although we failed to observe a cognitive impairment induced by sensitization, rats subjected to the same sensitization regimen do exhibit deficits in attention-based set shifting which can be ameliorated by local infusion of a D1 agonist into the PFC (Fletcher et al. 2005). Increases in PFC DA are observed in animals who perform the D5WSsh proficiently at a 30-min delay (Phillips et al. 2004) and local microinfusion of a D1 agonist into the PFC increases errors under these conditions (Floresco & Phillips, 2001). Under experimental conditions designed to impair performance on this task, such as imposing an unexpectedly long delay, the resulting
increase in errors were mitigated by local infusion of a D1 agonist (Floresco & Phillips, 2001). In the present study, an unexpected extension of the delay increased the number of errors in Veh-treated non-sensitized animals and this was significantly attenuated by D,L-Gov treatment prior to the recall test (Fig. 6). In contrast to previous findings with the specific DA D1 agonist SKF-38393, we did not observe a memory deficit when D,L-Gov was administered to rats performing optimally at a 30-min delay. Although these data are consistent with a pro-cognitive profile, functional binding studies do not confirm a direct DA D1 agonist action for D,L-Gov unlike L-SPD (Natesan et al. 2008). In-vitro functional binding data did not yield an increase in cAMP production expected of a direct DA D1 agonist, therefore, the pro-cognitive effects of D,L-Gov can only be mediated by an indirect agonist action at the DA D1 receptor or via affinity for other receptor classes.

Further study is required to understand how D,L-Gov improves these measures of cognition; however, the present behavioural findings coupled with the microdialysis data suggest that this compound may provide a viable therapeutic option capable of addressing a range of symptoms typically observed in schizophrenia. These data also add to a growing literature supporting further development of THPBs as a promising class of compounds that may provide novel treatment options for schizophrenia.

Acknowledgements

This work was supported by grants from CIHR, IRAP (A.G.P.), and a NARSAD young investigator award (C.C.L.). D,L-Gov was provided by Panora Pharmaceuticals.

Statement of Interest

A. G. Phillips is a director of Allon Pharmaceuticals and has received payment for serving on the board of this company. J. J. Miller is President of Panora Pharmaceuticals.

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