JPET Fast Forward. Published on August 31, 2018 as DOI: 10.1124/jpet.118.251116
This article has not been copyedited and formatted. The final version may differ from this version.

Title Page

Inhibitors of the neutral amino acid transporters ASCT1 and ASCT2 are effective in *in vivo* models of schizophrenia and visual dysfunction

Yong-Xin Li, Jia-Ying Yang, Miguel Alcantara, Grigor Abelian, Ashutosh Kulkarni, Ursula Staubli, Alan C Foster

Department of Biological Sciences, Allergan, Inc., 2525 Dupont Drive, Irvine, CA 92612, USA

Running Title Page:

Running Title: Effects of ASCT1/ASCT2 Transporter Inhibitors *in vivo*

Corresponding author:

Yong-Xin Li, Ph.D., Department of Biological Sciences, Allergan, Inc., 2525 Dupont Drive, Irvine, CA 92612, USA; Telephone: 714-246-6775; Email: li_yongxin@allergan.com

Co-Corresponding author: Alan C Foster, Ph.D.

Number of text pages: 34

Number of tables: 4

Number of figures: 6

Number of reference: 28

Number of words in the Abstract: 172

Number of words in the Introduction: 498

Number of words in the Discussion: 1286

Abbreviations: ASCT1, alanine/serine/cysteine transporter 1; ASCT2, Alanine/serine/cysteine transporter 2; CNS: central nervous system; IP, intraperitoneal; IV, intravenous; L-4ClPG, L-4-chlorophenylglycine; L-4FPG, L-4-fluorophenylglycine; L-4OHPG, L-4-hydroxyphenylglycine; LTP, long term potentiation; PG, phenylglycine; NMDA, N-methyl-D-aspartate; SC, subcutaneous; VEP, visual evoked potential

Recommended section: Neuropharmacology

Abstract

The NMDA receptor co-agonist D-serine is a substrate for the neutral amino acid transporters ASCT1 and ASCT2 which may regulate its extracellular levels in the CNS. We tested inhibitors of ASCT1 and ASCT2 for their effects in rodent models of schizophrenia and visual dysfunction which had previously been shown to be responsive to D-serine. L-4-fluorophenylglycine (L-4FPG), L-4-hydroxyPG (L-4OHPG) and L-4-chloroPG (L-4ClPG) all showed high plasma bioavailability when administered systemically to rats and mice. L-4FPG showed good brain penetration with brain:plasma ratios of 0.7-1.4, however values for L-4OHPG and L-4ClPG were lower. Systemically administered L-4FPG potently reduced amphetamine-induced hyperlocomotion in mice, whereas L-4OHPG was 100-fold less effective and L-4ClPG inactive at the doses tested. L-4FPG and L-4OHPG did not impair visual acuity in naïve rats, and acute systemic administration of L-4FPG significantly improved the deficit in contrast sensitivity in blue-light treated rats caused by retinal degeneration. The ability of L-4FPG to penetrate the brain makes this compound a useful tool to further evaluate the function of ASCT1 and ASCT2 transporters in the CNS.

Introduction

Hypofunction of the N-methyl-D-aspartate (NMDA) sub-type of glutamate receptors has been implicated in psychiatric and sensory disorders. The contribution of NMDA receptor hypofunction to schizophrenia was hypothesized initially on the schizophrenia-like state in otherwise normal human subjects induced by NMDA receptor antagonists such as ketamine and phencyclidine (Javitt et al., 2012; Balu, 2016). Subsequently, administration of the NMDA receptor co-agonist D-serine was shown to relieve schizophrenic symptoms in patients when co-administered with antipsychotics (Heresco-Levy et al., 2005; Kantrowitz et al., 2010), presumably by restoring NMDA receptor tone. In the visual system, NMDA receptors are important for responses along the visual pathway (Hartveit and Hegglund, 1990; Scharfman et al., 1990; Simon et al., 1992; Stevens et al., 2003), and we have shown previously that administration of D-serine can restore visual performance impaired by retinal degeneration (Staubli et al., 2016). Consequently, therapies based on restoring NMDA receptor tone may be effective in relieving schizophrenia symptoms and restoring visual function after retinal degeneration.

Although D-serine is a potent and effective co-agonist of the NMDA receptor (Kleckner and Dingledine, 1988; Matsui et al., 1995), large doses are required to produce central effects after systemic administration due to poor blood-brain barrier permeability of this polar amino acid (Smith et al., 2009). In addition, high systemic levels of D-serine pose the risk of renal toxicity (Krug et al., 2007; Kantrowitz et al., 2010). We have previously shown that D-serine is transported by the neutral amino acid transporters, ASCT1 (SLC1A4) and ASCT2 (SLC1A5; Foster et al., 2016) and that inhibition of these

transporters can potentiate NMDA receptor-mediated neuronal plasticity in a D-serine-dependent manner (Foster et al., 2017). This suggests that these predominantly glial transporters may play an important role in regulating extracellular D-serine in the brain and thereby NMDA receptor function. Another approach has been to target the high affinity transporters for the alternative endogenous NMDA receptor co-agonist glycine. However, despite promising preclinical data inhibitors of GlyT1 have not shown clinically meaningful benefits to date in schizophrenia patients (Singer et al, 2015; Bugarski-Kirola et al, 2016).

We previously identified phenylglycine (PG) analogs that are inhibitors of ASCT1 and ASCT2 (Foster et al., 2017) and here we tested their effects in animal models of schizophrenia and visual dysfunction to evaluate if systemically-administered inhibitors of these transporters had efficacy in these models of NMDA receptor hypofunction. The compounds evaluated were L-4-fluoroPG (L-4FPG) and L-4-chloroPG (L-4ClPG) which are relatively potent and selective ASCT1/2 inhibitors, and L-4-hydroxyPG (L-4OHPG) which additionally inhibits asc-1 (SLC7A10) (Foster et al., 2017), and has previously been tested in humans (Bergman et al., 1980). We previously showed that L-4FPG, L-4OHPG and L-4ClPG have IC_{50}'s for inhibition of astrocyte-mediated ASCT1 and ASCT2 transport of 44, 283 and 25 μM, respectively (Foster et al, 2017). They were not active in a range of ionotropic and metabotropic glutamate receptor assays and L-4ClPG and L-4FPG were tested and found to be inactive in a broader selectivity screen including 137 receptor, enzyme and transporter recognition sites (Foster et al, 2017).

Materials and Methods

Animals

All experiments were approved by the Allergan IACUC and carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Materials

L-4FPG and L-4OHPG were from Sigma-Aldrich and L-4ClPG was from Ark Pharm Inc. All other laboratory agents were purchased from Sigma-Aldrich.

Pharmacokinetic studies

Pharmacokinetic studies in rat for L-4FPG and L-4OHPG were performed by Allergan Pharmacokinetics Group. Medicilon Inc. performed studies in mice for L-4FPG, L-4OHPG, and L-4ClPG.

Subcutaneous (SC) administration of L-4FPG, L-4OHPG, L-4ClPG to mice: All compounds for SC administration were prepared for SC injection by dissolving them in water to yield a nominal concentration of 3 and 0.3 mg/mL (free form, pH=7) for L-4OHPG, 0.3 and 0.03 mg/mL for L-4FPG (free form, pH=7), and 60 mg/mL and 30 mg/mL (free form, pH=7) for L-4ClPG. A total of 108 male C75BL/6 mice from Shangai Laboratory Animal Center were SC administered a bolus dose of 10 mL/kg of varying concentrations ranging from 0.03 mg/mL to 60 mg/mL based on the compound. Plasma and brain tissue were subsequently collected and analyzed. Blood samples were collected at 15 min, 30 min, 1 h, 2 h, 4 h, and 6 h postdose. Following centrifugation, the resulting

plasma samples were transferred to clean tubes and stored frozen at -80°C pending bioanalysis. After the blood collection, the brain of each animal was collected at 0.5 and 6 h post dose. Prior to brain collection, a perfusion with ice cold normal saline was performed from the left ventricle. After the perfusion, the brains were harvested, rinsed with saline, dried with filter paper, and placed per animal into a tube and frozen in dry ice then stored at -80 °C until bioanalysis. The concentrations of both compounds in matrix were determined using a high-performance liquid chromatography/mass spectrometry (HPLC/MS/MS) method. Individual and mean plasma and brain concentrations were reported and WinNonlin® Professional 5.2. was used to calculate pharmacokinetic parameters ($AUC_{(0-t)}$, $AUC_{(0-\infty)}$, $MRT_{(0-\infty)}$, $T_{1/2}$, T_{max}, C_{max}).

Administration of L-4FPG and L-4OHPG to rat: Both compounds were prepared for SC, intraperitoneal (IP), and intravenous (IV) injection by dissolving them in deionized water to yield a nominal concentration of 2.5 mg/mL. A total of 18 pre-cannulated fasted male Sprague-Dawley rats per compound were dosed with a bolus injection of 10mg/kg by IV, IP, or SC for L-4FPG and 10mg/kg by IV or 30 mg/kg by SC or IP for L-4OHPG. Plasma and brain tissues were subsequently collected and analyzed. Blood samples following IV dosing were collected at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, and 8 h post dose. Blood samples following SC and IP dosing were collected at 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, and 8 h post dose. Approximately 0.3 mL of blood at each time point was collected via the femoral cannula and placed into a tube with K3EDTA. The blood was centrifuged to separate plasma and harvested into a 96-well transfer plate. Brain samples were collected at 30 minutes post dose. Prior to brain collection, each rat was terminated

via exsanguination under anesthesia. Brain was collected individually and weight was recorded. The concentrations of both compounds in matrix were determined using a HPLC/MS/MS. Individual and mean plasma and brain concentrations were reported and WinNonlin® Professional 5.2. was used to calculate pharmacokinetic parameters ($T_{1/2}$, CL, V_{ss}, and $AUC_{0\text{-}inf}$, were calculated for the IV dose group and C_{max}, T_{max}, $T_{1/2app}$, and %F for the SC and IP dose groups).

Amphetamine-induced hyperlocomotion

C57B/6 male mice (n=5-10 per group) were placed in an open field apparatus (PAS-Open Field, San Diego Instruments) and their activity was measured for 30 minutes. At 30 minutes, they received a vehicle injection (0.9% NaCl) or injection of test compound (SC) followed by an injection of amphetamine (2 mg/kg, SC) at minute 50. Their activity levels were subsequently measured for another 2 hours following amphetamine injection. Total activity over the period from 50 to 120 min was used to establish an activity curve. Pre-amphetamine activity was measured over the period from 30 to 50 min. When testing D-serine or the phenylglycine analogs, vehicle-injected animals were aways run concurrently.

Rats sweep VEP recording

The methods were described in a previous paper (Staubli, Rangel-Diaz et al., 2016). The recording electrodes were permanently implanted into the right visual cortex of Long Evans rats at lambda and 4.5 mm lateral to the midline, to a depth of 800 microns (layer III/IV). A reference electrode was placed epidurally on the midline 1.2 mm anterior to

bregma. All recordings were conducted in awake rats starting at least two weeks after recovery from surgery. PowerDiva software from Anthony Norcia (Smith Kettlewell Institute of Visual Sciences) was used for data acquisition and analysis. Visual stimuli were presented on a CRT computer monitor and consisted of full-field horizontally-oriented sine-wave gratings at 80% contrast, reversing at 6.25 Hz. VEPs were elicited by horizontally oriented gratings. The display was positioned 24 cm in front of the rat and centered at the vertical meridian. Mean luminance was held constant at 20 candelas (cd). To determine visual acuity thresholds, the sweep consisted of 15 spatial frequencies increasing from 0.03 to 1.8 cycles of grating per degree of visual angle (cpd) in 15 linear steps at 80% contrast. To determine contrast thresholds in blue light treated rats, a trial consisted of a contrast sweep increasing from 2.5% to 70% in 15 logarithmic steps, with the spatial frequency kept constant at pre-determined values, typically at 0.575 cpd. The sweeps were averaged until the signal to noise ratio was at least 3 or above. Contrast sensitivity was calculated as the inverse of the contrast threshold.

Blue-light treated rats: Young adult Long Evans rats previously implanted with sweep VEP electrodes were used. They were dark-adapted for twenty-four hours prior to exposing them to blue light consisting of 6000–8000 lx (~460 nm) for 8 h. A subset of animals served as littermate controls and were returned to regular room light following dark adaptation. During the blue light exposure, all animals had access to gel food (blue light) or food and water ad libitum (room light). After blue light/room light exposure, the animals were again dark adapted for 72 h.

Statistical Analysis

Statistical analyses were performed using a one-way ANOVA with Dunnett's test for pairwise comparisons or with Student's t-test.

Results

Pharmacokinetic studies with PG analogs

The pharmacokinetics of PG analogs were examined in mice and rats, the species used for evaluation of the effects of these compounds in models of schizophrenia and visual function. In addition to the assessment of bioavailability, a key question for these polar amino acid analogs was whether they could cross the blood-brain barrier sufficiently to influence nervous system function.

In rats, IV administration of L-4FPG indicated a relatively short plasma half-life (< 1 hr.), and rapid clearance; in comparison, the half-life of L-4OHPG was longer, with slower clearance and as a resulting plasma AUC that was greater than that of L-4FPG (Figure 1; Table 1). Administration to rats by the SC route showed high plasma bioavailability with F values for both L-4FPG and L-4OHPG close to 1 (Figure 1; Table 2). Administration of either compound by the IP route also showed high bioavailability (F values >0.8; Table 2). Measurement of brain levels 30 min after IV administration at 10 mg/kg indicated approx. 6μg/g for L-4FPG (Figure 1) with a brain to plasma ratio of 1.45; for L-4OHPG, lower brain levels (approx. 2μg/g, Figure 1) and brain to plasma ratio (0.36) was observed (Table 3). At 8 h post-dose, both compounds showed low brain levels (Figure 1). Brain to plasma ratios measured after SC or IP administration in the rat also showed good brain penetration for L-4FPG, but lower values for L-4OHPG (Table 3).

In mice, SC administration of L-4FPG and L-4OHPG gave half-life and C_{max} values for the two compounds that were consistent with the rat pharmacokinetics, and resulted in a high plasma bioavailability via this route (data not shown). Measurement of brain levels 30 min after SC administration in mice again indicated good brain exposure with a brain to plasma ratio of 0.73 for L-4FPG, and a lower value of 0.26 for L-4OHPG (Table 4). L-4ClPG was tested in mice by the IP route and found to have pharmacokinetic parameters similar to those for L-4FPG SC including high plasma bioavailability (data not shown); however, the brain to plasma ratio for L-4ClPG was low (0.13), compared with L-4FPG (Table 4). These data indicate a high bioavailability by the IP or SC routes in rats and mice for the PG analogs and a more substantial brain penetration for L-4FPG compared to the other compounds. For L-4OHPG brain penetration was more limited but substantial brain exposure could be achieved by increasing the dose. By contrast, L-4ClPG had poor brain penetration. Consequently, the pharmacokinetic studies confirmed that single systemic doses of PG analogs were adequate for evaluation of their effects in rodent models of schizophrenia and visual function where effects were monitored up to 2 h post-dose.

Effects of PG analogs in amphetamine-induced hyperlocomotion

In clinical studies, D-serine has been shown to be effective in relieving the symptoms of schizophrenia (Kantrowitz et al., 2010). The PG analogs were tested in a mouse model of schizophrenia, where amphetamine induces hyperlocomotion. First, D-serine was tested by SC administration. Mice were allowed to habituate in the activity chamber for 30 min, at which time D-serine was injected SC followed 20 min later by amphetamine at 2

mg/kg SC and activity monitored for an additional 120 mins. As shown in Figure 2, hyperlocomotion induced by amphetamine was reduced in a dose-dependent manner by D-serine (overall treatment effect was significant by one-way ANOVA, $F(4,51) = 2.944$; $p = 0.029$); a post-hoc Dunnett's test indicated that this effect was significant at the highest dose ($p = 0.007$). The high doses of D-serine required may reflect its low brain penetration and are similar to those reported previously in other *in vivo* rodent paradigms (Smith et al., 2009). To control for the possibility that D-serine may suppress locomotor activity in the absence of amphetamine, activity counts were summed during the 30-50 min period following D-serine dosing and prior to amphetamine administration. As shown in Figure 2, D-serine suppressed locomotor activity during this period (overall treatment effect was significant by one-way ANOVA, $F(4,51) = 3.988$; $p = 0.007$) and a post-hoc Dunnett's test indicated that this effect was significant at the highest dose ($p = 0.001$). This suggests that some, if not all, of the effect of D-serine on amphetamine-induced locomotion may be a non-specific effect of D-serine to suppress locomotor activity. This highlights the difficulty in interpreting data with a compound like D-serine that has poor brain bioavailability, requiring high systemic exposure that may result in confounding non-specific effects.

Using the same treatment paradigm, L-4FPG and L-4OHPG were tested for their effects on amphetamine-induced hyperlocomotion. For L-4FPG (Figure 3), a dose-dependent reduction was observed (overall treatment effect was significant by one-way ANOVA, $F(4,34) = 3.07$; $p = 0.03$) with a lowest effective dose for L-4FPG of 0.3 mg/kg (post-hoc Dunnett's test, $p = 0.023$); at 3 mg/kg the effect was not significant indicating a U-shaped

dose-response relationship exists. Unlike D-serine, there was no effect of L-4FPG on the pre-amphetamine locomotor activity (overall treatment effect was non-significant by one-way ANOVA, $F(4,34) = 0.753$; $p = 0.563$; Figure 3). Similarly, L-4OHPG (Figure 4) showed a dose-dependent reduction of amphetamine-induced locomotor activity (overall treatment effect was significant by one-way ANOVA, $F(2,35) = 3.30$; $p = 0.049$) with a lowest effective dose for L-4OHPG of 30 mg/kg (post-hoc Dunnett's test, $p = 0.032$). There was no effect of L-4OHPG on the pre-amphetamine locomotor activity (overall treatment effect was non-significant by one-way ANOVA, $F(2,35) = 0.790$; $p = 0.462$; Figure 4). L-4ClPG was tested at 0.3 and 1 mg/kg in the amphetamine model, the dose range where L-4FPG was shown to be effective. As shown in Figure 5, there was no significant effect (unpaired, 2-tailed Student's t-test) of L-4ClPG at 0.3 or 1 mg/kg on amphetamine-induced or pre-amphetamine locomotor activity.

Effects of PG analogs in models of visual function

Since we have previously shown that systemically-applied D-serine can have beneficial effects on visual function (Staubli et al., 2016), L-4FPG and L-4OHPG were tested in naïve rats for their ability to influence normal vision (Figure 6). Compared with vehicle, L-4FPG (10 mg/kg IP) produced a non-significant trend to improve visual acuity (2-tailed paired Student's t-test, vehicle: $p = 0.973$; L-4FPG: $p = 0.199$). L-4OHPG produced a small but significant improvement in visual acuity (2-tailed paired Student's t-test, vehicle: $p = 0.566$; L-4OHPG: $p = 0.011$). These data indicate that, at the doses tested, the PG analogs do not impair but may enhance visual acuity in naïve rats.

We have previously reported that rats exposed to blue light have long-lasting and stable deficits in contrast sensitivity that can be acutely improved by systemic administration of D-serine (Staubli et al., 2016). In the current experiments, a group of rats were used 53 weeks after blue light exposure that had a stable contrast sensitivity deficit of approximately 6 (Figure 6C); values for rats with intact vision under these experimental conditions are approximately 10 (Staubli et al., 2016). Administration of a single dose of L-4FPG (10 mg/kg, IP) 53 weeks after blue light exposure when a stable visual deficit was present produced an acute improvement in contrast sensitivity measured 30 min post-dose (2-tailed paired Student's t-test, vehicle: $p = 0.832$; L-4FPG: $p = 0.039$; Figure 6C). These data suggest that inhibition of ASCT1/2 in the visual system can acutely ameliorate the loss of visual function that results from retinal degeneration.

Discussion

The data presented here provide evidence that systemically-administered inhibitors of ASCT1 and ASCT2 have effects in the brain and can ameliorate the NMDA receptor hypofunctional state that occurs in animal models of schizophrenia and visual dysfunction. Further optimization of PG analogs or other chemotypes reported to inhibit ASCT1 or ASCT2, such as benzylserines (Grewer and Grabsch, 2004), glutamine analogs (Esslinger et al., 2005; Schulte et al., 2015) and hydroxyprolines (Farnsworth et al., 2015) may lead to improved molecules with considerable therapeutic potential.

A concern at the outset of these studies was whether the PG analogs, which have an alpha-amino acid structure and are polar in nature, would penetrate the blood-brain barrier to a degree that would allow them to be active *in vivo* after systemic administration. Pharmacokinetic studies in rats and mice indicated high bioavailability by IP or SC routes, with half-lives that allowed evaluation of compound effects by single acute administration in the animal models. Surprisingly, L-4FPG showed good blood-brain barrier penetration, with brain to plasma ratios ranging from 0.7-1.4 in the rat and 0.7-0.9 in the mouse. L-4OHPG and L-4ClPG had lower values. The 4-hydroxy moiety of L-4OHPG makes this the most polar of the three analogs, however based on lipophilicity, L-4ClPG would be expected to have the best blood-brain barrier penetration (logP values calculated from the SwissADME website (http://www.swissadme.ch/index.php) are L-4FPG = 0.09; L-4ClPG = 0.32; L-4OHPG = -0.65). Consequently, the favorable brain penetration of L-4FPG is likely due to factors

other than lipophilicity. One intriguing possibility is that this molecule uses an amino acid transport system to cross the blood-brain barrier. A strong candidate is system L, a transporter for neutral amino acids present at the blood-brain barrier that is responsible for transporting several neuroactive amino acids including L-DOPA and gabapentin (Kageyama et al., 2000; Dickens et al., 2013). PG analogs are substrates for system L (Reichel et al., 2000), and we previously showed that L-4FPG interacted with system L in astrocytes, whereas L-4OHPG and L-4ClPG were less active (Foster et al., 2017). The IC_{50} values of 36, 233 and 500 μM for L-4FPG, L-4OHPG and L-4ClPG respectively for system L (Foster et al., 2017) correspond to the relative extent of brain penetration for these compounds, suggesting that system L may be an important factor in their blood-brain barrier permeability.

D-serine has been shown to alleviate symptoms in schizophrenic patients (Kantrowitz et al., 2010) and has effects in preclinical animal models that may be predictive of clinical utility for schizophrenic symptoms (Smith et al., 2009; Contreras, 1990; Lipina et al., 2009). In addition, knock-out of D-amino acid oxidase, the degradative enzyme for D-serine, results in elevated brain D-serine and an antipsychotic profile in mice (Hashimoto, et al., 2008). In rats, Smith et al. (2009) showed that a high dose of D-serine (1280 mg/kg) attenuated amphetamine-induced hyperlocomotion without effect on habituation in the absence of amphetamine. Our studies in mice showed a similar effect of high dose D-serine (1200 mg/kg) on amphetamine-induced hyperlocomotion, however this was accompanied by a reduction in spontaneous locomotor activity. This highlights a potential species difference in the effects of D-serine on spontaneous locomotor activity,

but also raises the possibility that the effects of D-serine on amphetamine-induced locomotor activity were non-specific and not related to central NMDA receptor activity. High systemic doses of D-serine are required to elevate central D-serine (Smith et al., 2009) because of the poor blood-brain barrier penetration of this polar amino acid. The good brain penetration of L-4FPG allowed this compound to be tested at relatively low doses in the mouse model. Indeed, L-4FPG showed a dose dependent and potent effect on amphetamine-induced locomotor activity, with an optimal dose of 0.3 mg/kg. This was not accompanied by any change in spontaneous locomotor activity. As the dose was increased to 3mg/kg the effect was reduced; the reason for this is unclear. L-4OHPG also attenuated amphetamine-induced locomotor activity without an effect on spontaneous locomotor activity, but required a 100 times higher dose than L-4FPG. This may be due to reduced brain penetration combined with a weaker effect on ASCT1/2 transport (see below). At the same dose range where L-4FPG was effective, L-4ClPG (0.3 and 1 mg/kg) had no effect on amphetamine-induced or spontaneous locomotor activity. Since L-4ClPG is equally effective with L-4FPG as an inhibitor of ASCT1/2, this suggests that the poor blood-brain barrier penetration of L-4ClPG (possibly reflecting that it is a poor substrate for system L, see above) did not allow it to show activity at the doses tested. Overall, the effects of the PG analogs in the mouse locomotor activity model support the idea that L-4FPG has central effects when applied systemically consistent with its blood-brain barrier penetration and inhibition of ASCT1/2 transport.

The brain levels of L-4FPG and L-4OHPG in mice (measured at 30 min post-dose) required to produce a significant reduction of amphetamine-induced locomotion were 90

ng/mL (at 0.3 mg/kg L-4FPG) and 4520 ng/mL (at 30 mg/kg L-4OHPG) (Table 4). These equate to brain concentrations of approx. 0.5 and 30 μM, respectively. We have previously shown that L-4FPG and L-4OHPG enhance LTP in rat visual cortex slices in a dose- and D-serine-dependent manner (Foster et al., 2017). In this paradigm, L-4FPG significantly increased LTP at 1 μM with a trend at 0.3 μM, and L-4OHPG significantly elevated LTP at 100 μM with a trend at 10 μM, reflecting their differing affinities for the ASCT1/2 transporters. Since the brain levels that were active in the amphetamine-induced locomotor activity assay are close to the concentrations of L-4FPG and L-4OHPG required to enhance NMDA receptor-dependent neuronal plasticity, it suggests that inhibition of ASCT1/2 and subsequent enhancement of extracellular D-serine to enhance NMDA receptor activity is a plausible mechanism by which these compounds were effective in this *in vivo* model. For L-4ClPG, the lowest concentration producing a significant effect on LTP was 1μM (Foster et al., 2017). Extrapolating from the brain levels in mice achieved after IP injection of 10mg/kg L-4ClPG (Table 4), the brain levels at the doses tested in the mouse amphetamine model are approximately 0.35 μM at 1mg/kg and 0.11 μM at 0.3 mg/kg. Consequently, it appears that the brain levels achieved with the tested doses of L-4ClPG may be too low to inhibit ASCT1/2 sufficiently to produce an effect *in vivo*.

Our previous data with systemically-administered D-serine provided evidence for an acute restoration of visual performance in animals with impaired vision resulting from retinal insults (Staubli et al., 2016). As an initial step in testing PG analogs, L-4FPG and L-4OHPG were tested with IP administration for effects on visual acuity in naïve rats.

Neither compound impaired vision and L-4FPG showed a trend towards improvement whereas L-4OHPG significantly improved visual acuity (Figure 6). Subsequently, L-4FPG was tested in rats with retinal impairment that have a long-term stable deficit in contrast sensitivity. A single dose of 10 mg/kg IP showed a significant improvement in contrast sensitivity measured 30 min post-dose (Figure 6). These data suggest that L-4FPG has the potential to improve visual function following retinal impairment at a dose that does not affect normal vision. This effect could be mediated through enhancement of NMDA receptor function in central visual pathways or could be mediated at the level of the retina, where D-serine also has beneficial effects (Staubli et al., 2016).

In conclusion, we have provided evidence that PG analogs that are inhibitors of the neutral amino acid transporters ASCT1 and ASCT2 have central effects when administered systemically in animal models of schizophrenia and visual dysfunction. In particular, L-4FPG has good brain penetration which may be due transport across the blood-brain barrier by system L. Compounds like L-4FPG or analogs with improved properties may have therapeutic potential to treat disorders of NMDA receptor hypofunction.

Authorship contributions

Participated in research design: Li, Kulkarni, Staubli, Foster

Conducted experiments: Li, Yang, Alcantara, Kulkarni

Performed data analysis: Li, Yang, Alcantara, Abelian, Kulkarni, Staubli, Foster

Wrote or contributed to the writing of the manuscript: Li, Abelian, Kulkarni, Staubli, Foster

References

Balu DT (2016) The NMDA Receptor and Schizophrenia: From Pathophysiology to Treatment. *Adv Pharmacol* **76**: 351–382.

Bergman G, Atkinson L, Metcalfe J, Jackson N, and Jewitt DE (1980) Beneficial effect of enhanced myocardial carbohydrate utilisation after oxfenicine (L-hydroxyphenylglycine) in angina pectoris. *Eur Heart J* **1**:247-253.

Bugarski-Kirola D, Iwata N, Sameljak S, Reid C, Blaettler T, Millar L, Marques TR, Garibaldi G and Kapur S (2016) Efficacy and safety of adjunctive bitopertin versus placebo in patients with suboptimally controlled symptoms of schizophrenia treated with antipsychotics: results from three phase 3, randomized, double-blind, parallel-group, placebo-controlled, mutlicentre studies in the SearchLyte clinical trial programme. *Lancet Psychiatry* **3**:1115-1128.

Contreras PC (1990) D-serine antagonized phencyclidine- and MK-801-induced stereotyped behavior and ataxia. *Neuropharmacology* **29**:291–293.

Dickens D, Webb SD, Antonyuk S, Giannoudis A, Owen A, Rädisch S, Hasnain SS, and Pirmohamed M (2013) Transport of gabapentin by LAT1 (SLC7A5). *Biochem Pharmacol* **85**:1672-1683.

Esslinger CS, Cybulski KA, and Rhoderick JF (2005) Ngamma-aryl glutamine analogues as probes of the ASCT2 neutral amino acid transporter binding site. *Bioorg Med Chem* **13**:1111-1118.

Farnsworth JC, Lind GE, Lyda BR, Natale NR, and Kavanaugh MP (2015) SLC1A4 and SLC1A5 mediate transport of D-serine in brain. *Soc For Neurosci Abs* 571.03/B25.

Foster AC, Farnsworth J, Lind GE, Li YX, Yang JY, Dang V, Penjwini M, Viswanath V, Staubli U, and Kavanaugh MP (2016) D-serine is a substrate for neutral amino acid transporters ASCT1/SLC1A4 and ASCT2/SLC1A5, and is transported by both subtypes in rat hippocampal astrocyte cultures. *PLoS One* **11**(6):e0156551.

Foster AC, Rangel-Diaz N, Staubli U, Yang JY, Penjwini M, Viswanath V, and Li YX (2017) Phenylglycine analogs are inhibitors of the neutral amino acid transporters ASCT1 and ASCT2 and enhance NMDA receptor-mediated LTP in rat visual cortex slices. *Neuropharmacology* **126**:70-83.

Grewer C, and Grabsch E (2004) New inhibitors for the neutral amino acid transporter ASCT2 reveal its Na+-dependent anion leak. *J Physio* **557**:747-759.

Hartveit E, and Heggelund P (1990) Neurotransmitter receptors mediating excitatory input to cells in the cat lateral geniculate nucleus. II. Nonlagged cells. *J Neurophysiol* **63**:1361-1372.

Hashimoto A, Konno R, Yano H, Yoshikawa M, Tamaki R, Matsumoto H and Kobayashi H (2008) Mice lacking d-amino acid oxidase activity exhibit marked reduction of methamphetamine-induced stereotypy. *Eur J Pharmacol* **586**:221-225.

Heresco-Levy U, Javitt DC, Ebstein R, Vass A, Lichtenberg P, Bar G, Catinari S, and Ermilov M (2005) D-serine efficacy as add-on pharmacotherapy to risperidone and olanzapine for treatment-refractory schizophrenia. *Biol Psychiatry* **57**:577-85.

Javitt DC, Zukin SR, Heresco-Levy U, and Umbricht D (2012) Has an angel shown the way? Etiological and therapeutic implications of the PCP/NMDA model of schizophrenia. *Schizophr Bull* **38**:958-966.

Kageyama T, Nakamura M, Matsuo A, Yamasaki Y, Takakura Y, Hashida M, Kanai Y, Naito M, Tsuruo T, Minato N, and Shimohama S (2000) The 4F2hc/LAT1 complex transports L-DOPA across the blood-brain barrier. *Brain Res* **879**:115-121.

Kantrowitz J, Malhotra AK, Cornblatt B, Silipo G, Balla A, Suckow RF, D'Souza C, Saksa J, Woods SW, and Javitt DC (2010) High dose D-serine in the treatment of schizophrenia. *Schizophr Res* **121**:125-30.

Kleckner NW, and Dingledine R (1988) Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. *Science* **241**:835-837.

Krug AW, Völker K, Dantzler WH, and Silbernagl S (2007) Why is D-serine nephrotoxic and alpha-aminoisobutyric acid protective? *Am J Physiol Renal Physiol* **293**:F382-F390.

Lipina T, Labrie V, Weiner I, and Roder J (2005) Modulators of the glycine site on NMDA receptors, D-serine and ALX 5407, display similar beneficial effects to clozapine in mouse models of schizophrenia. *Psychopharmacology (Berl)* **179**:54–67.

Matsui T, Sekiguchi M, Hashimoto A, Tomita U, Nishikawa T, and Wada K (1995) Functional comparison of D-serine and glycine in rodents: the effect on cloned NMDA receptors and the extracellular concentration. *J Neurochem* **65**:454 –58.

Reichel A, Begley D, and Abbott NJ (2000) Carrier-mediated delivery of metabotropic glutamate receptor ligands to the central nervous system: structural tolerance and potential of the L-system amino acid transporter at the blood-brain barrier. *J Cereb Blood Flow Metab* **20**:168-174.

Scharfman HE, Lu SM, Guido W, Adams PR, and Sherman SM (1990) N-methyl-D-aspartate receptors contribute to excitatory postsynaptic potentials of cat lateral geniculate neurons recorded in thalamic slices. *Proc Natl Acad Sci USA* **87**:4548-4552.

Schulte ML, Dawson ES, Saleh SA, Cuthbertson ML, and Manning HC (2015) 2-Substituted Nγ-glutamylanilides as novel probes of ASCT2 with improved potency. *Bioorg Med Chem Lett* **25**:113-116.

Simon DK, Prusky GT, O'Leary DD, and Constantine-Paton M (1992) N-methyl-D-aspartate receptor antagonists disrupt the formation of a mammalian neural map. *Proc Natl Acad Sci USA* **89**:10593-10597.

Singer P, Dubroqua S and Yee BK (2015) The yellow brick road to new schizophrenia therapy. *Curr Pharm Des* **21**:3771-3787.

Smith SM, Uslaner JM, Yao L, Mullins CM, Surles NO, Huszar SL, McNaughton CH, Pascarella DM, Kandebo M, Hinchliffe RM, Sparey T, Brandon NJ, Jones B, Venkatraman S, Young MB, Sachs N, Jacobson MA, and Hutson PH (2009) The behavioral and neurochemical effects of a novel D-amino acid oxidase inhibitor compound 8 [4H-thieno [3,2-b]pyrrole-5-carboxylic acid] and D-serine. *J Pharmacol Exp Ther* **328**:921–930.

Staubli U, Rangel-Diaz N, Alcantara M, Li YX, Yang JY, Zhang KM, and Foster AC (2016) Restoration of visual performance by d-serine in models of inner and outer retinal dysfunction assessed using sweep VEP measurements in the conscious rat and rabbit. *Vision Res* **127**:35-48.

Stevens ER, Esguerra M, Kim PM, Newman EA, Snyder SH, Zahs KR, Miller RF (2003) D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. *Proc Natl Acad Sci USA* **100**:6789-6794.

Footnotes

Li, Yang, Alcantara, Abelian, Kulkarni, Staubli and Foster are employees of Allergan, Inc. The authors declare that they have no conflicts of interest.

Figure Legends

Figure 1: Pharmacokinetics of L-4FPG and L-4OHPG in the rat following IV or SC administration. Plasma concentration-time profile of L-4FPG (A) and L-4OHPG (B) and brain concentration-time profile of L-4FPG (C) and L-4OHPG (D). PG analogs were administered at the indicated dose and route and plasma and brain samples taken at various time points for measurement of drug levels. Values are plasma or brain drug concentration and are the mean ± SD of 3 animals per group.

Figure 2: Effects of SC D-serine on spontaneous and amphetamine-induced locomotor activity in mice. Animals were placed in the activity monitor and injected with D-serine or vehicle SC at 30 min followed by amphetamine (2 mg/kg SC) at 50 min. and activity monitored for an additional 120 min. (A) Activity counts for all groups over time; (B) Total locomotor activity for vehicle and D-serine at 100, 300, 600 and 1200 mg/kg SC after amphetamine administration (50-120 min). Values are mean ± SEM of the number of animals in parentheses. (C) Total locomotor activity for vehicle and D-serine at 100, 300, 600 and 1200 mg/kg SC prior to amphetamine administration (30-50 min). Values are mean ± SEM of the number of animals in parentheses. P values are from post-hoc Dunnett's test.

Figure 3: Effects of SC L-4FPG on spontaneous and amphetamine-induced locomotor activity in mice. Animals were placed in the activity monitor and injected with L-4FPG or vehicle SC at 30 min followed by amphetamine (2 mg/kg SC) at 50 min.

and activity monitored for an additional 120 min. (A) Activity counts for all groups over time; (B) Total locomotor activity for vehicle and L-4FPG at 0.03, 0.1, 0.3 and 3 mg/kg SC after amphetamine administration (50-120 min). Values are mean ± SEM of the number of animals in parentheses. (C) Total locomotor activity for vehicle and L-4FPG at 0.03, 0.1, 0.3 and 3 mg/kg SC prior to amphetamine administration (30-50 min). Values are mean ± SEM of the number of animals in parentheses. P values are from post-hoc Dunnett's test.

Figure 4: Effects of SC L-4OHPG on spontaneous and amphetamine-induced locomotor activity in mice. Animals were placed in the activity monitor and injected with L-4OHPG or vehicle SC at 30 min followed by amphetamine (2 mg/kg SC) at 50 min. and activity monitored for an additional 120 min. (A) Activity counts for all groups over time; (B) Total locomotor activity for vehicle and L-4OHPG at 3 and 30 mg/kg SC after amphetamine administration (50-120 min). Values are mean ± SEM of the number of animals in parentheses. (C) Total locomotor activity for vehicle and L-4OHPG at 3 and 30 mg/kg SC prior to amphetamine administration (30-50 min). Values are mean ± SEM of the number of animals in parentheses. P values are from post-hoc Dunnett's test.

Figure 5: Effects of SC L-4ClPG on spontaneous and amphetamine-induced locomotor activity in mice. Animals were placed in the activity monitor and injected with L-4ClPG or vehicle SC at 30 min followed by amphetamine (2 mg/kg SC) at 50 min. and activity monitored for an additional 120 min. Two independent experiments were conducted as shown in A and B. (A) Upper panel: total locomotor activity for

vehicle and L-4ClPG at 0.3 mg/kg SC after amphetamine administration (50-120 min); lower panel: total locomotor activity for vehicle and L-4ClPG at 0.3 mg/kg SC prior to amphetamine administration (30-50 min). (B) Upper panel: total locomotor activity for vehicle and L-4ClPG at 1 mg/kg SC after amphetamine administration (50-120 min); lower panel: total locomotor activity for vehicle and L-4ClPG at 1 mg/kg SC prior to amphetamine administration (30-50 min). Values are mean ± SEM of the number of animals in parentheses. P values are from unpaired 2-tailed t-test.

Figure 6: Effects of L-4FPG and L-4OHPG on visual function in rats. (A) Visual acuity (cpd) was measured at baseline and then 30 min after administration of vehicle or L-4FPG at 10 mg/kg, IP or (B) vehicle or L-4OHPG at 30 mg/kg, IP. (C) Rats were exposed to blue light to induce retinal degeneration and a stable deficit in contrast sensitivity (1/c where c is contrast). 53 weeks later, contrast sensitivity was measured at baseline and then 30 min after administration of vehicle or L-4FPG at 10mg/kg IP. Values are mean ± SEM of the number of animals in parentheses. P values are from paired 2-tailed t-test.

Table 1: Plasma pharmacokinetics of PG analogs in the rat with IV administration. PG analogs were administered at the indicated dose and plasma samples taken at various time points for measurement of drug levels. $t_{1/2}$: plasma half-life: CL: clearance; Vd: volume of distribution; $AUC_{0\text{-}inf}$: area under the curve. Values are the mean ± SD of 3 animals per group.

Compound	**Dose, Route** (mg/kg)	**$t_{1/2}$** (h)	**CL** (ml/min/kg)	**Vd** (ml/kg)	**AUC** (ng.hr/mL)
L-4FPG	10, IV	0.82 ± 0.12	16.2 ± 3.7	889 ± 48.0	10600 ± 2380
L-4OHPG	10, IV	1.30 ± 0.09	11.6 ± 1.1	986 ± 110	14500 ± 1360

JPET Fast Forward. Published on August 31, 2018 as DOI: 10.1124/jpet.118.251116
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2: Plasma pharmacokinetics of PG analogs in the rat with SC and IP administration. PG analogs were administered at the indicated dose and plasma samples taken at various time points for measurement of drug levels. $t_{1/2}$: plasma half-life: C_{max}: maximum concentration; T_{max}: time of peak plasma concentration; AUC: area under the curve; F: bioavailability compared to IV dosing. Values are the mean ± SD of 3 animals per group.

Compound	**Dose, Route** (mg/kg)	**$t_{1/2}$** (h)	**C_{max}** (ng/mL)	**T_{max}** (h)	**$AUC_{0\text{-}inf}$** (ng.hr/mL)	**F**
L-4FPG	10, SC	0.68 ± 0.07	7070 ± 1070	0.25 ± 0.0	10200 ± 1070	0.97 ± 0.24
L-4FPG	10, IP	1.04 ± 0.78	3940 ± 3230	1.58 ± 2.10	5800 ± 3880	0.93 ± 0.62
L-4OHPG	30, SC	1.38 ± 0.04	21900 ± 4490	0.33 ± 0.14	42300 ± 10500	0.96 ± 0.10
L-4OHPG	30, IP	NC	27400 ± 2050	1.24 ± 0.05	NC	0.83 ± 0.08

NC: Not calculable because the terminal phase was not captured accurately

Table 3: Brain penetration of PG analogs in rat after various routes of dosing. PG analogs were administered at the indicated dose and route and plasma and brain samples taken at 30 min for measurement of drug levels. The brain to plasma ratio was calculated from the mean drug levels at 30 min. Data are from 3 animals per group.

Compound	**Dose, Route** (mg/kg)	**Brain: Plasma**
L-4FPG	10, IV	1.45
L-4FPG	10, SC	0.66
L-4FPG	10, IP	1.04
L-4OHPG	10, IV	0.36
L-4OHPG	30, SC	0.18
L-4OHPG	30, IP	0.06

Table 4: Brain penetration of PG analogs in mouse after various routes of dosing.

PG analogs were administered at the indicated dose and route and plasma and brain samples taken at 30 min for measurement of drug levels. Values for brain levels are mean ± SD of 3 animals per group. The brain to plasma ratio was calculated from the mean drug levels at 30 min.

Compound **Mouse**	**Dose,** **Route** (mg/kg)	**Brain** (ng/mL)	**Brain:** **Plasma**
L-4FPG	3, SC	919 ± 198	0.73
L-4FPG	0.3, SC	90 ± 21	0.86
L-4OHPG	30, SC	4520 ± 529	0.26
L-4ClPG	10, IP	653 ± 260	0.13

JPET Fast Forward. Published on August 31, 2018 as DOI: 10.1124/jpet.118.251116
This article has not been copyedited and formatted. The final version may differ from this version.

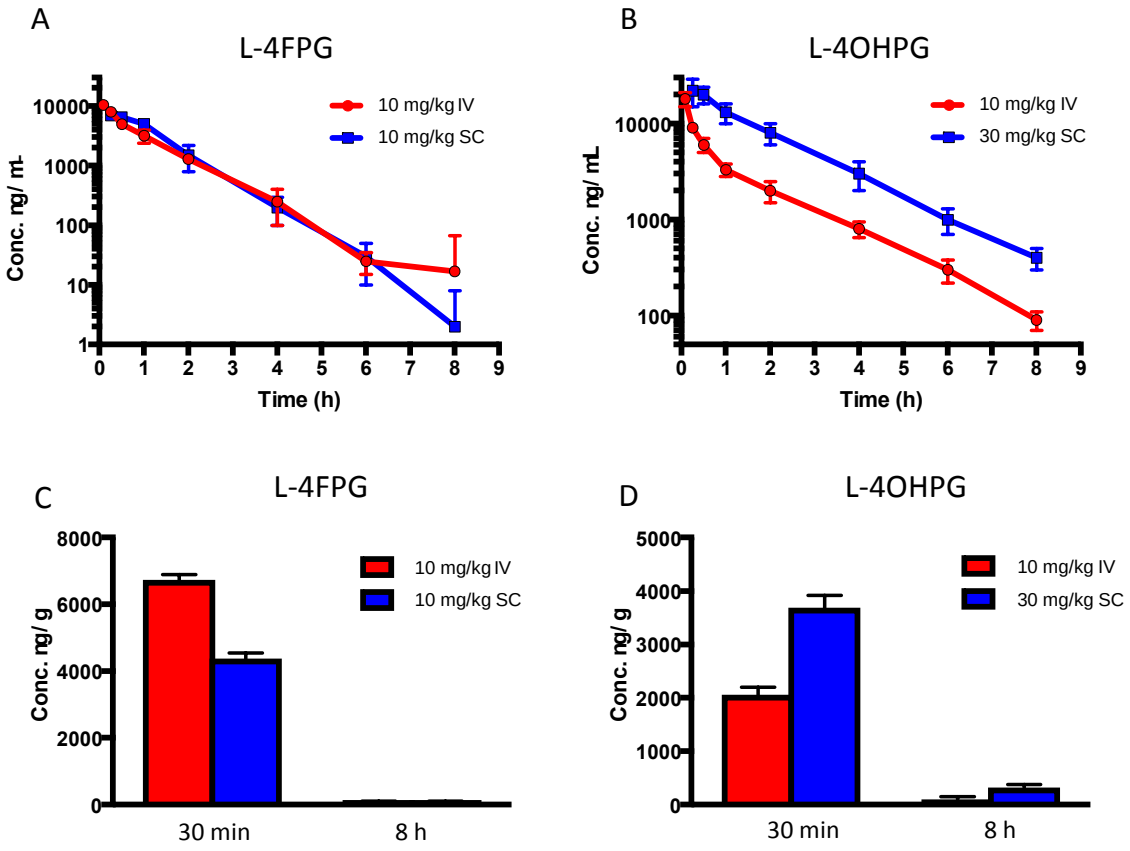

Figure 1

JPET Fast Forward. Published on August 31, 2018 as DOI: 10.1124/jpet.118.251116
This article has not been copyedited and formatted. The final version may differ from this version.

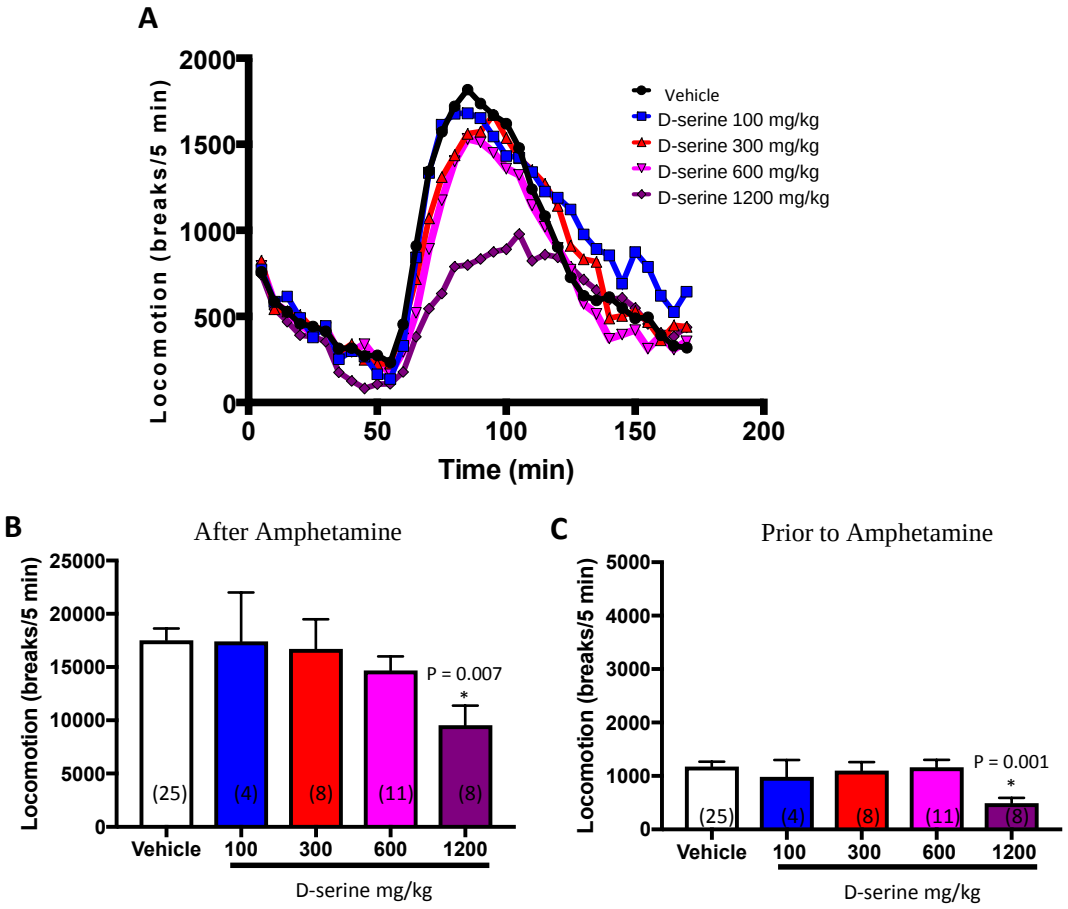

Figure 2

JPET Fast Forward. Published on August 31, 2018 as DOI: 10.1124/jpet.118.251116
This article has not been copyedited and formatted. The final version may differ from this version.

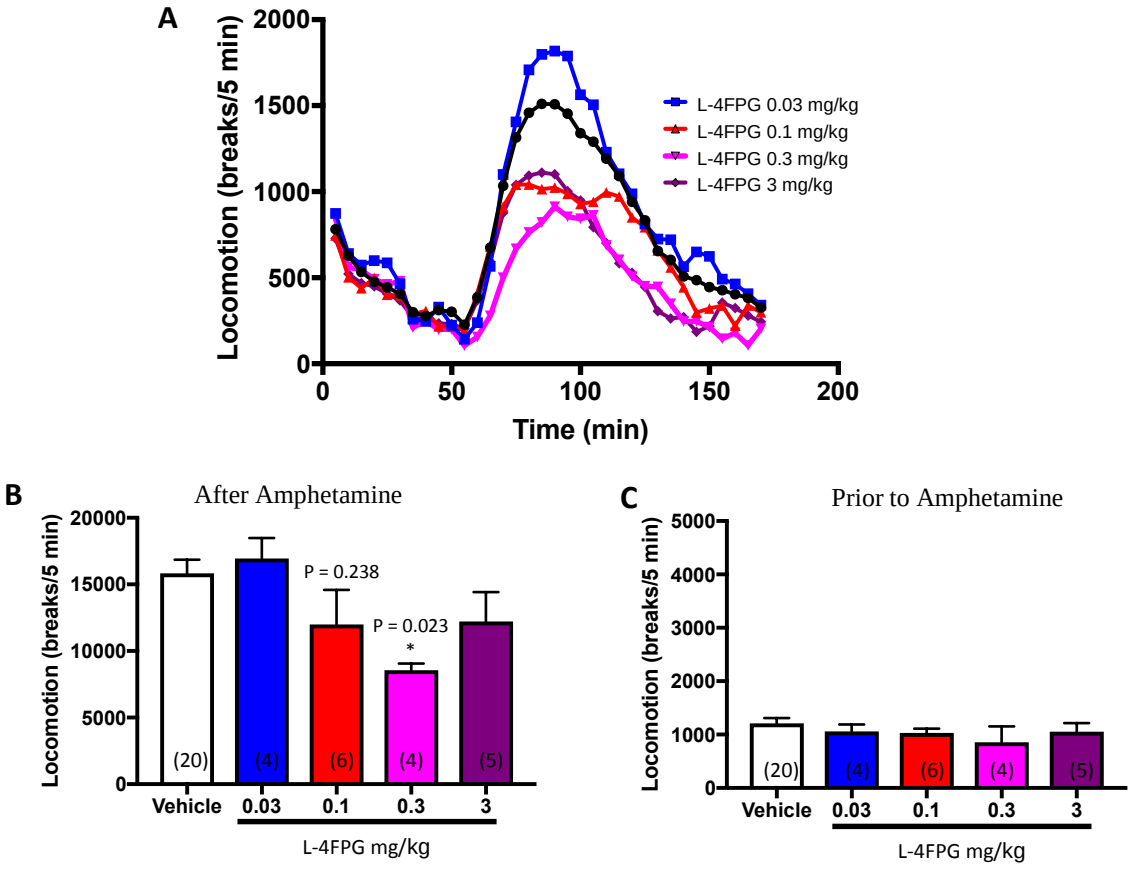

Figure 3

JPET Fast Forward. Published on August 31, 2018 as DOI: 10.1124/jpet.118.251116
This article has not been copyedited and formatted. The final version may differ from this version.

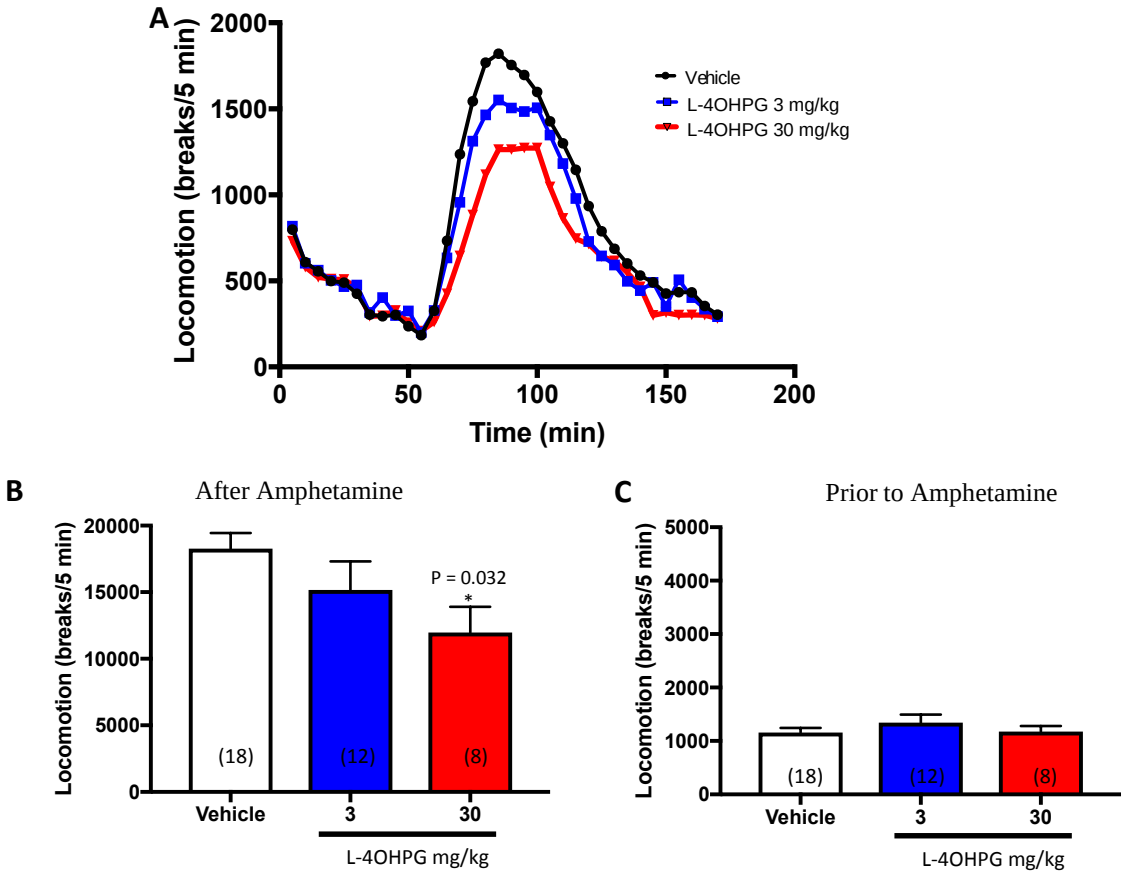

Figure 4

JPET Fast Forward. Published on August 31, 2018 as DOI: 10.1124/jpet.118.251116
This article has not been copyedited and formatted. The final version may differ from this version.

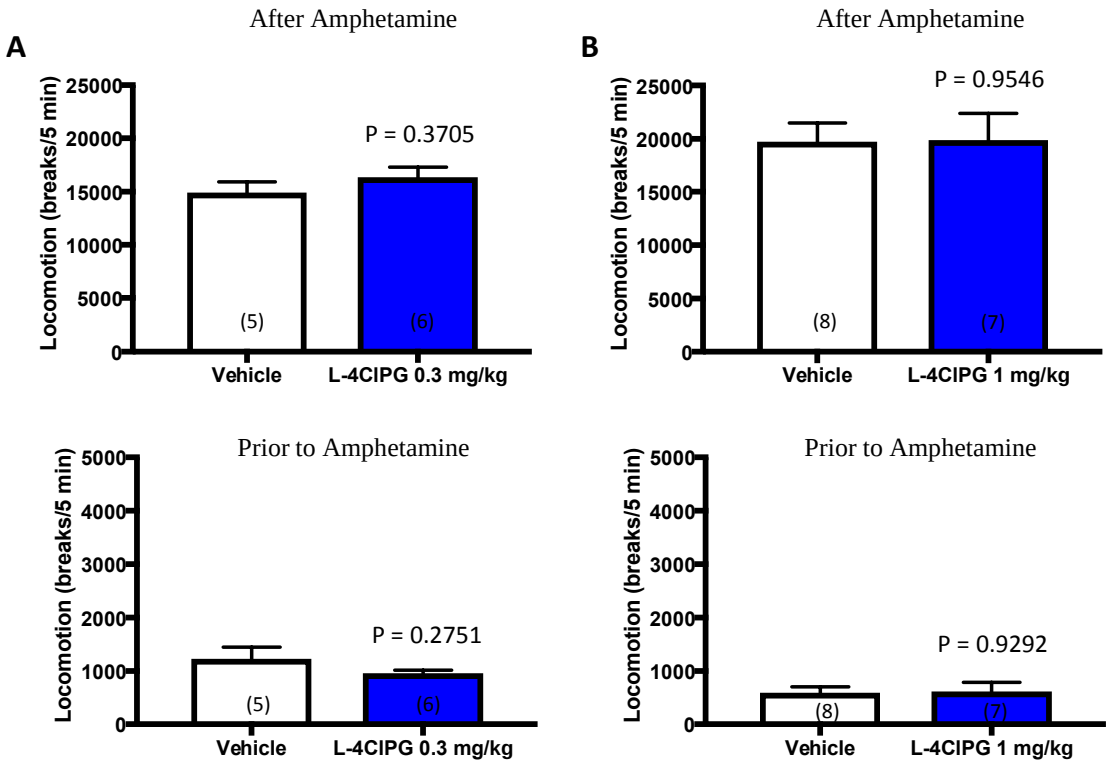

Figure 5

JPET Fast Forward. Published on August 31, 2018 as DOI: 10.1124/jpet.118.251116
This article has not been copyedited and formatted. The final version may differ from this version.

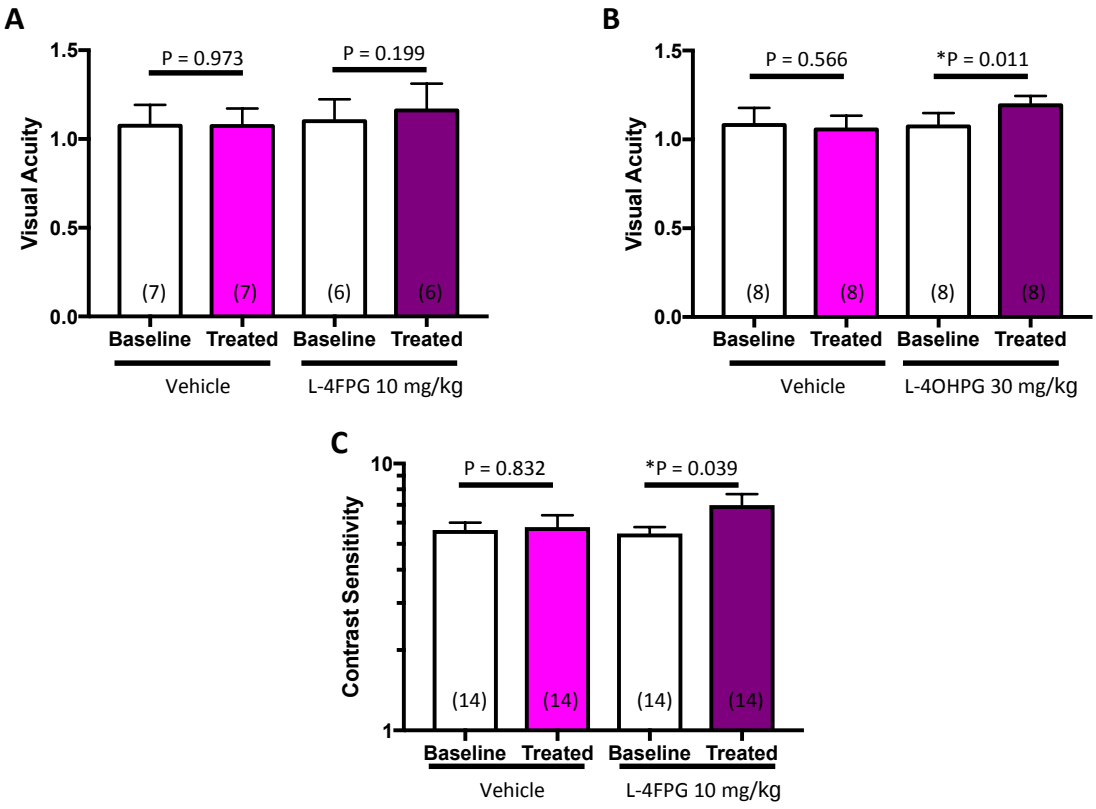

Figure 6