JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521

Title Page

Regulation of Extracellular Glutamate in the Prefrontal Cortex: Focus on the Cystine Glutamate Exchanger and Group I Metabotropic Glutamate Receptors

Roberto I. Melendez, Jompobe Vuthiganon, and Peter W. Kalivas[1]

Department of Neurosciences, Medical University of South Carolina,

Charleston, SC 29425

Running Title Page

Running Title: Prefrontal Regulation of Extracellular Glutamate

Corresponding Author: Roberto I. Melendez, Ph.D. Medical University of South Carolina Department of Neurosciences, 173 Ashley Avenue, Suite 403 BSB, Charleston, SC 29425, USA. Tel: (843) 792-4272 Email: melendez@musc.edu

Text pages: 30
Number of tables: 1
Number of figures: 6
Number of references: 37
Abstract: 237 words
Introduction: 414 words
Discussion: 1282 words

List of Nonstandard abbreviations:

AIDA, RS-1-aminoindan-1,5-dicarboxylic acid

AP5, 2-Amino-5-phosphonopentanoic acid

APDC, (2R,4R)-4-aminopyrrolidine-2,4-dycarboxylate

CPG, (S)-4-carboxyphenylglycine

DHPG, (RS)-3,5-dihydroxy-phenylglycine

EAAT 1-3, excitatory amino acid transporters 1-3

GVIA, ω-conotoxin GVIA

HCA, homocysteic acid

mGluR, metabotropic glutamate receptor

MPEP, 2-methyl-6-(phenylethynyl) pyridine

MVIIC, ω-conotoxin MVIIC

NMDA, N-methyl-D-aspartate

TBOA, DL-threo-β-benzy-loxyaspartate

TTX, tetrodotoxin

X^-_{AG}, Na^+-dependent glutamate uptake

x_c-, cystine-glutamate exchanger

Section: Neuropharmacology

JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Microdialysis was used to determine the *in vivo* processes contributing to extracellular glutamate levels in the prefrontal cortex of rats. Reverse dialysis of a variety of compounds proved unable to decrease basal levels of extracellular glutamate, including Na^+ and Ca^{+2} channel blockers, cystine/glutamate exchange (x_c^-) antagonists, and group I (mGluR1/5) and group II (mGluR2/3) metabotropic glutamate receptor (mGluR) agonists or antagonists. In contrast, extracellular glutamate was elevated by blocking Na^+-dependent glutamate uptake (X^-_{AG}) with DL-threo-β-benzyloxyaspartate (TBOA) and stimulating group I mGluRs with (RS)-3,5-dihydroxy-phenylglycine (DHPG). The accumulation of extracellular glutamate produced by blocking X^-_{AG} was completely reversed by inhibiting system x_c^- with 4-carboxyphenylglycine (CPG), but not by Na^+ and Ca^{+2} channel blockers. Because CPG also inhibits group I mGluRs, two additional group I antagonists were examined, LY367385 and RS-1-aminoindan-1,5-dicarboxylic acid (AIDA). While LY 367385 also reduced TBOA-induced increases in extracellular glutamate, AIDA did not. In contrast, all three group I antagonists reversed the increase in extracellular glutamate elicited by stimulating mGluR1/5. In vitro evaluation revealed that similar to CPG, LY 367385 inhibited x_c^-, and that stimulating or inhibiting mGluR1/5 did not directly affect [^{3}H]-glutamate uptake via x_c^- or X^-_{AG}. These experiments reveal that although inhibiting x_c^- cannot reduce basal extracellular glutamate in the prefrontal cortex, the accumulation of extracellular glutamate following blockade of X^-_{AG} arises predominately from x_c^-. The accumulation of glutamate elicited by mGluR1/5 stimulation does not appear to result from modulating $X^-_{AG,}$ x_c^- or synaptic glutamate release.

JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Glutamate is the primary excitatory neurotransmitter in the central nervous system, and alterations in glutamate transmission are implicated in pathologies ranging from neurotoxicity to neuropsychiatric disorders (Choi, 1988; Tsai and Coyle, 2002; Kalivas and Volkow, 2004). Because there is no extracellular enzymatic inactivation of glutamate, cellular uptake is necessary to remove glutamate released into the extracellular space by vesicular and nonvesicular mechanisms (Danbolt, 2001). Glutamate uptake transporters (EAAT1-5) are members of the X^-_{AG} family of electrogenic, Na^+ dependent amino acid transporters. Another glutamate transport system, referred to as system x_c^-, mediates the Na^+-independent exchange of one intracellular glutamate for one extracellular cystine molecule. Thus, rather than eliminating extracellular glutamate, x_c^- is a nonvesicular source of extracellular glutamate (Cho and Bannai, 1990; Sato et al., 1999; Patel et al., 2004) and *in vivo* microdialysis in the nucleus accumbens revealed that x_c^- is responsible for 50-70% of basal extracellular glutamate (Baker et al., 2002).

Glutamate is continuously being released from a variety of sources to the extracellular fluid. In addition to x_c^-, extracellular glutamate is derived from vesicular synaptic and nonvesicular glial release, both of which are calcium-dependent (Danbolt, 2001; Haydon, 2001). Although most studies to date have focused on synaptic release of glutamate from nerve terminals, the primary source of extracellular glutamate measured outside of the synaptic cleft appears to be x_c^-. Implicating x_c^-, Jabaudon et al. (1999) showed that inhibition of X^-_{AG} leads to extracellular accumulation of glutamate from sources that

were insensitive to blockade of voltage-dependent Na^+ and Ca^{2+} channels, or the administration of toxins which cleave proteins essential for exocytosis.

The *in vivo* concentration of extracellular glutamate has also been shown to be regulated by group I and group II metabotropic glutamate receptors (mGluR1/5 and mGluR2/3, respectively). *In vivo* microdialysis studies reveal that stimulation of group I or inhibition of group II mGluRs elevates extracellular glutamate level in various brain regions, including the nucleus accumbens (Swanson et al., 2001; Xi et al., 2002; Baker et al., 2002), parietal cortex (Moroni et al., 1998), and prefrontal cortex (Melendez et al., 2004).

The present study used *in vivo* microdialysis and *in vitro* glutamate uptake to determine the contribution of system x_c^- in regulating extracellular glutamate in the prefrontal cortex. The prefrontal cortex was examined because of its postulated role in drug addiction and schizophrenia, and the emerging concept that these neuropsychiatric disorders may involve pathological adaptations in the cellular processes regulating extracellular glutamate levels (Choi, 1988; Tsai and Coyle, 2002; Kalivas and Volkow, 2005).

JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

*Animals***.** Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250-300 g upon arrival were individually housed in an AALAC-approved animal facility with access to food and water *ad libitum*. Rooms were set on a 12:12-hr light cycle, with lights on at 7:00 A.M and all experimentation was conducted during the light period. All protocols were approved by the Institutional Animal Care and Use Committee in compliance with NIH's Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington D.C., USA, 1996).

Surgery. Rats were anesthetized with a combination of ketamine (100mg/kg, IP) and xylazine (3 mg/kg, ip). Using coordinates derived from the Paxinos and Watson (1998) atlas (in mm; + 2.7 anterior; + 1.1 lateral, - 2.0 ventral at a 6 degree angle from vertical), bilateral microdialysis guide cannulae (20 gauge, 14mm; Plastic One, Miami Lakes, FL) were implanted above the prefrontal cortex. Guide cannulae were secured to the skull using four skull screws (Small Parts, Roanoke, VA) and dental acrylic. After surgery, rats were permitted at least 5 days to recover before testing.

Microdialysis. Microdialysis probes were constructed with both the inlet and outlet tubing consisting of fused silica (Baker et al., 2002). The active region of the dialysis membrane was 3 mm in length and ~ 0.22 mm in diameter. The night before the dialysis experiment, a probe was inserted unilaterally through the guide cannulae into the prefrontal cortex. The next morning, dialysis buffer (in mM: 5 glucose, 5 KCl, 140 NaCl, 1.4 $CaCl_2$, and 1.2 $MgCl_2$, and 0.5% phosphate-buffered saline was added to a pH of 7.4) was advanced through the probe at a rate of 2 µl/min via syringe pump (Bioanalytial

Systems, West Lafayette, IN). Two h later, baseline samples were collected. The standard protocol used for microdialysis experiments involved the collection of five 20 min baseline samples, followed by three additional 20 min samples for each concentration of a given drug. Thus, multiple doses of each compound were administered in each rat. Liquid switches were used to minimize the pressure fluctuations while changing dialysis buffers with varying drug concentrations.

Dosage ranges of the various drugs were based upon the relative EC_{50} or IC_{50} values for binding to the respective receptors (Shimamoto et al., 1998; Schoepp et al., 1999; Gochenauer and Robinson, 2001) or concentrations shown effective in previous microdialysis studies (Swanson et al., 2001; Baker et al., 2002). All drugs were purchased from Tocris (Ballwin, MO) and were freshly prepared on the day of the experiment. RS-1-aminoindan-1,5-dicarboxylic acid (AIDA), (2R,4R)-4-aminopyrrolidine-2,4-dycarboxylate (APDC), (S)-4-carboxyphenylglycine (CPG), (RS)-3,5-dihydroxyphenylglicine (DHPG), LY 367385, 2-methyl-6-(phenylethynyl)pyridine (MPEP), and DL-threo-β-benzyloxyaspartate (TBOA) were initially dissolved in 0.1 N NaOH and neutralized with 0.1 N HCl. Working concentrations were then made by diluting with filtered dialysis buffer. 2-amino-5-posphonopentanoic acid (AP-5), diltiazem, ω-conotoxin GVIA (GVIA), homocysteic acid (HCA), ω-conotoxin MVIIC (MVIIC), EGTA, and tetrodotoxin (TTX) were dissolved in filtered dialysis buffer.

*Quantification of Glutamate***.** Microdialysis samples were collected into vials containing 10 µl of 0.05 M HCl. The concentration of glutamate in the dialysis samples

was determined using HPLC with fluorometric detection. Precolum derivitization of glutamate with *O*-phthalaldehyde was performed using a Gilson 231 XL autosampler (Middleton, WI). The mobile phase consisted of 11% acetonitrile (v/v), 100 mM Na_2HPO_4, 0.1 mM EDTA, pH 6.04. Glutamate was separated using a reversed-phase column (3 µm; 100 x 4.2 mm; Bioanalytical Systems, West Lafayette, IN) and was detected using a Shimadzu (Columbia, MD) 10RF-A fluorescence detector with an excitation wavelength of 320 nm and an emission wavelength of 400 nm. The concentration of glutamate in the dialysis samples was quantified by comparing peak heights from samples and external standards.

*Histology***.** After completion of the microdialysis experiments, rats were deeply anesthetized using CO_2 inhalation. The brains were removed and stored in 1% formalin for at least 1 week before sectioning. The tissue was then blocked, and coronal sections (100 µM) were cut and stained with cresyl violet to verify probe placements. Only animals with probes located in the prefrontal cortex were included in the data analysis.

*L-[^{3}H] Glutamate uptake assay***.** Rats were decapitated and the prefrontal cortex was rapidly dissected and cut into 350 x 350 µm prism-shaped slices using a McIllwan tissue chopper (Vibratome, St. Louis, MO). The slices were washed for 30 min at 37°C in oxygenated Kreb's-Ringer's solution phosphate buffer (KRP) (in mM: 140 NaCl, 1.3 $CaCl_2$, KH_2PO_4, 5 HEPES, 10 glucose, and 1 $MgCl_2$) with a final pH of 7.4. Glutamate uptake measurements were initiated by adding L- [^{3}H] glutamate (250 nM, 51 Ci/mmol;

PerkinElmer, Boston, MA) in the presence of 10 μM unlabeled L-[^{3}H] glutamate in a final volume of 250 μL of oxygenated buffer. After incubation at 37°C for 15 min, the uptake was terminated by washing the slices in ice-cold nonradioactive choline-containing buffer. Na^+-independent uptake was measured by replacing NaCl with equal concentrations of choline chloride. Under these conditions, [^{3}H]-glutamate uptake was shown to be Na^+-, time-, temperature-, and concentration-dependent (data not shown). Slices were then solubilized using 1% SDS, and the level of radioactivity was determined using a liquid scintillation counter. Protein content in the slices was measured using the Bradford assay.

*Immunoblotting and immunocytochemistry***.** Dissected nucleus accumbens and prefrontal cortex tissues were homogenized with a handheld tissue grinder in homogenization medium (0.32 M sucrose, 2 mM EDTA, 1% SDS, 50 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin; pH 7.2), subjected to low-speed centrifugation (2000 x g, to remove insoluble material), and frozen at 80°C. Protein determinations were performed using the Bio-Rad Dc protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Samples (30 μg) were subjected to SDS-PAGE using a minigel apparatus (Bio-Rad; 6%), transferred via semidry apparatus (Bio-Rad) to nitrocellulose membrane, and probed for the proteins of interest (one gel per protein per brain region). The rabbit anti-rat antibody against xCT was generated at the Medical University of South Carolina and used at a dilution of 1:500. Characterization of this antibody is described in detail elsewhere (Szumlinski et al., 2004). For immunocytochemistry, brains were fixed with 4% paraformaldehyde via intracardiac perfusion and stored overnight in 2%

paraformaldehyde. Coronal slices of prefrontal cortex (30 μm thick) were made with a vibratome and incubated overnight at room temperature in xCT antibody (1:2500). The sections were washed three times in phosphate-buffered saline and sequentially incubated with biotinylated anti-rabbit secondary antibody and preformed avidin: biotinylated enzyme complex according to product guidelines (Vectastain, Vector laboratories, Burlingam, CA). Tissues were then stained with diaminobenzidine and examined with light microscopy.

Statistical Analysis. A one-way ANOVA with repeated measures over dose was used to determine the effect of individual drugs on extracellular glutamate levels. A two-way ANOVA with repeated measures over time was used to compare glutamate between treatments and within treatments over time. Post hoc comparisons were made use Fisher's least significant difference test. Differences in the effects drugs on [^{3}H]-glutamate uptake were analyzed by one-way ANOVA.

Results

Basal level of extracellular glutamate levels in the prefrontal cortex. Figure 1A and table 1 summarize the results of the microdialysis experiments in which attempts were made to alter the basal level of extracellular glutamate in the prefrontal cortex using various concentration of drugs that target specific mechanisms postulated to contribute to glutamate release or elimination. Using relatively high concentration of drugs, various Ca^{2+} channel blockers failed to alter basal extracellular glutamate levels, including ω–

conotoxin MVIIC (N,P,Q-type), ω–conotoxin GVIA (N-type), diltiazem (L-type), or EGTA (chelator of extracellular Ca^{2+}). Similarly, the voltage-dependent Na^{+} channel blocker, TTX, or the NMDA-gated Ca^{2+} channel blocker, AP-5, was without effect.

Previous studies using *in vivo* microdialysis have shown that the inhibitors of x_c^-, CPG or HCA, and the mGluR 2/3 agonist, APDC, decreased basal levels of extracellular glutamate in the nucleus accumbens (Baker et al., 2002; Xi et al., 2002). In the present study, CPG (0.5 - 500 μM) failed to alter the extracellular levels of glutamate in the prefrontal cortex. Surprisingly, HCA (100 μM) produced a significant increase in extracellular glutamate (Figure 1A). However, HCA is also an NMDA agonist, and Figure 1D shows that the HCA-induced increase in glutamate was reversed by co-perfusion with the NMDA antagonist AP-5 (500 μM). Also, the reduction observed in glutamate by stimulating mGluR2/3 in the accumbens (Baker et al., 2002) was not observed in the prefrontal cortex (Figure 1A) following application of APDC (0.5-500 μM).

In contrast to the distinction between the accumbens and prefrontal cortex regarding compounds that decrease basal levels of extracellular glutamate, similar to earlier reports in the accumbens (Swanson et al., 2001; Xi et al., 2002; Baker et al., 2002) reverse dialysis of compounds that stimulate the accumulation of extracellular glutamate were effective in prefrontal cortex. First, blockade of X^-_{AG} by TBOA elevated extracellular glutamate in the prefrontal cortex to nearly 375% of baseline (Figures 1A and B). Also, the group I agonist DHPG produced a significant elevation in extracellular glutamate

levels (Figures 1A and C). However, reverse dialysis of the group I antagonists LY 367385 (0.5 - 50 μM) and AIDA (50 - 500 μM), failed to alter basal extracellular glutamate levels in the prefrontal cortex (Figure 1A), suggesting a lack of endogenous tone on group I mGluRs.

*Source of accumulated extracellular glutamate in the prefrontal cortex following blockade of X^-_{AG} with TBOA***.** Figure 2A illustrates that the TBOA (300 μM)-induced accumulation of glutamate in the prefrontal cortex was not altered by 1 μM TTX or 10 μM ω–conotoxin MVIIC, a broad spectrum antagonist of voltage-dependent Ca^{2+} channels. However, the TBOA-induced accumulation was reversed by co-perfusion with 0.5 - 50 μM CPG (Figure 2A and 2B). Although reversal by CPG indicates that the accumulation of glutamate may result from activity of x_c^-**,** in addition to blocking x_c^-**,** CPG is a mGluR1/5 antagonist (Schoepp et al., 1999). In order to determine if the effect of CPG was via mGluR1/5, additional mGluR1/5 antagonists were examined. While the antagonist AIDA (150 - 500 μM) was without effect (Figure 2A and 2C), LY 367385 (0.5 – 50 μM) abolished the TBOA-induced elevation in glutamate in a manner akin to CPG (Figure 2A and 2B). The fact that both LY 367385 and CPG reduced TBOA-induced extracellular glutamate, while AIDA was without effect may be related to their chemical structure; LY 367385 and CPG are phenylglycine derivatives, AIDA is not (Figure 2D).

*Source of accumulated extracellular glutamate in the prefrontal cortex following stimulation of mGluR1/5 by DHPG***.** Similar to the accumulation following blockade of X^-_{AG}, the increase in extracellular glutamate elicited by perfusing DHPG (50 μM) into the

prefrontal cortex was not affected by TTX (1 μM) or ω–conotoxin MVIIC (10 μM; Figure 3A). Also similar to the effect of TBOA, the rise in glutamate by DHPG (50 μM) was reversed by CPG (0.5 - 50 μM) and LY367385 (0.5 – 50 μM) (Figure 3A – 3C). However, in contrast to TBOA (Figure 2C), the rise in glutamate levels produced by DHPG was also reversed AIDA (150 – 500 μM; Figure 3D).

*Lack of interaction between x_c^- or X^-_{AG} and mGluR1/5***.** Although mitigated in part by the fact that the mGluR1/5 antagonist AIDA was without effect on the TBOA-induced rise in glutamate (figure 2C), the capacity of CPG and LY367385 to bind both x_c^- and mGluR1/5 (Schoepp et al., 1999; Gochenauer and Robinson, 2001), could indicate an interaction between x_c^- and mGluR1/5. To directly evaluate interactions between mGluR1/5 and x_c^- or $X^-_{AG,}$ Na^+-independent and -dependent [^{3}H]-glutamate uptake, respectively, was examined in tissue slices from the prefrontal cortex. Figure 4A verifies that Na^+-dependent [^{3}H]-glutamate uptake was inhibited in a dose-dependent manner by TBOA (5 – 500 μM), while Na^+-independent [^{3}H]-glutamate uptake was blocked by CPG (5 – 500 μM). Similarly, application of cystine (250 - 5000 μM) dose-dependently inhibited system x_c^- (maximal inhibition: 23% of baseline; data not shown). CPG (500 μM) did not alter Na^+-dependent [^{3}H]-glutamate uptake, and TBOA (500 μM) did not affect Na^+-independent [^{3}H]-glutamate uptake (Figure 4A). Consistent with the structural similarity between CPG and LY 367385 (see Figure 2D), LY 367385 (5 – 500 μM) caused a dose-dependent reduction in Na^+-independent [^{3}H]-glutamate uptake similar to CPG (Figure 4A), while AIDA (50 – 1500 μM) was without effect (Figure 4B). The mGluR1/5 antagonist MPEP (1 – 100 μM) was also without effect on Na^+-independent

[^{3}H]-glutamate uptake (Figure 4B). To directly verify that the effect of CPG on system x_c^- and X^-_{AG} did not result from blocking mGluR1/5, DHPG (500 μM) was co-applied with CPG (500 μM) or TBOA (500 μM). As shown in Figure 4C, DHPG did not reverse the CPG-induced decrease in Na^+-independent [^{3}H]-glutamate uptake (Figure 4C). DHPG (5 - 500 μM) alone did not alter Na^+-independent and –dependent [^{3}H]-glutamate uptake**.**

*Identification of x_c^- in the prefrontal cortex***.** Using an antibody against the catalytic subunit of x_c^- (xCT; Szumlinski et al., 2004), figure 5 shows immunhistochemical and immunoblotting evidence that x_c^- is present in the prefrontal cortex. Figure 5A shows that most, if not all cells in the prefrontal cortex express xCT and the higher magnification micrograph in figure 5B shows that some of the immunoreactivity appears to be present in clusters that are in or adjacent to the cell membrane. Figure 5C shows immunoblots comparing the level of xCT in tissue dissected from the prefrontal cortex or nucleus accumbens. Similar amounts of xCT were found in both regions.

Histology. Figure 6 verifies the location of dialysis probes used in this study. The majority of probes spanned the prelimibic cortex with a portion of the active membrane region in either the anterior cingulate or infralimbic cortex (Paxinos and Watson, 1998).

Discussion

The present study demonstrates that *in vivo* pharmacological blockade of Na^+-dependent glutamate transporters increases the basal concentration of extracellular glutamate in the prefrontal cortex, and that the accumulation of glutamate does not result from synaptic or

JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.

Ca^{2+}-dependent glutamate release. Rather, the accumulation of extracellular glutamate resulted from the release of glutamate by system x_c^-. However, the basal level of extracellular glutamate was not affected by voltage-dependent channel blockers, or inhibitors of x_c^-.

*Basal levels of glutamate***.** Akin to previous *in vivo* studies examining both cortical and subcortical brain regions, the basal extracellular level of glutamate was not affected by blocking various voltage-dependent ion channels associated with vesicular glutamate release (Timmerman and Westerink, 1997; Del Arco et al., 2003). However, the inability to reduce extracellular glutamate in the prefrontal cortex with the x_c^- antagonist CPG in the prefrontal cortex is in contrast with the effect of CPG in the nucleus accumbens (Baker et al., 2002). The cellular basis of the difference between the two brain regions is unclear given the presence of substantial immunoreactive xCT (catalytic subunit of x_c^-; Shih and Murphy, 2001) in the prefrontal cortex. However, the basal extracellular concentration of glutamate determined by no net flux dialysis is about two-fold greater in the nucleus accumbens than in the prefrontal cortex, and CPG reduces the level of basal glutamate in the accumbens to approximately the level of basal glutamate in the prefrontal cortex (Baker et al., 2002; Baker et al., 2003; Xi et al., 2002). Thus, in either structure, inhibition of x_c^- did not reduce glutamate levels below approximately 2 μM, and the remainder of glutamate was unaffected by inhibitors of synaptic glutamate release. One possible contribution to the 2 μM extracellular glutamate that is unaffected by x_c^- antagonists or voltage-dependent ion channel blockers is from decreasing efficacy of X^-_{AG} to eliminate glutamate. Although estimates of the Km of glutamate uptake vary

greatly between preparations, most typical values range between 5 and 30 μM (Danbolt, 2001). Moreover, in some studies the rate of glutamate unbinding from EAAT1 was found to exceed the rate of translocation into the cell, indicating that as the extracellular concentration is reduced, glutamate may be maintained in part by unbinding from transporters (Wadiche and Kavanaugh, 1998). Another possible contributor is the vesicular glutamate transporter that has been proposed to leak glutamate into the extracellular space when present in the plasma membrane, or other relatively poorly characterized transmembrane diffusion processes postulated to permit diffusion of glutamate into the extracellular space (Danbolt, 2001). Of particular interest for *in vivo* microdialysis estimates of extracellular glutamate is the release of glutamate produced by osmotic challenge (Kimelberg et al., 1990; Strange et al., 1996). Although the perfusion buffer employed in dialysis studies is designed to be isosmotic, unavoidable perturbations of the extracellular space by the probe may result in cell swelling and subsequent release of glutamate.

Another interesting distinction between the accumbens and prefrontal cortex was the fact that over the dosage range employed, HCA in the accumbens acts predominately as an x_c^- antagonist to reduce basal levels of glutamate (Baker et al., 2003), but in the prefrontal cortex HCA elevated glutamate. It was possible that this elevation resulted from HCA acting as a false cystine substrate in the prefrontal cortex, and thereby stimulating heteroexchange with intracellular glutamate (Patel et al., 2004). However, the elevation in glutamate by HCA was abolished by co-perfusion with the NMDA blocker AP-5, arguing that the increase resulted entirely from the known efficacy of HCA as an agonist

at NMDA receptors (Lehmann et al., 1988). The preferential effect of HCA on NMDA receptors relative to x_c^- may result from the relative abundance of NMDA receptors in the cortex compared with the nucleus accumbens (Monaghan and Cotman, 1986) or perhaps different phosphorylation states of xCT may result in reduced capacity of HCA to inhibit x_c^- (Gochenauer and Robinson, 2001; Baker et al., 2003; Tang and Kalivas, 2003).

Nonvesicular origin of extracellular glutamate accumulated by blocking X^-_{AG}. It was previously shown using in vitro electrophysiological estimates of extracellular glutamate that the accumulation of glutamate following blockade of X^-_{AG} with TBOA was not derived from synaptic release since it was unaffected by blockade of Na^+ or Ca^{2+} channels, or by the administration of peptide toxins that cleave proteins required for vesicular release (Jabaudon et al., 1999). Consistent with this elegant in vitro study, the accumulation of extracellular glutamate *in vivo* was unaffected by compounds that inhibit synaptic glutamate release. In contrast, the inhibition of x_c^- by CPG completely reversed the elevation in extracellular glutamate produced by TBOA. In addition to inhibiting x_c^-, CPG blocks mGluR1/5 (Ye et al., 1999), and CPG also reversed the increase in extracellular glutamate produced by reverse dialysis of the mGluR1/5 agonist DHPG. However, a selective effect on x_c^- by CPG was revealed by the fact that another mGluR1/5 antagonist AIDA inhibited the effect of DHPG but did not reduce the increase in glutamate by TBOA. The lack of effect by AIDA to inhibit x_c^- has been further verified in vitro using Na^+-independent [^{3}H]-glutamate (Figure 4) or [^{35}S]-cystine uptake in tissue slices (Baker et al., 2002).

JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.

Although the present experiments demonstrated that the effect of CPG on TBOA-induced accumulation of extracellular glutamate *in vivo* resulted from the inhibition of x_c^-, it is important to note that TBOA-induced accumulation of glutamate may not reflect a physiological function of x_c^-. Moreover, the microdialysis experiments could not exclude a role for x_c^- in the elevation of glutamate elicited by stimulating mGluR1/5. The accumulation of glutamate by DHPG was apparently nonsynaptic since it was unaffected by blocking voltage-dependent Na^+ or Ca^{2+} channels. However, since the effective x_c^- antagonists, CPG and LY 367385, also are mGluR1/5 antagonists it remained possible that stimulating mGluR1/5 with DHPG could have altered x_c^- via mGluR1/5-mediated cell signaling. For example, mGluR1/5 is positively coupled to phospholipase C intracellular signaling via calcium-dependent protein kinase (PKC) and inositol triphosphate, which have been proposed previously to underlie the ability of DHPG to release glutamate (Cochilla and Alford, 1998; Schwartz and Alford, 2000; Swanson et al., 2001). Moreover, stimulating PKC inhibits [^{35}S]-cystine uptake in astrocytes (Tang and Kalivas, 2003). However, arguing against mGluR1/5 regulation of x_c^-, DHPG did not affect Na^+-independent [^{3}H]-glutamate uptake in prefrontal cortical slices (Figure 4C).

LY 367385 is as novel inhibitor of system x_c^-. As has been the case with Na^+-dependent transporters, the most potent inhibitors of x_c^- identified to date are conformationally constrained analogues (Patel et al., 2004). By restricting bond rotations, the functional groups on the molecule can potentially be locked in a configuration that mimics the endogenous substrate. This strategy, which is commonly accomplished by introducing

ring systems into the carbon backbone, often results in both increased potency and specificity (Chamberlin et al., 1998). CPG, a confirmationally restricted compound, has been shown to be one of the most potent and selective inhibitors of system x_c^- (Gochenauer and Robinson, 2001; Patel et al. 2004). Unlike the group I antagonists AIDA and MPEP, LY 367385 is a confirmationally restricted compound and phenylglycine derivative of CPG (see Figure 2D). In the present study, LY 367385, but not AIDA and MPEP, dose-dependently reduced system x_c^-. Furthermore, LY 367385 was effective in reducing the TBOA-induced accumulation of extracellular glutamate in the prefrontal cortex as measured by *in vivo* microdialysis. To our knowledge, this is the first study to date showing the *in vivo* and *in vitro* effects of LY 367385 on system x_c^- activity.

Summary. The majority of extracellular glutamate measured in the prefrontal cortex by *in vivo* microdialysis originates from unidentified nonvesicular, nonsynaptic sources. The accumulation of extracellular glutamate produced by inhibiting X^-_{AG} was also shown to nonvesicular in origin, but system x_c^- was identified as the source of accumulated glutamate.

References

Amara SG and Fontana AC (2002) Excitatory amino acid transporters: keeping up with glutamate. *Neurochem Int* **41**:313-318.

Baker DA, McFarland K, Lake RW, Shen H, Tang XC, Toda S and Kalivas PW (2003) Neuroadaptations in cystine-glutamate exchange underlie cocaine relapse. *Nat Neurosci* **6**:743-749.

Baker DA, Xi ZX, Shen H, Swanson CJ and Kalivas PW (2002) The origin and neuronal function of *in vivo* nonsynaptic glutamate. *J Neurosci* **22**:9134-9141.

Bannai S. (1986) Exchange of cystine and glutamate across plasma membrane of human fibroblasts. *J Biol Chem* **261**:2256-2263.

Chamberlin AR, Koch HP, Bridges RJ (1998) Design and synthesis of conformationally constrained inhibitors of high-affinity, sodium-dependent glutamate transporters. *Methods Enzymol* **296**:175-189.

Cho Y and Bannai S (1990) Uptake of glutamate and cysteine in C-6 glioma cells and in cultured astrocytes. *J Neurochem* **55**:2091-2097.

Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous systems. *Neuron* **1**:623-634.

Cochilla AJ and Alford S (1998) Metabotropic glutamate receptor-mediated control of neurotransmitter release. *Neuron* **20**:1007-1016.

Danbolt NC (2001) Glutamate uptake. *Prog Neurobiol* **65**:1-105.

Del Arco A, Segovia G, Fuxe K and Mora F (2003) Changes in dialysate concentrations of glutamate and GABA in the brain: an index of volume transmission mediated actions? *J Neurochem* **85**:23-33.

Gochenauer GE and Robinson MB (2001) Dibutyryl-cAMP (dbcAMP) up-regulates astrocytic chloride-dependent L-[3H]glutamate transport and expression of both system xc(-) subunits. *J Neurochem* **78**:276-286.

Haydon P (2001) Glia: listening and talking to the synapse. *Nature Neuroscience* **2**:185-191.

Jabaudon D, Shimamoto K, Yasuda-Kamatani Y, Scanziani M, Gahwiler B and Gerber U (1999) Inhibition of uptake unmasks rapid extracellular turnover of gluamate of nonvesicular origin. *Proc.Natl.Acad.Sci. (USA)* **96**:8733-8738.

Kalivas PW and Volkow ND (2005) The neurobiology of addiction: A pathology of motivation and choice. *Am J Psychiatry* **in press**.

Kimelberg HK, Goderie SK, Higman S, Pang S and Waniewski RA (1990) Swelling-induced release of glutamate, aspartate and taurine from astrocyte cultures. *J Neurosci* **10**:1583-1591.

Lehmann J, Tsai C and Wood PL (1988) Homocysteic acid as a putative excitatory amino acid neurotransmitter: I. Postsynaptic characteristics at N-methyl-D-aspartate-type receptors on striatal cholinergic interneurons. *J. Neurochem.* **51**:1765-1770.

McBean GJ (2002) Cerebral cystine uptake: a tale of two transporters. *Trends Pharmacol Sci* **23**:299-302.

Melendez RI, Gregory ML, Bardo MT, Kalivas PW (2004) Impoverished rearing environment alters metabotropic glutamate receptor expression and function in the prefrontal cortex. *Neuropsychopharmacology* **29**:1980-1987.

Monaghan DT, Cotman CW (1986) Identification and properties of N-methyl-D-aspartate receptors in rat brain synaptic plasma membranes. *Proc Natl Acad Sci U S A* **83**:7532-7536.

Moroni F, Cozzi, A, Lombardi, G, Sourtcheva, S, Leonardi, P, Carfi, M and Pellicciari, R (1998) Presynaptic mGlu1 type receptors potentiate transmitter output in the rat cortex. *Eur.J.Pharmacol.* **347**:189-195.

Murphy TH, Miyamoto M, Sastre A, Schnaar RL, Coyle JT (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* **2**:1547-1558.

Patel SA, Warren BA, Rhoderick JF and Bridges RJ (2004) Differentiation of substrate and non-substrate inhibitors of transport system xc(-): an obligate exchanger of L-glutamate and L-cystine. *Neuropharmacology* **46**:273-284.

Paxinos G and Watson C (1998) *The rat brain in stereotaxic coordinates*. Academic Press, New York.

Sato H, Tamba M, Ishii T and S B (1999) Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J Biol Chem* **274**:11455-11458.

Schoepp DD, Jane DE and Monn JA (1999) Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology* **38**:1431-1476.

Schwartz NE and Alford S (2000) Physiological activation of presynaptic metabotropic glutamate receptors increases intracellular calcium and glutamate release. *J Neurophysiol* **84**:415-427.

Shih AY and Murphy TH (2001) xCt cystine transporter expression in HEK293 cells: pharmacology and localization. *Biochem Biophys Res Commun* **282**:1132-1137.

Shimamoto K, Lebrun B, Yasuda-Kamatani Y, Sakaitani M, Shigeri Y, Yumoto N and Nakajima T (1998) DL-threo-beta-benzyloxyaspartate, a potent blocker of excitatory amino acid transporters. *Mol Pharmacol* **53**:195-201.

Strange K, Emma F and Jackson PS (1996) Cellular and molecular physiology of volume-sensitive anion channels. *Am J Physiol* **270**:C711-C730.

Swanson C, Baker D, Carson D, Worley P and Kalivas P (2001) Repeated cocaine administration attenuates group I metabotropic glutamate receptor-mediated glutamate release and behavioral activation: A potential role for Homer 1b/c. *J Neuroscience* **21**:9043-9052.

Szumlinski KK, Dehoff MH, Kang SH, Frys KA, Lominac KD, Klugmann M, Rohrer J, Griffin W 3rd, Toda S, Champtiaux NP, Berry T, Tu JC, Shealy SE, During MJ, Middaugh LD, Worley PF, Kalivas PW (2004) Homer proteins regulate sensitivity to cocaine. *Neuron* **43**:401-413.

Tang X-C and Kalivas PW (2003) Bidirectional modulation of cystine/glutamate exchanger activity in cultured cortical astrocytes. *Ann NY Acad. Sci.* **1003**:472-475.

Timmerman W and Westerink BH (1997) Brain microdialysis of GABA and glutamate: what does it signify? *Synapse* **27**:242-261.

Tsai G and Coyle JT (2002) Glutamatergic mechanisms in schizophrenia. *Annu Rev Pharmacol Toxicol* **42**:165-179.

Wadiche JI and Kavanaugh MP (1998) Macroscopic and microscopic properties of a cloned glutamate transporter chloride channel. *J Neurosci* **18**:7650-7661.

Xi Z-X, Shen H, Carson D, Baker D and PW Kalivas (2002) Inhibition of glutamate release by group II metabotropic glutamate receptors. *J Pharmacol Exp Ther* **300**:162-171.

Ye Z-C, Rothstein JD and Sontheimer H (1999) Compromised glutamate transport in human glioma cells: reduction-mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine-glutamate exchange. *J Neuroscience* **19**:10767-10777.

Footnote

1. This research was supported in part by the National Institute of Health Grants MH-40817, DA-07288, and AA 007474.

JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1**.** Microdialysis of glutamate in the rat prefrontal cortex. A, the maximal percentage baseline data of extracellular glutamate levels in the rat prefrontal cortex following local application (i.e., reverse microdialysis) of various pharmacological agents. As summarized in Table 1, all drug doses were selected according to their IC_{50} or EC_{50} values as described previously by various investigators (e.g., Shoepp et al. 1999; Gochenauer and Robinson, 2001; Shimamoto et al., 1998). Various receptor channel blockers including compounds that blocked voltage-dependent Na^+- and Ca^{2+}-channels (see Table 1) failed to alter the extracellular levels of glutamate. Inhibition of Na^+-dependent glutamate uptake with TBOA (1000 μM) increased the extracellular levels of glutamate (see Figure 1B). Inhibition of Na^+- independent glutamate uptake (i.e., system x_c^-) with CPG, failed to alter the extracellular levels of glutamate, whereas HCA (100 μM) significantly increased extracellular glutamate. However, the HCA induced increase is more likely attributed to NMDA receptor activation (see Figure 1D). In the case of mGluRs, only the group I agonist, DHPG (50 μM) increased extracellular glutamate levels (see Figure 1C). Extracellular glutamate levels are expressed as percentage $\pm$ standard error of the mean (S.E.M) of basal pretreatment levels (calculated from 5 samples before treatment). B, One-way ANOVA with repeated measures over doses revealed that TBOA (n=5), dose-dependently increased the extracellular levels of glutamate in the prefrontal cortex [$F_{13,52} = 19.7$, $p < 0.05$]. C, the group I agonist, DHPG (n=7), dose-dependently increased the extracellular levels of glutamate in the prefrontal cortex dose [$F_{13,78} = 4.0$, $p < 0.05$]. D, the excitatory effect of 100 μM HCA [$F_{12, 49} = 2.7$, $p < 0.05$] was competitively reversed by co-application of the NMDA antagonist APV

(500 μM; n=5). The black lines indicate the beginning of local perfusion (i.e., reverse dialysis) of the respective drugs. Glutamate levels are expressed as pmol/sample ± SEM of basal pretreatment levels. * $p < 0.05$ compared to baseline.

Figure 2. EAAT-induced accumulation of extracellular glutamate is inhibited by CPG and LY 367385, but not, AIDA. A, The significant [$F_{1,9} = 105.9$, $p < 0.05$] accumulation of extracellular glutamate induced by TBOA (300 μM; n=5; was not inhibited by co-application of 1 μM TTX (n=4), 10 μM MVIIC (n=4), or 500 μM AIDA (n=5). However, the TBOA-induced accumulation was inhibited by co-application of CPG (n = 5) and LY 367385 (n = 5) [$F_{1,9} < 3.1$, $p > 0.1$]. Glutamate levels are expressed as percentage ± SEM of basal pretreatment levels. Panels B and C illustrate the time course used to sample extracellular glutamate in the prefrontal cortex before and after reverse microdialysis. The black lines indicate the beginning of local perfusion (i.e., reverse dialysis) of the respective drugs. B, Two-way ANOVA with repeated measures over time revealed that the excitatory effect of 300 μM TBOA (n=4) was competitively reversed by co-application of 0.5 - 50 μM CPG (n=5) or LY 367385 (n=5) [$F_{17,119} > 12.1$, $p < 0.05$]. C, The excitatory effect of 300 μM TBOA (n=4) was not competitively reversed by co-application of 150 - 500 μM AIDA (n=4). D, chemical structure comparisons between CPG and LY 367385 (conformationally constrained analogues and phenylglycine derivatives) versus AIDA. * $p < 0.05$ compared to baseline; + $p < 0.05$ compared to TBOA alone.

Figure 3. DHPG-induced elevation of extracellular glutamate levels is inhibited by CPG, LY 367385, and AIDA. A, The significant increase [$F_{1,9}$ = 18.5, $p < 0.05$] in extracellular glutamate induced by DHPG (50 μM; n=4) was not inhibited by co-application of 1 μM TTX (n=5) and 10 μM MVIIC (n=5). However, the DHPG-induced increase in extracellular glutamate was significantly inhibited by co-application of 50 μM CPG (n = 5), 50 μM LY 367385 (n = 5), and 500 μM AIDA [$F_{1,9} < 3.1$, $p > 0.1$]. Glutamate levels are expressed as percentage $\pm$ SEM of basal pretreatment levels. Panels B - D illustrate the time course used to sample extracellular glutamate in the prefrontal cortex before and after reverse microdialysis. The black lines indicate the beginning of local perfusion (i.e., reverse dialysis) of the respective drugs. B, Two-way ANOVA with repeated measures over time revealed that the excitatory effect of 50 μM DHPG (n=4) was competitively reversed by co-application of 0.5 - 50 μM CPG (n=5; time x group interaction ($F_{17,119}$ = 2.5, $p < 0.05$). C, the excitatory effect of 50 μM DHPG (n=4) was competitively reversed by co-application of 0.5 - 50 μM LY 367385 (n=4; time x group interaction ($F_{17,102}$ = 9.7, $p > 0.05$). D, the excitatory effect of 50 μM DHPG (n=4) was competitively reversed by co-application of 150 - 500 μM AIDA (n=5; time x group interaction ($F_{14,98}$ = 11.8, $p < 0.05$). * $p < 0.05$ compared to baseline; # $p < 0.05$ compared to DHPG alone.

Figure 4. System X^-_{AG} and x_c^- uptake of [^{3}H]-glutamate in prefrontal slices. A (left panel), TBOA (5 – 500 μM) dose-dependently decreased X^-_{AG} (Na^+-dependent) glutamate uptake ($F_{3,9}$ = 10.8, $p < 0.05$). Application of CPG (500 μM) failed to alter glutamate uptake. System x_c^- is a Na^+-independent cystine/glutamate exchanger that can be

monitored as the Na^+-independent uptake of glutamate, in which case, extracellular glutamate is exchanged for intracellular glutamate (Bannai, 1986; Murphy et al., 1989; Patel et al., 2004). A (right panel), CPG (5 - 500 μM) dose-dependently decreased x_c^- (Na^+-independent) glutamate uptake ($F_{3,9} = 18.9$, $p < 0.05$). Application of TBOA (500 μM) failed to alter x_c^-. Data are expressed as pmol/mg protein/min of 3-4 independent observations performed in triplicates. B, LY 367385 (5 – 500 μM) dose-dependently decreased x_c^- [$F_{3,6} = 44.1$, $p <0.05$], whereas AIDA (50 – 1500 μM) and MPEP (10 - 1000 μM) failed to significantly alter x_c^-. Data are expressed as pmol/mg protein/min of 3-4 independent observations performed in triplicates. C, Co-application of 500 μM DHPG failed to block the TBOA-induced decrease in X^-_{AG} and the CPG-induced decrease in x_c^-. Data are expressed as percent change of basal uptake values of 3-4 independent observations performed in triplicates. * $p < 0.05$ compared to baseline (paired sample t-test).

Figure 5. The catalytic subunit (xCT) of x_c^- is present in the prefrontal cortex. A, Low magnification of dorsal prefrontal cortex showing immunoreactive xCT in most cells. Bar= 50 μm. B, High magnification micrograph showing immunoreactive xCT in clusters that appear in or adjacent to the cell membrane. Bar= 10 μm. C, Immunoblots showing relative amounts of xCT in the prefrontal cortex (PFC) and nucleus accumbens (NA). Thirty μg of protein was loaded in each lane.

Figure 6. Location of the microdialysis probes in the prefrontal cortex. The numbers indicate millimeters rostral to bregma according to the atlas of Paxinos and Watson

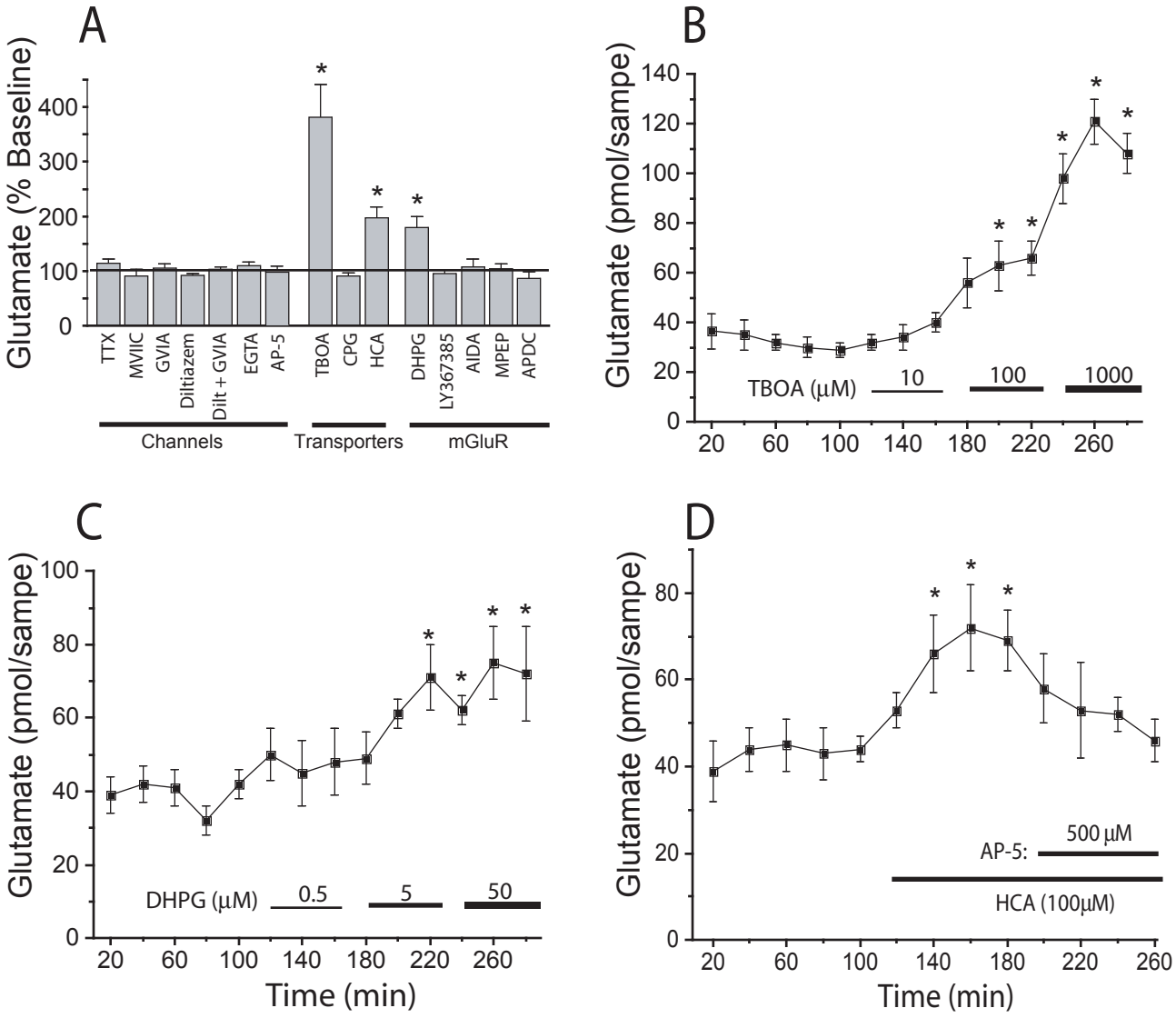
(1998). Lines indicate the active portion of the microdialysis membrane. Probe placements revealed that the probes traversed the dorsal (anterior cingulate and prelimbic) and ventral (infralimbic) region of the medial prefrontal cortex.

JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.

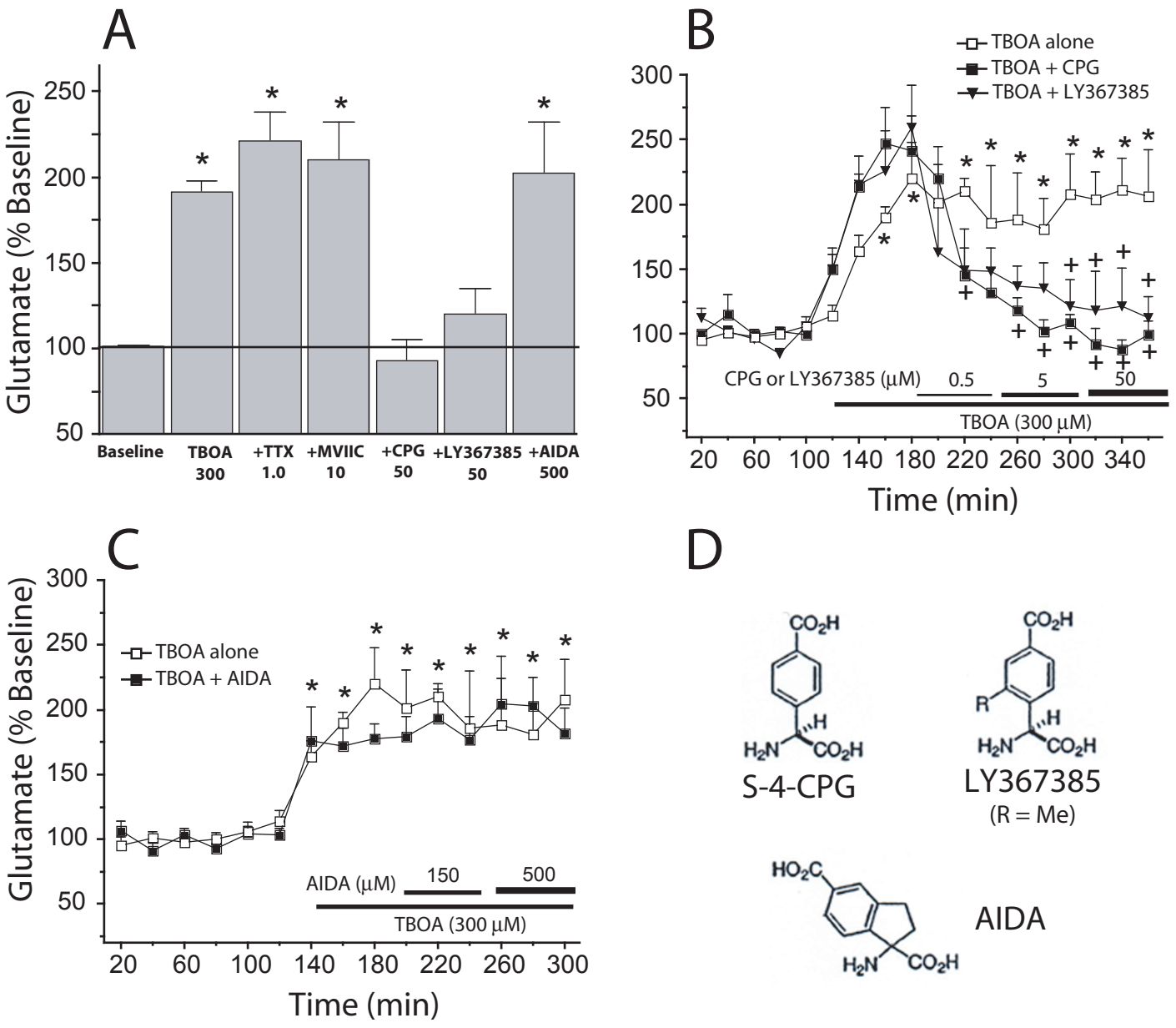
Table 1. Summary of the pharmacological agents employed and basal extracellular glutamate levels in Figure 1A measured in the rat prefrontal cortex by *in vivo* microdialysis

Drug	Mechanism of Action	Dose (μM)	Basal Glutamate (pmol/sample) (n)
Channels			
TTX	Na^+ channel antagonist	1-10	58 ± 10 (4)
MVIIC	N/P/Q type Ca^{2+} channel antagonist	10	42 ± 3 (4)
GVIA	N type Ca^{2+} channel antagonist	10	66 + 8 (4)
Diltiazem	L type Ca^{2+} channel antagonist	10	34 ± 9 (4)
EGTA	Ca^{2+} channel antagonist	100	46 ± 6 (4)
AP-5	NMDA antagonist	50 – 500	58 ± 8 (5)
Transporters			
TBOA	EAAT 1-3 antagonist	10 – 1000	30 ± 4 (5)
CPG	mGluR-1/5 and x_c^- antagonist	0.5 – 50	55 ± 6 (7)
LY 367385	mGluR-1/5 antagonist	0.5 – 50	72 ± 5 (6)
HCA	NMDA agonist and and x_c^- antagonist	1.0- 100	41 ± 4 (5)
mGluRs			
DHPG	mGluR-1/5 agonist	5 – 50	38 ± 3 (7)
AIDA	mGluR-1/5 antagonist	50 – 500	45 ± 6 (4)
MPEP	mGluR-5 antagonist	5 – 150	43 ± 9 (5)
APDC	mGluR-2/3 agonist	50 – 500	53 ± 8 (6)

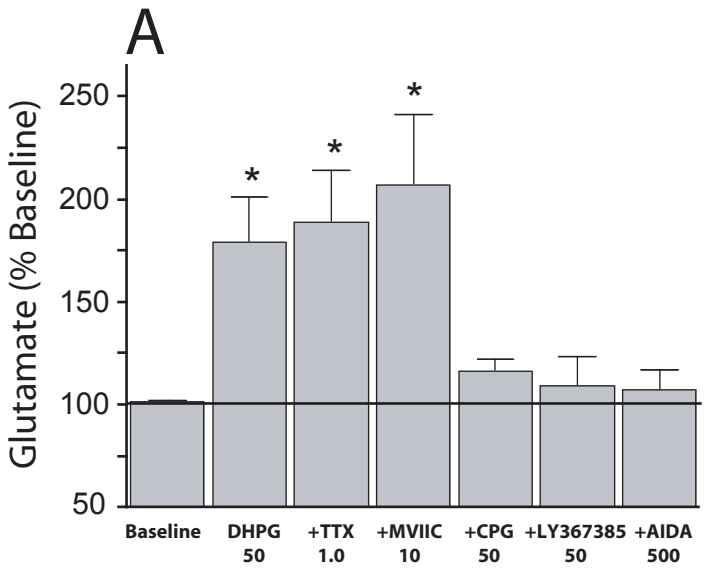
JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.

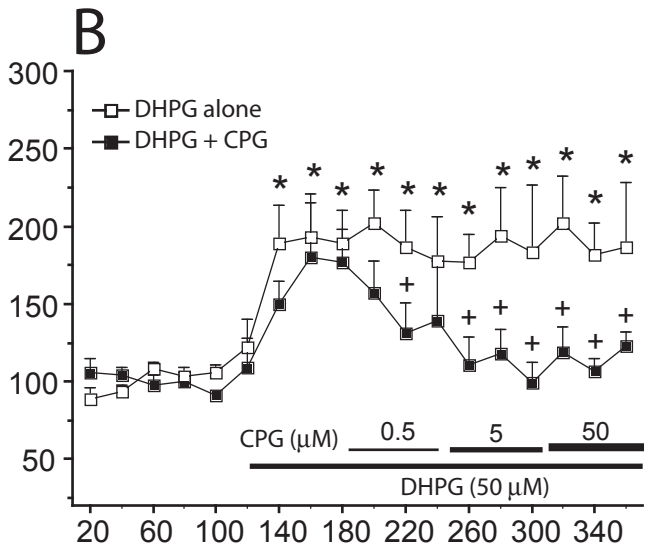


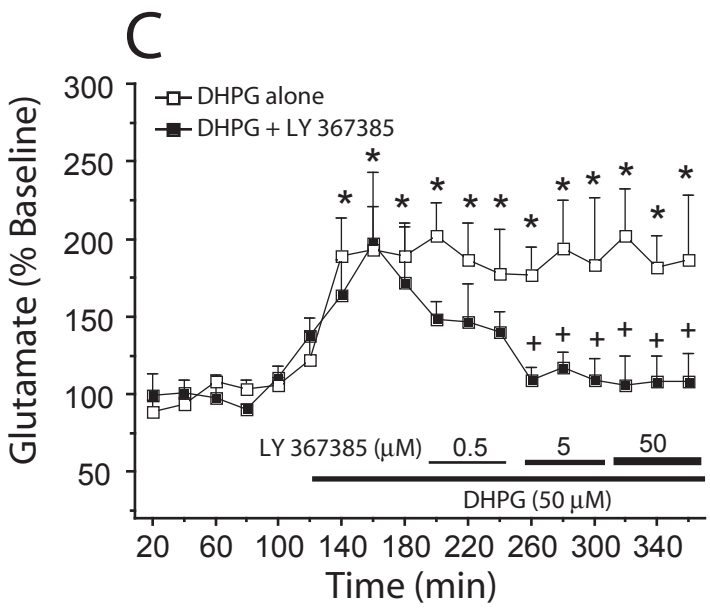
JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.

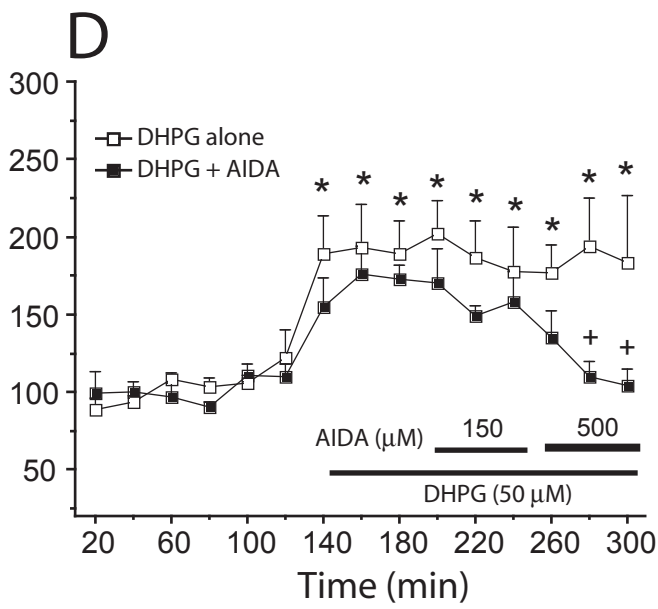


JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.

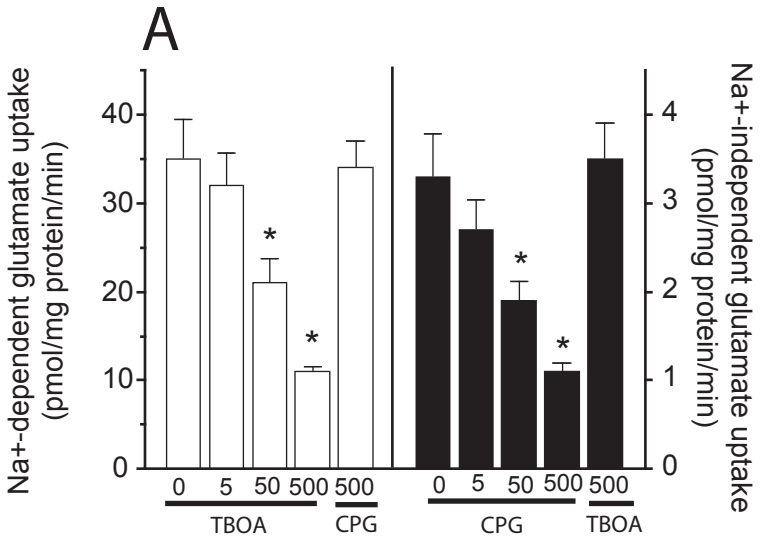


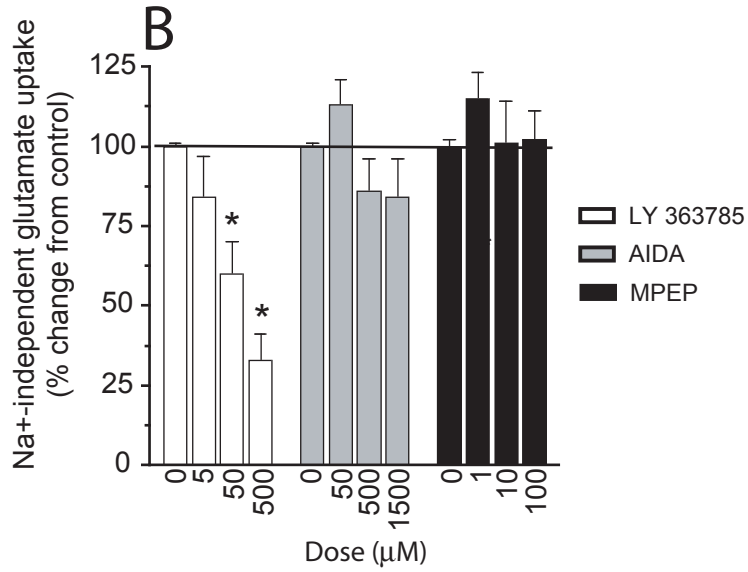


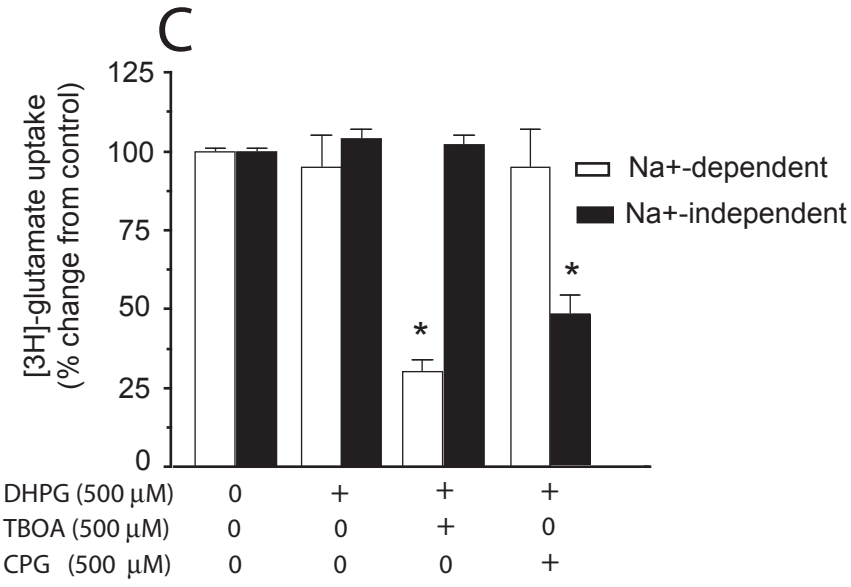




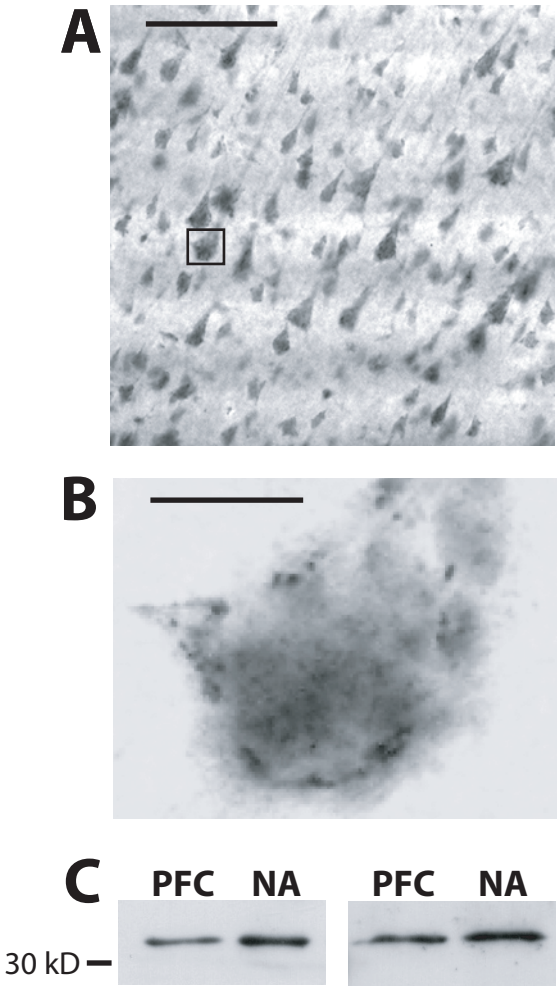
JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.







JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521



JPET Fast Forward. Published on March 15, 2005 as DOI: 10.1124/jpet.104.081521
This article has not been copyedited and formatted. The final version may differ from this version.