Oral Administration of a Specific Kynurenic Acid Synthesis (KAT II) Inhibitor Attenuates Evoked Glutamate Release in Rat Prefrontal Cortex

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Abstract

Cognitive deficits represent core symptoms in schizophrenia (SZ) and predict patient outcome; however, they remain poorly treated by current antipsychotic drugs. Elevated levels of the endogenous alpha7 nicotinic receptor negative allosteric modulator and NMDA receptor antagonist, kynurenic acid (KYNA), are commonly seen in post-mortem tissue and cerebrospinal fluid of patients with SZ. When acutely or chronically elevated in rodents, KYNA produces cognitive deficits similar to those seen in the disease, making down-regulation of KYNA, via inhibition of kynurenine aminotransferase II (KAT II), a potential treatment strategy. We determined, in adult Wistar rats, if the orally available KAT II inhibitor BFF816 a) prevents KYNA elevations in prefrontal cortex (PFC) after a systemic kynurenine injection and b) reverses the kynurenine-induced attenuation of evoked prefrontal glutamate release caused by stimulation of the nucleus accumbens shell (NAcSh). Systemic injection of kynurenine (25 or 100 mg/kg, i.p.) increased KYNA levels in PFC (532% and 1104% of baseline, respectively). NMDA infusions (0.15 μg/0.5 μL) into NAcSh raised prefrontal glutamate levels more than 30-fold above baseline. The two doses of kynurenine reduced evoked glutamate release in PFC (by 43% and 94%, respectively, compared to NMDA alone). Co-administration of BFF816 (30 or 100 mg/kg, p.o.) with kynurenine (25 mg/kg, i.p.) attenuated the neosynthesis of KYNA and dose-dependently restored NMDA-stimulated glutamate release in the PFC (16% and 69%, respectively). The ability to prevent KYNA neosynthesis and to normalize evoked glutamate release in PFC justifies further development of KAT II inhibitors for the treatment of cognitive deficits in SZ.

Keywords

glutamate; KAT II; kynurenic acid; microelectrode array; prefrontal cortex; schizophrenia

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1.0 Introduction

Schizophrenia (SZ), a devastating psychiatric disorder that can be traced to a combination of genetic vulnerabilities and environmental causes, affects approximately 1% of the world's population (Lewis and Lieberman, 2000). One symptom cluster of the disease, cognitive deficits, includes impairments in executive functions, such as attention, working memory, and cognitive flexibility (Keefe, 2007; Kerns et al., 2008). These are considered core symptoms (Elvevag and Goldberg, 2000) because they emerge during the prodromal period, can often be seen in first-degree relatives of patients, and correlate with the long-term functional outcome of the patient (Gold, 2004). Unfortunately, currently available antipsychotic drugs produce, at best, minor improvements in these cognitive impairments (Keefe et al., 2007a; Keefe et al., 2007b).

The cause(s) of the cognitive deficits seen in SZ patients remains unknown, but could be related to abnormally high levels of kynurenic acid (KYNA), a neuroinhibitory metabolite of the kynurenine pathway of tryptophan degradation (see Muller et al., 2011; Schwarcz et al., 2012 for review). KYNA is a potent negative modulator of the alpha7 nicotinic acetylcholine receptor (α7nAChR; Hilmas et al., 2001) and a competitive antagonist of the glycine co-agonist site of the N-methyl-D-aspartate receptor (NMDAR; Parsons et al., 1997), and its levels are elevated in post-mortem brain tissue and cerebrospinal fluid of patients with SZ (Erhardt et al., 2001; Schwarcz et al., 2001), irrespective of antipsychotic medication (Ceresoli-Borroni et al., 2006). In rodents, even relatively modest KYNA increases acutely reduce basal extracellular levels of glutamate (Konradsson-Geuken et al., 2010; Wu et al., 2010), GABA (Beggiato et al., 2014) and dopamine (Rassoulpour et al., 2005) in the prefrontal cortex (PFC) as well as in other brain areas (Rassoulpour et al., 2005; Pocivavsek et al., 2011; Beggiato et al., 2013). Notably, elevations in KYNA during sensitive periods of late gestation, have also been shown to reduce the gain of GABAergic transmission that occurs post-adolescence in the maturing PFC (Thomases et al., SFN 2014). In line with these neurochemical and electrophysiological effects, acute elevation of KYNA levels in adults, or chronic elevations of KYNA during sensitive periods of development, causes cognitive deficits that resemble those seen in persons with SZ, including diminished cognitive flexibility (Alexander et al., 2012; Alexander et al., 2013; Pershing et al., 2015), trace fear conditioning (Pershing et al., 2016), spatial working memory, and conditioned avoidance tasks (Pocivavsek et al., 2012).

In the mammalian brain, KYNA is formed mainly by the irreversible enzymatic transamination of its immediate bioprecursor kynurenine (Schwarcz et al., 2012). Of several kynurenine aminotransferases (KATs), KAT II is preferentially responsible for the neosynthesis of easily mobilizable KYNA (Guidetti et al., 2007) and has therefore been characterized in considerable detail (Han et al., 2008; Passera et al., 2011; Nematollahi et al., 2016). Inhibition of this enzyme provides a means to down-regulate cerebral KYNA production and may result in an attenuation of cognitive deficits (Schwarcz et al., 2012). Experimental proof-of-concept was provided in studies with KAT II knockout mice, which show improved performance in three hippocampus-related cognitive paradigms and a significant increase in hippocampal long-term potentiation (Potter et al., 2010). Additionally, the first-generation KAT II inhibitor *S*-ESBA demonstrates pro-cognitive effects following

intracerebral infusion in intact rats (Pocivavsek et al., 2012). Second-generation KAT II inhibitors were recently developed that are active in brain following systemic administration (Tuttle et al., 2013; Dounay et al., 2013; Koshy Cherian et al., 2014; Kozak et al., 2014). One of these compounds, the orally available BFF816, was not only found to reduce extracellular KYNA and increase extracellular glutamate levels in the rat hippocampus, but also to improve the performance of intact rats in the Morris water maze (Wu et al., 2014).

In the present study, we take advantage of BFF816 to determine, for the first time, if the effect of increased KYNA levels to reduce *evoked* glutamate release in PFC can be reversed by inhibiting the synthesis of KYNA. To this end, we utilized an experimental paradigm in which the release of glutamate was evoked by an infusion of NMDA into the shell region of the nucleus accumbens (NAcSh; Bortz et al., 2014; Bortz et al., 2016). This procedure results in an increase in cortical acetylcholine (ACh) release from basal forebrain and, subsequently, a local α7nAChR-dependent increase in prefrontal glutamate levels (Bortz et al., 2016). Notably, stimulation of the NAcSh in this manner facilitates the filtering of distractors during a sustained attention task in rodents, indicating that prefrontal glutamate, evoked under these conditions, has a positive impact on cognitive performance (St Peters et al., 2011). Thus, the restoration of *stimulated* prefrontal glutamate levels in PFC by BFF816 would represent a proof of principle for the use of KAT II inhibitors for the treatment of cognitive dysfunctions produced, in part, by elevations in brain KYNA levels.

2.0 Materials and Methods

2.1 Animals

Male Wistar rats (65-90 days of age, 280-420 g) were maintained in a temperature- and humidity-controlled room on a 12:12-hour light:dark cycle (lights on at 06:00 a.m.), and housed in pairs (pre-surgery) in plastic cages lined with corn cob bedding (Harlan Teklad, Madison, WI, USA). After implantation of the microelectrode array (MEA), animals were singly housed with *ad libitum* access to food and water. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to consider alternatives to *in vivo* techniques. Procedures involving animals were approved by the Institutional Animal Care and Use Committees of The Ohio State University and the University of Maryland School of Medicine, in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2 Reagents and test compounds

The following reagents were used to prepare and calibrate the glutamate-sensitive MEAs: m-phenylenediamine dihydrochloride (purchased from Acros Organics, NJ, USA), L-ascorbic acid, dopamine, L-glutamate monosodium salt, glutaraldehyde (25% solution in water), bovine serum albumin, and hydrogen peroxide (all obtained from Sigma Aldrich Corp., St. Louis, MO, USA) and L-glutamate oxidase (purchased from United States Biological; Salem, MA, USA).

For administration to animals *in vivo*, NMDA (Sigma Aldrich) was prepared in artificial cerebrospinal fluid (aCSF; pH 7.1-7.4; see Zmarowski et al., 2007), L-kynurenine sulfate

(purity 94%; Sai Advantium, Hyderabad, India) was dissolved in physiological saline (pH 7.1-7.4), and BFF816 (provided by Mitsubishi-Tanabe, Yokohama, Japan) was suspended in a solution of hydroxyl-propyl-beta-cyclodextrin (HPBCD; Sigma Aldrich; 20%; pH 7.1-7.4).

2.3 Microdialysis studies

Rats were anesthetized (chloral hydrate, 360 mg/kg, i.p.) and placed in a stereotaxic frame. A guide cannula (0.65 mm o.d.; Carnegie Medicin, Stockholm, Sweden) was positioned unilaterally over the medial PFC (AP: 3.2 mm anterior to bregma, ML: $\pm$ 0.6 mm from midline, DV: 2.0 mm below dura; hemispheres counterbalanced) and secured to the skull with acrylic dental cement. On the next day, a microdialysis probe (CMA/10, membrane length: 3.0 mm, Carnegie Medicin) was inserted through the guide cannula and connected to a microperfusion pump set to a speed of 1 μL/min. The freely moving animals were perfused with Ringer solution containing (in mM): NaCl, 144; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 1.7; pH 6.7. Microdialysis samples were collected every 30 min for a total of 8 hrs. Data were not corrected for recovery from the microdialysis probe.

2.3.1 Measurement of KYNA in dialysate—The content of KYNA in microdialysate samples was determined by high performance liquid chromatography. To this end, KYNA was isocratically eluted with a mobile phase containing zinc acetate (200 mM) and acetonitrile (5%; pH 6.2) and detected fluorimetrically (excitation wavelength: 344 nm; emission wavelength: 398 nm), as described (Wu et al., 2010).

2.4 Microdialysis studies: experimental procedures

2.4.1 Experiment 1: Effect of kynurenine on extracellular KYNA levels—
Following the establishment of a stable baseline of KYNA (~2 hrs), rats received an i.p. injection of 25 mg/kg or 100 mg/kg kynurenine (n = 6/ group). These and subsequent i.p. and p.o. injections were delivered at a volume of 0.1% body weight. Dialysates were collected every 30 min for a total of 8 hrs.

2.4.2 Experiment 2: Effect of BFF816 on basal and kynurenine-induced elevations of KYNA—Five groups of rats (n = 6/ group) were tested following the establishment of a stable baseline of KYNA (~2 hrs). To assess the effects of KAT II inhibition on *basal* levels of KYNA, BFF816 was administered (p.o.) at 30 mg/kg (Group 1) or 100 mg/kg (Group 2). To assess the effects of BFF816 on *elevated* KYNA levels, three groups of animals received a systemic injection of kynurenine (25 mg/kg, i.p.) immediately following a p.o. administration of HPBCD (vehicle for BFF816; Group 3), 30 mg/kg BFF816 (Group 4) or 100 mg/kg BFF816 (Group 5). In all groups, dialysates were collected every 30 min for a total of 8 hrs.

2.5 Biosensor studies

2.5.1 Preparation of glutamate-sensitive MEAs—MEAs were composed of a ceramic paddle with a stainless steel tip bearing four 15×333 μm platinum recording sites and a region that interfaces with the preamplifier. Each pair of recording sites (Figure 1A) was designated to be either glutamate-sensitive (Gluox) or not (sentinel; see Rutherford et al., 2007 for further details on MEA assemblage). This coating design (Figure 1B) permits

the isolation of the electrical signal driven solely by the oxidation of glutamate by subtracting the sentinel current from that from of the Gluox channel (i.e. self-referencing; Burmeister and Gerhardt, 2001; Rutherford et al., 2007, Konradsson-Geuken et al., 2009; Bortz et al., 2014). MEAs were calibrated *in vitro* using the FAST-16 MKII electrochemical recording system just prior to implantation (Figure 1C). Calibration criteria for each sensor were determined as previously described (Burmeister and Gerhardt, 2001; Rutherford et al., 2007 Konradsson-Geuken et al., 2009; Bortz et al., 2014), and all sensors used for analysis met these criteria.

2.5.2 Implantation of MEAs and infusion cannulae—Animals were anesthetized using isoflurane (2%, 0.8 L/min) and implanted with an MEA unilaterally in the PFC (in mm from bregma, midline and dura: AP: +2.7, ML: ±0.65, DV: –3.9; hemispheres counterbalanced), and a stainless steel guide cannula (Plastics One, Roanoke, VA) ipsilaterally in the NAcSh (at a 10° angle, in mm: AP: +0.4, ML: ±0.7, DV: –7.4; Figure 1D). The Ag/AgCl reference electrode was implanted in the contralateral side at a site distant from the recording area. All coordinates were determined using a stereotaxic atlas (Paxinos and Watson, 1998).

2.5.3 In vivo glutamate recordings—On the first day after MEA implantation, rats were placed in a wooden, electrically- shielded testing box (H 58 cm; W 342 cm; L 317 cm), without connecting them to the preamplifier, to allow habituation to the test environment prior to recording. On the second day, recordings of cortical glutamate began (3 consecutive test days). At the onset of each test day, the animals, allowed to move freely, were connected to the preamplifier and then remained undisturbed for 1–3 hours in order to obtain a stable baseline.

2.6 Biosensor studies: experimental procedures

2.6.1 Experiment 3: Effect of kynurenine on the evoked release of glutamate—Following the baseline period, animals in Group 1 (n = 6) received (counterbalanced sessions) an injection of either saline or kynurenine (25 mg/kg, i.p.). Two hrs later, NMDA (0.15 μg in 0.5 μL) was delivered over 2 sec into the NAcSh using an infusion cannula attached to a Hamilton PB600-1 manual dispenser (Hamilton Company, Reno, NV, USA), and phasic increases in extracellular glutamate levels were recorded. Animals in Group 2 (n = 6) underwent the same protocol but received saline or the higher dose of kynurenine (100 mg/kg, i.p.).

2.6.2 Experiment 4: Effect of BFF816 on evoked glutamate release—A single group of rats (n = 5) was tested in 3 counterbalanced test sessions. Following the baseline period, animals received an oral dose of vehicle for BFF816 (HPBCD), BFF816 (30 mg/kg), or BFF816 (100 mg/kg). Two hrs later, NMDA (0.15 μg) was infused into the NAcSh as described in Experiment 3, and phasic increases in extracellular glutamate levels were recorded.

2.6.3 Experiment 5: Effect of BFF816 + kynurenine on evoked glutamate release—Two groups of rats were tested in 3 counterbalanced sessions. Following the

baseline period, animals in Group 1 (n = 6) received one of three drug combinations: a) saline (i.p.) and HPBCD vehicle (p.o.); b) kynurenine (25 mg/kg, i.p.) and HPBCD vehicle (p.o.); or c) kynurenine (25 mg/kg, i.p.) and BFF816 (30 mg/kg, p.o.). Rats in Group 2 (n = 6) were administered identical drug combinations but received a higher dose of BFF816 (100 mg/kg, p.o.). Two hrs later, on each testing day, rats in both groups received an intra-NAcSh infusion of NMDA (0.15 μg), and phasic increases in extracellular glutamate levels were recorded.

2.7 Histology

At the conclusion of each experiment, animals received a sub-lethal dose of sodium pentobarbital and were transcardially perfused with 200 ml of heparinized saline following by 100 ml of 10% formalin. Cryostat-sectioned coronal brain slices (70 μm) were mounted on gelatin-coated slides, stained using cresyl violet, and examined under a light microscope for verification of microelectrode and cannula placement (Figure 1D).

2.8 Data analysis

The microdialysis data from Experiments 1 & 2 were analyzed using an initial 2-way omnibus ANOVA with time-point as a within-subjects condition and drug combination or drug dose as a between-subjects condition. If significance was determined in any omnibus ANOVA, *post-hoc* multiple comparisons were performed using the Bonferroni-corrected t-test.

The glutamate signal (Experiments 3-5), initially measured in pA, was transformed to a concentration equivalent (μM) on the basis of each sensor's individual calibration slopes, generated immediately before surgery. Measurements (taken after self-referencing) included: (i) basal glutamate levels (μM) in the PFC (calculated as the average of 10 sampling points prior to the marking of an event), (ii) maximal prefrontal glutamate increase (μM and % change from basal) following stimulation of the NAcSh, (iii) effect of kynurenine as measured by the % reduction of NMDA-evoked glutamate release; (iv) effect of BFF816 as measured by the % recovery of kynurenine-induced inhibition of evoked glutamate; (v) the latency (sec) between NMDA infusion and the onset in the rise of the glutamate peak; and (vi) time to clear 80 percent (T_{80}) of the glutamate increase from its maximal amplitude (sec). Comparisons were performed using the IBM SPSS statistics program (version 22, IBM Corporations, Armonk, NY, USA). An initial, omnibus ANOVA was performed separately for each dependent measure in each experiment. If significance was determined, *post-hoc* multiple comparisons were performed using the Bonferroni-corrected t-test.

3.0 Results

3.1 MEA, cannula, and probe placements

All subjects included in this analysis had histologically confirmed microdialysis probe, MEA, and cannula placements (Figure 1D). Notably, animals that had errant cannula placements (for example in the NAc core) in the biosensor experiments did not show any stimulation-evoked glutamate increase in the PFC (data not shown).

3.2 Experiments 1 & 2: Systemically administered kynurenine increases KYNA levels in the PFC: attenuation by co-administration of BFF816

In line with previous reports (Konradsson-Geuken et al., 2010; Alexander et al., 2012), systemically administered kynurenine, but not its vehicle, increased extracellular KYNA levels in the PFC in a dose- and time-dependent fashion (dose × time interaction; $F_{32,176} =$ 23.33, $P < 0.001$; Figure 2). Both doses significantly increased KYNA levels compared to vehicle (timepoints 2.5-8, P's < 0.05). The higher dose of kynurenine (100 mg/kg) was more effective than the lower dose (25 mg/kg) at 2.5 - 6.5 hrs (all P's < 0.05).

Oral administration of BFF816 (30 and 100 mg/kg), but not its vehicle (HPBCD), significantly reduced *basal* KYNA levels in the PFC (Figure 3; maximum decrease from baseline: 30 mg/kg: 22.8 ± 7.7%; 100 mg/kg: 29.1 ± 3.8%; dose × time interaction: $F_{32,176} =$ 2.303, $P = 0.004$).

Both doses of BFF816 also reduced *experimentally elevated* levels of KYNA in the PFC. Compared to the effect of treatment with kynurenine + HPBCD (the vehicle for BFF816), BFF816 (30 mg/kg) attenuated kynurenine (25 mg/kg)-induced KYNA increases over an extended period of time ($F_{(32,192)} = 49.487$, $P < 0.001$). There was a trend for the higher dose of BFF816 (100 mg/kg) to be more effective than the lower dose, particularly in the first hours after application (Figure 4), but there was no significant difference between the two doses overall ($P > 0.05$).

3.3 Experiment 3: Kynurenine attenuates evoked but not basal glutamate release in the PFC

Systemic administration of kynurenine dose-dependently reduced *stimulated* glutamate release in PFC following an infusion of NMDA into the NAcSh. Figures 5A–C illustrate the MEA tracings from a single representative animal. Figure 5A depicts current/time recordings from the individual Gluox and sentinel recording sites, whereas Figures 5B and 5C show the self-referenced current tracings that are due specifically to the oxidation of glutamate (following 25 and 100 mg/kg kynurenine, respectively). NAcSh stimulation with NMDA resulted in phasic increases in PFC glutamate (left section of traces in Figures 5B and 5C), which were similar in amplitude to those reported previously (Bortz et al., 2014). However, when NMDA was infused 2 hours after kynurenine (25 or 100 mg/kg; right section of traces in Figures 5B and 5C), the stimulated glutamate increases in the PFC were attenuated, with the higher dose of kynurenine producing a significantly greater degree of attenuation. These individual effects were replicated across the group data (Figure 5D; 25 mg/kg group: NMDA + saline = 6.22 ± 1.47 μM; NMDA + kynurenine = 3.32 ± 0.97 μM, 42.5 ± 10.1% decrease of evoked release; 100 mg/kg group: NMDA + saline = 3.77 ± 0.56 μM; NMDA + kynurenine = 0.24 ± 0.13 μM, 94.2 ± 4.4% decrease of evoked release**;** n = 6 each, 25 mg/kg vs. 100 mg/kg, $F_{1,11} = 24.97$, $P = 0.001$). Glutamate peak onset (overall mean = 53.2 ± 10.3 sec) and clearance ($T_{80,}$ overall mean = 7.5 ± 2.5 sec) after NMDA infusion were stable across all experiments and not affected by kynurenine or BFF816 pre-treatment (all P values > 0.05). Therefore, these values will not be discussed in subsequent experiments.

In contrast to the effects on *stimulated* glutamate release, kynurenine treatment produced only a small attenuation of *basal* glutamate levels that did not reach statistical significance (25 mg/kg kynurenine group: saline = 1.67 ± 1.25 μM, kynurenine = 1.36 ± 0.98 μM; 100 mg/kg kynurenine group: saline = 0.80 ± 0.45 μM, kynurenine = 0.56 ± 0.25 μM, P > 0.05).

3.4 Experiment 4: Orally administered KAT II inhibitor does not affect basal or evoked glutamate release in the PFC under endogenous levels of KYNA

We next determined the impact of inhibition of *endogenous* KYNA synthesis via administration of BFF816 (30 and 100 mg/kg), on basal and stimulated glutamate release. BFF816 did not significantly affect *basal* glutamate levels (saline = 1.42 ± 1.09 μM; 30 BFF816 = 1.55 ± 1.11 μM; 100 BFF816 = 1.69 ± 1.26 μM; n = 5, $F_{2,14}$ = 0.012, P = 0.988) in the absence of elevated kynurenine levels. Similarly, the decrease in *endogenous* KYNA levels produced by BFF816 (see Figure 3) did not significantly change NMDA-stimulated glutamate levels in the PFC (NMDA + saline = 5.11 ± 1.57 μM; NMDA + 30 BFF816 = 8.80 ± 1.64 μM; NMDA + 100 BFF816 = 7.53 ± 1.81 μM; n = 5, $F_{2,14}$ = 1.249, P = 0.322).

3.5 Experiment 5: Effect of BFF816 + kynurenine on basal and evoked glutamate release in the PFC

The final set of experiments was designed to examine whether BFF816 could restore *stimulated* glutamate levels that had been attenuated following kynurenine administration (i.e. elevated levels of KYNA). Figures 6A–B illustrate the self-referenced recordings from individual animals. NMDA (0.15 μg) infusion into the NAcSh, 2 hours after i.p. saline (vehicle for kynurenine) + p.o. HPBCD (vehicle for BFF816), resulted in a phasic increase in PFC glutamate (Figure 6A) similar to that seen in Experiment 3. Again similar to Experiment 3, kynurenine (25 mg/kg), administered to the same animals in a separate test session, significantly attenuated the stimulated glutamate release (from 3.10 to 1.33 μM; Figure 6A middle section). 30 mg/kg BFF816 reduced the magnitude of the kynurenine-suppressed glutamate release by 14%, but this did not reach statistical significance (Figure 6A right section). In contrast, rats that received kynurenine and the higher dose of BFF816 (100 mg/kg) showed a partial, but significant, restoration of the evoked glutamate release relative to the kynurenine-induced suppression (by 41%; Figure 6B right section). These findings were replicated across a group of animals (Figure 6C), revealing a main effect of the two drug combinations ($F_{2,20}$ = 31.505, P = 0.001) that interacted with the dose of BFF816 ($F_{2,20}$ = 4.621, P = 0.022). Specifically, 30 mg/kg BFF816 partially prevented the suppressing effects of kynurenine on NMDA-evoked glutamate release (30 mg/kg BFF816 group: NMDA = 6.01 ± 1.57 μM; NMDA + kynurenine = 3.04 ± 1.36 μM, NMDA + kynurenine + BFF = 4.00 ± 0.87 μM, 15.6 ± 3.0% recovery from NMDA + kynurenine; $F_{1,11}$ = 15.055, P = 0.003). Following the administration of kynurenine + 100 mg/kg BFF816, glutamate levels were significantly rescued (Figure 6C) and no longer significantly different from saline + HPBCD controls (100 mg/kg BFF816 group: NMDA = 4.76 ± 0.88 μM, NMDA + kynurenine = 0.79 ± 0.44 μM, NMDA + kynurenine + BFF = 4.01 ± 1.61 μM, 68.8 ± 19.4% rescue from NMDA + kynurenine; $F_{(1,11)}$ = 0.906, P = 0.364). Similar to previous experiments, *basal* glutamate levels were not significantly affected by kynurenine or BFF816 treatment (30 mg/kg BFF816 group: saline + HPBCD = 0.45 ± 0.18 μM, kynurenine + HPBCD = 0.31 ± 0.11 μM, kynurenine + BFF = 0.34 ± 0.08 μM; 100 mg/kg

BFF816 group: saline + HPBCD = 0.44 ± 0.18 μM, kynurenine + HPBCD = 0.47 ± 0.15 μM, kynurenine + BFF = 0.50 ± 0.21 μM, P > 0.05).

4.0 Discussion

The present experiments generated several novel results. First, systemic administration of kynurenine dose-dependently attenuates *stimulated* glutamate released in the PFC following an infusion of NMDA into the NAcSh. Second, BFF816, a selective KAT II inhibitor that can be administered orally and crosses the blood-brain barrier (Wu et al., 2014), substantively counteracts, in a dose-dependent manner, kynurenine-induced increases in extracellular KYNA levels. Finally, administration of BFF816 partially restores s*timulated* glutamate release in the PFC, and the magnitude of this effect is dependent upon the BFF816 dose used. Taken together, these data indicate that increases in cortical KYNA levels disrupt glutamate transmission in the PFC, and that both KYNA levels and glutamate transmission can be partially normalized via selective inhibition of KAT II activity. The following sections will address several issues raised by these experimental results and discuss the implications with respect to the etiology and therapeutics of SZ.

4.1 Intra-accumbens stimulation of prefrontal glutamate release

Infusions of NMDA into the NAcSh consistently elevate extracellular glutamate levels in the PFC, as demonstrated using rapid *in situ* electrochemistry (i.e. the MEA; Bortz et al., 2014; Bortz et al., 2016). The glutamate signal measured by this method has been referred to as *phasic* based on its high temporal resolution and faster kinetics (Burmeister & Gerhardt, 2001; Bortz et al., 2014; Parikh et al., 2008; Pocivavsek et al., SFN abstract 2014). It is not known, however, if this signal is caused by a single release event or by several signals that are captured under a single symmetrical wave form due to limited temporal resolution. In fact, the glutamate wave recorded in the present study was occasionally clearly split into what appeared to be multiple release events (Figure 6).

The extended latency (40-120 sec) between the NAcSh stimulation by NMDA and the increase in the glutamate signal in the PFC was unexpected, as trans-synaptic activation of the presumed neural circuit (NAcSh to basal forebrain to PFC), which is believed to mediate this effect (Zaborszky and Cullinan, 1992; Zmarowski et al., 2007; St Peters et al., 2011; Bortz et al., 2016), would take only msec to occur. The delayed release of prefrontal glutamate following intra-NAcSh NMDA therefore suggests a more complex mechanism. We are currently testing the hypothesis that protracted inhibitory processes initiated by interneurons within the NAcSh and/or the basal forebrain contribute to this phenomenon.

The increase in prefrontal glutamate levels following intra-accumbens infusions of NMDA has also been seen using microdialysis/HPLC methodology (Valentini et al., SFN abstract 2016). Measured by either MEA or microdialysis, this effect is secondary to an initial activation of local α7nAChRs (Bortz et al., 2016; Valentini et al., SFN abstract 2016), which are located on both neurons (Wang et al., 2006; Livingstone et al., 2010) and astrocytes (Duffy et al., 2011). Both cell types participate in the regulation of glutamatergic neurotransmission in the PFC (Wang et al., 2006; Vollbrecht et al., 2014), however, the relative roles of neuronal and astrocytic mechanisms remain to be determined. Notably,

diverse cellular origins of glutamate may also account for the currently unexplained, different temporal profiles observed by microdialysis and MEA. Thus, glutamate levels measured by dialysis remained elevated for ~40 min after the infusion of NMDA into the NAcSh (Valentini et al., SFN abstract, 2014), whereas evoked glutamate levels determined by the sensor typically returned to baseline values in ~30-45 sec. Future studies will also need to elucidate why basal levels of glutamate are affected by kynurenine or BFF816 when measured by dialysis (Pocivavsek et al., 2016; Wu et al., 2010; Wu et al., 2014) but not by the MEA (Sections 3.3-3.5).

4.2 Effects of KAT II inhibition on KYNA and glutamate levels in the PFC

In line with our recent microdialysis study (Wu et al., 2014), BFF816 decreased *basal* extracellular KYNA levels in the PFC relatively modestly, to a nadir of 30%, following administration of the higher dose of the KAT II inhibitor (100 mg/kg). Interestingly, BFF816 was far more effective following an acute, kynurenine-induced elevation of KYNA levels, causing a reduction by almost 75% (Figures 3 and 4). Thus, as discussed in more detail recently (Pocivavsek et al., 2016), KAT II inhibition appears to preferentially affect a rapidly mobilizable pool of KYNA.

We demonstrated previously that the kynurenine-induced reduction of *basal* extracellular glutamate levels was secondary to the neosynthesis of KYNA (Konradsson-Geuken et al., 2010; Wu et al., 2010; Wu et al., 2014). Thus, we assumed that inhibition of KYNA production by BFF816 was responsible for the KAT II inhibitor's ability to counteract the reduction of *stimulated* glutamate levels by kynurenine, as demonstrated in Experiment 5. However, Experiment 4 revealed that BFF816, when delivered alone, i.e. in the absence of exogenously supplied kynurenine, was insufficient to produce a significant increase in stimulated glutamate release, *as assessed by MEA*. This finding was unexpected, because both *S*-ESBA, perfused locally (Wu et al., 2010), and BFF816, delivered orally (Wu et al., 2014), increase *basal* glutamate levels in the PFC, as measured via microdialysis. We therefore conclude that endogenous KYNA does not regulate *stimulated* glutamate release unless KYNA levels are elevated. This notion is supported by another study where a systemically-administered KAT II inhibitor, PF-04859989, was shown to restore nicotine-induced glutamate transients in PFC after kynurenine injections, but had no effect in the absence of exogenous kynurenine (Koshy Cherian et al., 2014).

4.3 Translational implications

One of the key findings of the present study was that an acute elevation of KYNA inhibits NAcSh- mediated glutamate release in the PFC. As NAcSh stimulation via the same experimental protocol used here facilitates attentional processing in rodents (St Peters et al., 2011), the prefrontal glutamate release measured in these experiments may have cognitive relevance. Notably, glutamate transmission in the PFC is critical for several higher order cognitive functions that are deficient in SZ (Keefe, 2007; Kerns et al., 2008) such as cognitive flexibility (Stefani and Moghaddam, 2005), working memory (Aultman and Moghaddam, 2001), and sustained attention (Parikh et al., 2008). Thus, the present data provide further support to the notion, posited previously (see Muller et al., 2011; Schwarcz et al., 2012 for review), that the elevated KYNA levels seen in patients (Erhardt et al., 2001;

Schwarcz et al., 2001) may play a role in the occurrence of cognitive deficits in SZ via its interference with cortical glutamate transmission.

If elevations in KYNA levels indeed contribute to the cognitive deficits in SZ, pharmacological interventions leading either to reduced uptake of kynurenine into the brain (Sekine et al., 2016) or to a reduction in KYNA neosynthesis within the brain may result in cognitive enhancement. Using the latter approach, pro-cognitive effects were indeed demonstrated with intracerebral application of the KAT II inhibitor *S*-ESBA (Pocivavsek et al., 2011), which does not cross the blood-brain barrier. Systemically active KAT II inhibitors such as BFF816 and PF-04859989 therefore represent excellent tools for hypothesis testing in pre-clinical studies (Dounay et al., 2015). BFF816 indeed improves performance in the Morris water maze after acute administration in intact rats (Wu et al., 2014). Moreover, preliminary results indicate that BFF816 is able to reverse cognitive deficits in a well-validated neurodevelopmental animal preparation that duplicates several cognitive impairments seen in SZ (Pocivavsek et al., SFN abstract 2014).

4.4 Conclusions

We showed here that acute elevation of KYNA dose-dependently inhibits *stimulated* glutamate release in the PFC. Co-administration of BFF816 reduced prefrontal KYNA levels and concurrently partially rescued glutamate release. The ability to both regulate KYNA production and attenuate the (often detrimental) effects of elevated KYNA in the PFC bodes well for the development of novel KAT II inhibitors (Lu et al., 2016), as well as other conceptually related, innovative approaches (Zakrocka et al., 2016), for the purpose of treating cognitive deficits in individuals with SZ.

Acknowledgments

Abbreviations

ACh	acetylcholine
α7nAChR	alpha7 nicotinic acetylcholine receptor
Gluox	glutamate-sensitive
HPBCD	hydroxyl-propyl-beta-cyclodextrin
KYNA	kynurenic acid
KAT II	kynurenine aminotransferase-II
MEA	microelectrode array
NMDAR	N-methyl-D-aspartate receptor
NAcSh	nucleus accumbens shell
PFC	prefrontal cortex

| **SZ** | schizophrenia |

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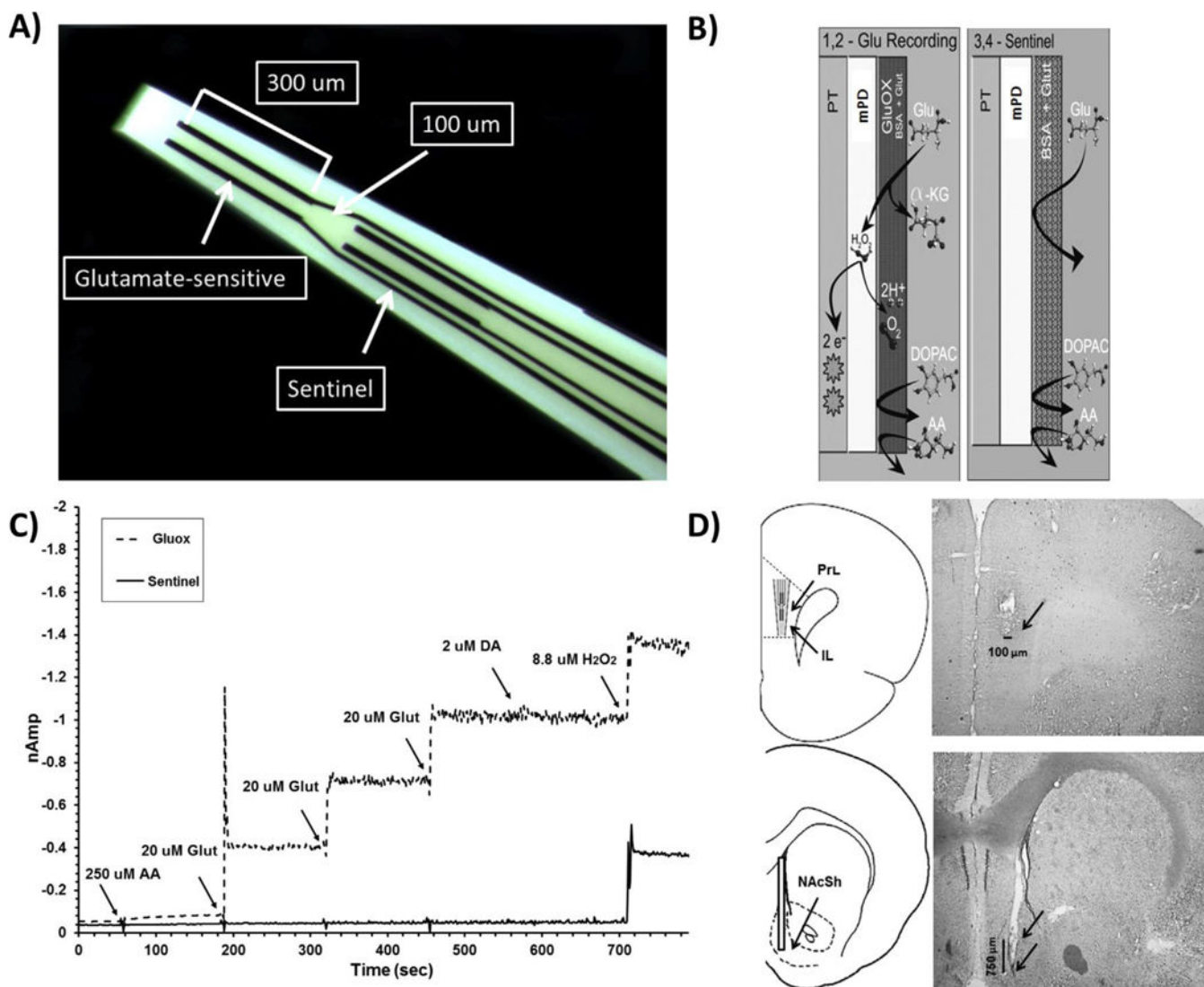
Figure 1. MEA design, signal transduction scheme, *in vitro* calibration, and representative placement

(**A**): Four platinum recording channels near the tip of the MEA allow for the detection and isolation of glutamate within a single brain sub-region (see Burmeister and Gerhardt, 2001 for further detail on self-referencing); (**B***):* Glutamate is detected *in vivo* when a glutamate molecule (Glu) contacts the platinum surface (PT) coated with glutamate oxidase (Gluox; see Rutherford et al., 2007) and is oxidized to the reporting molecule hydrogen peroxide (H_2O_2). The oxidation of H_2O_2 generates a current that is proportional to the concentration of glutamate at the surface of the MEA; (**C***): In vitro* calibrations of the MEA are conducted immediately before implantation into the PFC. The solid line tracing is from the glutamate-sensitive (Gluox) recording channel, and the dashed tracing is from the sentinel background channel. Arrows correspond to the addition of ascorbic acid (AA, 500 μL), glutamate (glut, 3 × 40 μL), dopamine (DA, 40 μL), and H_2O_2 (40 μL) into the stirred calibration beaker. Current (nAmp) is depicted along the y-axis, and time (sec) along the x-axis. The successive additions of glutamate (20 μM/aliquot) produce a linear increase on Gluox, but not sentinel

channels. Important for self-referencing, the Gluox and sentinel channels respond equally to every addition, except glutamate; (**D**): The cartoon and the corresponding photomicrograph depict the desired target location for both the MEAs (between the prelimbic (PrI) and infralimbic (IL) cortices) and infusion cannulae (within the nucleus accumbens shell). Arrows indicate the ventral termination of the MEA, guide, and infusion cannula within the respective photomicrographs. NAcc: Nucleus accumbens core; CC: Corpus callosum; aca: anterior commissure; NAcSh: Nucleus accumbens shell.
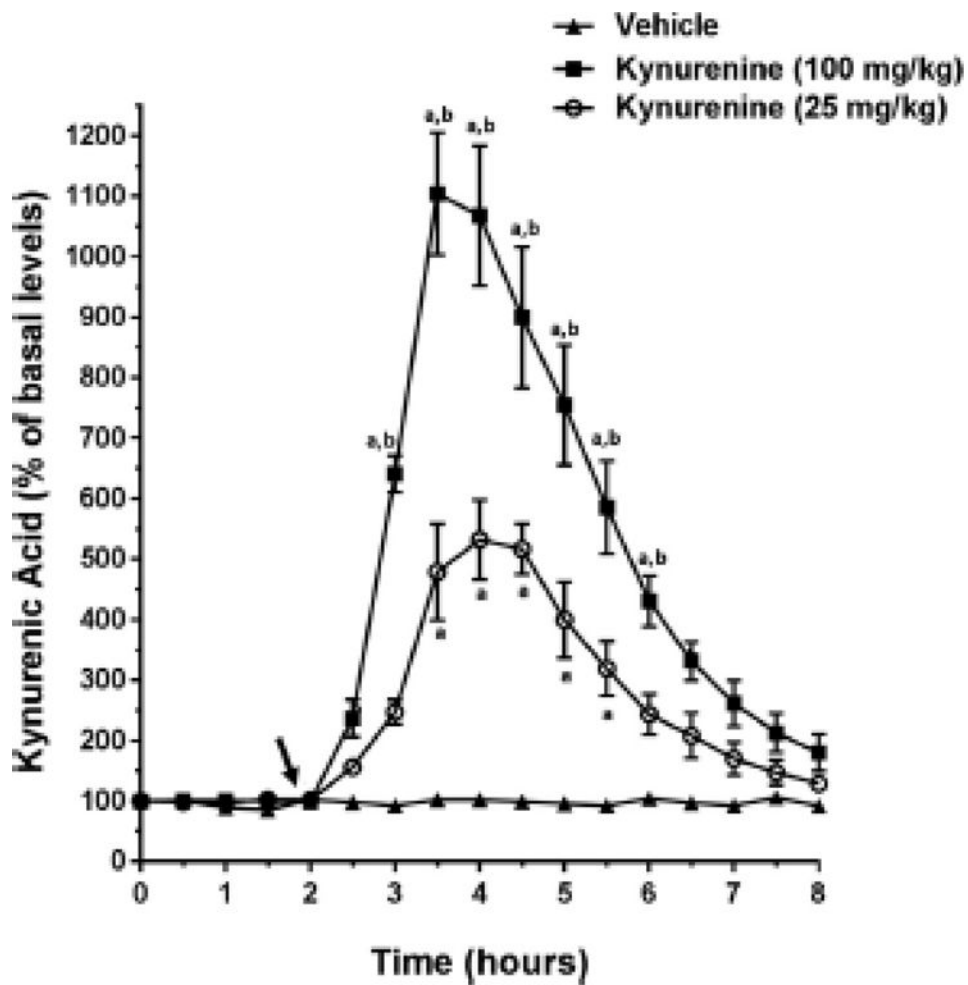
Figure 2. Systemic kynurenine administration increases extracellular KYNA levels in the PFC
Microdialysis samples were collected every 30 min after subjects received one of two doses of kynurenine (25 or 100 mg/kg, i.p.) or its vehicle solution (saline). The arrow indicates the timing of the injection. Post-hoc multiple comparisons were conducted using a Bonferroni adjusted t-test. [a] P < 0.05 vs. baseline values; [b] P < 0.05 vs. 25 mg/kg kynurenine.
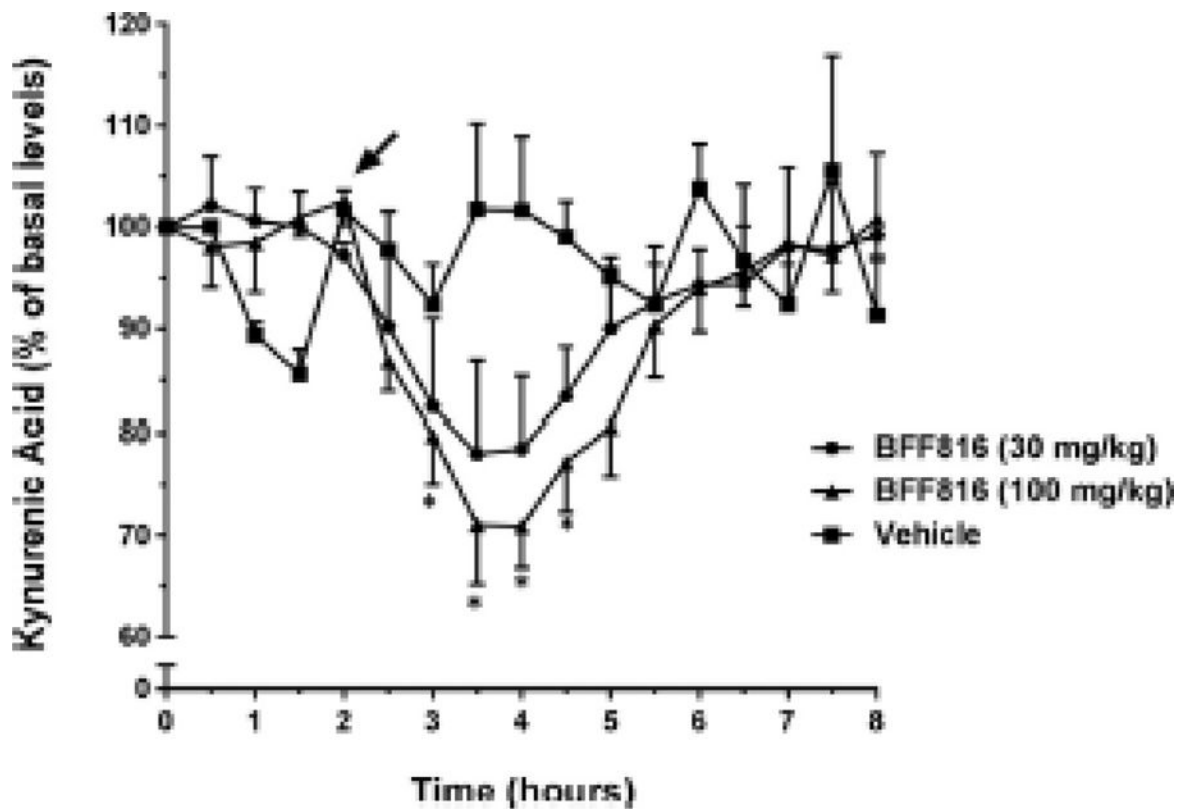
Figure 3. Oral administration of BFF816 reduces extracellular KYNA levels in the PFC
Microdialysis samples were collected every 30 min. The arrow indicates the timing of the oral administration of BFF816 (30 or 100 mg/kg, p.o.) or its vehicle solution HPBCD. * P < 0.05 vs. baseline values (Bonferroni adjusted t-test).
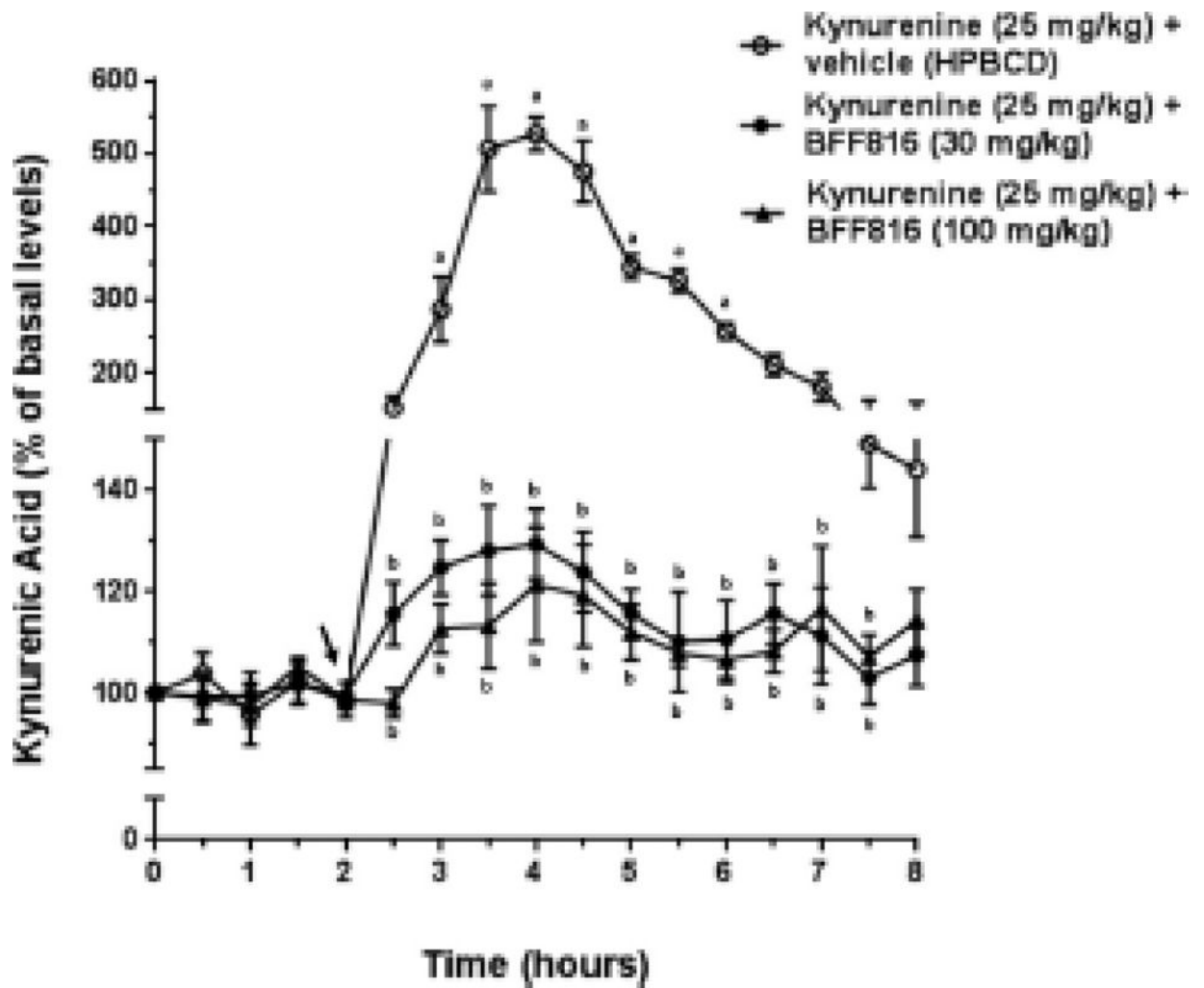
Figure 4. BFF816 attenuates the kynurenine-induced increase in extracellular KYNA levels in the PFC

Microdialysis samples were collected every 30 min. The timing of the administration of vehicle or BFF816 (30 or 100 mg/kg, p.o.), followed immediately by kynurenine (25 mg/kg, i.p.), is indicated by the solid arrow. [a] P < 0.05 vs. baseline values; [b] P < 0.05 vs. kynurenine alone (Bonferroni adjusted t-test).
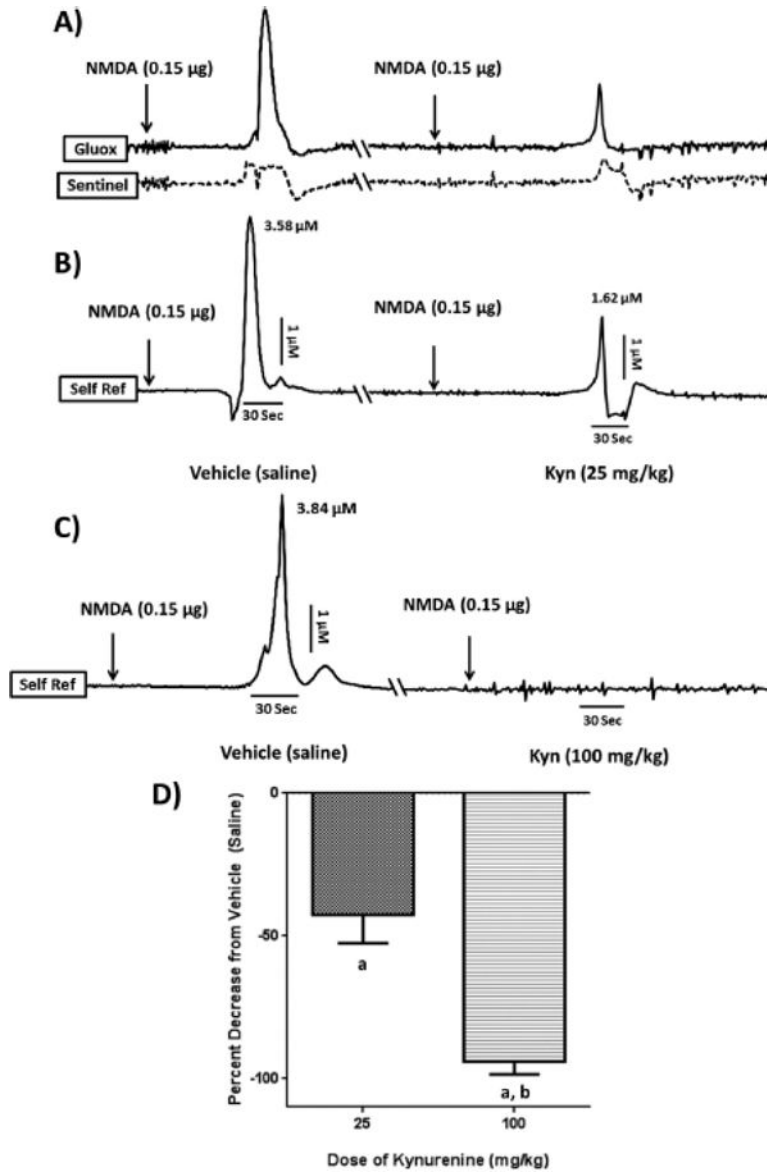
Figure 5. Systemic kynurenine decreases evoked glutamate levels in the PFC
Each set of representative tracings (panels **A–C**) was recorded from the PFC of the same
intact animal following an infusion of NMDA (0.15 μg in 0.5 μL) into the NAcSh 2 hrs after
a systemic injection of either vehicle or kynurenine. Each tracing is separated by at least 24
hrs (cut \\ in the axis), and baselines are displayed uniformly for ease of comparison (see text
for actual values). The vertical dimension represents changes in concentration (μM), whereas
the horizontal dimension reflects the passage of time (sec). The timing of NMDA infusions
relative to the glutamate peaks are indicated by arrows. (**A**): The solid-line tracing represents
the signal from the glutamate-sensitive channel (Gluox), whereas the dashed tracing
represents the signal from its adjacent sentinel channel; (**B/C**): Self-referenced (Self Ref)
signals derived from subtracting the sentinel channel from the Gluox channel, thereby
isolating the signal obtained exclusively from the oxidation of extracellular glutamate.
NMDA causes a robust increase in PFC glutamate, which is attenuated after an injection of

25 mg/kg kynurenine (i.p.) (**B**) and essentially abolished after an injection of 100 mg/kg kynurenine (i.p.) (**C**); (**D**): Group data (n = 6/ group). [a] P < 0.05 vs. vehicle; [b] P < 0.05 vs. 25 mg/kg kynurenine.
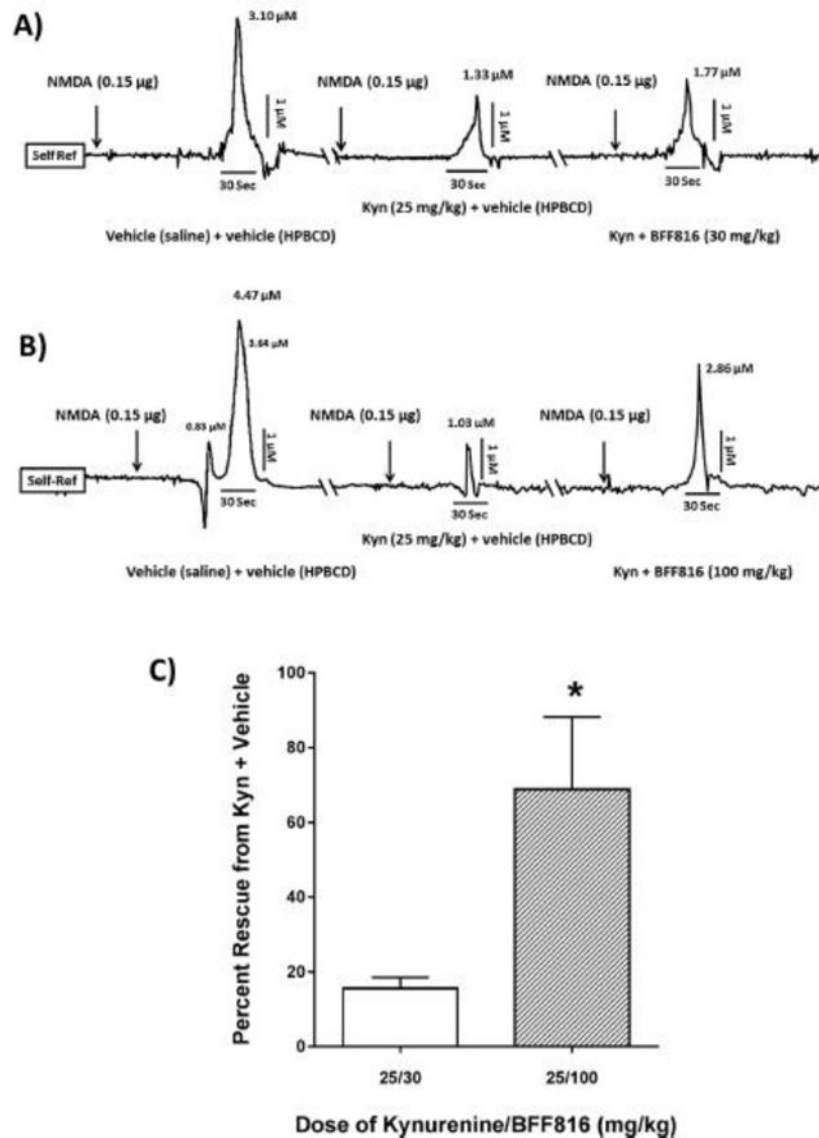
Figure 6. BFF816 counteracts the attenuating effect of kynurenine on evoked glutamate levels in the PFC

Two sets of self- referenced tracings (**A,B**) are taken from the same animal, separated by 24 hrs (cut \\ in the axis). (**A**): NMDA infused into the NAcSh after saline/HPBCD causes a robust increase in glutamate in the PFC (similar to Figure 5B). Administration of kynurenine (25 mg/kg, i.p.) + HPBCD 2 hrs prior to NMDA decreases stimulated glutamate release by 57%. Kynurenine + BFF816 (30 mg/kg, p.o.) modestly reduces this effect by 17%; (**B**): Using the same paradigm in a separate animal, a higher dose of BFF816 (100 mg/kg, p.o.) attenuates the kynurenine-induced reduction in evoked glutamate release by 41%; (**C**): Group data (n = 6/ group) illustrating percent rescue from kynurenine + vehicle. Percent rescue = BFF/kynurenine – HPBCD/kynurenine divided by HPBCD/saline. * $P < 0.05$ vs. the lower dose of BFF816 (Bonferroni adjusted t-test).