

CORTICAL KYNURENIC ACID BI-DIRECTIONALLY MODULATES PREFRONTAL GLUTAMATE LEVELS AS ASSESSED BY MICRODIALYSIS AND RAPID ELECTROCHEMISTRY

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Abstract—Using two *in vivo* methods, microdialysis and rapid *in situ* electrochemistry, this study examined the modulation of extracellular glutamate levels by endogenously produced kynurenic acid (KYNA) in the prefrontal cortex (PFC) of awake rats. Measured by microdialysis, *i.p.* administration of KYNA's bioprecursor L-kynurenine dose-dependently elevated extracellular KYNA and reduced extracellular glutamate (nadir after 50 mg/kg kynurenine: 60% decrease from baseline values). This dose-dependent decrease in glutamate levels was also seen using a glutamate-sensitive microelectrode array (MEA) (31% decrease following 50 mg/kg kynurenine). The kynurenine-induced reduction in glutamate was blocked (microdialysis) or attenuated (MEA) by co-administration of galantamine (3 mg/kg *i.p.*), a drug that competes with KYNA at an allosteric potentiating site of the $\alpha 7$ nicotinic acetylcholine receptor. In separate experiments, extracellular glutamate levels were measured by MEA following the local perfusion (45 min) of the PFC with kynurenine (2.5 μ M) or the selective KYNA biosynthesis inhibitor S-ethylsulfonylbenzoylalanine (S-ESBA; 5 mM). In agreement with previous microdialysis studies, local kynurenine application produced a reversible reduction in glutamate (nadir: -29%), whereas perfusion with S-ESBA increased glutamate levels reversibly (maximum: $+38\%$). Collectively, these results demonstrate that fluctuations in the biosynthesis of KYNA in the PFC bi-directionally modulate extracellular glutamate levels, and that qualitatively very similar data are obtained by microdialysis and MEA. Since KYNA levels are elevated in the PFC of individuals with schizophrenia, and since prefrontal glutamatergic and nicotinic transmission mediate cognitive flexibility, normalization of KYNA levels in the PFC may constitute an effective treatment strategy for alleviating cognitive

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Key words: $\alpha 7$ nicotinic receptor, astrocytes, microelectrode array, schizophrenia.

Abnormal neurotransmitter interactions within the prefrontal cortex (PFC) play an important role in the deficits in executive function seen in patients with schizophrenia (SZ; Volk and Lewis, 2002; Barch, 2005; Tan et al., 2007). Pharmacological and genetic studies suggest that these cognitive impairments, which include abnormal attention and working memory, are critically shaped by dysregulations in cortical glutamatergic neurotransmission (Krystal, 2003; Lewis and Moghaddam, 2006; Robbins and Murphy, 2006). There is also good evidence for cholinergic dysfunction in SZ (for reviews, see Hyde and Crook, 2001; Sarter et al., 2005), including reductions in the expression of nicotinic (Guan et al., 1999; Mathew et al., 2007) and muscarinic (Dean et al., 2002; Scarr et al., 2009) receptors in the PFC, and an association between polymorphisms in the gene regulating nicotinic acetylcholine receptors (nAChR) and disease transmission (Mexal et al., 2010). Together, these impairments may constitute key components of the neurochemical disarray underlying prefrontal cognitive deficits in individuals with SZ (Sarter et al., 2005).

Elevated levels of kynurenic acid (KYNA) in the PFC may contribute to the abnormal glutamatergic and nicotinic function in SZ (Schwarcz et al., 2001; Erhardt et al., 2009). This concept is in part based on the finding that endogenous KYNA, an astrocyte-derived metabolite of the kynurenine pathway of tryptophan degradation (Kiss et al., 2003), acts preferentially as a non-competitive antagonist of the $\alpha 7$ nAChR (Hilmas et al., 2001). In the PFC, as elsewhere in the brain, these receptors are present in multiple locations, including glutamatergic and cholinergic nerve terminals (Lambe et al., 2003; Dickinson et al., 2008; Duffy et al., 2009), interneurons (Alkondon et al., 2000; Krenz et al., 2001) and astrocytes (Patti et al., 2007). It is likely, in particular, that the close association of cholinergic and glutamatergic terminals in the PFC accounts for the fact that $\alpha 7$ nAChR activity regulates prefrontal glutamate release (Marchi et al., 2002; Rousseau et al., 2005; Wang et al., 2006; Konradsson-Geuken et al., 2009). By inhibiting $\alpha 7$ nAChRs at a site that is very similar to that occupied by the pro-cognitive, allosteric potentiator galantamine (Samochocki et al., 2003; Lopes et al., 2007), elevated KYNA levels may therefore cause and/or exacerbate pre-

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Abbreviations: AA, ascorbic acid; ACh, acetylcholine; aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; BSA, bovine serum albumin; DA, dopamine; GluOx, glutamate oxidase; HPLC, high-performance liquid chromatography; KAT, kynurenine aminotransferase; KYNA, kynurenic acid; MEA, microelectrode array; *m*-PD, *meta*-phenylenediamine; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PFC, prefrontal cortex; Pt, platinum; S-ESBA, S-ethylsulfonylbenzoylalanine; SZ, schizophrenia.

frontal hyponicotinergic and – secondarily—hypoglutamatergic transmission in SZ.

In the course of our studies investigating possible behavioral consequences of abnormal KYNA levels in the PFC, we recently began to examine the effects of acute KYNA manipulations on prefrontal glutamate release in intact, awake rats. In these experiments, we stimulated KYNA formation in the PFC locally by administering kynurenine, the immediate metabolic precursor of KYNA (Speciale et al., 1990; Swartz et al., 1990). In complementary studies, cortical KYNA levels were *decreased* pharmacologically by locally applied S-ethylsulfonylbenzoylalanine (S-ESBA), a selective but not brain-penetrable inhibitor of kynurenine aminotransferase II (KAT II), the major biosynthetic enzyme of KYNA in the mammalian brain (Pellicciari et al., 2006; Guidetti et al., 2007a,b). Using *in vivo* microdialysis, we observed that elevations and reductions in cortical KYNA levels resulted in reciprocal changes in extracellular glutamate levels in the PFC (Wu et al., 2010). These results were reminiscent of the bi-directional effects of fluctuating KYNA concentrations on extracellular dopamine and acetylcholine (Wu et al., 2006; Zmarowski et al., 2009).

The present study was designed to explore the relationship between KYNA and glutamate in the PFC in greater depth, using additional experimental approaches. Thus, we investigated the effects of *systemically* administered kynurenine, which raises KYNA levels throughout the brain and therefore better duplicates the situation in SZ (Rassoulpour et al., 2006). Moreover, we compared *in vivo* monitoring by microdialysis with a glutamate-sensitive microelectrode array (MEA), which allows the detection of extracellular glutamate levels with a temporal and spatial resolution not otherwise achievable (Burmeister and Gerhardt, 2001; Rutherford et al., 2007).

EXPERIMENTAL PROCEDURES

Animals

Male, adult Sprague–Dawley (Charles River Laboratories, Kingston, NY, USA) or Wistar rats (Charles River, Wilmington, MA, USA) weighing 250–450 g were used in our experiments. Animals were maintained in a temperature- and humidity-controlled room on a 12:12-h light/dark cycle (lights on at 0600 h) and had access to food and water *ad libitum*. All procedures involving animals were approved by the University of Maryland or The Ohio State University Institutional Animal Care and Use Committees in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Materials

L-Kynurenine (sulfate form; “kynurenine”), KYNA, L-ascorbic acid (AA), dopamine (DA), L-glutamate (monosodium salt), glutaraldehyde [25% (w/v) in water], bovine serum albumin (BSA), chloral hydrate, and H₂O₂ were obtained from Sigma Aldrich Corp. (St. Louis, MO, USA). Galantamine was a research gift from Janssen Pharmaceutica (Beerse, Belgium). L-Glutamate oxidase (GluOx; EC 1.4.3.11) was purchased from Seikagaku America, Inc. (East Falmouth, MA, USA). S-ESBA was synthesized as described previously (Pellicciari et al., 2006). *Meta*-phenylenediamine (*m*-

PD) was purchased from Acros Organics (Fairlawn, NJ, USA). All solutions were prepared using distilled, deionized water.

Microdialysis

Rats used in Experiment 1 were anesthetized (chloral hydrate, 360 mg/kg *i.p.*) and placed in a stereotaxic frame. A guide cannula (SciPro, Inc., Sanborn, NY, USA) was positioned unilaterally over the medial PFC (AP: 3.2 mm anterior to bregma, L: ± 0.6 mm from midline, DV: 2.0 mm below dura; hemispheres counterbalanced) and secured to the skull with acrylic dental cement. Coordinates were determined from the atlas of Paxinos and Watson (1985). On the next day, a microdialysis probe (CMA/10, membrane length: 3.0 mm, Carnegie Medicin, Stockholm, Sweden) was inserted and connected to a microperfusion pump set to a speed of 1 μ L/min. The freely moving animals were perfused with Ringer solution containing (in mM) NaCl, 144; KCl, 4.8; MgSO₄, 1.2; CaCl₂, 1.7; pH 6.7. After the establishment of a stable baseline, test compounds were administered systemically. Microdialysis fractions were collected every 30 min, and KYNA and glutamate were determined in the same samples. Data were not corrected for recovery from the microdialysis probe.

HPLC-based measurements of KYNA and glutamate from dialysates

The content of KYNA and glutamate in microdialysate samples was determined according to established high performance liquid chromatography (HPLC) procedures (Swartz et al., 1990; Quarta et al., 2004). KYNA was measured following isocratic elution with a mobile phase containing 200 mM zinc acetate and 5% acetonitrile (pH 6.2) and detected fluorimetrically (excitation wavelength: 344 nm; emission wavelength: 398 nm). Glutamate (o-phthalaldehyde/ β -mercaptoethanol derivatization; excitation wavelength: 390 nm; emission wavelength: 460 nm) was determined fluorimetrically after gradient elution.

The glutamate-sensitive microelectrode and the detection of glutamate-generated signals

The MEA for freely-moving animals consists of a ceramic paddle containing, at its tip, four platinum (Pt) recording sites, interfaced with a pre-amp headstage (see Konradsson-Geuken et al., 2009, for details on this assemblage). The four 15 \times 333 μ m Pt recording sites are arranged in two pairs beginning approximately 100 μ m from the electrode tip. One pair of recording sites was designated to be sensitive to glutamate plus other endogenous electroactive species and was coated with GluOx (2%, 1 unit/1 μ L, 100 nL), BSA (1%) and glutaraldehyde (0.125%). The remaining pair served as a background or sentinel site, sensitive to the oxidation of the same electroactive molecules except for glutamate. This coating arrangement and the design of the MEA allows for a self-referencing procedure in which the current derived exclusively from the oxidation of glutamate can be isolated (Day et al., 2006; Rutherford et al., 2007; Konradsson-Geuken et al., 2009). Coated MEAs were allowed to dry for ≥ 2 days at room temperature (25 °C) and low humidity prior to *in vitro* calibration. *m*-PD (5.0 mM) was then electropolymerized onto all sites in order to reduce access of potential electroactive interferents, including AA and catecholamines, to the Pt recording sites (Mitchell, 2004). Electroplating was conducted in bubbled nitrogen using cyclic voltammetry (peak-to-peak amplitude of 0.25 V every 0.05 s for 25 min).

The enzyme detection scheme for generating the current derived by the selective oxidation of glutamate is more fully described elsewhere (Day et al., 2006; Rutherford et al., 2007; Konradsson-Geuken et al., 2009). Briefly, at the glutamate-sensitive sites, glutamate is oxidized by GluOx, generating α -ketoglutarate and H₂O₂. Because the MEA is maintained at a constant potential (+0.7 V vs. Ag/AgCl reference), the H₂O₂ reporting

molecule is oxidized, yielding two electrons. The resulting current is then amplified and recorded by a FAST-16 *mkI* recording system (Quanteon, LLC, Nicholasville, KY, USA). On the sentinel channels, extracellular glutamate reaches the Pt surface but in the absence of GluOx no oxidation current is generated. Any current detected at these sites is due to endogenous electroactive molecules other than glutamate.

In vitro calibration of microelectrodes

MEAs were calibrated prior to implantation. Calibrations were performed in a stirred solution of phosphate-buffered saline (PBS; 0.05 M, 40 mL, pH 7.4, 37 °C). After stabilization, AA (250 μ M), glutamate (3 \times 20 μ M), DA (2 μ M), and H₂O₂ (8.8 μ M) were sequentially added to the calibration beaker. Amperometric signals were acquired at a rate of 2.0 Hz. The slope (sensitivity, pA/ μ M glutamate), limit of detection (μ M glutamate), selectivity (ratio of glutamate over AA), and linearity (R^2) were calculated. In order to be used for subsequent *in vivo* recordings, the MEAs had to conform to the following calibration criteria (single electrode mode): (i) similar background current (i.e. no greater than a 20 pA difference between the glutamate-sensitive and sentinel channels), (ii) linear response to increasing concentrations of glutamate ($R^2 > 0.998$), (iii) a minimum slope of -3.0 pA/ μ M glutamate, (iv) a limit of detection of ≤ 0.5 μ M, and (v) a high selectivity for glutamate over either AA or DA (i.e. $> 50:1$).

Implantation of MEAs and dialysis probes

In Experiment 2, rats received, under isoflurane anesthesia, unilateral implants of MEAs in the PFC (AP: 2.7 mm anterior to bregma, L: ± 0.6 mm from midline, DV: 3.9 mm below dura; hemispheres counterbalanced). In some animals (Experiment 2B), a guide cannula for microdialysis probes (see above) was implanted 200 μ m anterior to the MEA such that the membrane tip of the probe (3.0 mm) could be used for the intra-cortical delivery of test compounds in close proximity to the MEA. A dummy cannula was positioned in the guide cannula and extended 1.0 mm beyond the tip of the guide. An Ag/AgCl reference electrode was implanted in a contralateral site distant from the recording area.

In vivo recordings and intra-cortical perfusions

All electrochemical recordings were conducted in freely moving rats in a large wooden box (57.2 cm H \times 41.9 cm W \times 17.0 cm L) 2–6 days after implantation of the MEA (Experiment 2A) or MEA/dialysis probes (Experiment 2B). Animals were placed in the recording box and connected to the head stage. Stable baseline signals were recorded following a 3–4 h habituation period. In Experiment 2A, the effects of systemic administration of test compounds on cortical glutamate were determined. In the first study, rats were treated with kynurenine (25 or 50 mg/kg i.p.; $n=5$ /dose). In a second study, we tested the hypothesis that the effect of kynurenine was mediated by the antagonism of $\alpha 7$ nAChRs. Thus, we injected galantamine (3.0 mg/kg i.p.), a positive allosteric modulator that binds at a site of the $\alpha 7$ nAChR that is very similar to that targeted by KYNA (Lopes et al., 2007), 5 min prior to kynurenine (50 mg/kg, $n=5$). Recordings continued for 6 h post-injection. Another group of rats ($n=5$) was injected with both kynurenine and galantamine except that, in this case, the administration of galantamine was delayed until 3 h post-kynurenine.

In Experiment 2B, the effects of local changes in KYNA on cortical glutamate levels were determined using counterbalanced perfusions of kynurenine (2.5 μ M; to increase KYNA) or S-ESBA (5.0 mM; to decrease KYNA) on consecutive recording days ($n=5$). A stable glutamate baseline was achieved during which artificial cerebrospinal fluid (aCSF, pH=7.2; Zmarowski et al., 2009) was locally perfused (1.25 μ L/min) for 45 min. Kynurenine

or S-ESBA (solutions adjusted to pH 7.1–7.4) was then perfused at the same rate for 45 min, followed by a return to control aCSF perfusion for 45 min. Finally, in a separate group of rats, we determined the effects of S-ESBA (5.0 mM) on glutamate with the MEA potential reduced to +0.25 V (from +0.7 V). As glutamate-derived H₂O₂ does not oxidize at this lower potential, the elimination of the self-referenced signal at 0.25 V supports the interpretation that differences in current at the GluOx vs. sentinel sites reflected current derived from extracellular glutamate.

Histology

At the conclusion of each experiment, animals were anesthetized with isoflurane and then given an overdose of pentobarbital. Brains were removed and stored in formalin (10%) for at least 24 h, and then transferred to a sucrose solution (30%) for at least 3 days. Brains were sectioned using a cryostat; and coronal and sagittal sections (50 μ m) were mounted on gelatin-coated slides, stained using Cresyl Violet and examined under a light microscope for verification of MEA and dialysis probe placements.

Data analysis

Microdialysis data were expressed as a percent change from baseline as baseline values did not differ significantly among treatment groups. The time-dependent effects of drug administration were determined using two-way analysis of variance (ANOVA) with DOSE and TIME as factors. A Huynh-Feldt correction was utilized in order to reduce Type I errors associated with repeated measures ANOVAs (Vasey and Thayer, 1987). Individual post hoc comparisons were conducted using *t*-tests between dependent means and a Bonferroni adjustment of α level. For the microelectrode experiments, group comparisons of absolute glutamate levels or various temporal dimensions of the glutamate signal were analyzed using one- and two-way ANOVAs. In some instances, when only percentage data were being compared, *t*-tests between independent means were utilized. Significance was defined as $P < 0.05$. All statistical tests were performed using SPSS for Windows (Version 17.0; Chicago, IL, USA).

RESULTS

Experiment 1: effects of systemic kynurenine on cortical KYNA and glutamate levels as measured by microdialysis

The dose- and time-dependent effects of systemically administered kynurenine on extracellular levels of KYNA and glutamate in the PFC are illustrated in Fig. 1. The higher dose (50 mg/kg i.p.) produced greater and longer-lasting elevations of KYNA than the lower dose (25 mg/kg i.p.) (Panel A). These differences were evident as main effects of DOSE ($F_{1,9}=142.34$, $P < 0.001$), TIME ($F_{15,135}=107.86$, $P < 0.001$), and DOSE \times TIME interaction ($F_{15,135}=29.54$, $P < 0.001$). Panel B demonstrates that kynurenine also caused a dose-dependent reduction in prefrontal glutamate levels that was a mirror image of the elevations seen in KYNA. Again, the effect of the two doses on glutamate differed in terms of the magnitude of the decrease ($F_{1,9}=13.05$, $P=0.006$), its duration ($F_{15,135}=17.06$, $P < 0.001$), and their interaction ($F_{15,135}=4.01$, $P < 0.001$).

The effects of kynurenine on cortical glutamate levels are hypothesized to reflect increased $\alpha 7$ nAChR inhibition by elevated levels of KYNA (Wu et al., 2010). This was tested by applying galantamine, an $\alpha 7$ nAChR agonist that targets a very similar site of the $\alpha 7$ nAChR as KYNA

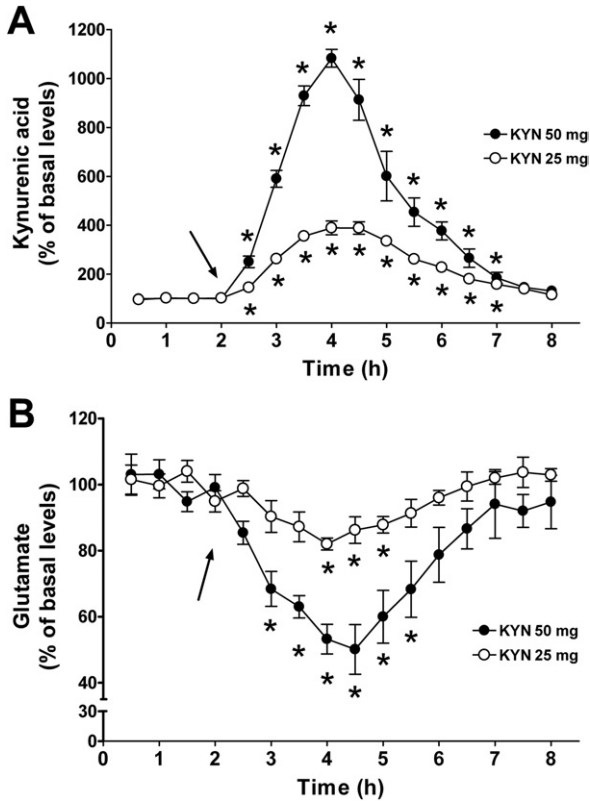


Fig. 1. Effects of systemically administered kynurenine (25 and 50 mg/kg i.p.; arrows) on the extracellular levels of KYN and glutamate in the rat PFC, assessed by *in vivo* microdialysis. KYN and glutamate were determined in the same samples, as described in the text. Kynurenine produces a dose- and time-dependent increase in KYN levels (A), which is closely mirrored by dose-dependent reductions in glutamate levels (B). Baseline values, average of four samples, were KYN: 2.7 ± 0.2 nM; glutamate: 1.9 ± 0.1 μ M, which were obtained from all animals. Data are the mean \pm SEM of 5 (25 mg/kg) and 6 (50 mg/kg) rats, respectively. * significantly different from last baseline value.

(Lopes et al., 2007), in conjunction with kynurenine (Fig. 2). Co-administration of galantamine (3.0 mg/kg i.p.) with kynurenine (50 mg/kg) had no effect on the ability of kynurenine to elevate KYN levels (cf. Figs. 2A and 1A; $F_{1,10}=0.94$, $P=0.36$). Despite this persistent elevation of KYN levels, galantamine completely blocked the ability of kynurenine to reduce cortical glutamate levels (cf. Figs. 2B and 1B, $F_{1,10}=23.22$, $P=0.001$). Injection of galantamine (3.0 mg/kg i.p.) alone had no effect on cortical levels of KYN (Fig. 2A) or glutamate (Fig. 2B).

Experiment 2A: effects of systemic kynurenine on cortical glutamate levels as measured by the microelectrode array

The following experiments utilized the glutamate-sensitive MEA to examine the effects of fluctuating KYN levels on prefrontal glutamate. Prior to implantation, MEAs were calibrated in a beaker with known concentration of potential analytes. Fig. 3 illustrates the results of a representative *in vitro* calibration. Current tracings following the administration of AA, glutamate, DA and H₂O₂ (indicated by

arrows) are shown from each of the four channels. The top two tracings (GluOx) depict current at the glutamate-sensitive channels, whereas the bottom two tracings (sentinel) illustrate current from the sentinel or background channels. The addition of AA to the beaker produced only minor increases in oxidation current, which were, importantly, comparable on all four channels. Serial additions of glutamate (20 μ M aliquots) produced large, reproducible and linear increases in current at the GluOx sites as the concentration of glutamate in the beaker was progressively elevated. In contrast, the signal on the sentinel channels did not change as a result of the addition of glutamate. Addition of DA produced only a slight change in current, indicating the effectiveness of *m*-PD, and this modest increase was comparable across the four channels. Finally, all four recording sites exhibited similar sensitivity to the reporter molecule, H₂O₂, which is a necessary condition for the self-referencing procedure used to isolate the cur-

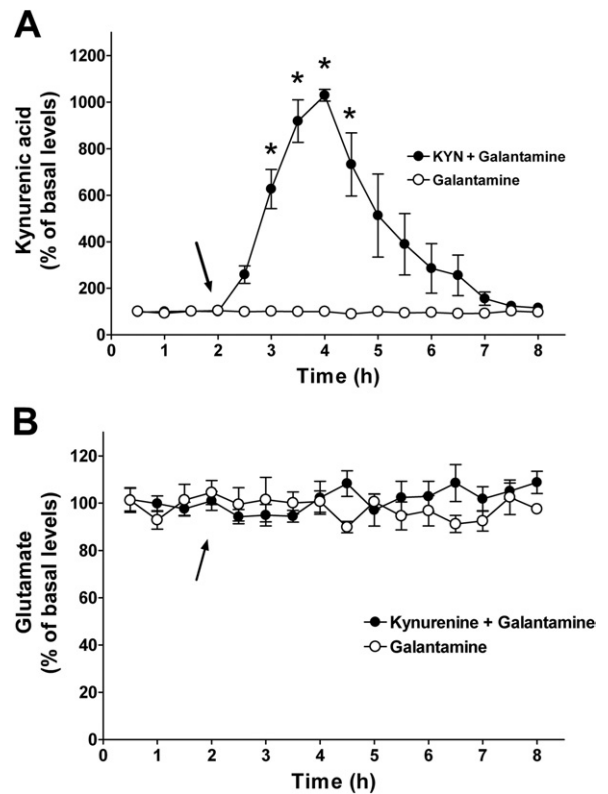


Fig. 2. Effects of systemically administered kynurenine (50 mg/kg i.p.)+galantamine (3 mg/kg i.p.) or galantamine alone (arrows) on the extracellular levels of KYN and glutamate in the rat PFC, assessed by *in vivo* microdialysis. KYN and glutamate were determined in the same samples, as described in the text. Co-administration of galantamine has no effect on kynurenine’s ability to elevate KYN (values and time course comparable to Fig. 1A) (A) but abolishes the ability of kynurenine to reduce glutamate (cf. Fig. 1B) (B). Galantamine alone has no effect on either KYN or glutamate levels. Baseline values, average of four samples, were KYN: 2.5 ± 0.2 nM; glutamate: 1.7 ± 0.2 μ M, which were obtained from all animals. Data are the mean \pm SEM of 6 (kynurenine+galantamine) and 4 (galantamine alone) rats, respectively. * significantly different from last baseline value.

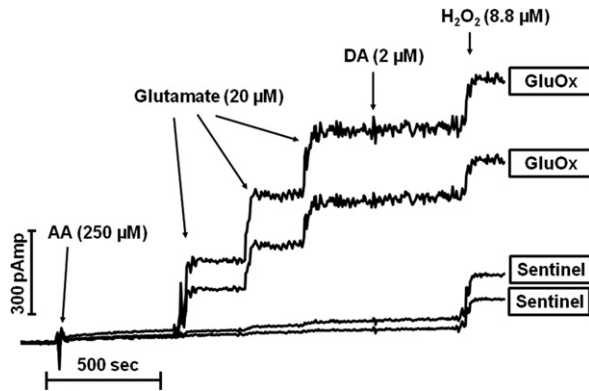


Fig. 3. Representative *in vitro* calibration of the glutamate-sensitive MEA immediately prior to implantation into the PFC. Top two tracings: glutamate-sensitive (GluOx) recording channels. Bottom two tracings: sentinel background channels. Arrows indicate addition of various substances to the calibration beaker. Current (pAmp) is depicted along the vertical axis, and time (sec) along the horizontal axis. Successive additions of glutamate (raising concentration by 20 μM /aliquot) produce a linear increase in the glutamate signal. No glutamate-related changes in current are detected on the two sentinel channels. Note equivalent sensitivities to the reporter molecule H_2O_2 on all four channels. *meta*-Phenylenediamine (*m*-PD) (see Methods) coating blocked access of negatively charged *in vivo* interferents such as ascorbic acid (AA) or dopamine (DA).

rent change caused by the oxidation of glutamate (Burmeister and Gerhardt, 2001).

Fig. 4 shows a representative MEA tracing, obtained in the PFC, following the systemic administration of kynurenine (50 mg/kg *i.p.*). After the establishment of a stable baseline, kynurenine produced a gradual and protracted reduction in signal at the glutamate-sensitive site (top tracing). There was only a minor negative drift in the current output at the background sentinel site (middle tracing). Self-referencing the sentinel from the GluOx sites yielded a signal that isolated current changes due only to the oxidation of glutamate (third tracing). The kynurenine-induced decrease in extracellular glutamate reached a maximum of $-2.1 \mu\text{M}$ (-23%) from baseline by the end of the 6 h recording session. Importantly, a control procedure conducted at the end of the session verified that the MEA had maintained its ability to record changes in extracellular glutamate levels. Thus, an infusion of exogenous glutamate (0.25 mM) at the nadir of the current output elicited a rapid and pronounced increase in signal. Control recordings conducted at $+0.25 \text{ V}$ eliminated any differences between the GluOx and sentinel sites, consistent with the interpretation that the self-referenced signal is generated by the oxidation of glutamate-derived H_2O_2 (data not shown). The bottom tracing in Fig. 4 illustrates a representative self-referenced record from an animal receiving a control injection of saline (0.9%, *i.p.*). This provides evidence that the injection *per se* did not lead to significant changes in current and that the signal was relatively stable over the entire recording session.

Table 1 summarizes group data on the magnitude and timing of the changes in the glutamate signal. Overall, kynurenine (50 mg/kg, $n=5$) caused a significant decrease (-31%) compared to basal glutamate levels ($t_4=4.67$,

$P=0.009$). This reduction was evident within 6 min of the injection and reached a maximum 5 h later. This effect of kynurenine persisted throughout the 6 h recording session.

As in the microdialysis study (Fig. 1B), the ability of systemic kynurenine to decrease prefrontal glutamate levels was dose-dependent. Fig. 5 illustrates the tracings from a representative animal treated with 25 mg/kg kynurenine (*i.p.*) ($n=5$). While the baseline glutamate signal was similar to that seen when studying the effects of the higher dose, administration of the lower dose produced an effect that was smaller in magnitude ($-1.3 \mu\text{M}$, -12% from baseline) and of shorter duration (return to baseline within 4 h) than the reduction caused by the higher dose (cf. Fig. 4). As summarized in the group data (Table 1), 25 mg/kg kynurenine caused a significant decrease (-11%) from

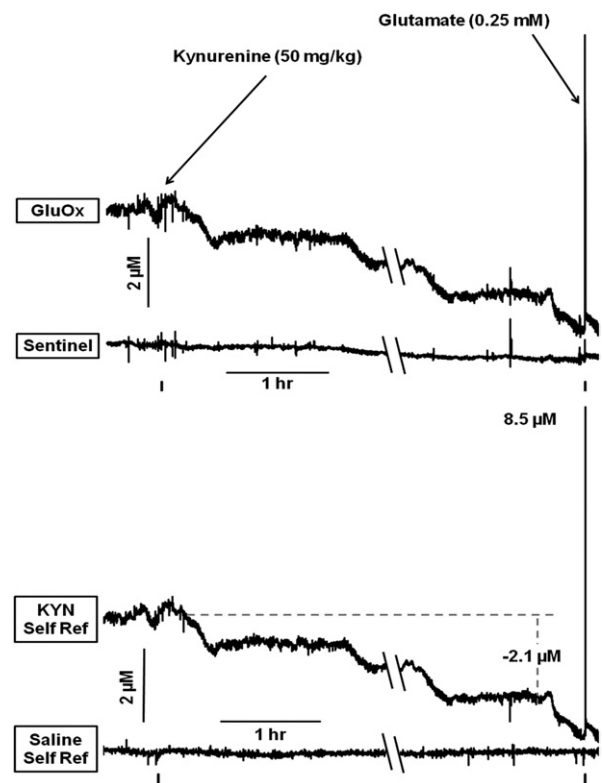


Fig. 4. Representative MEA tracings, obtained in the PFC, from a rat receiving kynurenine (KYN; 50 mg/kg *i.p.*). Top two tracings: MEA signal from the glutamate-sensitive site (GluOx) and its adjacent background sentinel. The third trace from the top reflects the self-referenced signal (Self Ref) of the GluOx channel against the sentinel background. Deflections along the vertical axis reflect changes in concentration (μM), and the horizontal axis depicts time (hr). The total recording session extended for 6 h after kynurenine administration (note 3 h break in time axis). Kynurenine produces a steady decline in the glutamate signal, reaching a maximum decrease of $2.1 \mu\text{M}$ by the end of the recording session (see Table 1 for group data). A control local infusion of glutamate (0.25 mM) rapidly elicits the characteristic phasic glutamate signal ($8.5 \mu\text{M}$ increase), demonstrating that the MEA remained sensitive to changes in glutamate levels at the nadir of the kynurenine effect. Note that the abrupt drop in baseline, prior to the glutamate infusion, is an artifact reflecting disruption of background current due to the removal of the dummy cannula and the insertion of the infusion cannula. Bottom tracing: self-referenced signal from an animal that received a control saline injection (0.9%, *i.p.*), demonstrating stability of the basal glutamate signal.

Table 1. Prefrontal glutamate levels following systemic injections: group microelectrode data

Measure	Kynurenine (25 mg/kg)	Kynurenine (50 mg/kg)	Galantamine (3 mg/kg)+kynurenine (50 mg/kg)
Basal glutamate (μM)	6.0 \pm 1.3	6.2 \pm 1.4	6.5 \pm 1.6
Post-injection glutamate ($\mu\text{M}\%$ change from basal)	5.4 \pm 1.2 (–10.6 \pm 1.1 ^a)	4.4 \pm 1.1 (–30.8 \pm 5.0)	5.7 \pm 1.4 (–10.8 \pm 2.3 ^a)
Time to effect (min)	16.2 \pm 4.9	5.4 \pm 3.6	16.3 \pm 8.9
Time to max effect (min)	111.2 \pm 23.3 ^a	314.0 \pm 20.0	77.8 \pm 20.2 ^a
Duration of effect (min)	210.3 \pm 21.6 ^a	>6 h	146.6 \pm 13.4 ^{a,b}

All pharmacological treatments were administered via i.p. injections. Values are means \pm SEM with $n=5$ rats/treatment group. ^a significantly different from kynurenine (50 mg/kg). ^b significantly different from kynurenine (25 mg/kg).

basal glutamate levels ($t_4=3.83$, $P=0.019$). Glutamate levels were reduced within 16 min after kynurenine and returned to basal values within 3½ h of the injection. 50 mg/kg kynurenine produced greater reductions in glutamate levels than 25 mg/kg ($F_{1,8}=7.71$, $P=0.02$). While there was no significant dose-dependent difference in the onset of the effect, the time to reach maximal effect ($F_{1,9}=44.70$, $P<0.001$) and effect duration ($F_{1,9}=47.99$, $P<0.001$) were significantly longer for the higher dose than for the smaller dose.

The kynurenine-induced reduction of prefrontal glutamate levels reflected the ability of enhanced KYNA to antagonize the $\alpha 7$ nAChR. Thus, as in the microdialysis experiment (Fig. 2B), pre-treatment with galantamine (3.0 mg/kg i.p.) markedly attenuated the effects of 50 mg/kg kynurenine. Fig. 6 illustrates a representative current tracing from the galantamine/kynurenine combination. The

third trace from the top depicts the self-referenced glutamate signal following the combined treatment. Compared to the profile seen following 50 mg/kg kynurenine alone (Fig. 4), the combination of the two compounds produced a smaller maximal effect on glutamate (–1.3 μM), which reverted more quickly to basal levels (2½ h). The group data listed in Table 1 show that the combination of galantamine and kynurenine ($n=5$) led to smaller decreases in glutamate ($F_{1,9}=13.34$, $P=0.006$) and also shorter durations of the inhibition ($F_{1,9}=255.36$, $P<0.001$) relative to kynurenine alone. In fact, pre-treatment with galantamine rendered the effects of 50 mg/kg kynurenine similar to the profile seen following the application of 25 mg/kg kynure-

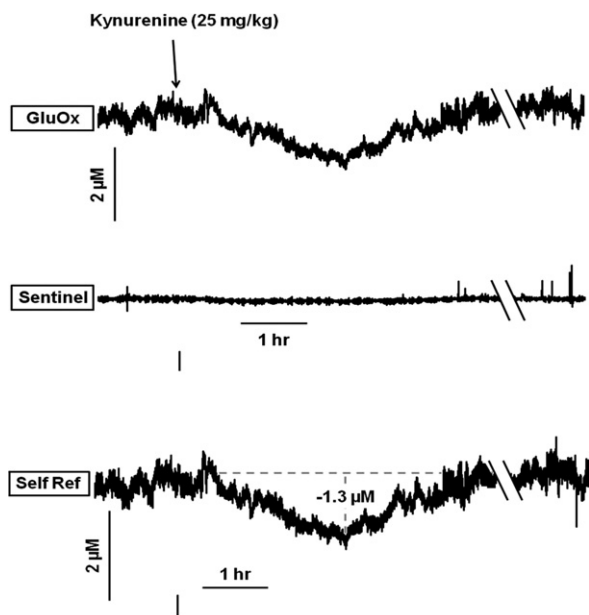


Fig. 5. Representative MEA tracings, obtained in the PFC, from a rat receiving an injection of kynurenine (25 mg/kg i.p.). The self-referenced glutamate signal (bottom tracing; Self Ref; note 3 h break in time axis) reveals that 25 mg/kg kynurenine produces a smaller maximal decrease in glutamate (1.3 μM) than 50 mg/kg of kynurenine (cf. Fig. 4). Glutamate levels return to basal values within 3½ h (see Table 1 for group data).

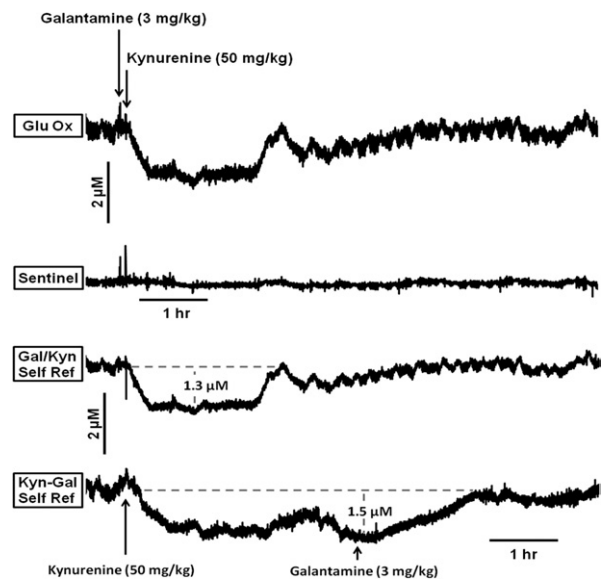


Fig. 6. Representative MEA tracings, obtained in the PFC, from a rat receiving an injection of galantamine (3 mg/kg i.p.), followed, 5 min later, by kynurenine (Kyn; 50 mg/kg i.p.). Top two tracings: MEA signal from the GluOx and its adjacent background sentinel sites. The third tracing, illustrating the self-referenced (Self Ref) glutamate signal, reveals that galantamine attenuates the amplitude of the decline and the time course to values similar to those seen following 25 mg/kg kynurenine (cf. Fig. 5, and Table 1). Bottom tracing: self-referenced record from an animal that first received kynurenine (50 mg/kg, arrow) and then, 3 h later at the nadir of glutamate levels, galantamine (Gal; 3 mg/kg, arrow). The glutamate signal gradually returns to basal values following delayed galantamine administration, suggesting that the kynurenine-induced attenuation of glutamate release is consistently mediated by $\alpha 7$ nAChRs.

nine alone. However, treatment with galantamine reduced the time to maximum effect relative to either dose of kynurenine ($F_{1,9}=6.28$, $P=0.04$).

The bottom trace in Fig. 6 (“Kyn/Gal”) illustrates an experiment in which the administration of galantamine was delayed until 3 h after the injection of kynurenine (50 mg/kg), i.e. a time when the inhibitory effect of kynurenine on glutamate was pronounced. Galantamine caused a clear reversal of the glutamate signal, which returned to baseline values within 90 min. These results demonstrated that the kynurenine-induced decrease in glutamate is consistently mediated by the $\alpha 7$ nAChR.

Experiment 2B: effects of local KYNA changes on cortical glutamate levels as measured by the microelectrode array

The next series of experiments determined the effects of local changes in KYNA on the prefrontal glutamate signal. In these studies, 2.5 μM kynurenine was perfused directly into the PFC to elevate KYNA levels. We had shown previously by microdialysis that this treatment causes a 125% increase in KYNA levels in the PFC (Wu et al., 2010). In the present experiment, perfusion of kynurenine (45 min in duration) produced a clear decrease in glutamate levels (Fig. 7). The isolated glutamate signal (bottom trace) from this representative animal revealed that glutamate was reduced by 1.7 μM (–25% from baseline) following kynurenine application. The decrease was apparent as soon as 6 min following the onset of the perfusion with the KYNA precursor and persisted for 5½ h. Analysis of the group data ($n=5$) revealed that basal glutamate levels

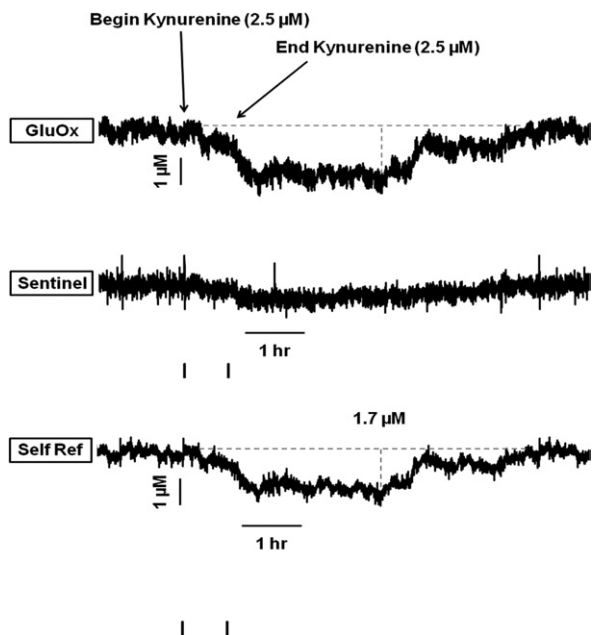


Fig. 7. Representative MEA tracings, obtained in PFC, from a rat receiving an intracortical perfusion of kynurenine (2.5 μM , 45 min duration). The self-referenced glutamate signal (Self Ref) shows a kynurenine-induced decline in glutamate that begins within 6 min of the start of the perfusion, reaches a maximal decrease of 1.7 μM (25% decrease from baseline), and persists for 5½ h.

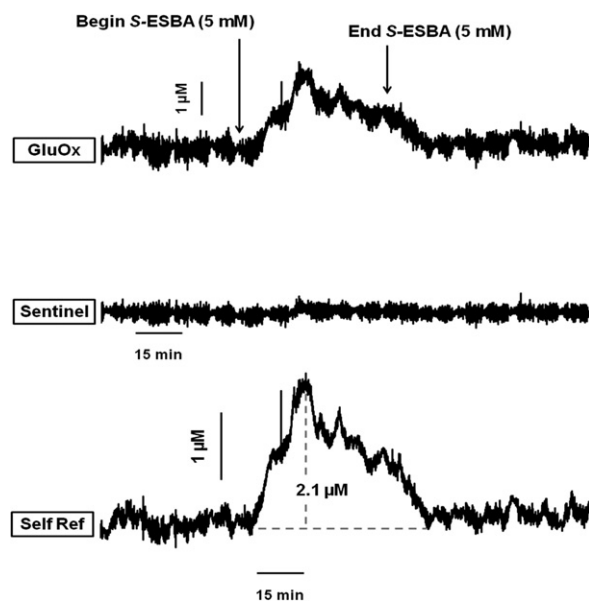


Fig. 8. Representative MEA tracings, obtained in the PFC, from a rat receiving an intracortical perfusion of the KAT II inhibitor S-ESBA (5 mM, 45 min duration). The bottom trace illustrates the self-referenced glutamate signal (Self Ref) and reveals an S-ESBA-induced increase in glutamate levels, which is apparent 5 min after the start of the perfusion. The increase reaches a maximum of 2.1 μM (37% above baseline) and persists for approximately 1 h.

(7.7 ± 2.2 μM) were comparable to those reported in Table 1. Local administration of kynurenine decreased basal glutamate levels by $28.8 \pm 8.5\%$ (mean \pm SEM; $t_4=3.41$, $P=0.04$). As a group, the decreases occurred within 13 ± 7 min, reached a nadir by 112 ± 30 min and returned to baseline by 320 ± 38 min after the start of the kynurenine perfusion.

In contrast, local perfusion of S-ESBA (5.0 mM), an inhibitor of KYNA's biosynthetic enzyme KAT II, produced the opposite effect on basal glutamate levels in the PFC. Previous microdialysis experiments had demonstrated that local perfusion of the PFC with 3.0 mM S-ESBA causes a 35% reduction in extracellular KYNA levels and a concomitant significant increase in extracellular glutamate (Wu et al., 2010). In line with these results, the synthesis inhibitor produced a rapid and marked elevation in prefrontal glutamate levels measured by the MEA. Fig. 8 depicts a representative tracing from an S-ESBA recording session. Following a stable baseline, the 45 min perfusion of S-ESBA led to a clear increase in signal on the GluOx site (top trace) with very little change on the sentinel site (middle trace). The relative stability of the sentinel site confirmed the effectiveness of the *m*-PD exclusion layer. This was especially important since S-ESBA also stimulates extracellular DA levels in the PFC (Wu et al., 2006). The resultant, self-referenced glutamate signal (bottom trace) revealed a rapid increase to a maximum of 2.1 μM (37% from baseline). The increase was apparent as early as 5 min from the start of the infusion and persisted for approximately 1 h. As a group ($n=5$), basal glutamate levels (8.0 ± 2.8 μM) were similar to those reported in Experiment 2A (cf. Table 1). Local perfusion of S-ESBA increased

basal glutamate levels by $38.4 \pm 13.7\%$ (mean \pm SEM; $t_4 = -3.61$, $P = 0.02$). On average, the increase occurred within 4 ± 1 min, reached a maximum by 26 ± 4 min and returned to baseline by 65 ± 4 min after the start of perfusion with the enzyme inhibitor.

DISCUSSION

The present study revealed several novel facets of the recently described modulation of extracellular glutamate levels by endogenous KYNA (Wu et al., 2010). First, we showed that significant reductions in prefrontal glutamate are seen following the systemic administration of KYNA's precursor kynurenine. Second, the reduction of extracellular glutamate following the peripheral application of kynurenine was prevented by the co-application of galantamine, indicating that $\alpha 7$ nAChRs constitute a crucial link between excessive KYNA and glutamate. Third, qualitatively very similar results were obtained when the effect of systemic kynurenine administration was monitored by microdialysis or with a selective glutamate MEA. Finally, as measured by the MEA, local perfusion of the specific KYNA synthesis inhibitor S-ESBA produced an unexpectedly rapid elevation in prefrontal glutamate levels. Taken together, these results, which have functional implications for the cognitive deficits seen in schizophrenia patients, shed new light on the mechanisms that underlie the regulation of cortical glutamate release by endogenous KYNA.

KYNA is an astrocyte-derived neuromodulator

KYNA, initially described as a broad spectrum antagonist of ionotropic glutamate receptors with low potency (Perkins and Stone, 1982), inhibits the glycine co-agonist site of the N-methyl-D-aspartate (NMDA) receptor (NMDAR) competitively with an IC_{50} of ~ 8 – $15 \mu M$ in the absence of glycine. In the presence of glycine, the IC_{50} of KYNA increases to $239 \mu M$ (Kessler et al., 1989; Hilmas et al., 2001). More recently, electrophysiological and biochemical studies revealed that KYNA antagonizes $\alpha 7$ nAChRs, albeit non-competitively, at physiological (i.e. nanomolar) concentrations (Hilmas et al., 2001). $\alpha 7$ nAChRs are therefore assumed to constitute an important, and possibly preferential, target of endogenous KYNA *in vivo* (Stone, 2007; see below).

In the rat brain, KAT II is the major enzyme catalyzing the irreversible transamination of kynurenine to KYNA (Guidetti et al., 2007a). Brain KAT II is almost exclusively localized in astrocytes (Guidetti et al., 2007b), and *in vitro* studies have demonstrated that newly produced, astrocyte-derived KYNA is readily released into the extracellular milieu (Kiss et al., 2003) where it can modulate neuronal $\alpha 7$ nAChRs and NMDARs. Notably, extracellular KYNA levels are elevated when synthesis is driven by kynurenine and reduced when KAT II activity is compromised by pharmacological or genetic means (Turski et al., 1989; Swartz et al., 1990; Yu et al., 2004; Pellicciari et al., 2006). In the present study, manipulations with either kynurenine or the specific KAT II inhibitor S-ESBA (which does not enter the brain after peripheral administration) were used exper-

imentally to explore the link between KYNA and glutamate in the PFC.

In contrast to KYNA, which penetrates the blood-brain barrier only poorly due to its polar nature, kynurenine enters the brain promptly after systemic application (Gál and Sherman, 1978) using the large neutral amino acid carrier that also transports more abundant, competing amino acids such as tryptophan and phenylalanine (Fukui et al., 1991). In agreement with a previous study in the rat striatum (Swartz et al., 1990), we demonstrated here that peripheral administration of kynurenine dose-dependently raised extracellular KYNA levels in the PFC. In quantitative terms, the increase in cortical KYNA levels following the systemic application of 25 mg/kg kynurenine was comparable to the effect caused by a local perfusion of $2.5 \mu M$ kynurenine, which we described recently (Wu et al., 2010). Of note, this dose of systemic kynurenine also duplicated the reduction in extracellular glutamate seen after a local perfusion of kynurenine in the PFC. Increases in KYNA in other brain areas therefore do not appear to interfere with the control of prefrontal glutamate by KYNA.

Regulation of prefrontal glutamate by KYNA: the role of $\alpha 7$ nAChRs

Although there is consensus that local application of nicotine enhances cortical glutamatergic transmission, the relative roles of specific cholinergic receptor subtypes appear to be intricate. Thus, initial electrophysiological and microdialysis studies implied both muscarinic and nicotinic (Toth et al., 1993; Vidal and Changeux, 1993) receptors, whereas subsequent work with genetic models and specific pharmacological agents provided convincing evidence that $\alpha 4\beta 2$ nAChRs play a crucial role in nicotine-induced glutamate release in the PFC (Gioanni et al., 1999; Lambe et al., 2003; Parikh et al., 2008).

Work from several laboratories has shown that selective activation of $\alpha 7$ nAChRs, too, stimulates prefrontal glutamate release (Rousseau et al., 2005; Wang et al., 2006; Konradsson-Geuken et al., 2009). We posit that this effect is responsible for the bi-directional modulation of extracellular glutamate by endogenous KYNA described here. Our conclusion is based on the ability of galantamine, which functions as a selective allosteric potentiator of the $\alpha 7$ nAChR at the dose used (3 mg/kg i.p.; Samochocki et al., 2003; Geerts et al., 2005), to attenuate the kynurenine-induced reduction in extracellular glutamate without affecting KYNA neosynthesis (cf. Figs. 1A and 2A). This effect of galantamine, which was observed here with either of the two monitoring procedures used, is most readily explained by the fact that the drug acts as an agonist at a site of the $\alpha 7$ nAChR that is very similar to the one that is inhibited by KYNA (Lopes et al., 2007). Notably, our experiments using the MEA revealed that $\alpha 7$ nAChRs are continuously involved in the KYNA-induced reduction in prefrontal glutamate levels, since administration of galantamine at the point of maximal kynurenine-induced decrease resulted in a reversal of glutamate levels toward basal values (Fig. 6).

In vivo experiments in other areas of the rat brain, using choline, methyllycaconitine and α -bungarotoxin as specific pharmacological tools (Rassoulpour et al., 2005; Lopes et al., 2007; Konradsson-Geuken et al., 2009), had previously also indicated that $\alpha 7$ nAChRs rather than NMDA receptors are the primary target of KYNA. The events linking fluctuations in extracellular KYNA levels, $\alpha 7$ nAChR activity, and glutamatergic transmission are complex, however. Thus, a recent ultrastructural immunogold study demonstrated that the majority (65%) of $\alpha 7$ nAChRs in the rat PFC are localized within dendrites and dendritic spines. These structures are receptive to axon terminals that lack $\alpha 7$ nAChRs but form asymmetric excitatory-type (i.e. probably glutamatergic) synapses. In contrast, a significant proportion (22%) of $\alpha 7$ nAChRs was found to be associated with presynaptic cholinergic nerve terminals, suggesting that acetylcholine release in the PFC is auto-regulated through these receptors (Duffy et al., 2009). These anatomical data, together with earlier studies (Csillik et al., 1998; Alkondon et al., 2000; Krenz et al., 2001), do not support the proposition that acetylcholine (ACh) regulates glutamate release in the PFC solely by activating $\alpha 7$ nAChRs situated on glutamatergic nerve terminals (Marchi et al., 2002; Dickinson et al., 2008; Livingstone et al., 2010). Thus, the effect of KYNA on extracellular glutamate described here likely involves inhibition of heterogeneously distributed $\alpha 7$ nAChRs receptors—either within the PFC or in brain areas with reciprocal links to the PFC (Del Arco and Mora, 2005, 2008; Biton et al., 2007; Couey et al., 2007).

Convergence between microdialysis and microelectrode methods

The present study utilized two methods to assess extracellular glutamate levels, i.e. conventional microdialysis and rapid electrochemistry using a glutamate-sensitive MEA. Overall, both methods revealed qualitatively similar, $\alpha 7$ nAChR-mediated (galantamine-sensitive) reductions in prefrontal glutamate following systemically administered kynurenine. Moreover, the increase in cortical glutamate levels seen using the microelectrode following local perfusion of S-ESBA paralleled our recent microdialysis study (Wu et al., 2010). This convergence of results obtained with microelectrode and microdialysis/HPLC methodologies not only substantiates our experimental results but also provides additional indirect validation of the specificity of the self-referenced glutamate signal (Day et al., 2006; Rutherford et al., 2007).

The use of the microelectrode, which provides second-by-second resolution, allowed for a precise quantification of the onset and offset of our experimental interventions—in contrast to the temporal constraint imposed by the 30 min collection intervals in the microdialysis studies. These experiments demonstrated that systemic administration of kynurenine (50 mg/kg) causes a significant reduction in prefrontal glutamate within 5 min, and local perfusion of the KYNA synthesis inhibitor S-ESBA produces a similarly timed elevation (within 4 min) of glutamate levels. Although our methodological limitations did

not permit the measurement of extracellular KYNA in 5-min intervals, these findings show that astrocytes can be efficiently manipulated to adjust KYNA production and, in functional terms, that KYNA can be rather rapidly mobilized to influence glutamatergic tone.

While the two methods yielded qualitatively identical patterns of glutamate modulation by KYNA, there were quantitative differences. For example, compared to microdialysis, MEA analyses showed a smaller response to kynurenine (50 mg/kg), yet the effect was longer in duration. Also, the increase in prefrontal glutamate levels following perfusion of S-ESBA was more pronounced when assessed by microdialysis (Wu et al., 2010) than by MEA. The nature and significance of the differences between the two *in vivo* monitoring techniques, which have also been noted by others (Rutherford et al., 2007; Van der Zeyden et al., 2008), are currently under investigation in our laboratories.

Functional implications

Although the bi-directional modulation of prefrontal glutamate levels by KYNA is of obvious relevance to brain physiology, our findings may be especially pertinent for the etiology and treatment of several of the cognitive deficits seen in SZ. Individuals with SZ have abnormally high KYNA levels in the PFC (Schwarcz et al., 2001) and exhibit deficits in several prefrontally-mediated executive functions such as working memory (Barch and Smith, 2008), attention (Nuechterlein et al., 2009) and cognitive flexibility (Leeson et al., 2009). Dysregulations in cortical glutamatergic and cholinergic transmission have been proposed to underlie these cognitive deficits and may, in fact, be causally related to increased KYNA function (see Introduction). Consistent with this hypothesis, experimentally-induced elevations in brain KYNA levels in animals are associated with performance deficits in several behavioral tasks that require cognitive operations similar to those that are impaired in SZ (Shepard et al., 2003; Erhardt et al., 2004; Chess and Bucci, 2006; Chess et al., 2007).

Collectively, these data suggest that strategies designed to reduce the production of KYNA in brain may constitute efficacious adjunctive therapies for the treatment of cognitive deficits in SZ (Wonodi and Schwarcz, 2010). This approach would be in line with recent clinical studies, which showed modest pro-cognitive effects in SZ patients treated with partial $\alpha 7$ nAChR agonists, positive $\alpha 7$ nAChR modulators such as galantamine (Schubert et al., 2006; Freedman et al., 2008; Thomsen et al., 2010) or various glutamatergic agonists (Coyle, 2006). In light of the present results and the recent demonstration that KAT II-deficient mice show enhanced cognitive behaviors (Potter et al., 2010), we propose that selective, systemically active KAT II inhibitors might be especially efficacious as cognition enhancing agents in patients.

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