

**Regulation of GABAergic inputs to CA1 pyramidal neurons by nicotinic receptors and
kynurenic acid**

Jyotirmoy Banerjee, Manickavasagom Alkondon, Edna F. R. Pereira, Edson X. Albuquerque

Division of Translational Toxicology, Department of Epidemiology and Public Health,
University of Maryland School of Medicine, Baltimore, MD

Running title: Kynurenic acid suppresses nAChR-dependent GABA release

Corresponding author: Edson X. Albuquerque, Division of Translational Toxicology,
Department of Epidemiology and Public Health, University of Maryland School of Medicine, 10
S. Pine Street, suite 900, Baltimore, MD 21201. Phone: 410-706-7065. Fax: 410-706-4200.
Email: ealbuquerque@som.umaryland.edu

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Abbreviations: α -BGT, α - Bungarotoxin; ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; KAT II, kynurenine aminotransferase II; KYNA, kynurenic acid; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; PSC, postsynaptic current; TTX, Tetrodotoxin.

Abstract

Impaired $\alpha 7$ nicotinic receptor (nAChR) function and GABAergic transmission in the hippocampus and elevated brain levels of kynurenic acid (KYNA), an astrocyte-derived metabolite of the kynurenine pathway, are key features of schizophrenia. KYNA acts as a non-competitive antagonist with respect to agonists at both $\alpha 7$ nAChRs and NMDA receptors. Here, we tested the hypothesis that in hippocampal slices tonically active $\alpha 7$ nAChRs control GABAergic transmission to CA1 pyramidal neurons and are sensitive to inhibition by rising levels of KYNA. The $\alpha 7$ nAChR-selective antagonist α -bungarotoxin (α -BGT, 100 nM) and methyllycaconitine (MLA, 10 nM), an antagonist at $\alpha 7$ and other nAChRs, reduced by $51.3 \pm 1.3\%$ and $65.2 \pm 1.5\%$, respectively, the frequency of GABAergic PSCs recorded from CA1 pyramidal neurons. MLA had no effect on miniature GABAergic PSCs. Thus, GABAergic synaptic activity in CA1 pyramidal neurons is maintained, in part, by tonically active $\alpha 7$ nAChRs located on the somatodendritic and/or preterminal region of axons of interneurons that synapse onto the neurons under study. L-kynurenine (20 or 200 μ M) or KYNA (20-200 μ M) suppressed concentration dependently the frequency of GABAergic PSCs; the inhibitory effect of 20 μ M L-kynurenine had an onset time of about 35 min and could not be detected in the presence of 100 nM α -BGT. These results suggest that KYNA levels generated from 20 μ M kynurenine inhibit tonically active $\alpha 7$ nAChR-dependent GABAergic transmission to the pyramidal neurons. Disruption of nAChR-dependent GABAergic transmission by mildly elevated levels of KYNA can be an important determinant of the cognitive deficits presented by patients with schizophrenia.

Introduction

Hippocampal dysfunction and a specific defect in hippocampal interneurons are consistent findings in the brains of patients that suffer from schizophrenia (Tamminga et al, 2010; Konradi et al, 2011), a disease that afflicts approximately 1% of the population worldwide. The contribution of hippocampal dysfunctions to the complex phenotype of the disease is underscored by numerous pharmacological studies. For instance, microinfusion of the GABA_A receptor antagonist picrotoxin in the ventral hippocampus of rats impairs prepulse inhibition of the startle reflex response, a measure of sensorimotor gating that is deficient in patients with schizophrenia (Bast et al., 2001). Intraventricular administration of the $\alpha 7$ nicotinic receptor (nAChR) antagonist α -bungarotoxin to rats also impairs sensory gating as measured by the disruption of the attenuation of amplitude of second auditory-evoked potential in a pair of stimuli in the CA3 region of the hippocampus (Luntz-Leybman et al., 1992). Finally, systemic treatment of rats with NMDA receptor antagonists recapitulates major clinical features of schizophrenia that are ameliorated by treatment with nicotine and $\alpha 7$ nAChR-selective agonists (see references in Timofeeva and Levin, 2011). Among these features are deficits of working memory and decreased levels of glutamic acid decarboxylase 67, one of the enzyme isoforms that catalyzes the synthesis of GABA, and parvalbumin, a Ca²⁺-binding protein expressed by interneurons, primarily in the *stratum pyramidale*.

Evidence also exists that a number of metabolic pathways is affected in the brains of patients with schizophrenia. The kynurenine pathway of the tryptophan metabolism is one of these pathways. Cerebral cortical levels of kynurenic acid (KYNA), an astrocyte-derived kynurenine metabolite, have been found to be higher in patients with schizophrenia than in age-matched control subjects (Schwarcz et al., 2001). KYNA is a neuroactive metabolite that

interacts with a multitude of molecular targets in the brain. At concentrations ranging from tens to hundreds micromolar, KYNA acts as a competitive antagonist of glycine at NMDA receptors. The IC_{50} s for KYNA to inhibit NMDA receptors is approximately 15 μ M in the absence of glycine and 230 μ M in the presence of 10 μ M glycine (Hilmas et al., 2001). With an IC_{50} of approximately 7 μ M, KYNA blocks non-competitively $\alpha 7$ nAChRs (Hilmas et al., 2001; Lopes et al., 2007). Finally, with EC_{50} s of 7 μ M and 39 μ M KYNA activates the rat and the human orphan G-protein receptor 35 (GPR35), respectively (Wang et al., 2006).

Studies of mice with a null mutation in the gene that encodes kynurenine aminotransferase II (KAT II), an enzyme responsible for more than 70% of the astrocytic synthesis of KYNA from kynurenine, have revealed the role of the metabolite in modulating hippocampal $\alpha 7$ nAChR activity (Alkondon et al., 2004). Decreased hippocampal KYNA levels in the *mKat-2*^{-/-} mice resulted in increased $\alpha 7$ nAChR activity in CA1 *stratum radiatum* interneurons and increased GABAergic transmission onto the CA1 pyramidal neurons (Alkondon et al., 2004). Additional support for the ability of KYNA to regulate the activity of multiple neurotransmitter systems was obtained from recent studies in which KYNA was synthesized *de novo* in hippocampal slices while synaptic transmission, action potentials, and nAChR activity were recorded from interneurons. Increasing *de novo* production of KYNA decreased agonist-induced activation of $\alpha 7$ nAChRs and inhibited tonically active NMDA receptors in *stratum radiatum* interneurons, in addition to reducing the rate of firing of these neurons (Alkondon et al., 2011a,b).

Tracing the consequences of increased production of KYNA on $\alpha 7$ nAChR activity and GABAergic synaptic transmission to CA1 pyramidal neurons, the major output of the hippocampus, is a significant step towards understanding the pathophysiology of schizophrenia.

Therefore, the present study was designed to test the hypothesis that, under resting conditions, in a fully functional neurocircuitry, activation of $\alpha 7$ nAChRs by basal levels of choline and/or acetylcholine in hippocampal slices contributes to maintain GABAergic synaptic activity in CA1 pyramidal neurons and is reduced by increasing *de novo* synthesis of KYNA. To test this hypothesis, GABAergic postsynaptic currents (PSCs) were recorded from the somata of CA1 pyramidal neurons in slices that were subjected to incubation and/or superfusion with nAChR subtype-selective antagonists and different concentrations of kynurenine, the precursor of KYNA.

Materials and Methods

Hippocampal slices. Male Sprague-Dawley rats at ages ranging between 30 and 35 days were used in this study. Animal care and handling were done strictly in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of the University of Maryland. Rats were euthanized by CO₂ narcosis followed by decapitation. Their brains were removed in ice-cold artificial cerebrospinal fluid (ACSF), which was composed of (in mM): NaCl, 125; NaHCO₃, 25; KCl, 2.5; NaH₂PO₄, 1.25; CaCl₂, 2, MgCl₂, 1; and dextrose, 25. The ACSF was bubbled with 95% O₂ and 5% CO₂. The hippocampi were dissected out and mounted on the stage of a Vibratome (Leica VT1000S, Leica Microsystems Inc., Bannockburn), which was used to cut transversal hippocampal slices of 300-350 μm thickness. Slices were stored at room temperature for at least 45 minutes in an immersion chamber containing ACSF continuously bubbled with 95% O₂ and 5% CO₂ before recordings. Some of the slices were transferred to a chamber containing ACSF with test compounds that was continuously bubbled with 95% O₂ and 5% CO₂. Slices were incubated with the test compounds for 2 to 5 h at room temperature, exception made to L-kynurenine-incubated slices, which were maintained at 30°C to maximize the activity of KAT II.

Electrophysiological recordings. PSCs were recorded from the soma of CA1 pyramidal neurons at a holding potential of 0 mV according to the standard whole-cell mode of the patch-clamp technique using an LM-EPC7 amplifier (List Electronic, Darmstadt, Germany). In the recording chamber, hippocampal slices were superfused with ACSF at 2 ml/min. All recordings were obtained in the presence of the muscarinic receptor antagonist atropine (0.5 μM), a concentration that does not affect α7 nAChR currents and does not modify KYNA-induced α7 nAChR

inhibition (Alkondon et al., 2011a; Lopes et al., 2007). Test compounds were applied to the slices via bath perfusion. Signals were filtered at 3 kHz, digitized at 10 KHz through Digidata 1322A (Molecular devices, USA), and recorded using the Clampex module of the pCLAMP9 software (Molecular Devices, Sunnyvale, USA). The frequency of PSCs in control condition remained nearly the same even at a sampling rate of 20 KHz, so we sampled at the rate of 10 KHz throughout for ease of handling the data.

Patch pipettes were pulled from borosilicate glass capillaries (OD 1.2 mm, World Precision Instruments, New Haven, CT) with a P-97 Flaming-Brown puller (Sutter Instruments, Novato, CA). When filled with internal solution the patch pipettes had resistances between 4 and 6 M Ω . The leak current was generally between 50 and 150 pA, and when it exceeded 200 pA, the data were not included in the analysis. The access resistance was monitored during the course of the experiments and ranged between 15 and 20 M Ω . Data from any neuron were not considered for analysis when the access resistance increased more than 20% from the initial values. The internal pipette solution contained 0.5% biocytin in addition to (in mM): ethylene-glycol bis(β -amino-ethyl ether)-N-N'-tetraacetic acid, 10; HEPES, 10; Cs-methane sulfonate, 130; CsCl, 10; MgCl₂, 2; and lidocaine N-ethyl bromide (QX-314), 5 (pH adjusted to 7.3 with CsOH). All recordings were done at room temperature (20–22°C). Only a single neuron was studied per slice. Therefore, the number of neurons represents the number of hippocampal slices analyzed. Biocytin staining was developed and the morphology of biocytin-filled neurons identified them as pyramidal neurons.

Data analysis and statistics. PSCs were analyzed in 5-min recordings using the Clampfit module of the pCLAMP 9.0 software (Molecular Devices, Sunnyvale, USA). Frequency, peak

amplitude, rise time (10–90%), and decay-time constant (τ_d) of synaptic events were measured. The threshold amplitude for detecting PSCs was set at 10 pA and the PSCs detected by the software were visually inspected to minimize errors. Events that did not show a typical synaptic waveform were rejected manually. For kinetic analysis, only single events with a sharp rising phase and an exponential decay were chosen during visual inspection of the recordings. Double- and multiple-peak currents were excluded for determination of PSC properties, but included for calculation of frequency of PSCs. Rise times and τ_d were determined during the analysis of the averaged chosen single events aligned at half rise time. Data are expressed as mean \pm SEM of results obtained from various animals and statistical significance was analyzed using one-way ANOVA or *t* test in Sigmaplot 11.0 (Systat Software, Inc., Chicago, IL). Further, the cumulative distributions of events in control versus treatment groups were compared using Kolmogorov-Smirnov test (K-S test). For this, events from different neurons in each group were pooled together and then subjected to the K-S test using Clampfit module of the pCLAMP 9.0 software.

Drugs. (-)Bicuculline methochloride, atropine sulfate, L-kynurenine sulfate (“kynurenine”), kynurenic acid (KYNA), N-(2,6-Dimethylphenylcarbamoylemethyl) triethylammonium bromide (QX-314), 2-amino-5-phosphonovaleric acid (APV), and tetrodotoxin (TTX) were purchased from Sigma Chemical Co. (St. Louis, MO). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) was purchased from Research Biochemicals International (Natick, MA). α -Bungarotoxin (α -BGT) was purchased from Biotoxins Inc. (St. Cloud, FL). Methyllycaconitine citrate (MLA) was a gift from Professor M. H. Benn (Dept. Chemistry, Univ. Calgary, Alberta, Canada). Kynurenine-containing ACSF was always prepared on the day of the experiments. A stock solution of 500 mM KYNA was made in 1 M NaOH and subsequently serially diluted in regular ACSF as

needed. The pH of KYNA-containing and all other ACSF solutions, measured within 2-3 min after bubbling with 95% O₂/ 5% CO₂, was approximately 7.4. Stock solutions of all other compounds were made in distilled water, kept frozen, and, subsequently, diluted in ACSF.

Results

Spontaneous GABAergic synaptic activity in CA1 pyramidal neurons

Spontaneous synaptic currents recorded from CA1 pyramidal neurons at 0 mV appeared as outward events (see traces in Figure 1A, B). Under control conditions, the frequency of these events ranged from 1.05 to 1.27 Hz (Mean \pm SEM = 1.18 ± 0.08 ; n = 94 neurons from 94 slices from 63 rats). These spontaneously occurring synaptic currents were completely blocked following 15-min superfusion of the hippocampal slices with ACSF containing 10 μ M bicuculline (Figure 1A, C), indicating that they were mediated via GABA_A receptors. The slow decay-time constant of the outward currents (about 35 ms, see Table 1) was consistent with the notion that they were GABAergic in nature. To examine the extent to which glutamatergic excitation of GABAergic interneurons that synapse onto the CA1 pyramidal neurons regulates the spontaneous GABAergic synaptic activity recorded from the latter, hippocampal slices were superfused with ACSF containing the AMPA and NMDA receptor antagonists CNQX (10 μ M) and APV (50 μ M). The frequency of GABAergic PSCs recorded from the CA1 pyramidal neurons in the presence of both CNQX and APV was comparable to that recorded under control conditions (Figure 1B, C). These results suggest that the spontaneous GABAergic synaptic activity recorded from the pyramidal neurons is not regulated by the basal glutamatergic activity in the slices.

Suppression of spontaneous GABAergic synaptic activity in CA1 pyramidal neurons exposed to $\alpha 7$ nAChR antagonists

To determine whether GABAergic transmission to CA1 pyramidal neurons is regulated by tonically active $\alpha 7$ nAChRs in interneurons, hippocampal slices were incubated for 2 h in

ACSF containing the $\alpha 7$ nAChR antagonist methyllycaconitine (MLA, 10 nM) and subsequently continuously superfused in MLA-containing ACSF. The concentration of MLA used in this study is sufficient to produce complete block of $\alpha 7$ nAChR currents (Alkondon et al., 2009). The cumulative plot of inter-event intervals recorded in the presence of MLA was displaced towards longer intervals compared to control (Figure 2A). In addition, the mean frequency of events recorded in the presence of MLA was significantly lower than that recorded under control conditions (Figure 2B). In contrast, MLA had no significant effect on the decay-time constant (τ_d), rise time, mean peak amplitude or cumulative distribution of the peak amplitude of GABAergic PSCs (Table 1).

In another set of experiments, spontaneous GABAergic PSCs were recorded from pyramidal neurons before and during their superfusion with MLA (10 nM)-containing ACSF. Under this experimental condition, MLA significantly reduced the mean frequency of PSCs (Figure 2C). The magnitude of the effect was nearly the same regardless of whether the slices were incubated for 2 h or superfused for 15 min with MLA-containing ACSF (Figure 2B, C).

The frequency of GABAergic PSCs was also significantly lower in slices that had been incubated for 1 h in ACSF containing a saturating concentration of the $\alpha 7$ nAChR antagonist α -BGT (100 nM) than in control slices. α -BGT caused a displacement of the cumulative distribution of inter-event intervals towards longer intervals (figure 3A) and reduced the mean frequency of PSCs (Figure 3B). α -BGT also decreased the frequency of PSCs in the presence of glutamate receptor blockers CNQX (10 μ M) and APV (50 μ M) (Figure 3B). The magnitude of the effect of combined bath application of MLA (10 nM) and α -BGT (100 nM) in slices that had been pre-incubated with α -BGT was significantly larger than that of α -BGT alone (Figure 3C).

Suppression of GABAergic synaptic activity in CA1 pyramidal neurons exposed to the Na⁺-channel blocker tetrodotoxin (TTX)

To isolate action potential-independent from action potential-dependent GABAergic PSCs, hippocampal slices were first incubated for 1 h in TTX (200 nM)-containing ACSF and subsequently superfused with the same solution. The cumulative distribution of inter-event intervals recorded in the presence of TTX was displaced towards longer intervals in comparison with control (Figure 4A). TTX also reduced the mean frequency and the mean amplitude of GABAergic events by $68.3 \pm 2.0 \%$ and $49.4 \pm 1.3 \%$, respectively (Figure 4B; Table 2). Neither the rise time nor τ_d of the GABAergic PSCs was affected by TTX (Table 2). These results are consistent with TTX-induced block of the action potential-dependent GABAergic transmission; only miniature PSCs (mPSCs) remained in the presence of TTX.

In the continuous presence of TTX, MLA affected neither the cumulative distribution of inter-event intervals (Figure 4A) nor the mean frequency of mPSCs (Figure 4B) recorded from CA1 pyramidal neurons. MLA was also devoid of any effect on the mean amplitude, rise time, and τ_d of mPSCs (Table 2).

GABAergic synaptic activity recorded from CA1 pyramidal neurons decreased in the presence of increasing concentrations of L-kynurenine

Studies from our laboratory and others have shown that incubation of rat hippocampal slices with L-kynurenine increases production of KYNA *in situ* (Scharfman et al., 1999; Alkondon et al., 2011a). To examine the effects of newly synthesized KYNA on GABAergic transmission in CA1 pyramidal neurons, hippocampal slices were first incubated for 2-5 h in ACSF containing 2 μ M, 20 μ M, or 200 μ M kynurenine. At the end of the incubation time, slices

were transferred to the recording chamber, where they were continuously superfused with kynurenine-containing ACSF while GABAergic PSCs were recorded from CA1 pyramidal neurons. Control slices were incubated and maintained in kynurenine-free ACSF.

Kynurenine caused a concentration-dependent reduction of the mean frequency of GABAergic PSCs (Figure 5A, 5B). At 20 and 200 μ M kynurenine also caused a significant displacement of the cumulative distribution of inter-event intervals towards longer intervals (Figure 5A). The mean peak amplitude, rise time, and τ_d of PSCs were not affected by the test concentrations of kynurenine (Table 3).

In the absence of pre-incubation, 15-min superfusion of hippocampal slices with ACSF containing kynurenine (200 μ M) had no significant effect on the frequency of GABAergic PSCs (Figure 5B). The slow onset of the action of L-kynurenine on GABAergic transmission is in agreement with the concept that this action is the result of the metabolic conversion of kynurenine into a neuroactive metabolite. The onset of inhibition was further assessed in experiments in which recordings were obtained from neurons before and during their superfusion with ACSF containing of 20 μ M kynurenine. As illustrated in Figure 5C, a significant reduction in the frequency of GABAergic PSCs was detected after 35 min of bath application of kynurenine. The percent inhibition increased from approximately 13% at 35 min to nearly 22% at 45 min, the maximum recording time within which there was no noticeable change in access resistance. The effect of 20 μ M kynurenine had not fully developed by 45 min, because the frequency of GABAergic PSCs recorded from CA1 pyramidal neurons in slices incubated for 2-5 h with 20 μ M kynurenine was approximately 32% lower than that recorded under control conditions (Figure 5B, 5C). Further, in the kynurenine (20 μ M)-incubated slices, the frequency of GABAergic PSCs was 0.76 ± 0.02 Hz ($n = 5$ neurons) at 2-3 h, and 0.74 ± 0.02 Hz at 3-5 h (n

= 5 neurons), suggesting that maximum inhibition was achieved by 2-3 h incubation with the drug.

Exogenously applied KYNA reduced GABAergic synaptic activity in CA1 pyramidal neurons

To analyze the effects of exogenously applied KYNA on GABAergic transmission to CA1 pyramidal neurons, hippocampal slices were first incubated for 2-5 h in ACSF containing a test concentration of KYNA (100 nM-200 μ M) and subsequently superfused with KYNA-containing ACSF. The mean frequency of GABA PSCs recorded in the continuous presence of ≥ 20 μ M KYNA was lower than that recorded under control conditions (Figure 6A). The magnitude of the effect increased with increasing concentrations of KYNA (Figure 5B 6A) and was comparable to that of equimolar concentrations of L-kynurenine, particularly at the highest concentrations. In the presence of 200 nM TTX, incubation of the slices with KYNA (20 μ M) had no significant effect on the frequency of mIPSCs (Figure 6B).

To determine the onset time for KYNA to suppress GABAergic PSCs, the frequency of events recorded from neurons before and during superfusion with ACSF containing 200 μ M KYNA was analyzed. In this set of experiments, slices were first exposed to ACSF containing CNQX (10 μ M) and APV (50 μ M) followed by the addition of KYNA (200 μ M). As shown in Figure 6C, the frequency of GABAergic PSCs was significantly reduced at 15 min of bath application of KYNA, and the magnitude of the effect increased further with time.

Effects of the admixture of L-kynurenine and the $\alpha 7$ nAChR antagonist α -BGT on GABAergic synaptic activity in CA1 pyramidal neurons

To determine whether $\alpha 7$ nAChRs contribute to the effects of L-kynurenine on GABAergic transmission, hippocampal slices were first incubated for 1 h in ACSF containing a saturating concentration of the $\alpha 7$ nAChR antagonist α -BGT (100 nM) and for an additional 2-5 h in ACSF containing both α -BGT (100 nM) and L-kynurenine (20 or 200 μ M).

The mean frequency of GABAergic PSCs recorded in the presence of α -BGT alone was not statistically different from that recorded in the presence of α -BGT-plus-20 μ M kynurenine (Figure 7A). α -BGT (100 nM) alone decreased the frequency of PSCs by 51.3 ± 1.3 % (Figure 7B). Following incubation of the slices with L-kynurenine (20 μ M) alone, the mean frequency of events was 30.7 ± 1.5 % lower than that recorded under control conditions (Figure 7B). The magnitude of the effect of the admixture of α -BGT (100 nM) and L-kynurenine (20 μ M) on the frequency GABAergic PSCs was comparable to that of α -BGT alone (Figure 7A, B), indicating that there is no additive inhibitory effect for the combined treatment.

Following 2-5-h incubation in ACSF containing 200 μ M L-kynurenine, the frequency of GABAergic PSCs was 49.2 ± 1.9 % lower than that recorded under control conditions (Figure 7B). The effect of the admixture of 100 nM α -BGT and 200 μ M kynurenine on the mean PSC frequency was larger than that of either chemical alone (Figure 7A, B). Likewise, the magnitude of the effect of the admixture of 10 nM MLA plus 200 μ M kynurenine on the frequency of GABAergic PSCs was larger than that seen with either 10 nM MLA or 200 μ M kynurenine (Figure 8). Both rightward displacement of the cumulative plot of inter-event intervals and reduction of the mean frequency of PSCs were more pronounced in the presence of both 10 nM MLA and 200 μ M kynurenine than in the presence of either chemical alone (Figure 8A, B). In addition, the admixture of 10 nM MLA and 200 μ M kynurenine caused a significant reduction of the amplitude of PSCs (Table 3).

Discussion

The results presented in this study demonstrate that i) under resting conditions, $\alpha 7$ nAChRs located on interneurons are active in hippocampal slices and contribute to maintain GABAergic synaptic input to CA1 pyramidal neurons, and ii) incubation of hippocampal slices with low micromolar concentrations of kynurenine, the precursor of KYNA, leads to the *de novo* synthesis of levels of KYNA that are sufficient to inhibit basal activation of $\alpha 7$ nAChRs, and, thereby, suppress $\alpha 7$ nAChR-dependent GABAergic synaptic transmission to the pyramidal neurons. The physiological and clinical relevance of these findings is discussed herein.

The use of nAChR antagonists reveals that there is a basal level of $\alpha 7$ nAChR activity in hippocampal slices that controls GABAergic synaptic inputs to CA1 pyramidal neurons

Glutamatergic activity provides major excitatory input to many neuron types, yet, in the present study, blockade of both AMPA and NMDA receptors in the slices had no significant effect on the frequency of GABAergic PSCs recorded from the pyramidal neurons (see Figure 1). These results strongly suggest that GABAergic synaptic activity recorded from CA1 pyramidal neurons is controlled primarily by factors other than the prototypical glutamate inputs.

In numerous studies, the use of nicotinic agonists and antagonists demonstrated that hippocampal interneurons express a number of pharmacologically distinct nAChR subtypes, the most prevalent being the α -BGT-sensitive $\alpha 7$ nAChRs (Jones & Yakel, 1997; Alkondon et al., 1997; Frazier et al., 1998; McQuiston & Madison, 1999; Ji and Dani, 2000). Electrical stimulation of specific hippocampal pathways have led to the identification of $\alpha 7$ nAChR-mediated synaptic transmission in small subsets of CA1 *stratum radiatum* and *stratum pyramidale* interneurons and in CA1 and CA3 pyramidal neurons (Alkondon et al., 1998; Frazier

et al., 1998; Stone, 2007; Hefft et al., 1999; Grybko et al., 2011; Albuquerque et al. 2009). The low probability of finding synaptic transmission mediated by $\alpha 7$ nAChRs in the hippocampus has been accounted for by the predominant non-synaptic localization of these receptors (Umbriaco et al., 1995; Aznavour et al., 2005). Here, the use of nAChR-subtype-selective antagonists led to the identification of hippocampal neurocircuitries in which basal levels of cholinergic transmitter activate $\alpha 7$ nAChRs to control GABAergic synaptic activity in the pyramidal neurons.

Two $\alpha 7$ nAChR antagonists were used in this study: α -BGT and MLA. The finding that α -BGT reduced the frequency without altering the amplitude of GABAergic PSCs recorded from CA1 pyramidal neurons demonstrated that in the hippocampal slices synaptic release of GABA onto the pyramidal neurons is maintained, in part, by $\alpha 7$ nAChRs that are activated by basal levels of ACh and/or choline. MLA also reduced the frequency of PSCs recorded from the pyramidal neurons, though to a greater extent than did α -BGT. The larger effect of MLA compared to that of α -BGT (see Figure 2 and 3) may be due to the ability of MLA to block some heteromeric $\alpha 7$ or non- $\alpha 7$ nAChRs. The $\alpha 7$ nAChR currents in hippocampal neurons that are sensitive to both α -BGT and MLA (Albuquerque et al., 2009) arise from single channels with conductance in the range of 73 pS to 91 pS (Castro and Albuquerque, 1993; Mike et al., 2000). In chick sympathetic neurons, however, Yu and Role (1998) described the existence of both homomeric and heteromeric $\alpha 7$ nAChRs with single-channel conductance of 18 pS being sensitive to α -BGT, and 35 pS channels being sensitive to MLA. As hippocampal interneurons co-express $\beta 2$ and $\alpha 5$ subunits in abundance with $\alpha 7$ subunits (Son and Winzer-Serhan, 2008; Sudweeks and Yakel, 2000), it is conceivable that MLA-sensitive/ α -BGT-insensitive $\alpha 7$ nAChRs that contain $\beta 2$ and/or $\alpha 5$ subunits contribute to regulation of the activity of GABAergic synaptic

inputs to CA1 pyramidal neurons seen here. Alternatively, some of the MLA effects may arise from blockade of non- $\alpha 7$ heteromers such as $\alpha 4\alpha 6\alpha 5(\beta 2)_2$ or $\alpha 3\alpha 4\alpha 5(\beta 2)_2$ nAChRs, as proposed by Klink et al. (2001) for some GABA neurons in the substantia nigra.

The frequency of miniature GABAergic PSCs recorded from the CA1 pyramidal neurons in the presence of TTX was not affected by MLA. Therefore, the $\alpha 7$ and non- $\alpha 7$ nAChRs that are sensitive to MLA and modulate GABAergic transmission to CA1 pyramidal neurons are not located on the presynaptic terminals of interneurons. Instead, these receptors are located on the somatodendritic and/or preterminal axon region of interneurons that synapse onto the pyramidal neurons. These receptors may also be located on the somatodendritic, preterminal, or axon regions of glutamatergic neurons that synapse onto interneurons that in turn synapse onto the pyramidal neurons from which recordings are obtained. The contribution of nAChRs present on glutamate neurons is limited because glutamate receptor blockers CNQX and APV had no significant effect on the frequency of spontaneous GABAergic PSCs (see Figure 1) and did not prevent the inhibitory effect of α -BGT (Figure 3B). *In situ* hybridization studies have demonstrated that the majority of the hippocampal interneurons express mRNA encoding the $\alpha 7$ nAChR, whereas subsets of *stratum oriens* interneurons express mRNA encoding the $\alpha 2$ nAChR subunit, and subsets of *stratum radiatum* and *stratum lacunosum moleculare* interneurons express mRNAs that encode the $\alpha 5$ nAChR subunit (Winzer-Serhan and Leslie, 2005; Son and Winzer-Serhan, 2008). Low levels of mRNA encoding the $\alpha 3$ nAChR subunit have also been detected in the hippocampus, but not in interneurons (Son and Winzer-Serhan, 2008). Functional nAChRs that have the pharmacological properties of $\alpha 3$ -containing nAChRs have been found in glutamatergic neurons/axons that synapse onto *stratum radiatum* interneurons (Alkondon et al., 2004).

Typically, CA1 pyramidal neurons receive GABAergic inputs from multiple interneuron types located in various layers of the CA1 region (Miles et al., 1996). Interneurons located in the *stratum radiatum* and *lacunosum moleculare* innervate distal regions of the apical dendrites. As a result, GABAergic inputs from these interneurons are of very low amplitude and slow rise times and are rarely detected in voltage-clamp recordings obtained from the soma of pyramidal neurons (Ouardouz and Lacaille, 1997; Buhler and Dunwiddie, 2002). Two more lines of evidence rule out the contribution of GABAergic PSCs from stratum radiatum interneurons. First, APV while able to suppress the excitability of stratum radiatum interneurons by 70% (Alkondon et al., 2011b) had no significant effect on the frequency of GABAergic PSCs (see Figure 1). Second, MLA, which is least effective in suppressing the spontaneous action potential frequency in the stratum radiatum interneurons (Alkondon et al., 2011b), was found to be highly effective in suppressing the frequency of GABAergic PSCs recorded from CA1 pyramidal neurons (see Figure 2). It is therefore conceivable that most of the GABAergic PSCs recorded from the pyramidal neurons in the present study arose from interneurons in the *stratum pyramidale* and in the *stratum oriens*. Interneurons in the *stratum pyramidale*, classified in other studies as basket cells, provide multiple innervation sites around the pyramidal cell soma and axon initial segment (Cope et al., 2002). Since nicotinic synaptic potentials sensitive to MLA and α -BGT have been detected in basket cells (Stone, 2007), it is likely that activation of α 7 nAChRs by basal levels of ACh and/or choline in the slices causes excitation of these basket cells, and, thereby, contributes to most of the nicotinic regulation of GABAergic synaptic activity in the CA1 pyramidal neurons.

KYNA generated by low micromolar concentrations of kynurenine disrupts GABAergic synaptic transmission in CA1 pyramidal neurons via inhibition of $\alpha 7$ nAChR activity: Clinical relevance

Astrocyte processes intermingle with neuronal structures thereby providing an ideal environment for multiple types of neuron-glia interaction (Black and Waxman, 1988; Theodosis and Poulain, 1999; Butt et al., 1994). The KAT II enzyme present primarily in astrocytes is actively involved in the conversion of the substrate kynurenine into KYNA (Guidetti et al., 2007). Previous studies have demonstrated that, *in vitro*, kynurenine can be converted into KYNA in hippocampal slices (Scharffman et al., 1999; Alkondon et al., 2011a). In the present study, incubation of hippocampal slices with kynurenine (20 or 200 μ M) resulted in a concentration-dependent suppression of the frequency of GABAergic PSCs in the CA1 pyramidal neurons. The slow onset of the effect of kynurenine (see Figure 5C) suggested that the effect was mediated by kynurenine-derived KYNA.

Following inhibition of the slices with α -BGT, 20 μ M kynurenine had no inhibitory effect on GABAergic synaptic transmission. This finding supported the contention that suppression of GABAergic transmission by levels of KYNA generated in slices incubated with low micromolar concentrations of kynurenine was the result of KYNA-induced inhibition of $\alpha 7$ nAChRs. On the other hand, the admixture of 200 μ M kynurenine with 100 nM α -BGT or 10 nM MLA produced more suppression of GABAergic transmission than each chemical alone. Therefore, levels of KYNA generated by exposure of the slices to high concentrations of kynurenine affect additional targets that contribute to the regulation of GABAergic synaptic activity in the pyramidal neurons. Indeed, it has been shown that concentrations of KYNA generated by exposure of hippocampal slices to 200 μ M kynurenine are sufficient to inhibit extrasynaptic NMDA receptors in CA1 interneurons (Alkondon et al., 2011a). Inhibition of these

receptors can decrease the excitability of the interneurons, and thereby, suppress GABAergic transmission to the pyramidal neurons. Also, the potential contribution of metabolites other than KYNA to the effects of high concentrations of kynurenine on GABAergic transmission cannot be ruled out.

The present demonstration that GABAergic transmission to CA1 pyramidal neurons is suppressed as a result of inhibition of $\alpha 7$ nAChRs by mild increases of *de novo* synthesis of KYNA has significant clinical relevance. Brain tissue levels of KYNA have been found to be higher in patients with schizophrenia as opposed to those detected in age-matched controls (Schwarcz et al., 2001). Disruption of hippocampal GABAergic inhibition, particularly originating from parvalbumin-positive interneurons, i.e. interneurons in the *stratum pyramidale* of the hippocampus, is a hallmark of the disease and seems to be a major determinant of the cognitive deficits that these patients present (Freedman et al., 2000; Freedman and Goldowitz, 2010; Timofeeva and Levin, 2011; Konradi et al, 2011). Thus, it is tempting to speculate that suppression of the activity of GABAergic synaptic inputs to CA1 pyramidal neurons due to KYNA-induced inhibition of $\alpha 7$ nAChRs, particularly in parvalbumin-positive interneurons, contributes to the pathophysiology of this catastrophic disorder.

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Authorship Contributions

Participated in research design: J. Banerjee, M. Alkondon, E.F.R. Pereira, E.X. Albuquerque.

Conducted experiments: J. Banerjee.

Contributed new reagents or analytic tools: None

Performed data analysis: J. Banerjee; E.F.R. Pereira.

Wrote or contributed to the writing of the manuscript: J. Banerjee, M. Alkondon, E.F.R. Pereira,
E.X. Albuquerque.

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Footnotes

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Reprint requests should be sent to Dr. Edna F.R. Pereira, Division of Translational Toxicology, Department of Epidemiology and Public Health, University of Maryland School of Medicine, 10 S. Pine Street, Suite 900, Baltimore, MD 21201. Phone: 410-706-7065. Fax: 410-706-4200. Email: EPereira@som.umaryland.edu

Figure Legends

Figure 1. Spontaneous GABAergic PSCs from CA1 pyramidal neurons. A. Sample recordings of spontaneous GABAergic PSCs at 0 mV obtained from CA1 pyramidal neurons of 30-day-old rats under control condition (top trace). The second and third trace show spontaneous PSCs at an expanded time scale under control condition. Bottom trace shows recordings at 0 mV 15 min following superfusion of the slice with ACSF containing GABA_A receptor antagonist bicuculline (10 μ M). **B.** Representative recordings of PSCs from another neuron at 0 mV before (top trace) and during superfusion of slices with ACSF containing glutamate receptor antagonists APV (50 μ M) and CNQX (10 μ M). Neurons had been superfused with the glutamate receptor antagonists for 15 min prior to beginning of analysis. **C.** Quantification of the effects of bicuculline and APV-plus-CNQX on the spontaneous PSCs recorded from CA1 pyramidal neurons at 0 mV. Graph and error bars represent mean and S.E.M., respectively, of data obtained from five neurons from four rats in bicuculline, and six neurons from three rats in CNQX + APV.

Figure 2. Effect of MLA on frequency of GABAergic PSCs. A. Cumulative probability plots of inter-event intervals and peak amplitude (inset) of PSCs recorded from control and MLA-incubated slices. Plots represent data from five neurons from four rats for control and eight neurons from four rats for MLA incubation. MLA caused a significant rightward displacement of the cumulative distribution of inter-event intervals ($p < 0.01$ according to K-S test). **B.** Mean frequency of GABAergic PSCs recorded (i) under control conditions, and (ii) in the continuous presence of MLA following 2-h incubation with MLA. Graph and error bars represent mean and

S.E.M., respectively, of data obtained from 10 neurons from five rats in control, and eight neurons from five rats in MLA. *** $p < 0.001$ compared to control according to unpaired t-test.

C. Mean frequency of GABAergic PSCs recorded under control condition followed by 15-min superfusion of the slices with MLA. Graph and error bars represent mean and S.E.M., respectively, of data obtained from seven neurons from seven rats. *** $p < 0.001$ compared to control according to paired t-test.

Figure 3. Effect of α -BGT on frequency of GABAergic PSCs. **A.** Cumulative probability of inter-event intervals of PSCs recorded under control conditions and following 1-h incubation in ACSF containing 100 nM α -BGT. Plots represent data from four neurons from four rats in control, and eight neurons from four rats in α -BGT. The cumulative distribution of inter-event intervals obtained in the presence of α -BGT was significantly displaced to the right in comparison to control ($p < 0.01$ according to K-S test). **B.** Quantification of the effects of α -BGT, CNQX-plus-APV, and CNQX-plus-APV-plus- α -BGT. Results obtained from control slices were compared to those obtained from slices following 1-h incubation with CNQX-plus-APV or 1-h incubation with CNQX-plus-APV-plus- α -BGT. Graph and error bars represent mean and S.E.M., respectively, of data obtained from five neurons from five rats in control condition, eight neurons from five rats in α -BGT incubation, five neurons from three rats in CNQX + APV incubation, and six neurons from three rats in CNQX-plus-APV-plus- α -BGT. ** $p < 0.01$ compared to control according to unpaired t test. ## $p < 0.01$ compared to CNQX-plus-APV by unpaired t test. **C.** Frequency of PSCs recorded in the continuous presence of α -BGT (100 nM) was compared to that recorded in the continuous presence of α -BGT (100 nM) plus MLA (10 nM). In these experiments, all slices were incubated for 1 h in α -BGT (100 nM)-

containing ACSF and subsequently superfused with ACSF containing only α -BGT (100 nM) or both α -BGT (100 nM) and MLA (100 nM). Graph and error bars represent mean and S.E.M., respectively, of data obtained from seven neurons from four rats in α -BGT, and seven neurons from four rats in α -BGT + MLA. ** $p < 0.01$ according to paired t-test.

Figure 4. Effect of TTX on frequency of GABAergic PSCs. A. Cumulative distribution of inter-event intervals of PSCs recorded under control conditions, in the continuous presence of 200 nM TTX, or in the continuous presence of 200 nM TTX plus 10 nM MLA. In comparison to control, the cumulative distribution of inter-event intervals obtained in the presence of TTX or TTX-plus-MLA was displaced to the right ($p < 0.001$ according to K-S test). **B.** Mean frequency of PSCs recorded under the same experimental conditions as in A. Graph and error bars represent mean and S.E.M., respectively, of data obtained from five neurons from five rats in control, 10 neurons from six rats in TTX, and six neurons from four rats in TTX + MLA. *** $p < 0.001$ compared to control according to one-way ANOVA followed by Dunnett post-hoc test.

Figure 5. Concentration dependent effect of kynurenine on frequency of spontaneous PSCs. A. Cumulative probability plot of inter-event intervals of PSCs recorded under control conditions or in the continuous presence of kynurenine (2-200 μ M) following 2-5-h incubation in ACSF containing the corresponding concentration of kynurenine. The plots were obtained from data in 11 neurons from 11 rats in control, seven neurons from four rats in 2 μ M kynurenine, 10 neurons from six rats in 20 μ M kynurenine, and six neurons from four rats in 200 μ M kynurenine. Cumulative distributions of inter-event intervals recorded from neurons in the presence of 20 μ M and 200 μ M kynurenine were significantly displaced to the right in comparison with control ($p <$

0.05 and 0.01, respectively, according to K-S test) compared to control. **B.** Mean frequency of PSCs recorded (i) under control conditions, (ii) in the continuous presence of 2 μM , 20 μM , or 200 μM kynurenine following 2-5 h incubation with the corresponding concentration of kynurenine, or (iii) during 15-min perfusion with 200 μM kynurenine. Compared to control, kynurenine reduced significantly the mean frequency of PSCs: *, $p < 0.05$; **, $p < 0.01$ according to one-way ANOVA followed by Dunnett post-hoc test. The magnitude of the effect of 200 μM kynurenine was larger than that of 20 μM kynurenine (#, $p < 0.05$ according to one-way ANOVA followed by Tukey post-hoc test). Graph and error bars represent mean and S.E.M., respectively, of data obtained from same number of neurons and rats as in **A**. Data from five neurons from three rats were used in 200 μM kynurenine bath application. **C.** Graph shows time-dependent percent reduction in PSC frequency by continuous superfusion of ACSF containing kynurenine (20 μM). The maximum inhibition obtained during an incubation protocol (data from **B**) is provided in the last column for comparison. The onset time for the effect kynurenine is ~35 min. *, $p < 0.05$; **, $p < 0.01$ according to one-way ANOVA followed by Dunnett post-hoc test.

Figure 6. Concentration-dependent effect of exogenously applied KYNA on the frequency of spontaneous PSCs. **A.** Mean frequency of PSCs recorded under control condition or in the continuous presence of KYNA (0.1-200 μM) following 2-5-h incubation in ACSF containing the corresponding concentration of KYNA. Graph and error bars represent mean and S.E.M., respectively, of data obtained from 17 neurons from 17 rats in control, four neurons from three rats in 100 nM KYNA, six neurons from three rats in 1 μM KYNA, eight neurons from five rats in 20 μM KYNA, six neurons from four rats in 50 μM KYNA, five neurons from three rats in

100 μ M KYNA, and six neurons from four rats in 200 μ M KYNA. *, $p < 0.05$; **, $p < 0.01$ compared to control according to one-way ANOVA followed by Dunnett post-hoc test. **B.** Graph showing time-dependent percent reduction in PSC frequency by continuous superfusion of ACSF containing KYNA (200 μ M). In this set of experiments, ACSF contained 10 μ M CNQX + 50 μ M APV during and 10 min prior to application of KYNA. The onset time for the effect KYNA is around 15 min. **, $p < 0.01$ according to one-way ANOVA followed by Dunnett post-hoc test. **C.** Mean frequency of PSCs recorded in the continuous presence of TTX (200 nM), or in the continuous presence of TTX (200 nM) plus KYNA (20 μ M). Graph and error bars represent mean and S.E.M., respectively, of data obtained from 12 neurons from eight rats in TTX, and five neurons from two rats in TTX + KYNA.

Figure 7. Effect of the admixture of kynurenine and α -BGT on the frequency of PSCs. A. Mean frequency of PSCs recorded (i) after 1-h incubation with α -BGT (100 nM), (ii) after 1-h incubation with ACSF containing α -BGT (100 nM) followed by 2-5 h incubation with α -BGT (100 nM) plus kynurenine (20 μ M) or (iii) after 1-h incubation with ACSF containing α -BGT (100 nM) followed by 2-5 h incubation with α -BGT (100 nM) plus kynurenine (200 μ M). In each experimental group, the ACSF used to superfuse the slices was the same as that used during the incubation time. Graph and error bars represent mean and S.E.M., respectively, of data obtained from 10 neurons from six rats in α -BGT, six neurons from four rats in α -BGT + 20 μ M kynurenine, and six neurons from four rats in α -BGT + 200 μ M kynurenine. The effect of admixture of α -BGT (100 nM) plus kynurenine (200 μ M) is significantly higher than that of α -BGT alone (**, $p < 0.01$ according to one-way ANOVA followed by Tukey post-hoc test). **B.** Graph shows the percent reduction of PSC frequency in the continuous presence of α -BGT (100

nM), kynurenine (20 μ M), α -BGT (100 nM) plus kynurenine (20 μ M), kynurenine (200 μ M), or α -BGT (100 nM) plus kynurenine (200 μ M). The magnitude of effect of admixture of α -BGT (100 nM) plus kynurenine (20 μ M) was significantly larger than kynurenine (20 μ M) alone and not different from that of α -BGT (100 nM). The effect of α -BGT (100 nM) plus kynurenine (200 μ M) was significantly larger than that of kynurenine (200 μ M) or α -BGT (100 nM) alone. **, ## $p < 0.01$ according to one-way ANOVA followed by Tukey post-hoc test.

Figure 8. Combined effect of kynurenine and MLA on the frequency of PSCs. A.

Cumulative probability plots of inter-event intervals of PSCs recorded under control condition and after 2-5-h incubation in ACSF containing different agents. In each experimental group, the ACSF used to superfuse the slices was the same as that used during the incubation time.

Compared to control, cumulative distributions of inter-event intervals were displaced to the right by 10 nM MLA ($p < 0.001$, according to K-S test), 200 μ M kynurenine ($p < 0.01$, according to K-S test), and 200 μ M kynurenine plus 10 nM MLA ($p < 0.001$, according to K-S test).

Cumulative probability plots of data obtained from 19 neurons from 19 rats in control, eight neurons from four rats in MLA, six neurons from four rats in 200 μ M kynurenine, and eight neurons from four rats in 200 μ M kynurenine plus 10 nM MLA. **B.** Mean frequency of PSCs recorded under the same experimental conditions as in **A.** Graph and error bars represent mean and S.E.M., respectively, of data obtained from the same number of neurons as in **A.** In the presence of 200 μ M kynurenine, 200 μ M kynurenine plus 10 nM MLA and 10 nM MLA, the mean frequency of PSCs was significantly lower than that of control (***, $p < 0.001$ according to one-way ANOVA followed by Dunnett post-hoc test). Mean frequency of PSCs recorded in the presence of 200 μ M kynurenine plus 10 nM MLA was significantly lower than that recorded in

the presence of kynurenine (200 μ M) or MLA (10 nM) alone (#, $p < 0.05$ according to one-way ANOVA followed by Tukey post-hoc test).

Table 1. Characteristics of GABAergic PSCs recorded from CA1 pyramidal neurons in hippocampal slices in the continuous presence of 10 nM MLA. Recordings were obtained either during 15-min superfusion of the slices with 10 nM MLA or during superfusion that followed 2-5-h incubation with MLA. Data are presented as mean \pm S.E.M. of results obtained from 10 neurons from five rats in ACSF, eight neurons from five rats in MLA incubation, and six neurons from six rats in MLA bath application.

Treatment	Amplitude (pA)	Rise time 10 to 90% (ms)	τ_d (ms)
ACSF	25.2 \pm 0.73	2.34 \pm 0.19	36.1 \pm 1.72
10 nM MLA (Bath Exposure)	20.4 \pm 0.44	1.67 \pm 0.19	42.1 \pm 3.51
10 nM MLA (Incubation)	19.9 \pm 0.92	1.78 \pm 0.18	40.7 \pm 2.63

Table 2. Characteristics of GABAergic PSCs recorded from CA1 pyramidal neurons in hippocampal slices incubated with TTX (200 nM) alone or together with MLA (10 nM). Data are presented as mean \pm SEM of results obtained from five neurons from five rats in control, ten neurons from six rats in TTX, and six neurons from four rats in TTX plus MLA. ** $p < 0.01$ compared to control according to one-way ANOVA followed by Dunnett post-hoc test.

Treatment	Amplitude (pA)	Rise Time 10 to 90% (ms)	τ_d (ms)
ACSF	26.5 \pm 0.69	2.19 \pm 0.18	34.8 \pm 1.59
200 nM TTX	13.4 \pm 0.52**	1.87 \pm 0.15	38.6 \pm 2.98
200 nM TTX plus 10 nM MLA	14.1 \pm 0.84**	1.81 \pm 0.24	39.9 \pm 3.04

Table 3. Characteristics of GABAergic PSCs recorded from CA1 pyramidal neurons in hippocampal slices incubated with kynurenine (200 μ M) alone or together with 10 nM MLA. Data are presented as mean \pm SEM of results obtained from eight neurons from eight rats in ACSF, six neurons from four rats in 200 μ M L-kynurenine, and eight neurons from four rats in 200 μ M L-kynurenine plus MLA. * $p < 0.05$ compared to control according to one-way ANOVA followed by Dunnett post-hoc test.

Treatment	Amplitude (pA)	Rise Time 10 to 90% (ms)	τd (ms)
ACSF	24.9 \pm 0.83	2.25 \pm 0.21	39.3 \pm 2.14
200 μM Kynurenine	19.9 \pm 0.96	2.07 \pm 0.12	43.5 \pm 2.25
200 μM Kynurenine plus 10 nM MLA	15.3 \pm 0.44*	1.73 \pm 0.18	52.4 \pm 2.98
200 μM KYNA	20.2 \pm 0.89	2.02 \pm 0.24	40.8 \pm 2.61

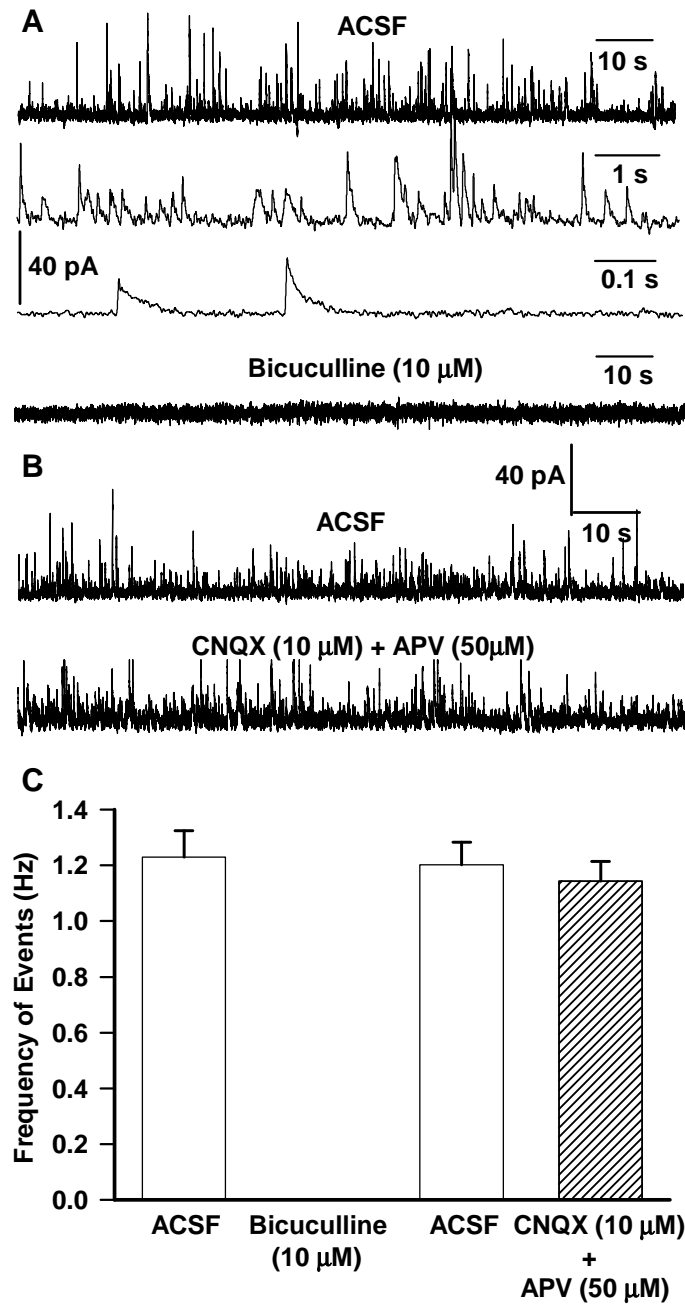


Figure 1

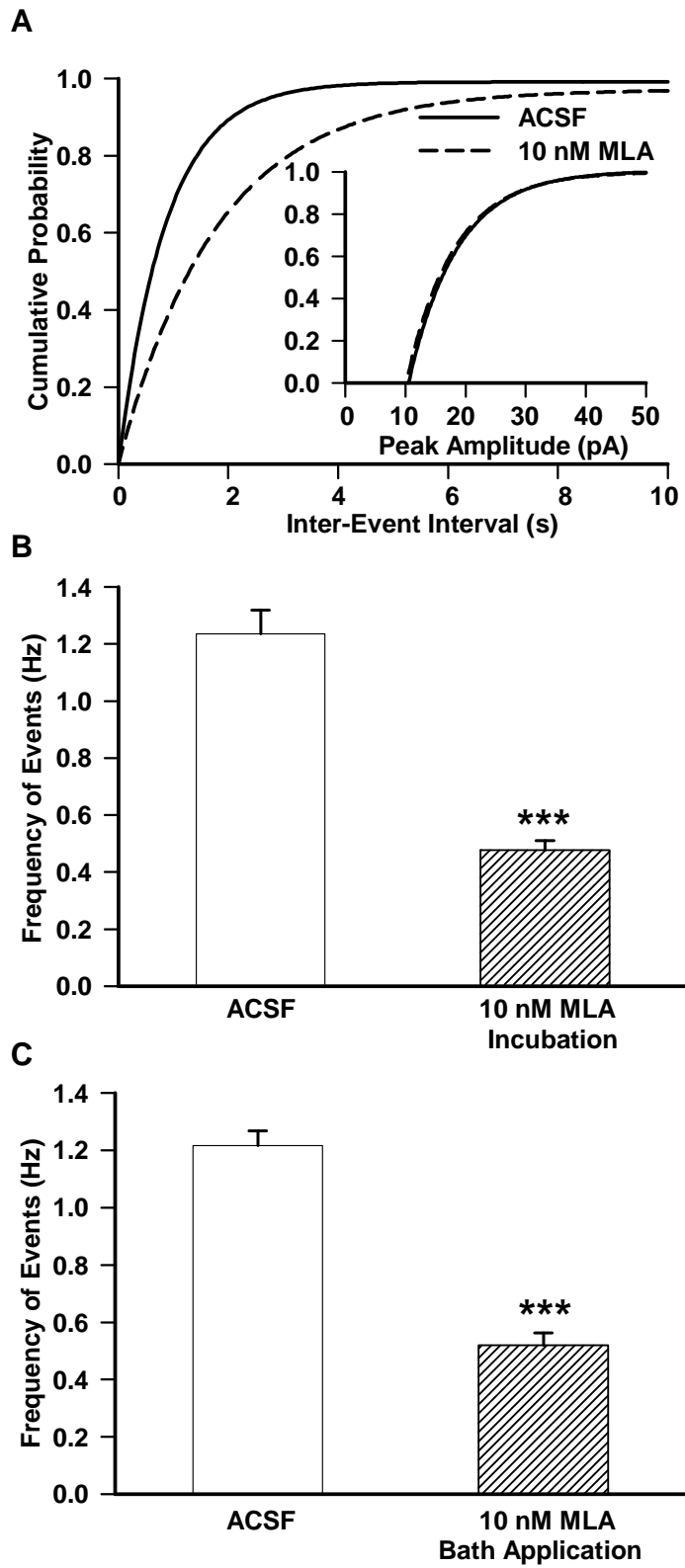


Figure 2

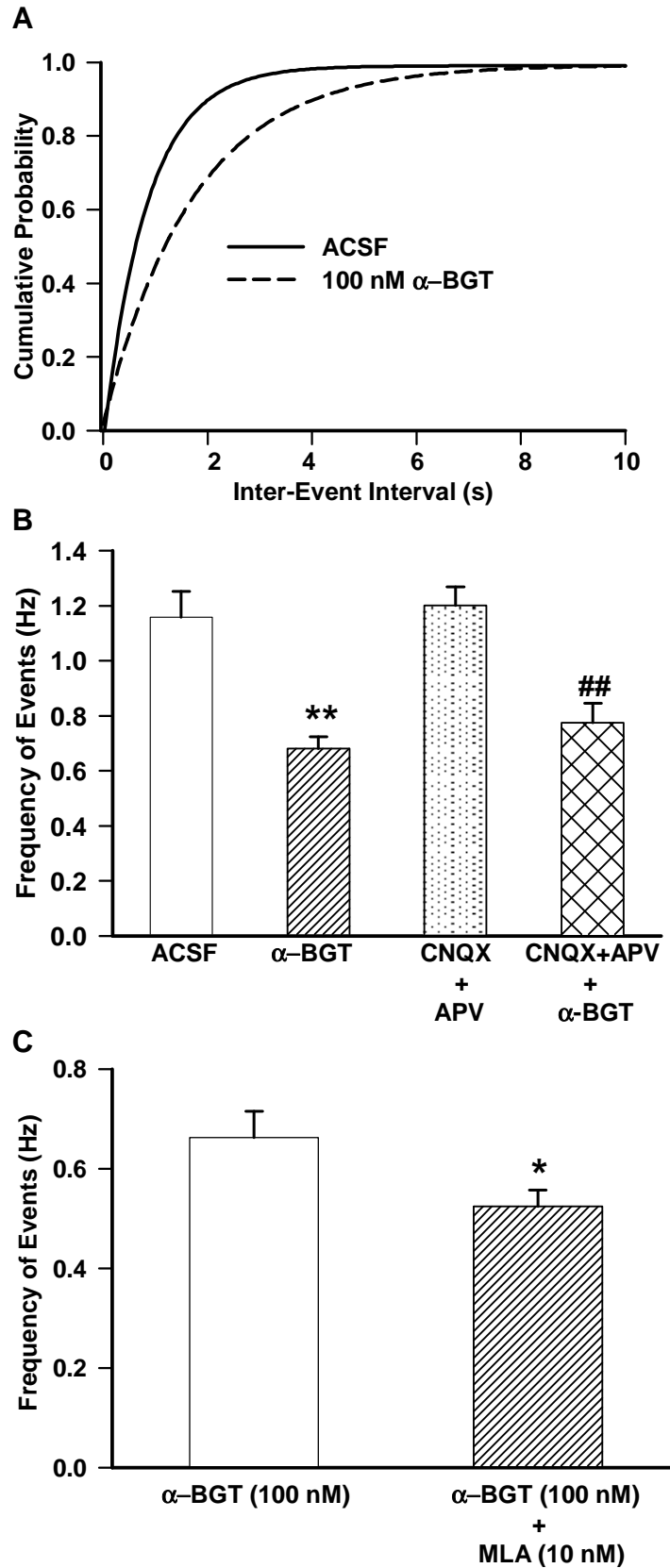


Figure 3

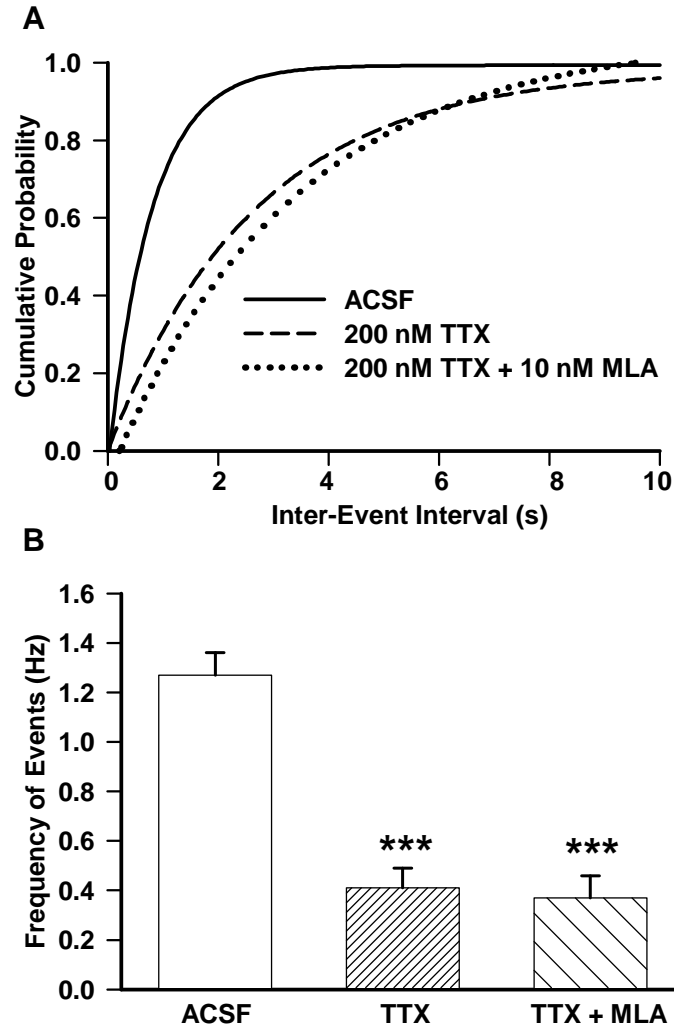


Figure 4

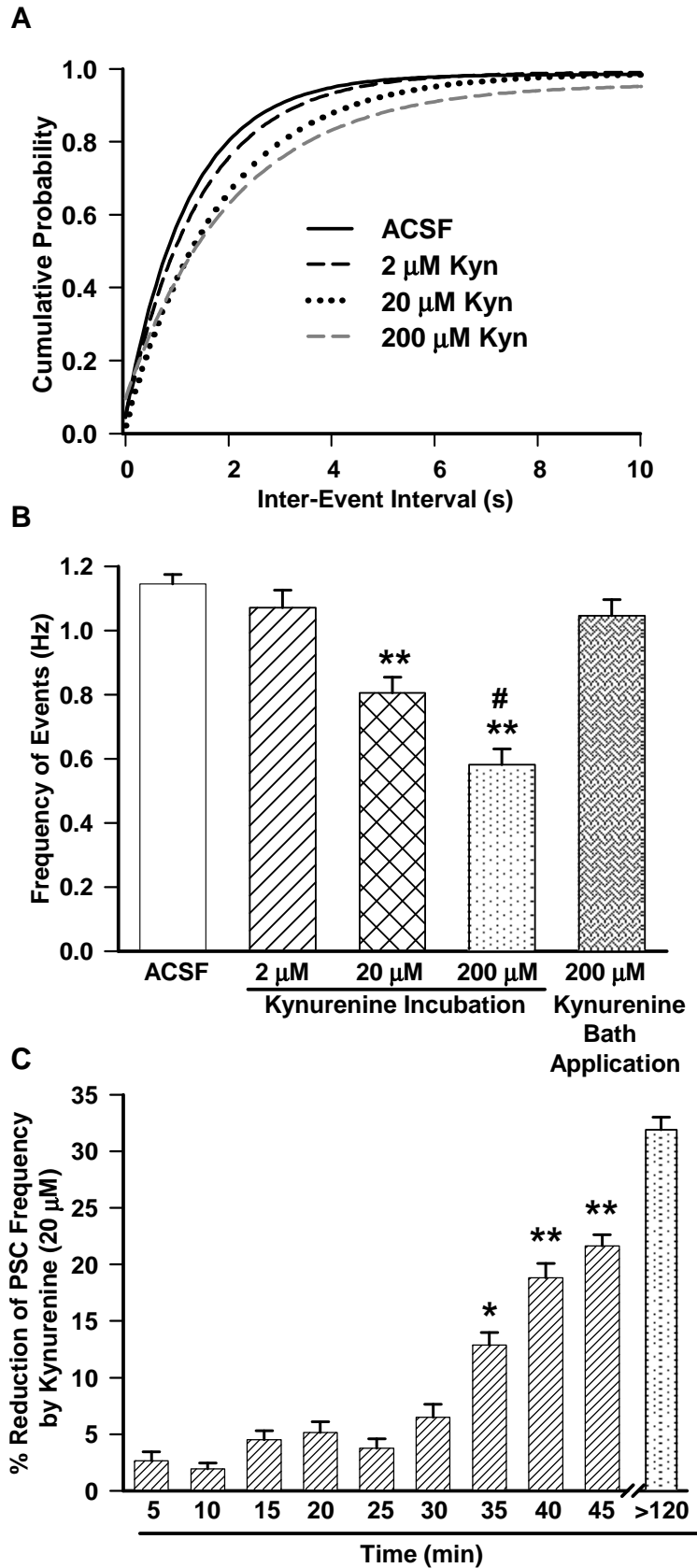


Figure 5

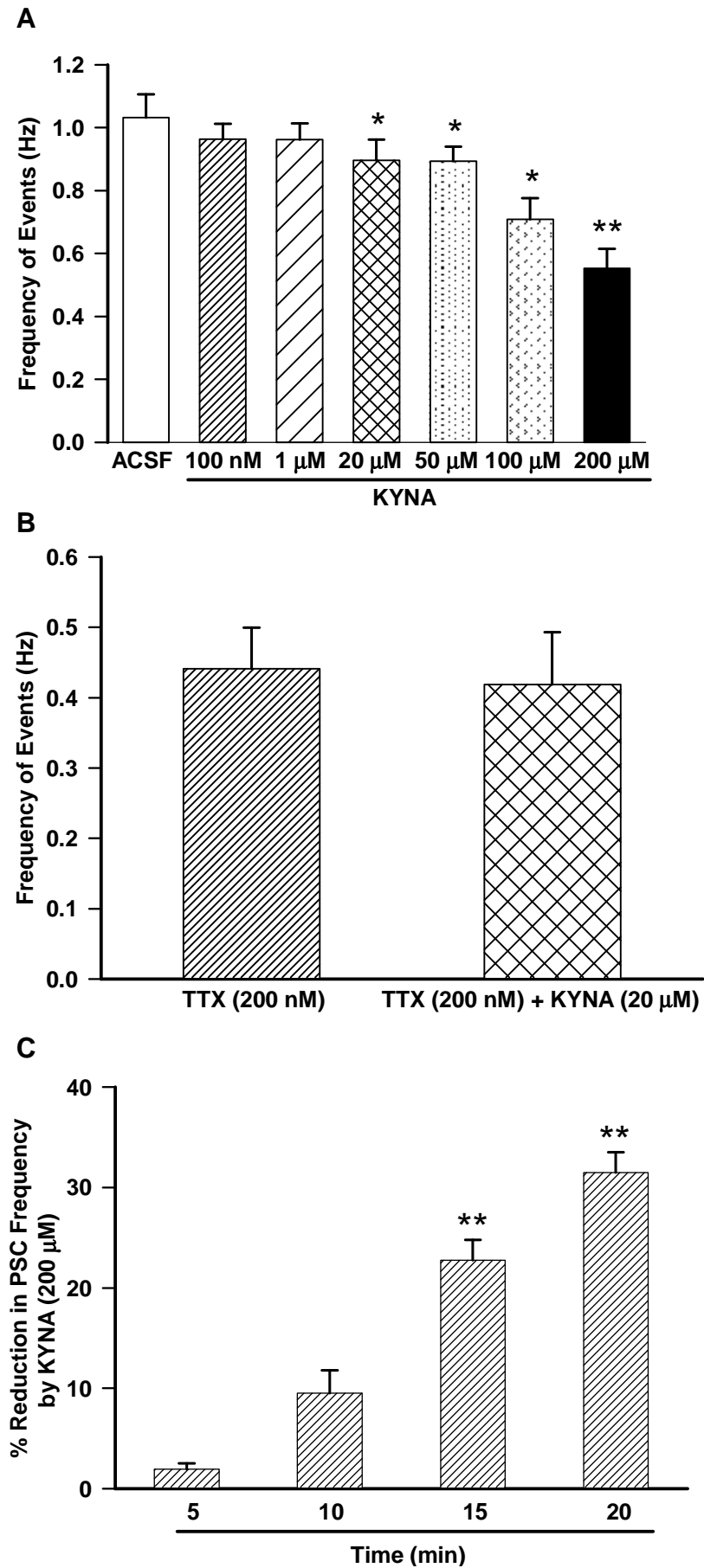


Figure 6

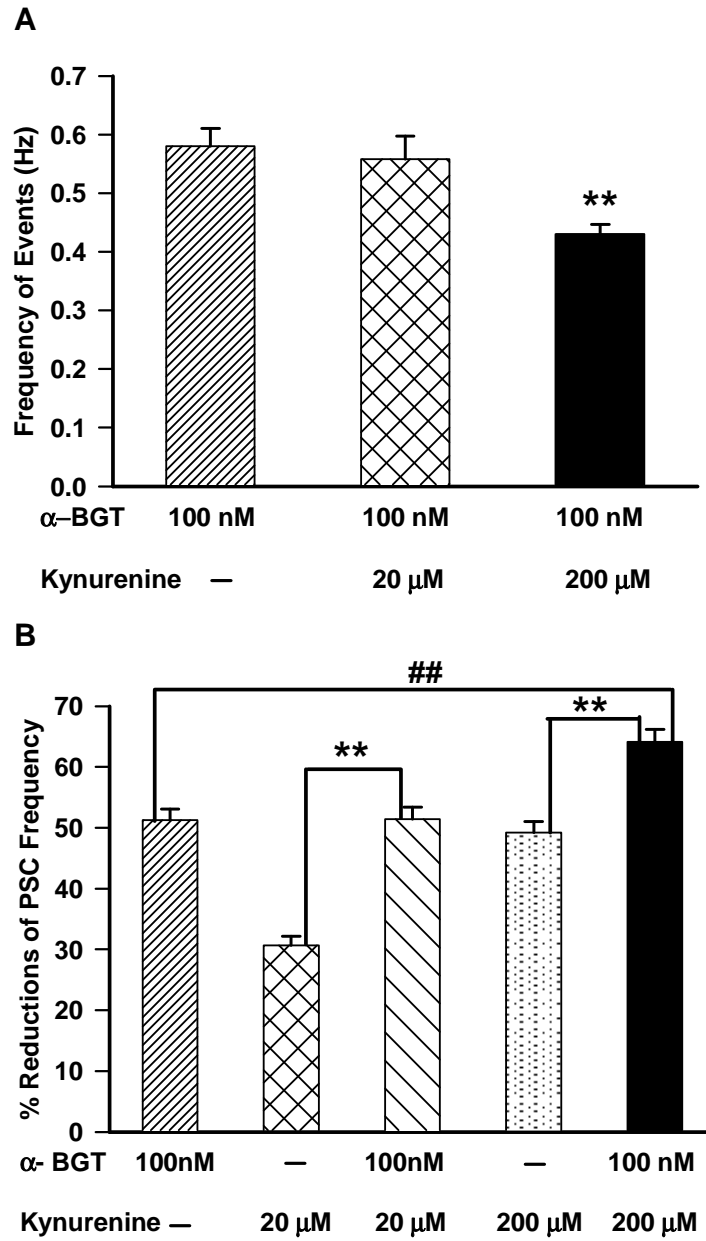


Figure 7

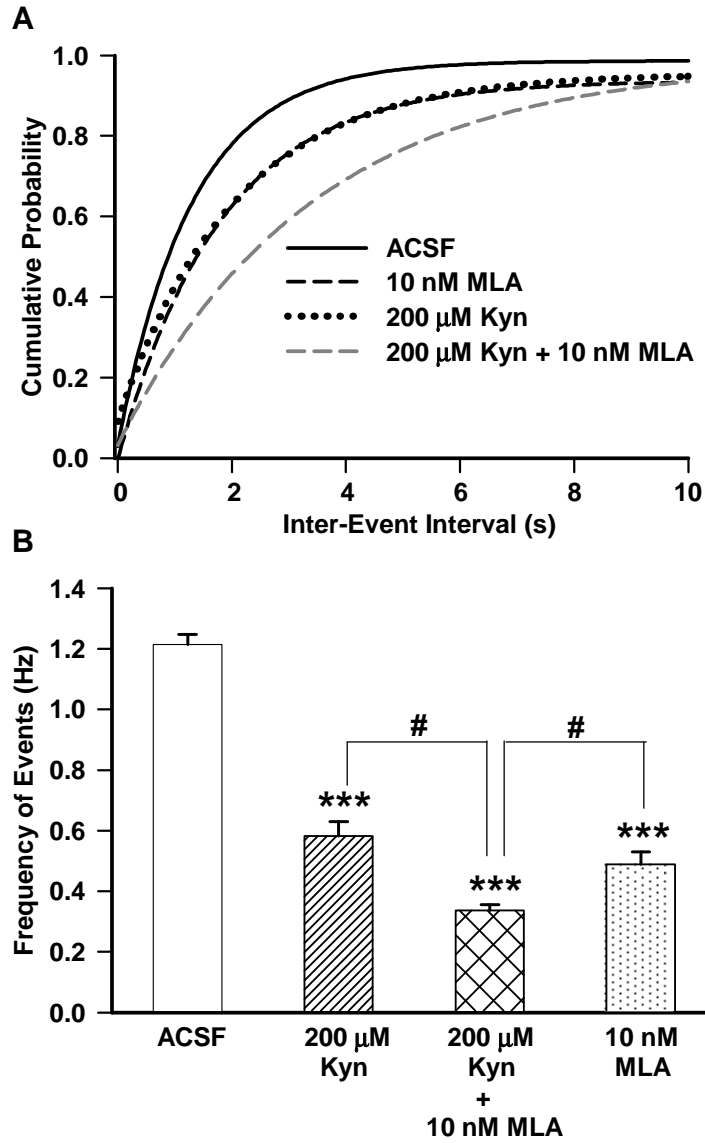


Figure 8