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ETHANOL INHIBITION OF KAINATE RECEPTOR-MEDIATED EXCITATORY NEUROTRANSMISSION IN THE RAT BASOLATERAL NUCLEUS OF THE AMYGDALA

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Abstract

The neurobiological mechanisms governing alcohol-induced alterations in anxiety-like behaviors are not fully understood. Given that the amygdala is a major emotional center in the brain and regulates the expression of both learned-fear and anxiety, neurotransmitter systems within the basolateral amygdala represent likely mechanisms governing the anxiety-related effects of acute ethanol exposure. It is well established that, within the glutamatergic system, *N*-methyl-D-aspartate (NMDA)-type receptors, are particularly sensitive to intoxicating concentrations of ethanol. However, recent evidence suggests that kainate-type glutamate receptors are sensitive to ethanol as well. Therefore, we examined the effect of acute ethanol on kainate receptor (KA-R)-mediated synaptic transmission in the basolateral amygdala (BLA) of Sprague Dawley rats. Acute ethanol decreased KA-R-mediated excitatory postsynaptic currents (EPSCs) in the BLA in a concentration-dependent manner. Ethanol also inhibited currents evoked by focal application of the kainate receptor agonist (R, S)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propanoic acid (ATPA), and ethanol inhibition of kainate EPSCs was not associated with a change in paired-pulse ratio, suggesting a postsynaptic mechanism of ethanol action. The neurophysiological consequences of this acute sensitivity were tested by measuring ethanol's effects on KA-R-dependent modulation of synaptic plasticity. Acute ethanol, like the GluR5-specific antagonist (RS)-3-(2-carboxybenzyl)willardiine (UBP 296), robustly diminished ATPA-induced increases in synaptic efficacy. Lastly, to better understand the relationship between KA-R activity and anxiety-like behavior, we bilaterally microinjected ATPA directly into the BLA. We observed an increase in measures of anxiety-like behavior, assessed in the light/dark box, with no change in locomotor activity. This evidence suggests that kainate receptors in the BLA are inhibited by pharmacologically relevant concentrations of ethanol and may contribute to some of the acute anxiolytic effects of this drug.

Keywords

kainate receptor; ethanol; electrophysiology; fEPSP; amygdala; rat

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Introduction

Animal and human studies have identified an important relationship between anxiety and alcohol-related disorders. For example, clinical and epidemiological studies have shown a significant degree of co-morbidity between anxiety disorders and alcoholism (Boyd et al., 1984). Moreover, acute alcohol exposure is known to decrease anxiety-related behaviors, while withdrawal from chronic alcohol abuse markedly increases anxiety (Costall et al., 1988; Hershon, 1973). Despite compelling evidence linking anxiety and alcoholism, the physiological substrates underlying this interaction are not well understood.

The amygdala serves as the center for regulation of specific aspects of fear and anxiety behaviors. Within the amygdala, the lateral/basolateral subdivision (BLA) receives sensory and cognitive information from thalamic and cortical inputs (McDonald, 1998; Pitkanen, 2000) and integrates these environmentally-driven stimuli in a poorly-understood process that ultimately results in the expression of anxiety-like or fearful behavioral responses (Campeau and Davis, 1995a, b). Suppression of glutamatergic receptors in the BLA has been shown to block bicuculline-induced anxiety (Sajdyk and Shekhar, 1997) as well as to prevent predator stress-induced increases in anxiety-like behavior (Adamec et al., 1999).

Alcohol is thought to act by a summation of interactions with a number of neurotransmitter systems that mediate fast excitatory and inhibitory synaptic transmission in the CNS. There are three major subtypes of ionotropic glutamate receptors: NMDA, AMPA and kainate-type (KA-R). While the physiological role of AMPA and NMDA receptors in the amygdala with respect to alcohol have been fairly well characterized, the role of KA-Rs in mediating the effects of ethanol has only begun to emerge with the development of pharmacological tools that have allowed the separation of AMPA- and KA-receptor mediated responses (reviewed in (Pinheiro and Mulle, 2006). For example, KA-Rs contribute to postsynaptic glutamatergic excitatory responses in the BLA (Li and Rogawski, 1998) and also mediate a form of long-lasting heterosynaptic facilitation in this brain region (Li et al., 2001). We have previously demonstrated that KA-Rs in the rat hippocampus are potently inhibited by acute ethanol (Carta et al., 2003; Weiner et al., 1999). In fact, the potency of these effects was, in some cases, four-fold greater than that of the well-characterized ethanol inhibition of NMDA receptors. Our goal in this study was to determine what role the actions of acute ethanol may have on KA-Rs for both amygdala-dependent neurophysiology and behavioral anxiety.

Methods

Animals

All animal procedures were performed in accordance with protocols approved by Wake Forest University School of Medicine Animal Care and Use Committee and were consistent with the NIH animal care and use policy. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) were between 120 and 140 grams at the beginning of the electrophysiological experiments described. Rats were housed in an animal care facility at 23°C with a 12-hour light/dark cycle and given food and water *ad libitum*.

Whole-cell patch-clamp electrophysiology

Drug naïve male Sprague-Dawley rats were anesthetized with isoflurane and euthanized by decapitation. 400µm coronal brain slices were prepared as described previously (Floyd et al., 2003). For *in vitro* slice preparations, 100µM ketamine was added to a modified aCSF containing (in mM): 180 Sucrose, 30 NaCl, 4.5 KCl, 1 MgCl₂•6H₂O, 26 NaHCO₃, 1.2 NaH₂PO₄, 10 glucose. Slices were then stored in standard oxygenated aCSF solution (in mM):

126 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 26 NaHCO₃, 10 glucose, and 2 CaCl₂·2H₂O at room temperature for at least 1 hour and up to 6 hours following preparation.

Methods for whole-cell recordings from rat BLA neurons within coronal slices were similar to those reported previously (DuBois et al., 2006). Briefly, electrodes were filled with an intracellular pipette solution containing (in mM): 122 CsOH, 17.5 CsCl, 10 HEPES, 1 EGTA, 5 NaCl, 0.1 CaCl₂, 4 Mg-ATP, and 0.3 Na-GTP, 2 QX-314 (Cl), pH adjusted to 7.2 with gluconic acid, osmolality ranged from 280–290mmol/kg with sucrose. EPSCs were evoked every 20 sec by brief (0.2 msec) square-wave electrical stimulation within the external capsule (EC) (Fig. 1A) using platinum/iridium concentric bipolar stimulating electrodes (FHC, Bowdoinham, ME) with an inner pole diameter of 25µm. Cells were voltage clamped at –60mV. *UBP 296 dose response*, KA-R-mediated synaptic currents were recorded using trains of electrical stimulation (3 stimuli at 100Hz) in the presence of a blocker cocktail that included 50µM GYKI 53655 (1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine, a non-competitive AMPA receptor antagonist; Tocris), 50µM APV (D(-)-2-amino-5-phosphonopentanoate, a competitive NMDA receptor antagonist; Tocris), and 20µM bicuculline methiodide (BMI, a GABA_A receptor antagonist; Tocris). Train stimulus amplitude was measured using the largest amplitude of the stimuli. To illustrate the specificity of UPB 296 (Tocris), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated EPSCs were also evoked at a holding potential of –60mV by single electrical stimuli delivered every 20 seconds via local stimulation within the BLA in the presence of increasing concentrations of UBP 296. *Kainate EPSC and acute ethanol experiments*, KA-R-mediated synaptic currents were recorded using trains of electrical stimulation (6 stimuli at 100Hz) in the presence of a blocker cocktail that included 30µM GYKI 53655, 50µM APV, and 20µM BMI. Train stimulus amplitude was measured using the largest amplitude of the stimuli. *ATPA-evoked, kainate-mediated whole-cell currents*, KA-R-mediated currents were evoked using bath application of 100µM ATPA. ATPA was applied directly to the soma of BLA neurons using a Picospritzer III (General Valve, Fairfield, NJ) in the presence of a blocker cocktail that included 30µM GYKI 53655, 100nM Tetrodotoxin (Tocris) and was completely blocked by 40µM DNQX. *Kainate-EPSC paired pulse*, KA-R-mediated synaptic currents were recorded in whole-cell mode using single, paired, square-wave electrical stimulation in the presence of a blocker cocktail that included 30µM GYKI 53655, 50µM APV, and 20µM BMI. *NMDA receptor experiments*, EPSCs were evoked at a holding potential of –30mV by single electrical stimuli delivered every 20 seconds via local stimulation within the BLA. Both responses were pharmacologically isolated using 40µM DNQX (AMPA/kainate receptor antagonist) and 20µM BMI. Recordings were acquired with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA), and digitized with a Digidata 1320 (Axon Instruments). Data were analyzed using pCLAMP 9.0 (Axon Instruments) and subjected to a t-test. Data are presented as the percent change in current amplitude, relative to control values.

Field Recording

Slices were placed in the recording chamber and were continuously superfused in aCSF (2ml/min) warmed to approximately 30°C (Lovinger and McCool, 1995). Electrodes were filled with an intracellular pipette solution containing 150mM NaCl. Ensemble postsynaptic potentials were evoked every 30 sec by brief (0.2 msec) square-wave electrical stimulation within the EC and were entirely blocked by 20µM DNQX (Fig. 4). These responses are referred to as field excitatory postsynaptic potentials (fEPSPs) throughout the manuscript. Maximal fEPSP responses were determined in every slice; and, the stimulus intensity that evoked 40–50% maximal amplitude was used for the synaptic plasticity experiments. All field experiments were recorded with 10µM bicuculline methiodide in the bath for the entire experiment. Slices were allowed to equilibrate in the chamber containing aCSF and bicuculline for 30min before

recording. Baseline fEPSPs were recorded for 10 minutes prior to application of drug. ATPA was applied for 15 minutes and then the slice was washed for 40 minutes. When ethanol or UBP 296 was applied, it was turned on after the baseline and 5 minutes before the application of ATPA. fEPSP recordings were acquired with an Axoclamp 200 amplifier (Molecular Devices/Axon Instruments, Foster City, CA) in the current-clamp mode and digitized with a Digidata 1322A D/A board (Axon Instruments). The 10–90% rise slope was measured using pCLAMP 9.0. Data were analyzed using one-way ANOVA across the treatment groups and are presented as mean percent changes $\pm$ SEM.

Behavioral analysis

Behavioral experiments were carried out on adult male Sprague-Dawley rats (309.1 ± 1.6 g) that were surgically implanted with guide cannulas inserted bilaterally into the BLA (coordinates: 2.8mm posterior to the bregma, 8.2mm ventral to bregma, and 5.0mm lateral to the midline). ATPA (25, 125, 250pmol) or vehicle (aCSF) were injected bilaterally via infusion pump (Harvard Apparatus, Holliston, MA) at a rate of 0.2 μ l/min (total volume = 0.5 μ l) using a microinjection procedure described previously (McCool and Chappell, 2007).

Two behavioral tests were used to assess the effects of ATPA injections into the BLA. Anxiety-like behavior was assessed using a custom-designed light/dark box (27 $\times$ 58 $\times$ 30cm). An area of 24 $\times$ 27cm was painted black and illuminated by a 40W red light bulb (dark side). The remaining area was painted white and was illuminated by a 40W incandescent light (light side) and the two chambers were separated by a partition that contained a 10 $\times$ 10cm doorway in the center to allow free access to the two sides. On test days, 5 minutes after ATPA or vehicle injections, subjects were placed on the light side of the chamber, facing away from the doorway. Test sessions lasted 5 minutes and activity was recorded on videotape for subsequent analysis by an investigator blinded to the experimental status of each subject. Anxiety-like behaviors scored included the latency for the rats to re-enter the light side for the first time, the total number of crosses to the light side, and the total time spent on the light side. Subjects were exposed to the test no more than two times, separated by at least one week, each subject was only tested with a single ATPA dose, and the order of testing (aCSF vs. ATPA) was randomized.

Locomotor activity was assessed using Omnitech Digiscan Animal Activity Monitors (model-RXYZCM, Columbus, OH). These acrylic plastic chambers (42 $\times$ 42 $\times$ 30cm) were enclosed in sound-attenuating cubicles equipped with exhaust fans that provided additional masking of extraneous noise. The monitors were equipped with infrared photo-detectors located in an array of eight photobeams on each wall of the chamber, arranged regularly along the length of the chamber 2.5cm above the floor. Five minutes following 125pmol ATPA or vehicle injections, subjects were placed in the center of the chambers and activity was assessed for 30 minutes. Locomotor measures evaluated included total horizontal activity, total distance traveled, and movement time. Each subject was only exposed to the activity box under a single experimental condition (ATPA or aCSF).

Following the behavioral testing, all subjects were sacrificed for histological confirmation of electrode placement using procedures described previously (McCool and Chappell, 2007). Only data from animals with confirmed bilateral cannula placements in the BLA were included in the analysis (17 of 20 animals).

Behavioral data were analyzed using either unpaired t-tests (locomotor activity) or ANOVA followed by the Dunnett's multiple comparison test (light/dark box) with a minimum level of significance of $p < 0.05$.

Results

Acute ethanol dose dependently decreased KA mediated EPSCs

A previous study reported that acute ethanol dose-dependently decreased KA-R-mediated synaptic currents in the hippocampus to a greater extent than that of NMDA and AMPA receptor-gated EPSCs (Weiner et al., 1999). To test whether similar differences are expressed in the BLA we investigated the effects of several concentrations of acute ethanol on KA-R-mediated EPSCs using whole-cell *in vitro* slice electrophysiology.

Previous studies have reported that KA EPSCs can be recorded by stimulation of the external capsule and the amplitude of these responses can be markedly enhanced by using short stimulus trains (Li et al., 2001; Li and Rogawski, 1998). Therefore, in our first experiments, KA EPSCs were evoked in BLA neurons by stimulus trains delivered to the external capsule (Fig. 1A). We confirmed our isolation of KA-R-mediated currents using a selective GluR5 antagonist, UBP 296. UBP 296 inhibited both KA-R- and AMPA-R-mediated synaptic responses in a dose-dependent fashion (Fig. 1B). The concentration-response relationship revealed an IC_{50} for KAR-mediated EPSCs of $21\mu M$ (Fig. 1C). While we were unable to test concentrations $>300\mu M$ due to solubility issues, the estimated UBP 296 IC_{50} for AMPAR-mediated synaptic responses was greater than $200\mu M$. We chose to use $10\mu M$ UBP 296 throughout the rest of the study since this concentration was close to the IC_{50} for KA-R-mediated responses ($\sim 40\%$ inhibition) but did not appear to inhibit AMPA-mediated synaptic responses to any appreciable extent.

We found that bath application of ethanol significantly decreased KA-R-mediated EPSCs by $25.9 \pm 6.8\%$ for $20mM$ ($p < 0.05$; $n = 14$); $47.1 \pm 5.9\%$ for $40mM$ ($p < 0.01$; $n = 11$); and $55.2 \pm 7.5\%$ for $80mM$ ($p < 0.01$; $n = 13$) (Fig. 2A&B). This inhibition was apparent within 2–3 minutes and readily reversed upon ethanol washout. In contrast to the relatively potent effect of ethanol on KA EPSCs, ethanol had much less of an effect on NMDA EPSCs, with significant inhibition only being observed at the highest concentration tested ($80mM$, $16.0 \pm 4.3\%$; $n = 19$) and this concentration had no effect on AMPA EPSCs ($3.7 \pm 2.9\%$; $n = 7$; $p > 0.05$) (Fig. 2A&B). KA-R-mediated synaptic currents were recorded in the presence of a maximally effective concentration of the selective AMPA receptor antagonist, GYKI 53655. Nevertheless, it was technically not possible to completely rule out the possibility that some AMPA-R activation contributed to KA EPSCs. However, the observations that ethanol had no effect on AMPA EPSCs but potently inhibited KA-R-mediated synaptic responses suggest that AMPA-Rs contributed minimal to KA EPSCs in these studies.

Acute Ethanol Inhibition of KA-R function is mediated postsynaptically

We next carried out two experiments to determine if ethanol inhibition of KA-R mediated EPSCs was mediated via a pre- or postsynaptic mechanism. First, we directly applied $100\mu M$ ATPA near the cell being recorded, via pressure application, to activate a postsynaptic KA receptor-gated current. ATPA-evoked currents were recorded every 60 seconds in the presence of $500nM$ TTX and $30\mu M$ GYKI 53655 to block voltage-gated sodium channels and AMPA receptors, respectively. Bath application of $80mM$ EtOH significantly inhibited the amplitude of postsynaptic ATPA-evoked currents ($46.4 \pm 6.1\%$; $n = 13$; $p < 0.01$) (Fig. 3A&B). In 5 cells, DNQX applied after ethanol wash was able to inhibit most of the remaining KA-R current ($81.1 \pm 3.5\%$).

In the second experiment, we evoked pairs of kainate EPSCs using single electrical stimuli at an inter-pulse interval of $50msec$ and compared the ratio of the second synaptic response to the first in the presence and absence of acute ethanol. At short inter-stimulus intervals, the ratio of synaptic current amplitudes following a pair of electrical stimuli is commonly believed to

be inversely related to the probability of neurotransmitter release (Andreasen and Hablitz, 1994; Katz et al., 1993). Although 80mM ethanol significantly inhibited the amplitude of kainate EPSCs (Fig. 4 A&B), ethanol inhibition was not associated with a change in the paired-pulse ratio (baseline PPR = 1.2 ± 0.2 , ethanol PPR = 1.2 ± 0.1 ; $p > 0.05$; $n = 12$) (Fig. 4C). Taken together with the ethanol inhibition of ATPA-induced currents, these findings are consistent with a postsynaptic mechanism of acute ethanol inhibition of kainate EPSCs in the rat BLA.

Acute ethanol and UBP 296 block ATPA induced Synaptic Plasticity

Field excitatory postsynaptic potentials, fEPSPs, are used widely in the literature to measure increases and decreases in synaptic strength (McKernan and Shinnick-Gallagher, 1997; Rogan and LeDoux, 1995; Rogan et al., 1997; Schroeder and Shinnick-Gallagher, 2005). The GluR5 agonist ATPA has been used to stimulate increases in synaptic facilitation and this has been suggested to take place through recruitment of additional excitatory synapses (Li et al., 2001). To examine the neurophysiological ramifications of acute ethanol inhibition of KA-R-mediated synaptic transmission, we used fEPSPs to examine increases in synaptic strength induced by the kainate receptor agonist ATPA. fEPSPs baseline was recorded for 10 minutes and ATPA was applied for 15 minutes. During the 15 minute ATPA application, the amplitude and slope of fEPSPs decreased below baseline, similar to that seen in other laboratories (Li et al., 2001). fEPSP amplitude and slope rapidly (in < 5 min) returned to baseline levels after removing ATPA from the slice. 15 min after the application of $5\mu\text{M}$ ATPA, fEPSP slope was increased by $285.2\% \pm 99.1\%$ ($n = 7$) (Fig.5). The ATPA-dependent increase in fEPSP slope was significantly attenuated by the KA-R antagonist $10\mu\text{M}$ UBP 296 ($140.3\% \pm 32.3\%$ of baseline; $n = 5$; $p < .05$). Likewise, pre-exposure of the slice with 80mM ethanol also significantly inhibited this ATPA-induced increase in synaptic strength ($122.4\% \pm 20.7\%$ of baseline; $n = 7$; $p < .05$). Similar findings were evident when considering the amplitude of fEPSPs. ATPA application increased the amplitude to $165.7 \pm 13.4\%$ of baseline, while ATPA + UBP 296 or +acute ethanol produced amplitudes of only $104.8 \pm 6.5\%$ ($p < 0.1$) and $113.2 \pm 7.8\%$ ($p < .01$) of baseline, respectively (Fig. 5B). These data suggest that UBP296 and acute ethanol inhibit KA-R-dependent synaptic plasticity.

ATPA injections lead to increased anxiety-like behavior in Sprague Dawley rats

We bilaterally microinjected a KA-R agonist, ATPA, into the BLA of Sprague Dawley rats to assess possible behavioral manifestations of KA-R activity in this brain region. We first assessed whether ATPA would lead to an increase in anxiety-like behaviors in these rats using a light/dark box. Bilateral microinjection of ATPA significantly and dose-dependently increased re-entry latency (time to re-enter the light side following the first egress to the dark side) ($F = 5.93$, $p < 0.005$) and decreased the number of crosses to the light side ($F = 6.1$, $p < 0.004$), consistent with increased anxiety-like behavior (Fig 6). Post-hoc analyses of these data revealed significant effects of 125pmol and 250pmol ATPA on each of these measures ($p < 0.05$, Dunnett's Test, relative to control). Time on the light side was also modestly decreased by ATPA microinjection, although this effect was not statistically significant.

In the next experiment, we assessed the effect of 125pmol ATPA, a dose that produced significant changes in anxiety-like behavior, on locomotor behavior. Five minutes after the microinjection treatment, animals were placed in sound-attenuated, darkened automated activity monitors for 30 minute sessions. ATPA microinjection had no effect on horizontal activity, total distance traveled, or total movement time (Fig 7). There was also no effect of ATPA on initial locomotor activity during the first five minutes of the sessions (data not shown).

Discussion

Our results are the first to suggest that acute ethanol inhibits KA-R synaptic responses in the BLA. In addition, initial behavioral evidence suggests that activation of KA-Rs in the BLA is anxiogenic. It is therefore reasonable to suggest that the inhibitory effects of acute ethanol on KA-Rs may contribute to the anxiolytic effects of acute ethanol in rats. Interestingly, the inhibition of KA-R mediated synaptic currents by ethanol was four-fold more efficacious/potent than ethanol inhibition of NMDA-receptor mediated currents, while it had no effect on AMPA-mediated EPSCs. In addition to inhibiting synaptic currents, we also show for the first time that acute ethanol inhibits KA-R dependent synaptic plasticity in the BLA. Since increased synaptic efficacy at BLA glutamatergic synapses is associated with increased expression of learned-fear behaviors (McKernan and Shinnick-Gallagher, 1997), ethanol inhibition of ATPA-induced synaptic plasticity suggests an additional mechanism for the anxiolytic effect of acute ethanol. This is supported by our behavioral data showing enhanced anxiety-like behavior induced by the KA-R agonist, ATPA.

Acute ethanol inhibits KA EPSCs via a postsynaptic mechanism

Kainate receptors are a major subtype of the excitatory glutamate receptor family and are present both pre- and postsynaptically at glutamatergic synapses. KA-Rs consist of five possible subunits, GLUR₅₋₇, needed for functional channels, and KA₁₋₂ (Braga et al., 2004). Receptors containing GLUR_{5,6} and KA₂ have been shown to be highly expressed in the BLA and contribute postsynaptically to glutamatergic excitatory postsynaptic potentials (EPSPs) (Li and Rogawski, 1998). KA-Rs expressed in neuronal cultures (Valenzuela et al., 1998; Valenzuela and Cardoso, 1999) and in the rat hippocampus (Weiner et al., 1999) are inhibited by acute ethanol exposure.

First, our results confirm previous findings (Li and Rogawski, 1998) that stimulation of the external capsule evokes synaptic responses mediated by KA-Rs in BLA neurons. These responses were specifically inhibited by the kainate receptor-selective antagonist UBP 296. Most importantly, we show that acute ethanol also significantly inhibits KA-R postsynaptic responses in BLA principal neurons. While we did not specifically address if presynaptic KA-Rs located on glutamatergic terminals contribute to these findings, our data indicate that acute ethanol exposure has no effect on glutamate release as ethanol inhibition of KA EPSCs was not associated with any changes in paired-pulse ratio. As previously observed in the hippocampus (Weiner et al., 1999), acute ethanol inhibited synaptically evoked postsynaptic amygdalar KA EPSCs more potently than either AMPA EPSCs, which were not affected by even the highest concentration of ethanol tested (80 mM), or NMDA receptor-mediated synaptic responses, which were only significantly inhibited at this highest concentration. These findings agree with other reports of postsynaptic inhibition of NMDA receptors by acute ethanol in isolated cells of the BLA (Floyd et al., 2003), in the hippocampus (Lovinger et al., 1989), central nucleus of the amygdala (Roberto et al., 2004) and the ventral bed nucleus of the stria terminalis (Kash et al., 2007). Acute ethanol has been shown to have no effect on AMPA receptors in the hippocampus (Ariwodola et al., 2003) with a small, 12% inhibition on composite AMPA and KA-R currents in cerebellar granular cells (Valenzuela et al., 1998). Therefore, our data are consistent with data for other glutamate receptors and with the hypothesis that acute ethanol inhibits KAR-mediated EPSCs via a postsynaptic mechanism.

ATPA-induced synaptic plasticity is inhibited by acute ethanol

Field excitatory postsynaptic potentials, fEPSPs, are used widely in the literature to measure increases and decreases in synaptic strength (McKernan and Shinnick-Gallagher, 1997; Rogan and LeDoux, 1995; Rogan et al., 1997; Schroeder and Shinnick-Gallagher, 2005). Using fEPSPs, we wanted to examine neurophysiological endpoints related to KA-R activation in the

BLA. Recently, the KA-R agonist ATPA was shown to increase synaptic strength in BLA principal neurons, potentially through recruitment of excitatory synapses (Li et al., 2001). Further, in the CA1 region of the hippocampus, long term ATPA application led to an enduring increase in the number of glutamatergic synapses in that region (Vesikansa et al., 2007). Using 5 μ M ATPA, a concentration that does not affect AMPA receptors in cortex (Stensbol et al., 1999), we were able to replicate the ATPA-induced facilitation in the current work and block the facilitation using a concentration of UBP 296 that was selective for KA-Rs in our preparation. Most significantly, acute ethanol also blocked the ATPA-induced synaptic plasticity. This suggests that acute ethanol can block KA-R dependent changes in synaptic strength in the BLA without affecting AMPA-mediated synaptic transmission. While our data do not exclude the potential activation of AMPA receptors by ATPA in our preparation, the acute sensitivity to low concentrations of UBP 296 and to inhibition by acute ethanol does rule out AMPA receptor participation in the ATPA-induced plasticity.

The KA-R agonist ATPA causes a dose-dependent increase in anxiety-like behavior measured in a light/dark box

Our data also suggest that the acute sensitivity of BLA KA-Rs to ethanol may be behaviorally relevant. We show that microinjection of the kainate agonist ATPA into the BLA can dose-dependently increase anxiety-like behavior measured in a light/dark box, while having no effect on general locomotor activity. These observations are consistent with other microinjection studies showing that increased excitability within the BLA is associated with increased behavioral indices of anxiety-like behavior (Lack et al., 2007; Menard and Treit, 1999; Sanders and Shekhar, 1995). It has also been recently shown that i.p. injection of LY382884, another KA-R antagonist, decreases anxiety-like behavior of rats (Alt et al., 2007). Furthermore, topiramate, a compound that has been shown to antagonize pharmacologically-isolated KA-R responses in the BLA, can inhibit ATPA induced seizures (Kaminski et al., 2004). Thus, the postsynaptic KA-Rs present on principal neurons seem to play a prominent role in regulating excitation within this brain region. This supports suggestions that GluR5 receptors in the BLA are a potential therapeutic target for anxiety related behaviors (Aroniadou-Anderjaska et al., 2007). As would be expected, antagonism of AMPA and NMDA receptors has also proven to be anxiolytic (Adamec et al., 1999; Gatch et al., 1999; Walker and Davis, 2002). However, our electrophysiological data demonstrates that BLA KA-Rs are particularly sensitive to ethanol inhibition, at least relative to these other subtypes of ionotropic glutamate receptors. Such findings, coupled with the observation that ATPA microinjection within the BLA may increase anxiety-related behaviors, are therefore consistent with the hypothesis that ethanol inhibition of BLA KA-R function may contribute to some of the acute anxiolytic effects of this drug.

Conclusions

Taken together, these novel data demonstrate that KA-R-mediated excitatory synaptic transmission in the BLA is potently inhibited by ethanol. In addition, we show that ethanol inhibits BLA synaptic plasticity induced by KA-R agonists and supported this finding using a KA-R selective antagonist. Our behavioral studies also suggest that KA-Rs in the BLA may represent a novel element of the neurophysiology underlying anxiety-like behaviors and potentially contribute to some of the acute anxiolytic effects of ethanol.

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Figure 1. UBP 296 is a selective antagonist for BLA kainate receptors

A, Slices were stimulated (single stimulus for AMPA current, train of 3 stimuli for kainate current) in the external capsule and the recording electrode was placed in the basolateral amygdala as shown (figure taken from (Paxinos and Watson, 1997)). **B**, The concentration-response curve for UBP 296 revealed the IC_{50} for KA-Rs in the BLA was approximately $21\mu M$, while the IC_{50} was approximately $\geq 200\mu M$ for AMPA receptors. **C**, Exemplar traces show the inhibition of AMPA receptor synaptic currents (top) and kainate receptor synaptic currents (bottom) by 10 and $30\mu M$ UBP296.

Figure 2. Acute ethanol significantly inhibits kainate receptor-mediated EPSCs in the rat basolateral amygdala

A, Traces are averages of 5–7 currents (for kainate current, largest amplitude of 6 train stimulations) recorded under control, 80 mM ethanol, and wash conditions and illustrate significant ethanol inhibition of NMDA- and KA-, but not AMPA receptor-mediated EPSCs. **B**, Bar graph summarizing the effect of ethanol on AMPA, NMDA, and KA-R EPSCs recorded from BLA pyramidal neurons, expressed as percent of control. Bath application of 20mM ($n = 14$), 40mM ($n = 11$), and 80mM ethanol ($n = 13$) significantly decreased KA-R-mediated EPSCs while NMDA receptor-mediated synaptic currents were only inhibited by the highest concentration (80 mM $n = 9$). Note that AMPA EPSCs were not inhibited by 80mM ethanol ($n = 7$). (*, $p < 0.05$; **, $p < 0.01$, t-test)

Figure 3. Ethanol inhibits ATPA-evoked currents in BLA pyramidal neurons

A, Time course from a representative recording illustrating the effect of 80 mM ethanol and DNQX on the amplitude of currents evoked by pressure application of 100 μ M ATPA onto a BLA pyramidal neuron in the presence of 500 nM TTX and 30 μ M GYKI 53655. ATPA-evoked currents were evoked every 60 seconds. Traces above the graph are averages of 4–6 sweeps evoked under the conditions corresponding to the letters in the time course. **B**, Bar graph summarizing the effect of 80mM EtOH and DNQX on AMPA-evoked currents. Numbers in brackets represent the number of cells tested under each experimental condition. *, $p < 0.05$, t-test).

Figure 4. Ethanol inhibition of kainate EPSCs is not associated with a change in paired-pulse ratio (PPR)

A, Representative traces of paired-pulse responses are averages of 6 KA-R EPSCs recorded under control conditions and in the presence of 80 mM ethanol. **B**, Bar graphs summarizing the effect of 80mM ethanol on the amplitude of pairs of KA EPSCs evoked at an inter-pulse interval of 50msec. **C**, Summary of the paired-pulse ratio, calculated as Peak 1 amplitude/Peak 2 amplitude, recorded under control conditions (CTL) and in the presence of 80 mM ethanol. Note that ethanol significantly inhibited the amplitude of Peak 1 and Peak 2 with no change in PPR. (N = 13; *, $p < 0.05$, relative to control, t-test).

Figure 5. Acute ethanol inhibits ATPA induced synaptic plasticity in the BLA

A, Acute ethanol and the kainate receptor competitive antagonist UBP 296 significantly inhibit ATPA-induced facilitation of the fEPSP slope. 20 minutes after 5μM ATPA application, fEPSP slope was significantly increased by almost 2-fold ($n = 7$ slices). This effect was significantly inhibited by pre-incubation with 10μM UBP 296 ($n = 5$ slices) or with 80mM ethanol ($n = 7$ slices). **B**, fEPSP amplitudes were also significantly inhibited by acute ethanol and UBP 296. **C**, Time course of ATPA-induced increase in fEPSP slope recorded from control slices or slices treated with 80mM ethanol. Slope values were binned across two-minute intervals (four fEPSPs) to better illustrate the synaptic plasticity. **D**, Exemplar traces are from a single representative slice and averaged from 20 baseline traces (first 10 minutes) and the last 20

traces (last 10 minutes) of wash out. Traces are shown for each condition as well as blockade of a fEPSP by the AMPA/kainate receptor antagonist DNQX (50 μ M). (*, $p < 0.05$; **, $p < 0.01$, one-way ANOVA followed by Dunnett's test, relative to control.)

Figure 6. Effect of bilateral microinjection of the kainate receptor agonist, ATPA, on anxiety-like behaviors assessed in the light/dark box

ATPA microinjection into the BLA resulted in a significant dose-dependent increase in latency to re-enter the light side (A), a significant dose-dependent decrease in the number of crosses to the light side (B), and a trend toward a decrease in time spent on the light side (C). (n = 11 for control, n = 5 for 25pmol ATPA, n = 3 for 125pmol, n=4 for 250pmol; *, p < 0.05, ANOVA followed by Dunnett's test, relative to control).

Figure 7. Bilateral microinjection of ATPA has no effect on locomotor activity measures measured in an open field

ATPA microinjection of 125pmol ATPA into the BLA (n = 5) had no effect on horizontal activity (A), total distance traveled (B), or movement time (C).