Nociceptin/Orphanin FQ Blockade of Corticotropin-Releasing Factor-Induced Gamma-Aminobutyric Acid Release in Central Amygdala is Enhanced after Chronic Ethanol Exposure

Supplemental Information

Supplemental Materials and Methods

Chronic Ethanol Treatment

Briefly, male Sprague-Dawley rats were housed 2-4 per cage with a 6 AM to 6 PM light cycle and with free access to food and water. Rats were placed into either ethanol vapor chambers (*n* = 49) or air-only chambers (naïve controls; *n* = 59). Ethanol-treated rats were intermittently exposed (14 h on, 10 h off) to ethanol vapors for 3-4 weeks (1). Naïve/control rats were exposed to air 24 h/day. We cut transverse slices (400-µm thick), incubated them in an interface configuration for ~30 min, then completely submerged and continuously superfused them (at 2-4 ml/min) with warm (31^{o}C), gassed artificial cerebrospinal fluid (ACSF) of the following composition in mM: NaCl, 130; KCl, 3.5; NaH_2PO_4, 1.25; $MgSO_4$•7H2O, 1.5; $CaCl_2$, 2.0; $NaHCO_3$, 24; glucose, 10. Drugs were added to the ACSF from stock solutions to obtain known concentrations in the superfusate.

Intracellular Recording

We superfused the slices with the glutamate receptor blockers 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 µM) and DL-2-amino-5-phosphonovalerate (APV; 30 µM), and the $GABA_B$ receptor antagonist CGP 55845A (1 µM). At the end of recording, we superfused 30 µM bicuculline (or 50 µM picrotoxin) to confirm the $GABA_A$ergic nature of the inhibitory postsynaptic potentials (IPSPs); these antagonists completely blocked the IPSPs. Data were acquired with an Axoclamp-2A preamplifier (Axon Instruments, Foster City, CA) and stored for later analysis using pClamp software (Axon Instruments). To determine the synaptic response parameters for each cell, we performed an input-output (I/O) protocol (1-2) consisting of a range of five current stimulations (50-250 mA; 0.125 Hz), starting at the threshold current required to elicit an IPSP up to the strength required to elicit the maximum amplitude. We normalized the three middle (omitting threshold and maximal) stimulus intensities of five equal steps as 1-3; these stimulus strengths and the half-maximal stimulus strength were maintained throughout the entire duration of the experiment. In Figure 1B, we averaged the effects of each dose of nociceptin over all three stimulus intensities. In the time-course studies, we applied four consecutive stimuli (at 30 sec intervals) at the half-maximal amplitude determined from the I/O relationship. We examined paired-pulse facilitation (PPF) using paired stimuli at 50 and 100 msec inter-stimulus intervals. The stimulus strength was adjusted such that the amplitude of the first IPSP was half-maximal. We calculated the PPF ratio as the ratio of the second IPSP amplitude over the first.

Whole-Cell Patch-Clamp Recording of Miniature Inhibitory Postsynaptic Currents

Patch pipettes (4-8 MΏ) were pulled from borosilicate glass (Warner Instruments, Hamden, CT) and filled with an internal solution containing (in mM): 135 KCl, 10 HEPES, 2 $MgCl_2$, 0.5 EGTA, 2 Na-ATP, 0.2 Na-GTP, pH 7.2-7.3, osmolarity 275-290 mOsm. We acquired data with an Axoclamp-2A preamplifier (Molecular Devices, Sunnyvale, CA) and analyzed them using Mini 5.1 software (Synaptosoft, Leonia, NJ). To assess the role of protein kinase A (PKA) in nociceptin and corticotropin-releasing factor (CRF) effects on miniature inhibitory postsynaptic currents (mIPSCs), we visualized 43 CeA neurons in brain slices (300 μm) using infrared differential interference contrast (IR-DIC) optics and CCD camera (EXi Aqua, QImaging, British Columbia, Canada) (3). A 60X water immersion objective (Olympus) was used for identifying and approaching central nucleus of the amygdala (CeA) neurons. Whole-cell voltage-clamp recordings of mIPSCs were made with a Multiclamp 700B amplifier (Molecular Devices), low-pass filtered at 2-5kHz, digitized (Digidata 1440A; Molecular Devices), and stored on a PC using pClamp 10 software (Axon Instruments). Drugs were constituted in ACSF and applied by bath superfusion.

Rp-adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt hydrate (Rp-cAMP, Sigma, St. Louis, MO) was made up as a concentrated stock solution (1,000x) in distilled water and stored at –20°C. Protein kinase inhibitor-(6–22)-amide (PKI, Tocris, Ellisville, MO) was made up as a concentrated stock solution (1,000x) in distilled water and was added to the pipette internal solution on the day of use.

In all recordings, series resistance (<10 MΩ) was continuously monitored with a 10 mV hyperpolarizing pulse and experiments with >20% change in series resistance were not included in final analysis. Frequency, amplitude and kinetics of mIPSCs were analyzed using semi-automated threshold based mini detection software (Mini Analysis, Synaptosoft Inc., Fort Lee, NJ) and were visually confirmed. To accurately determine the mIPSC amplitude, only mIPSCs that were >5 pA were accepted for analysis.

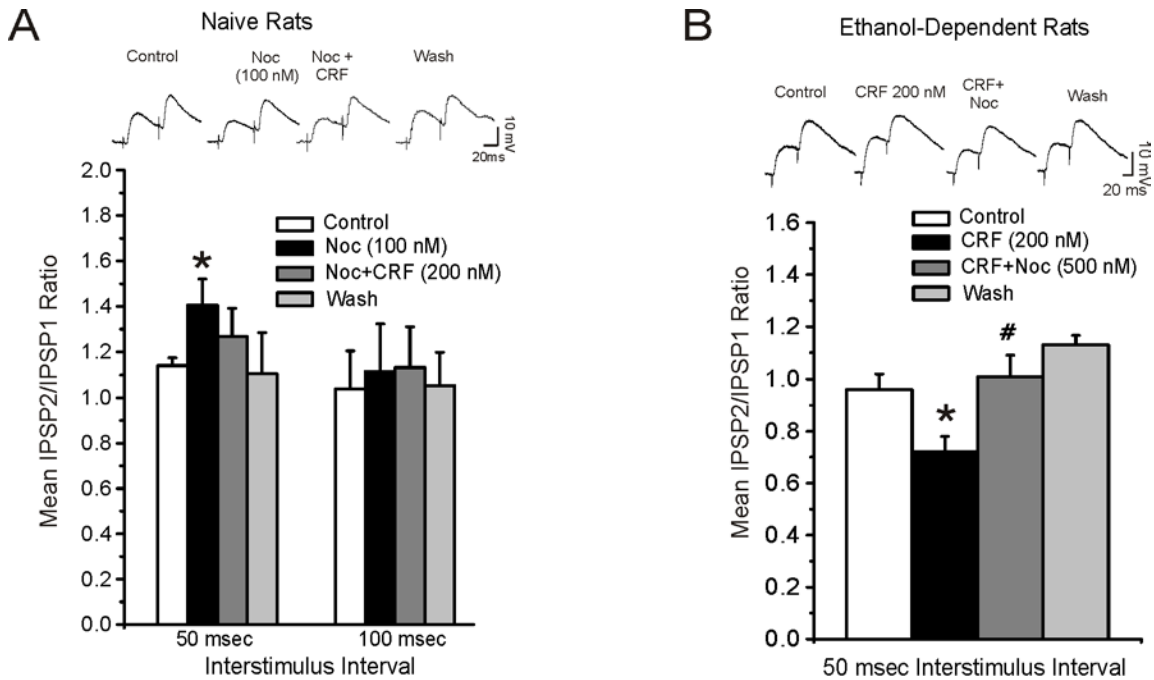

Figure S1. (A) Representative recordings of evoked 50 msec paired-pulse IPSPs in a CeA neuron from a naïve rat. Bottom Panel: Nociceptin significantly (*$p < 0.05$; $n = 7$) increases the 50 msec PPF ratio of IPSPs and blocks the CRF-induced decrease in PPF ratio. Nociceptin did not alter the 100 msec PPF in these neurons. **(B)** Top Panel: Representative recordings of evoked paired-pulse IPSPs in a CeA neuron from an ethanol-dependent rat. Bottom Panel: Pooled data of PPF ratios in CeA neurons of ethanol-dependent rats. CRF significantly (*$p < 0.05$; $n = 11$) decreases the PPF ratio of IPSPs to 75.0 ± 4.8% of control. Application of nociceptin in the presence of CRF reverses the CRF-induced decrease of PPF ratio and further increases the PPF ratio (#$p < 0.05$; $n = 11$), with recovery upon washout. CeA, central nucleus of the amygdala; CRF, corticotropin-releasing factor; IPSP, inhibitory postsynaptic potentials; Noc, nociceptin; PPF, paired-pulse facilitation.

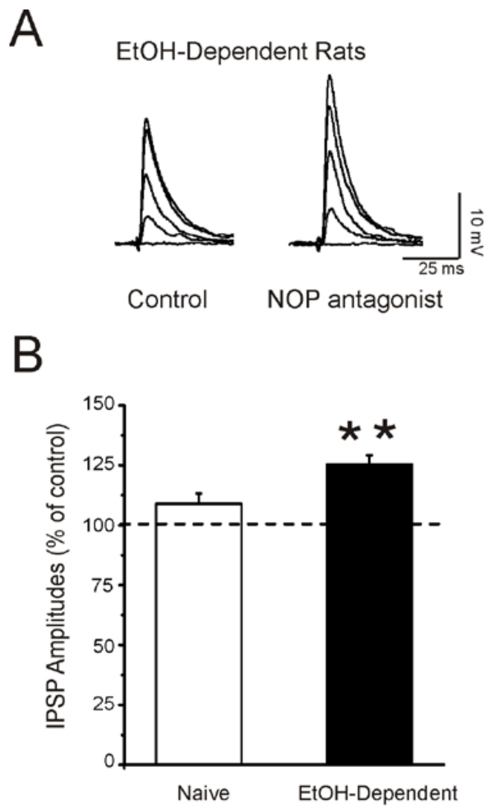

Figure S2. (A) Representative recordings of evoked IPSPs over the complete input-output protocol (which consists of a range of five current stimulations) in the CeA of a dependent rat during control recording (left) and during application of the selective nociceptin receptor antagonist [Nphe^1]Nociceptin(1–13)NH_2 (1 μM) for 15 min (right). The NOP antagonist increases evoked IPSP amplitudes in CeA neurons from ethanol-dependent rats. **(B)** Histograms representing percent change of mean ± SEM evoked IPSP amplitudes induced by the NOP antagonist [Nphe^1]Nociceptin(1–13)NH_2 (1 μM) in CeA neurons from naive and ethanol-dependent rats. The NOP antagonist significantly (*p < 0.05; n = 8) increases evoked IPSP amplitudes in CeA neurons of ethanol-dependent rats, but not in 6 CeA neurons of naïve rats. CeA, central nucleus of the amygdala; EtOH, ethanol; IPSP, inhibitory postsynaptic potentials; NOP, opioid N/OFQ receptor; SEM, standard error of the mean.

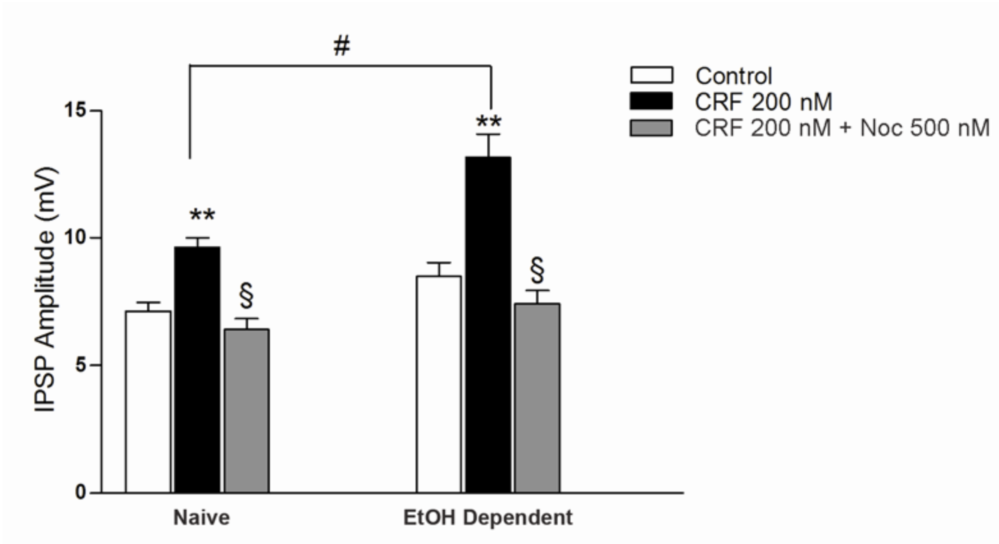

Figure S3. Cumulative statistical analysis of the effect of CRF and nociceptin in CeA neurons from naïve and ethanol dependent rats. Two-way RM ANOVA indicated that the increase in IPSP amplitude induced by CRF in dependent animals was significantly greater than the one induced in naïve animals [$F(2,20) = 5.17$, $p < 0.05$]. $^{\#}p < 0.05$ indicates significance between the CRF effect in naïve versus ethanol dependent rats. Two-way RM ANOVA also indicated a significant drug x ethanol exposure interaction effect [$F(2, 40) = 6.51$ $p < 0.01$]. Student Newman-Keuls post-hoc analyses revealed that CRF produced a significant ($p < 0.01$) increase in IPSP amplitude in both groups. $^{**}p < 0.01$ compared to control. Post-hoc analyses also demonstrated that the co-application of CRF and nociceptin significantly decreased IPSP amplitude, completely occluding the CRF effect and returning the amplitude to levels below baseline ($^{\S}p < 0.01$ compared to CRF). CeA, central nucleus of the amygdala; CRF, corticotropin-releasing factor; EtOH, ethanol; IPSP, inhibitory postsynaptic potentials; Noc, nociceptin; RM ANOVA, repeated measures analysis of variance.

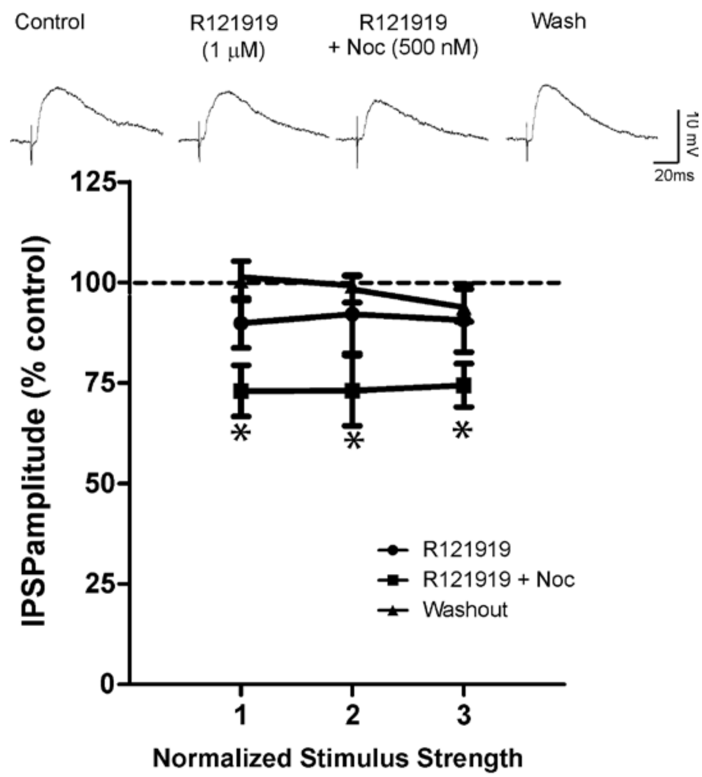

Figure S4. Top panel: Representative recordings of evoked IPSPs in a CeA neuron from a naïve rat. Bottom Panel: Application of the CRF_1 antagonist R121919 (1 μM) for 15 min slightly decreases evoked IPSP amplitudes. Co-application of 500 nM nociceptin significantly [$F(2,7) = 3.488$; $^*p < 0.05$] decreases evoked IPSP amplitudes, with recovery upon washout. CeA, central nucleus of the amygdala; CRF, corticotropin-releasing factor; EtOH, ethanol; IPSP, inhibitory postsynaptic potentials; Noc, nociceptin.

Supplemental References

1. Roberto M, Cruz MT, Gilpin NW, Sabino V, Schweitzer P, Bajo M, *et al.* (2010): Corticotropin releasing factor-induced amygdala gamma-aminobutyric acid release plays a key role in alcohol dependence. *Biol Psychiatry* 67:831-839.
2. Roberto M, Madamba SG, Moore SD, Tallent MK, Siggins GR (2003): Ethanol increases GABAergic transmission at both pre- and postsynaptic sites in rat central amygdala neurons. *Proc Natl Acad Sci U S A* 100:2053-2058.
3. Gilpin NW, Misra K, Herman MA, Cruz MT, Koob GF, Roberto M (2011): Neuropeptide Y opposes alcohol effects on gamma-aminobutyric acid release in amygdala and blocks the transition to alcohol dependence. *Biol Psychiatry* 69:1091-1099.