Glutamate receptor dysregulation during protracted withdrawal from intermittent ethanol vapor in rats

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Methods

Animals: All procedures were approved by the DMU IACUC (2018-09). Studies utilized male (~100g) and female (~75g) Sprague Dawley rats for all studies. All studies were approved by the Des Moines University Animal Care and Use Committee.

Ethanol Exposure: Animals were group housed (2-3/cage) inside custom built plexiglass ethanol exposure chambers. The chronic intermittent ethanol (CIE) exposure paradigm consisted of vaporized ethanol for 12h on/ 12hr off for 4 consecutive days followed by a 3-day intermittent withdrawal period. This 4d on/3d off pattern was repeated for 3 cycles. Following the last exposure animals were placed in their home cages and allowed to enter protracted withdrawal prior to experimental use. Blood ethanol concentrations were analyzed from tail blood samples collected once per exposure cycle (3x/animal). BEC levels for all animals included in the presented data are 196 mg/dl. Air levels of EtOH were measured daily.

Electrophysiology: Whole cell patch clamp recordings of basolateral amygdala (BLA) pyramidal neurons were conducted in coronal brain slices (400uM) in room temperature aCSF. For NMDAR recordings Picrotoxin (0.1 mM) and 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, 0.02 mM) were used to block GABAergic and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor mediated synaptic transmission, respectively. Patch pipettes (6-8MΩ) were filled with internal solution containing (in mM): 140 CsCl, 10 HEPES, 2 $MgCl_2$, 5 NaATP, 0.6NaGTP, 2 QX-314.

For AMPAR recordings, APV (0.05mM) and picrotoxin were used to block NMDA and GABAergic mediated synaptic transmission. Internal solution for AMPAR experiments also contained spermine (0.1mM)

For all electrophysiology studies the external capsule was the site of electrical stimulation using concentric bipolar electrodes (FHC).

Biotinylation, BS3, Western Blots: All treatments followed previously published methods. Following the dissection of the BLA, membrane surface protein labels NHS-SS-BIOTIN (APExBIO) or BS3 (Covachem) were added to the tissue. These membrane impermeant reagents bind to proteins on the cell membrane surface. This allowed for the separation of proteins in surface (membrane bound) and internal (unbound) fractions. Unbound fractions were not analyzed for Biotinylation studies. Tissue was then processed for western blot analysis. Tissue was separated by weight using 4-12% gradient gels and transferred using either wet or semidry transfer methods. Relative protein expression was quantified following membrane incubation in primary and secondary antibodies. List as requested.

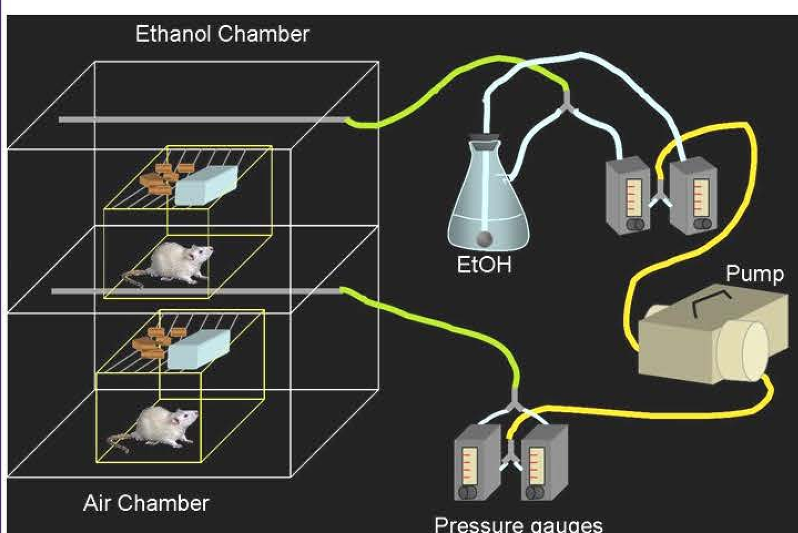

Figure 1. Diagram of vapor exposure equipment. Top chamber houses EtOH exposure animals. Bottom chamber houses Air exposure control (CON) animals.

Introduction

Functional dysregulation of the glutamatergic receptor system during withdrawal from chronic drug exposure is a primary driver of drug craving and relapse. Animal models of substance use have demonstrated dynamic alterations in both AMPA and NMDA receptor function and expression that contribute to drug seeking behaviors. Several studies have identified an increased contribution of atypical GluN3-containing triheteromeric NMDARs and GluA1-containing AMPARs. Short-term withdrawal (24h) from chronic ethanol exposure also demonstrates functional dysregulation of AMPA and NMDARs suggesting that similar mechanisms could regulate drug craving and relapse behaviors across drugs of abuse. We utilized an ethanol vapor exposure model to synchronize withdrawal time periods across animal cohorts. The goal of these studies was to measure alterations in receptor function and trafficking during protracted ethanol withdrawal. We utilized whole cell patch clamp methods to measure NMDA and AMPA receptor mediated synaptic responses in both air and ethanol exposed animals. We also utilized western blot techniques to measure relative protein expression in tissue samples treated with biotin or BS3. These treatments allow the quantification of expression in distinct surface and intracellular tissue fractions. Together, these techniques provide a more comprehensive picture of how glutamatergic receptors are regulated during protracted withdrawal.

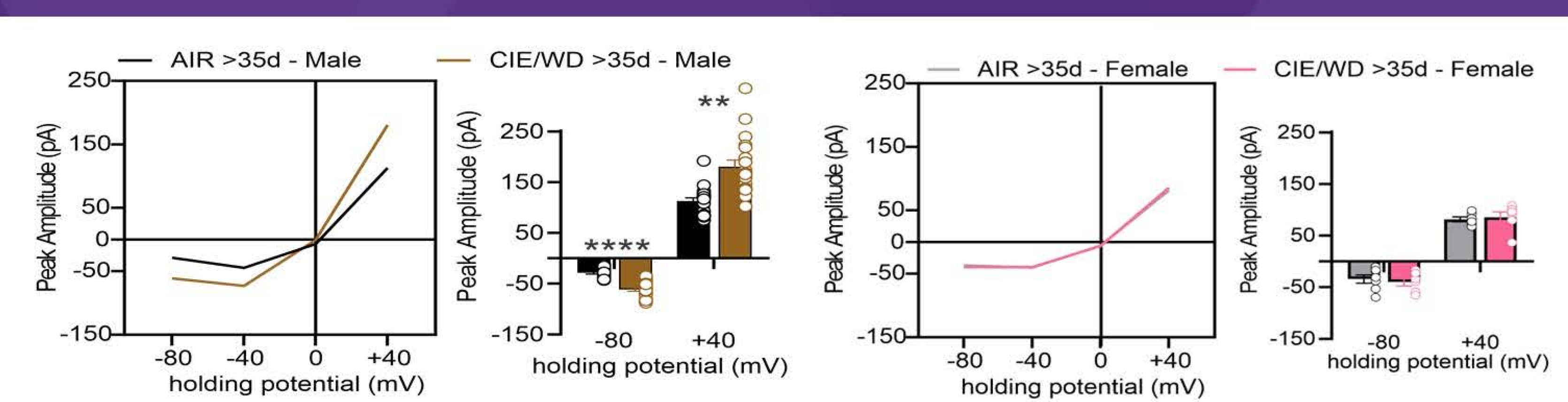

Figure 2. Sex dependent increase in NMDAR function during protracted withdrawal following 3 cycles of chronic intermittent ethanol vapor exposure. **A; Left)** IV curve plot demonstrating increased NMDAR mediated responses at -80mV and +40mV in male withdrawal rats **A, Right)** Bar graph of individual data points showing increased NMDAR responses during protracted withdrawal at -80 and + 40mV. CIE/WD Male (19 cells/11 Animals); AIR>35d (19 cells/13 Animals). **B Left)** IV curve plot demonstrating no change in NMDAR mediated responses in female rats. **B Right)** Bar graph of individual data points showing no significant change in NMDAR responses during protracted withdrawal at -80 and + 40mV. Female (CIE/WD; 6 cells/5 animals; Air; 6 cells/6 animals). **** = p<0.0001; ** p<0.01. Student t-test. Data suggests sex-specific regulation of NMDARs during protracted withdrawal from CIE exposure.

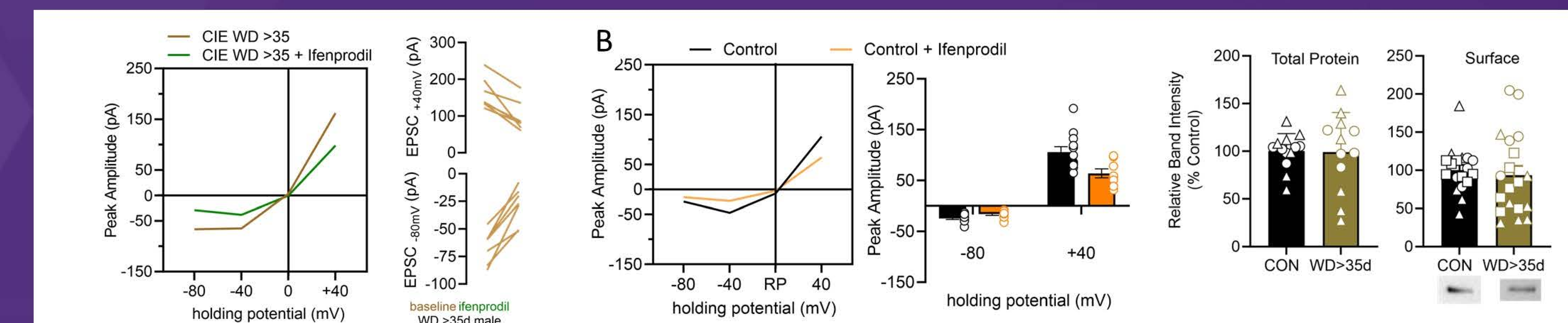

Figure 3. Increased NMDAR function is mediated by GluN2B-containing receptors. **A,Left)** IV Plot of evoked responses at various membrane holding potentials. Drug application was conducted onto single cells "within cell" in male rats during protracted withdrawal from CIE exposure. **A,Right**) Within-cell data showing effect of ifenprodil (5μM) application on response amplitudes at various membrane potential. paired t-test** = p>0.01, *** = p>0.001;. Effect at -80mV suggest atypical NMDARs (GluN1/GluN2B/GluN3) are present during protracted withdrawal from CIE in male rats. **B, Left)** IV plot of evoked responses at various holding potentials in control cells **B, Right)** Mean data at -80 and +40mV using "between" cell analysis. Effect at +40mV suggests basal expression of GluN2B in control animals (10 cells/8 animals vs Control + ifen (7 cells /7animals) Unpaired t-test, ** = p>0.01 **C),** Total and surface protein expression values for CON and WD tissue treated with biotinylation procedures to isolate surface bound vs unbound intracellular proteins. Aggregate data from 3 independent cohorts of animals. 18 CON, 19 WD>35d samples. Each cohort is represented by a shape.

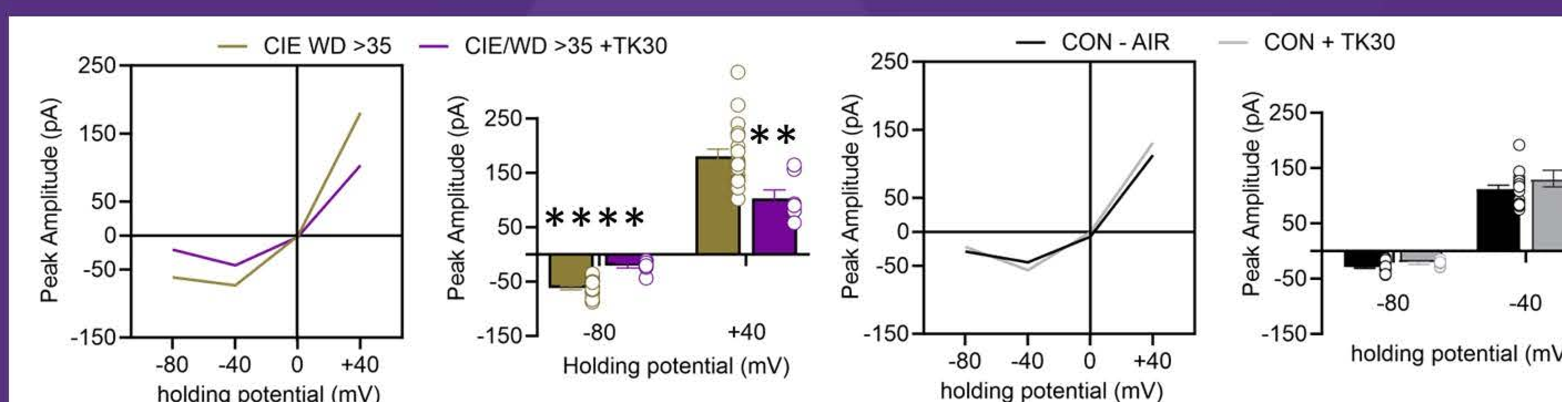

Figure 4. Increased NMDAR function mediated by GluN3-containing receptors. **A)** IV Plot of evoked responses at various membrane holding potentials. "Between Group" drug application (TK30, 30uM) in male rats during protracted withdrawal from CIE exposure. CIE/WD>35 (19 cells/11 Animals) data presented in Fig2. CIE/WD>35d + TK30 (n=7 cells/6 animals) Unpaired t-tests; **** = p>0.0001. **= p>0.01. **B)** IV Plot of evoked responses at various membrane holding potentials. "Between Group" drug application (TK30, 30uM) in male CON cells (19 cells/13 Animals) and Control cells + TK30 (6 cells/3 animals). No effect of TK30 in control cells. **C)** Protein analysis of total and surface expressed proteins following biotinylation treatment. No difference in total protein, with no significant increases (p=0.09) in surface expression of GluN3A. 1 cohort; CON (n=6), WD>35d (n=6).

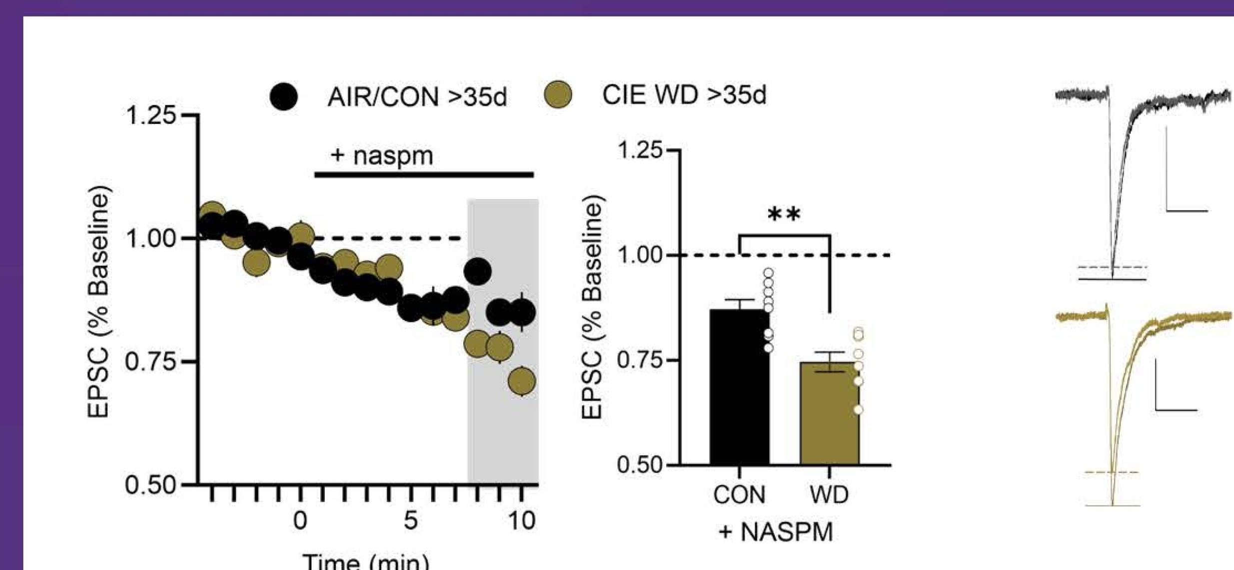

Figure 5. NASPM sensitivity during protracted withdrawal following 3 cycles of CIE vapor exposure in Male rats. A) Normalized EPSC of AMPAR mediated evoked responses during the application of naspm (100uM). Increased sensitivity to Naspm application in cells from animals in protracted withdrawal shown as the % decrease from baseline. t-test, ** = p<0.01, Air/Con (8 cells/5 animals) CIE/WD (8 cells/5 animals). Implicates an increase in the functional contribution of calcium permeable GluA1 containing AMPARs. **B)** Representative traces of baseline (5 min avg) and peak naspm effect (avg last 3 minutes). Top: CON cells baseline (solid/black) and last 3 min of naspm application (dashed/gray). Bottom: CIE/WD>35d cells baseline (solid/brown) and last 3 min of naspm application (dashed/gold). Scale bars: 25pA x 100ms.

CIE/WD in males induces an increase in both NMDA and AMPA receptor function.

Pharmacological studies implicate increased GluN2B (ifenprodil) and GluA1 (Naspm).

Characteristics of increased of GluN3 mediated functional expression. Functional contribution of GluN3 indicated by Pharmacology (TK30)

Supports the functional contribution of atypical GluN1/GluN2B/Glun3 NMDARs

CIE/WD in females did not induce any change in NMDAR mediated synaptic transmission suggests sex-dependent sensitivity to ethanol exposure and withdrawal paradigm.

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