1   Sex- and Subtype-Specific Adaptations in Excitatory Signaling Onto Deep-Layer Prelimbic

2   Cortical Pyramidal Neurons After Chronic Alcohol Exposure

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ABSTRACT

Long-term alcohol use results in behavioral deficits including impaired working memory, elevated anxiety, and blunted inhibitory control that are associated with prefrontal cortical (PFC) dysfunction. Preclinical observations demonstrate multiple impairments in GABAergic neurotransmission onto deep-layer principal cells (PCs) in prelimbic cortex that suggest dependence-related cortical dysfunction is the product of elevated excitability in these cells. Despite accumulating evidence showing alcohol-induced changes in interneuron signaling onto PCs differ between sexes, there is limited data explicitly evaluating sex-specific ethanol effects on excitatory signaling onto deep-layer PCs that may further contribute to deficits in PFC-dependent behaviors. To address this, we conducted electrophysiological and behavioral tests in both male and female Sprague-Dawley rats to evaluate the effects of chronic ethanol exposure. Among our observations, we report a marked enhancement in glutamatergic signaling onto deep-layer PCs in male, but not female, rats after alcohol exposure. This phenomenon was furthermore specific to a sub-class of PC, sub-cortically projecting Type-A cells, and coincided with enhanced anxiety-like behavior, but no observable deficit in working memory. In contrast, female rats displayed an alcohol-induced facilitation in working memory performance with no change in expression of anxiety-like behavior. Together, these results suggest fundamental differences in alcohol effects on cell activity, cortical sub-circuits, and PFC-dependent behaviors across male and female rats.

INTRODUCTION

Alcohol use disorders (AUDs) are a major public health hazard, with AUDs contributing to ~6% of preventable fatalities worldwide and costing nearly 3% of the gross domestic product in developed countries [1, 2]. Though of great concern, AUDs encompass a wide range of aberrant drinking behaviors that elicit myriad pathological peripheral and central nervous system adaptations, and the discrete mechanisms by which these behaviors manifest are therefore difficult to discern. Of particular interest to our laboratory is the pathogenesis of dependence-related alcohol consumption, as dependent individuals, though comprising only ~5-14% of alcohol abusers, account for the majority of the public health burden stemming from AUDs [3, 4]. Excessive alcohol consumption characteristic of dependence is specifically associated with diminished executive control of intake as well as elevated anxiety experienced during acute withdrawal that drives consumption [5]. These observations consistently correlate with notable structural and functional impairments in prefrontal cortical (PFC) function, leading models of addiction to include PFC dysfunction as a core component [6].

Nevertheless, much of the literature investigating alcohol effects on the PFC neglect to include females, despite comparable lifetime risks for developing AUDs among men and women (~36% and 22.7%, respectively [7]) as well as recent policy directives by the National Institutes of Health to include female subjects [8]. Indeed, the assumption that AUDs manifest comparably between sexes conflicts with available clinical and preclinical evidence demonstrating key differences in both the acquisition and progression of AUDs, finding that males escalate alcohol consumption sooner than females [9-11].

Our laboratory has used rat models to explore the molecular adaptations to chronic ethanol exposure that are associated with dependence-like symptoms such as increased seizure

60   susceptibility and anxiety-like behavior, finding latent sex differences in responses to alcohol.

61   For instance, we have previously reported dimorphic alterations in $GABA_A$ receptor subunit

62   expression, reporting that, while α1 subunit expression is reduced in both males and females

63   following chronic alcohol exposure [12, 13], α3 subunit expression is altered only in males [14].

64   Furthermore, elevations in N-methyl-D-aspartate (NMDA) receptor function and expression that

65   promote increased cell excitability are reliably observed with chronic alcohol exposure in male

66   rats [15, 16] also appear to be sex-specific [17].

67   More recently, we and others have specifically investigated alcohol effects on the

68   functional properties of cortical neurons between sexes, finding important similarities and

69   differences [18-20]. Specifically, in seeking to localize ethanol-induced $GABA_A$ receptor subunit

70   expression changes in cortex, our laboratory reported equivalent reductions in GABAergic

71   spontaneous inhibitory post-synaptic currents (sIPSCs) onto deep-layer principal cells (PCs), the

72   primary source of efferent signaling in PFC, after alcohol exposure in male and female rats.

73   Reductions in sIPSC frequency furthermore occurred in tandem with post-synaptic reductions in

74   α1 subunit expression, consistent with elevated PC excitability during acute withdrawal [12, 13].

75   Interestingly, though, interneuron recordings revealed that while fast-spiking interneurons

76   displayed equivalent reductions in excitability in both sexes consistent with reductions in sIPSC

77   frequency, Martinotti interneurons exhibited markedly divergent responses in excitability

78   between males and females [19]. It is unclear how such differential changes in intra-cortical

79   feedback inhibition affect local microcircuit function, and raise an important question as to

80   whether other functional adaptations induced by alcohol exposure in PFC are similarly sex-

81   dependent. Building on these observations of GABAergic signaling on deep-layer PCs we

82   therefore hypothesized that glutamatergic tone onto these cells from superficial layer PCs would

83   display similarly sex-specific responses to chronic ethanol exposure that further promote

84   pyramidal cell hyperexcitability. In addition, we sought to probe whether potential functional

85   changes correlated with alterations in PFC-dependent behavior, finding that chronic alcohol

86   exposure elicits complex functional and behavioral adaptations that do not occur uniformly

87   between sexes.

88

89   **MATERIALS AND METHODS**

90   *Animal Care and Exposure Paradigm*

91   Male and female Sprague Dawley rats were obtained from in-house breeding using

92   breeders sourced from Envigo (Indianapolis, IN) and pair-housed in a temperature and humidity

93   controlled vivarium under a 12 hour light/dark cycle and given free access to water and food

94   unless otherwise noted. All animals were PN90-130 at time of testing, and all procedures were

95   carried out in accordance with guidelines specified by the Institutional Animal Care and Use

96   Committee at the University of North Carolina at Chapel Hill. Ethanol exposure consisted of a

97   once-daily dose of ethanol (5.0 g/kg, 25% v/v) or water via intragastric gavage (i.g.)

98   approximately 2 hours into the light cycle that reliably elicits BECs in both male and female rats

99   of ~250-300 mg/dl ([13]; **Supplemental Figure S1**). Experimenters performing gavage

100   possessed extensive training with the technique and when performed correctly is not especially

101   stressful, eliciting comparable EPM observations as other forced-administration models [21, 22].

102   Animals received 15 consecutive days of exposure, all experiments performed 24 hours after the

103   last exposure, and behavioral and physiological experiments performed in separate animals. At

104   the conclusion of all experiments, rats were sacrificed by rapid decapitation and brains collected

105   for electrophysiological or biochemical analyses.

106   *Elevated Plus Maze (EPM) and Delayed Non-Match-to-Sample (NMS) T-Maze*

107   EPM was conducted 24 hours after the last water/ethanol exposure in a partially lit room

108   (~20 lux), using previously described methods [12]. The Delayed NMS T-maze task was

109   performed according to previously described procedures [23, 24]. Detailed description of

110   behavioral tasks and procedures can be found in **Supplementary Materials.**

111   *Electrophysiological Recordings*

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112   After sacrifice, brains were extracted and ~300 µM thick coronal slices containing the

113   mPFC were prepared in oxygenated ice-cold sucrose solution (in mM: 200 sucrose, 1.9 KCl, 1.2

114   NaH2PO4, 6 MgCl2, 0.5 CaCl2, 0.4 ascorbate, 10 glucose, 25 NaHCO3, osmolarity adjusted to

115   ~310 mOsm) using a Leica VT1000S vibratome (Buffalo Grove, IL). Slices were then

116   transferred to a continuously oxygenated holding chamber containing standard aCSF solution (in

117   mM: 125 NaCl, 2.5 KCl, 1.5 NaH2PO4, 1.3 MgCl2, 2.6 CaCl2, 0.4 ascorbate, 10 glucose, 25

118   NaHCO3, osmolarity adjusted to ~310 mOsm) warmed to 32-34° C and incubated for 30min,

119   after which the holding chamber was allowed to cool to room temperature for 1 hour. Whole-cell

120   recordings of prelimbic Layer V pyramidal cells were performed at room temperature using an

121   Axon Instruments Multiclamp 700A amplifier sampling at 10 kHz and filtered at 2 kHz.

122   Recordings were performed in standard aCSF solution supplemented with 50 µM dl-AP5 and 10

123   µM CGP-52432, and using 2-4 mOhm borosilicate glass filled with a K-gluconate-based internal

124   solution (in mM: 120 K-gluconate, 10 KCl, 2 MgCl2, 5 EGTA, 10 HEPES, 3 Na-ATP, 0.5 Na-

125   GTP, 2 phosphocreatine, pH 7.4, osmolarity adjusted to ~290 mOsm with sucrose). Following

126   gigaseal formation and breakthrough, cells were held at -70 mV and electrically-evoked

127   excitatory post-synaptic currents (eEPSCs) were obtained via a bipolar stimulating electrode

128   placed in Layers I-III of prelimbic cortex. Stimulation intensity was tuned to elicit a ~200-300

129   pA response, and baseline stimulation frequency-response recordings obtained. Additional

130   description of electrophysiological methods can be found in **Supplementary Materials**.

131   *Statistical Analysis*

132   Data are expressed as mean±SEM unless otherwise noted. Electrophysiological data were

133   analyzed using linear mixed modeling and Satterthwaite approximation of effective degrees of

134   freedom to model extraneous inter-animal variability as a random-effect with SAS 9.4 software

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135   (SAS Institute; Cary, NC) as previously reported [19, 25]. Behavioral data were analyzed by

136   unpaired t-tests with Welch's correction (EPM) and two-way ANOVA with Bonferroni post-hoc

137   tests (NMS T-maze) using GraphPad Prism 6.0 (San Diego, CA) and statistical power estimated

138   using the free software GPower 3.1. As direct statistical comparison of sex would require

139   prohibitively high n's for the present experiments, male and female datasets were analyzed

140   independently. For all analyses significance is defined as $p < 0.05$ (*<0.05, **<001, ***<0.001).

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RESULTS

Sex-Specific Enhancement of Excitatory Signaling onto Deep Layer Pyramidal Neurons After Ethanol Exposure

While post-synaptic changes in glutamatergic and GABAergic signaling onto deep-layer PCs during acute withdrawal have been described [13, 16, 26], it is unclear whether chronic alcohol exposure affects Layer II/III excitatory signaling onto deep-layer PCs, or whether such effects occur in both sexes. Therefore, we evaluated post-synaptic responses in deep-layer PCs to repeated electrical stimulations of superficial layer PCs, as this is a well-validated means of gauging presynaptic glutamate release probability [27-29]. As shown in **Figure 1**, alcohol exposure shifted post-synaptic responses to electrical stimulation from a facilitating profile towards repeated-pulse depression at both low- (3 Hz; **Figure 1A**) and high-frequencies (30 Hz; **Figure 1B**) in male rats, indicative of elevated glutamate release probability [3 Hz: $F_{(3,132)}$Interaction=8.87, p<0.0001; $F_{(3,132)}$Stimulation=8.16, p=<0.0001; $F_{(1,19.4)}$Treatment=22.55, p=0.0001: 30 Hz: $F_{(1,132)}$Interaction=3.05, p=0.031; $F_{(3,132)}$Stimulation=16.62, p<0.0001; $F_{(1,19.5)}$Treatment=4.8, p=0.041]. Female rats, however, displayed no apparent effect of chronic ethanol on repeated-pulse responses (RPR) at either frequency [**Figure 1D-E**: 3 Hz: $F_{(1,14.5)}$Treatment=2.39, p=0.143: 30 Hz: $F_{(1,16.2)}$Treatment=0.06, p=0.807]. The observation that this effect occurs irrespective of initial stimulation intensity further suggests a generalized enhancement of presynaptic release probability (**Figure 1C,F**). Additionally, application of picrotoxin (100 µM) did not alter responses, showing that changes in presynaptic release are likely not the product of local inhibitory processes [**Figure 1G-I**: $F_{(1,8.07)}$Treatment=10.3, p=0.012; $F_{(1,151)}$Picrotoxin=1.91, p=0.17].

Heterogeneity in Alcohol Effects on Principal Cell Excitability

165   Despite a relatively robust *n*, we observed substantial variability in RPRs in both males

166   and females that suggested the possibility of a non-homogeneous cell population, and indeed

167   distinct subtypes of deep-layer PCs have previously been described [30-33]. These cell subtypes,

168   termed Type-A and Type-B, are readily discriminable by physiological criteria including the

169   presence of a robust hyperpolarization-induced voltage sag (V_H; **Figure 2A**, red arrows; **Figure**

170   **2B-C**), differential facilitating responses to presynaptic stimulation (**Figure 2D-E**; representative

171   traces shown in **Figure 2F**), and intrinsic membrane characteristics (**Supplemental Table 1**).

172   Additional experiments were therefore conducted, revealing that subtyping according to these

173   criteria (e.g. total V_H >11.0 mV, facilitating RPR profile, and C_{Mem} >95.0 pF) reliably

174   distinguished Type-A from Type-B cells. As shown in **Figure 2G**, both cell types were observed

175   in approximately equal frequency in male and female rats, and irrespective of exposure group.

176   Thus, we next asked whether alcohol-induced changes in presynaptic glutamate release from

177   superficial-layer PCs may be preferential for either PC subtype.

178   As shown in **Figure 3A**, we observe a significant shift from facilitation towards RPR

179   depression after alcohol exposure in Type-A PCs of male rats that is not observed in females

180   (**Figure 3B**) [male: $F_{(3,100)}$Stimulation=10.2, p<0.0001; $F_{(1,8.84)}$Treatment=5.58, p=0.0344:

181   female: $F_{(3,80.1)}$Stimulation=6.28, p=0.0007; $F_{(1,7.08)}$Treatment=0.04, p=0.853]. Based on previous

182   reports that alcohol-induced changes in presynaptic release are calcium dependent [11, 34], we

183   hypothesized that reducing extracellular calcium would reduce release probability, observed as a

184   shift towards RPR facilitation, to a greater degree in alcohol-exposed PCs. **Figures 3C-E**

185   demonstrate that reducing extracellular calcium elicits a significant enhancement in RPR

186   facilitation in both male and female Type-A cells [males: $F_{(1,127)}$CalciumMod=93.4, p<0.0001:

187   females: $F_{(1,175)}$CalciumMod=81.26, p<0.0001] to equivalent levels between water/ethanol-

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188 exposed groups. Analyzing the % change in RPR between conditions, however, revealed

189 significantly higher modulation in male ethanol-exposed Type-A cells (**Figure 3D**), suggesting

190 alcohol effects on presynaptic release in males involve a calcium-dependent mechanism [males:

191 $F_{(1,13.2)}$Treatment=4.92, p=0.0473]. We further measured current-evoked spiking, and alcohol

192 exposure resulted in a significant left-ward shift (e.g. >100% increase at 150 pA) in intrinsic

193 activity in male, but not female, Type-A cells that further underscores the ethanol-induced bias

194 toward hyperexcitability in this population [**Figure 3G-H**; males: $F_{(1,13.2)}$Treatment=4.92,

195 p=0.0446: females: $F_{(1,10.4)}$Treatment=0.11, p=0.744].

196 Evaluation of RPRs in Type-B cells revealed no apparent differences between water and

197 ethanol-treated groups in either male or female rats (**Figure 4A-B**) [males:$F_{(1,9.5)}$

198 Treatment=0.25, p=0.63; $F_{(2,38.9)}$Stimulation=4.42, p=0.001: females: $F_{(1,9.2)}$Treatment=0.99,

199 p=0.35; $F_{(3,87.3)}$Stimulation=6.36, p=0.0006]. Reducing extracellular calcium similarly elevated

200 RPR in both male and female rats to equivalent levels (**Figure 4C,E**) [males:

201 $F_{(1,131)}$CalciumMod=49.71, p<0.0001; $F_{(1,10.1)}$Treatment=0.40, p=0.54: females:

202 $F_{(1,112)}$CalciumMod=55.72, p<0.0001; $F_{(1,5.3)}$Treatment=0.29, p=0.61], with no observable

203 difference in % change between conditions (**Figure 4D,F**) [males: $F_{(1,9.5)}$Treatment=0.48,

204 p=0.506: females: $F_{(1,4.9)}$Treatment=1.67, p=0.25]. Consistent with minimal ethanol effects on

205 these cells, we further observed no apparent difference between exposure groups on intrinsic

206 excitability in either sex (**Figure 4G-H**) [males: $F_{(1,9.8)}$Treatment=0.37, p=0.555: females:

207 $F_{(1,12.3)}$Treatment=0.72, p=0.411].

208 *Differential Effects of Alcohol Exposure on PFC-related Behaviors by Sex*

209 The vast interconnectivity of the PFC permits this region to influence a wide variety of

210 behaviors. As such, altering prelimbic PFC signaling elicits a number of cognitive impairments

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211   including deficits in working memory [23, 35] and the expression of anxiety-like behaviors [11,

212   12, 34, 36]. On this basis, we therefore sought to determine whether the observed subtype-

213   specific changes in deep-layer PCs after chronic alcohol exposure were associated with specific

214   changes in behavior. To gauge working memory, we employed the Delayed NMS T-maze task,

215   training rats for 7 consecutive days followed by 15 days of water/ethanol exposure and then 3

216   days of testing (**Figure 5A**). Both male and female rats reached a satisfactory level of

217   performance on the task by the end of training, and no significant differences in training between

218   groups were observed (**Figure 5-B,E**). While increasing delay time between forced-arm and

219   choice trials resulted in an expected decrease in choice accuracy, male rats displayed no apparent

220   difference in performance between water- and alcohol-exposed groups, either at 24 hr acute

221   withdrawal (**Figure 5C**) or over 3 days (**Figure 5D**) of cumulative testing [24 hr test two-way

222   ANOVA: $F_{(2,56)}$Time=7.38, p=0.0014; $F_{(1,28)}$Treatment=0.0438, p=0.836: 3 day test two-way

223   ANOVA: $F_{(2,56)}$Time=22.6, p<0.0001; $F_{(1,28)}$Treatment=0.732, p=0.399]. Female rats, however,

224   displayed a significant interaction between alcohol exposure and delay time at 24 hr acute

225   withdrawal (**Figure 5F**) [two-way ANOVA, Bonferroni post-test: $F_{(2,36)}$Interaction=5.609,

226   p=0.0076; $F_{(2,36)}$Time=10.3, p=0.0003; $F_{(1,18)}$Treatment =4.20, p=0.0553] and a robust main

227   effect of alcohol exposure over 3 days (**Figure 5G**) of cumulative testing [two-way ANOVA,

228   Bonferroni   post-test: $F_{(2,36)}$Interaction=4.249,   p=0.022; $F_{(2,36)}$Time=35.97,   p<0.0001;

229    $F_{(1,18)}$Treatment=10.84, p=0.004] that indicate ethanol exposure improved performance of female

230   rats on this task.

231   In contrast, measurement of anxiety-like behavior revealed significant effects in males,

232   but not females. Specifically, male ethanol-exposed rats displayed significant reductions in both

233   the percent time in open-arm and open-arm entries on EPM (**Figure 5H**), consistent with

234   elevated anxiety during 24 hr acute withdrawal [unpaired two-tailed T-test with Welch's

235   correction: % Open-Arm Time $t_{11.5}$=2.841, p=0.0155; Open-Arm Entries $t_{12.6}$=2.842, p=0.0143].

236   Female rats, on the other hand, exhibited no effect of alcohol exposure on either measure

237   (**Figure 5I**) [unpaired two-tailed T-test: % Open-Arm Time t_{19}=0.849, p=0.407; Open-Arm

238   Entries t_{19}=0.353, p=0.728]. That female rats displayed somewhat lower open arm time relative

239   to males raises the possibility of a floor effect precluding further effects of alcohol exposure.

240   However, since number of open-arm entries and immobility episodes (**Supplemental Figure S2**)

241   similarly did not differ we do not believe this to be the case, although future studies would

242   benefit from additional assays of anxiety-like behavior.

DISCUSSION

The present study sought to determine the extent to which chronic alcohol exposure altered inter-laminar glutamatergic tone onto deep-layer PCs in prelimbic PFC and whether such changes occurred equivalently between sexes. Among our observations, we report that chronic exposure results in significantly elevated glutamate release probability from Layer II-III pyramidal cells on deep-layer PCs in male, but not female, rats. Additional investigation revealed that both male and female rats display readily distinguishable deep-layer PC subpopulations (Type-A and –B) whose functional and behavioral significance are only beginning to be fully understood. Discriminating according to these subtypes, we further show that alcohol effects on top-down glutamatergic signaling appear specific to sub-cortically projecting Type A cells in male, but not female, rats. These changes co-occurred with elevated anxiety-like behavior with no apparent effects on working memory in male rats, implicating specific PFC PC subtype afferents in the expression of alcohol-induced anxiety-like behavior in males. Furthermore, the data suggests these cells are particularly vulnerable to the effects of alcohol. Interestingly, in addition to an apparent homeostatic recovery in top-down glutamate signaling onto deep-layer PCs, female rats also exhibited enhanced performance on the NMS T-maze working memory task that further underscores the necessity of including sex as a relevant biological variable in the study of alcohol use disorders.

Diversity in Deep-Layer Cortical Pyramidal Cells

The stratified layers of cortex host a rich milieu of excitatory and inhibitory neurons that adopt highly specialized functional roles within a cortical column [37-39], permitting significant computational power. We and others have investigated the effects of chronic ethanol exposure on a number of these cell subtypes, finding consistent adaptations that bias frontal cortical regions

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266   toward a hyperexcitable state [12, 13, 40]. While a number of studies have investigated

267   excitatory signaling within cortex in a variety of disease models, most neglect to parse pyramidal

268   cell subtype-specific changes despite a wealth of anatomic, genetic, and physiological data

269   demonstrating at least two apparent subtypes [32, 41, 42].

270   Type-A and -B PC subtypes are anatomically distinguishable by projection destination,

271   with Type-As preferentially distributing afferents to mid- and hind-brain structures via the

272   pyramidal tracts while Type-B cells predominantly project intra-telencephalically [31, 33, 42].

273   Functionally, Type-A neurons also display a characteristic hyperpolarization-activated cyclic

274   nucleotide gated channel (HCN)-mediated voltage sag when hyperpolarizing current is applied

275   that is notably modest in Type-B cells, and exhibit a distinct facilitating response to excitatory

276   signaling from superficial-layer pyramidal cells (**Figure 2A-B**) [30, 32]. Further characterization

277   of this phenomenon demonstrated an apparent predisposition for Type-A cells as coincidence

278   detectors of incoming excitation due to reliable facilitating synaptic responses over a narrow

279   temporal window (**Figure 2D-E**) [41]. Reducing the response selectivity of these neurons, for

280   instance via a generalized enhancement of presynaptic glutamate release probability (**Figure**

281   **3A**), would diminish the response specificity of these cells and reduce their capacity to filter out

282   otherwise "noisy" information. Given the preferential interconnectivity of these cells with

283   regions like the peri-aqueductal gray (PAG), shown to regulate fear and anxiety-like behaviors

284   [42, 43], it is reasonable to expect that doing so would elicit elevations in such behaviors to

285   otherwise innocuous stimuli (e.g. **Figure 5H**). In contrast, Type-B cells integrate excitatory

286   inputs from local PCs as well as regions including hippocampus over a wide temporal window

287   that includes the gamma frequency range [41]. This latter phenomenon in particular suggests a

288   specific role for these cells in the generation and maintenance of reverberant gamma oscillations

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289   characteristic of working memory [44, 45], and indeed clinical evidence demonstrates significant

290   correlations between aberrant gamma band patterns and impairments in working memory [46].

291   Based on these phenomena, it is tempting to postulate that alcohol-induced changes in Type-A or

292   -B cell activity would elicit concomitant changes in anxiety-like behavior or working memory

293   performance respectively, and the present observations off some insight into the validity of this

294   schema.

295   *Preferential Alcohol Effects on PFC-Dependent Behaviors Between Sexes*

296   Indeed, we report robust changes in Type-A cell function that specifically co-occur with

297   elevated anxiety-like behavior in males, suggesting these cells influence expression of anxiety-

298   like behavior and are preferentially affected by alcohol exposure. Consistent with this scenario

299   are recent observations showing that PAG-projecting PFC neurons exhibit physiological

300   properties characteristic of Type-A cells, and mediate the expression of both fear-conditioned

301   and alcohol drinking behaviors, appearing to function as a behavioral "brake" on consumption

302   [42]. Extended alcohol exposure, though, elicits a hyperactive state in Type-A cells during acute

303   withdrawal that coincides with heightened anxiety-like behavior, and one interpretation may be

304   that overactive Type-A cells projecting to regions like PAG are promoting expression of these

305   behaviors. Indeed, such a scenario is consistent with observations that specifically inactivating

306   the prelimbic PFC diminishes the expression of these behaviors [36]. That we observe no effect

307   of ethanol on either Type-A cell activity or anxiety-like behavior in females supports this

308   assertion, particularly in light of other evidence that females appear resilient to PFC-mediated

309   elevations in anxiety-like behavior after ethanol exposure [11]. Alternatively, we have previously

310   reported that PFC PCs projecting to CeA display reduced inhibitory tone, and while it is not

311   presently known if these cells are Type-A or Type-B, their heightened downstream signaling to

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312 CeA is nevertheless consistent with enhance expression of anxiety-like behavior in males [13].

313 Indeed, others report that increased PFC signaling into the adjacent basolateral amygdala (BLA)

314 during acute withdrawal is necessary and sufficient to elicit such behaviors [34], though again it

315 is unclear from which PC subclass these terminals emanate. Considered together, it seems

316 reasonable to hypothesize that hyperactivity specifically within Type-A cells diminishes the

317 specificity of PFC top-down control over the expression of fear/anxiety-like behaviors,

318 contributing to negative affect states characteristic of ethanol dependence. This raises the

319 question as to what predisposes this cell population to the effects of chronic alcohol exposure,

320 both pre- and post-synaptically. That deep-layer Type-A and –B PCs show apparent subtype-

321 specific genetic markers suggests the possibility of selectively targeting one or the other

322 population, and doing so represents fertile ground for additional study [47, 48].

323 Though the specific mechanism(s) governing alcohol effects on glutamate release

324 probability onto PC cells remain unclear, based on observations modulating extracellular calcium

325 (**Figure 3C-D**) it is likely such changes reflect alterations in calcium-dependent presynaptic

326 processes including voltage gated calcium channel (VGCC) expression and/or function, which is

327 further supported by data showing no effect of picrotoxin on this phenomenon (**Figure 1G-I**).

328 Reports demonstrating that adaptations in VGCCs as well as other proteins integral to

329 presynaptic vesicular release govern enhanced glutamate release probability after chronic ethanol

330 exposure support this hypothesis [49, 50]. Indeed, it is also not presently known whether inputs

331 to Type-A/-B cells differentially express VGCC subtypes, representing an additional avenue for

332 investigation. Another open question is the degree to which concomitant changes in glutamate

333 and GABA tone on deep-layer PCs [13, 26] specifically drive altered excitability in Type-A cells

334 (**Figure 3G**). Similar elevations in PC excitability have been observed in lateral orbital frontal

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335 cortex after chronic alcohol exposure due to reductions in small conductance calcium-activated

336 potassium (SK) channel-mediated conductance [40], and differential expression or activity of

337 these channels may contribute to our observations in PFC, warranting further investigation.

338 Though not addressed in the present work, chronic alcohol exposure has also been shown to

339 potently alter monoamine signaling in cortex [51, 52], and as Type-A PCs appear especially

340 sensitive to regulation by neuromodulators [30, 31], it would be of substantial interest to evaluate

341 how such changes coordinate with alcohol effects on synaptic scaling to influence excitability

342 between PC subtypes.

343   As a defining feature of Type-A cells, it is somewhat curious we did not observe any

344 apparent effects of ethanol exposure on HCN channel activity in these cells, particularly given

345 recent reports of significant modulation of these channels by alcohol exposure [19, 24].

346 Observations by Salling, Skelly [24], though, were conducted using an adolescent alcohol

347 exposure model that, due to extensive developmental regulation of HCNs during adolescence

348 [23], likely underlies incongruent findings between their report and this paper. As well, our

349 measurements are likely only detecting somatic or proximal dendrite-expressed HCNs, the

350 possibility these channels are undergoing extensive spatial remodeling or functional modification

351 cannot be ruled out. In any case, it is notable that we again observe higher V_H in female cells

352 than males, consistent with recent findings by our laboratory showing similarly elevated HCN-

353 mediated activity in Martinotti interneurons and HCN1 protein expression in PFC [19].

354 Importantly, that study further showed differential sex-specific changes in both Martinotti cell

355 excitability and HCN-mediated current that could explain female-specific alcohol-facilitated

356 working memory performance despite no apparent effects on Type-B PCs. Such a schema is

357 consistent with previous observations showing somatostatin-positive interneurons, which include

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358   Martinotti cells, are intimately involved in mediating working memory [53]. That elevated basal

359   levels of HCNs in PFC co-occur with somewhat higher performance during NMS T-maze

360   training in females compared to males (**Figure 5B,E**) additionally conforms to human data

361   showing similarly higher performance on working memory tasks in women, further supporting

362   the conclusion that cortical circuits and behaviors cannot be assumed equivalent between sexes

363   [54, 55]. Though sex-specific alterations in hippocampal input to PFC after alcohol exposure

364   may also underlie disparate effects on working memory [35], given the prominent role of HCNs

365   in cortical circuit function and working memory [23, 41, 45], investigating differential innate and

366   ethanol-induced expression patterns of cortical HCNs on working memory between sexes is a

367   particularly compelling avenue for future investigation.

368   *Conclusions*

369   In sum, the present work demonstrates evidence that chronic alcohol exposure promotes

370   differential adaptations in both prefrontal cortical cell excitability and PFC-dependent behaviors

371   between male and female rats that further underscores the need to consider sex as a relevant

372   biological variable in studies of AUDs. Additionally, we show that distinct deep-layer PC

373   subpopulations are apparent in both males and females, and display complex adaptations that

374   warrant further investigation. Future studies will seek to discern the role of these cells in

375   mediating alcohol-induced perturbations in select cortical microcircuits to elicit specific aberrant

376   behaviors.

377

378   **FUNDING AND DISCLOSURE**

381

382 **AUTHOR CONTRIBUTIONS**

383 Study design, data collection and analysis, and manuscript preparation were conducted by BAH.

384 TKO and GB assisted with EPM experiments shown in **Figure 5**. MAH and ALM assisted with

385 interpretation of results and preparation of the manuscript.

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524 **FIGURE LEGENDS**

525 **Figure 1:** Chronic alcohol exposure elevates superficial layer excitatory signaling onto deep-

526 layer pyramidal neurons in prelimbic PFC. **A-B**) Chronic alcohol exposure shifts electrically-

527 evoked AMPA receptor-mediated currents responses from repeated-pulse facilitation towards

528 depression in male rats across a range of stimulation frequencies [3 Hz: $F_{(1,19.4)}$ Treatment=22.55;

529 30 Hz: $F_{(1,19.5)}$ Treatment=4.8; n=20-22 cells from 10-12 rats/group]. **C**) Differential repeated-

530 pulse responses (RPR) between groups are not the product of altered sensitivity to electrical

531 stimulation. **D-E**) No effect of chronic ethanol exposure is observed on RPR in female PCs [3

532 Hz: $F_{(1,14.5)}$ Treatment=2.39; 30 Hz: $F_{(1,16.2)}$ Treatment=0.06; n=17-20 cells from 9-11

533 rats/group]. **F**) Similar to males, there was no apparent change in sensitivity of electrical

534 stimulation between exposure groups. **G-H**) Application of picrotoxin (100 μM) did not alter

535 RPR in either sex, suggesting this phenomenon is not activity-dependent. **I**) Representative

536 traces depicting electrically-evoked (30 Hz) AMPA currents pre- and post-application of

537 picrotoxin.

538

539 **Figure 2:** Deep-layer PCs exhibit distinct subtypes in both male and female rats. **A)** Simplified

540 circuit diagram showing differential innervation patters of Type-A and -B PC subtypes, as well

541 as representative current-clamp traces from each. Red arrows show presence of HCN-mediated

542 voltage characteristic of Type-A cells. **B-C**) Mean V_H (± S.D.) between cell subtypes in males

543 and females [males: Type-A Water=64.5±17.3; Type-A EtOH=58.4±17.1; Type-B

544 Water=27.0±5.5; Type-B EtOH=30.3±7.4: females: Type-A Water= 82.5±19.0; Type-A

545 EtOH=99.5±27.2; Type-B Water=37.5±10.7; Type-B EtOH=35.0±11.8]. Females displayed

546 somewhat greater V_H in all cells compared to males. **D-E)** Type-A and −B cells exhibit

25

547   differentiable repeated-pulse responses to electrical stimulation in both male and female rats. **F)**

548   Representative voltage-clamp traces showing repeated-pulse responses between cell subtypes. **G)**

549   PC subtypes were observed in approximately equal frequency in male and female rats.

550

551   **Figure 3:** Sex-specific alcohol enhancement of glutamate release probability onto Type-A PCs.

552   **A-B)** Type-A cells in males display alcohol-induced shift toward repeated-pulse depression that

553   is not observed in female rats, consistent with observations in **Figure 1** [males: $F_{(1,8.84)}$

554   Treatment=5.58, p=0.0344; n=15 cells from 7-8 rats/group: females: $F_{(1,7.08)}$ Treatment=0.04,

555   p=0.853; n=12-14 cells from 6-7 rats/group]. **C-D)** Reducing extracellular calcium concentration

556   reduces glutamate release probability, shown as a profound elevation in repeated-pulse

557   facilitation [$F_{(1,127)}$ CalciumMod=93.4, p<0.0001; n=8-10 cells from 5-6 rats/group].

558   Significantly larger pulse facilitation after modulating calcium concentration in ethanol-exposed

559   cells is indicative of alcohol-induced enhancement of glutamate release probability on these

560   neurons [$F_{(1,13.2)}$ Treatment=4.92, p=0.0473]. **E-F)** Females display similarly enhanced pulse

561   facilitation in low-calcium solution, with no difference observed between water- and alcohol-

562   exposed groups [$F_{(1,175)}$ CalciumMod=81.26, p<0.0001; n=12-13 cells from 6-7 rats/group]. **G-**

563   **H)** Alcohol exposure results in significantly elevated excitability of Type-A cells in male, but not

564   female, rats [males: $F_{(1,13.2)}$ Treatment=4.92, p=0.0446; n=15 cells from 7-8 rats/group].

565

566   **Figure 4:** Alcohol exposure does not alter glutamate release onto Type-B PCs in male or female

567   rats. **A-B)** Type-B PCs do not exhibit any appreciable change in repeated-pulse responses after

568   chronic ethanol exposure in either male or female rats (males; n=12-13 cells from 6-7 rats/group:

569   females; n=13-14 cells from 6-7 rats/group). **C-F)** Modulating extracellular calcium

26

570   concentration results in an expected shift toward pulse facilitation in both sexes, with no

571   difference between water- and alcohol-exposed groups [males: $F_{(1,131)}$ CalciumMod=49.71,

572   p<0.0001; n=9-10 cells from 6-7 rats/group: females: $F_{(1,112)}$ CalciumMod=55.72, p<0.0001;

573   n=7-9 cells from 4 rats/group]. **G-H)** Alcohol exposure does not alter excitability of Type-B cells

574   in either male or female rats.

575

576   **Figure 5:** Chronic ethanol exposure alters PFC-dependent behaviors in a sex-dependent manner.

577   **A)** Training and exposure paradigm for the delayed non-match-to-sample (NMS) T-maze task.

578   **B)** Male water- and ethanol-exposed rats perform equally during task training prior to alcohol

579   exposure. **C-D)** Increasing delay between forced and choice trails results in expected reductions

580   in task performance that does not differ between exposure groups at 24 hr acute withdrawal and

581   is consistent across 3 consecutive test days. **E)** Similar to males, female groups perform

582   equivalently during task training prior to exposure. **F-G)** Alcohol-exposed female rats exhibit

583   significantly higher performance on the delayed NMS T-maze task at 60 seconds delay

584   compared to water-exposed rats during 24 hr acute withdrawal [$F_{(2,36)}$ Interaction=5.609,

585   p=0.0076; Bonferroni post-test]. Cumulative performance across 3 consecutive test days reveals

586   a robust enhancement in task performance after alcohol exposure [$F_{(1,18)}$ Treatment=10.84,

587   p=0.004]. **H)** Chronic alcohol exposure in male rats results in significant elevations in anxiety-

588   like behavior on an elevated plus maze [Open Arm Time $t_{11.5}$=2.841, p=0.0155; Open Arm

589   Entries $t_{12.6}$=2.842, p=0.0143]. **I)** In contrast, female rats display no apparent effect of ethanol

590   exposure on expression of anxiety-like behavior.