

Supplementary Materials for:

**NEONATAL ETHANOL EXPOSURE TRIGGERS APOPTOSIS IN THE MURINE
RETROSPLENIAL CORTEX: ROLE OF INHIBITION OF NMDA RECEPTOR-DRIVEN
ACTION POTENTIAL FIRING**

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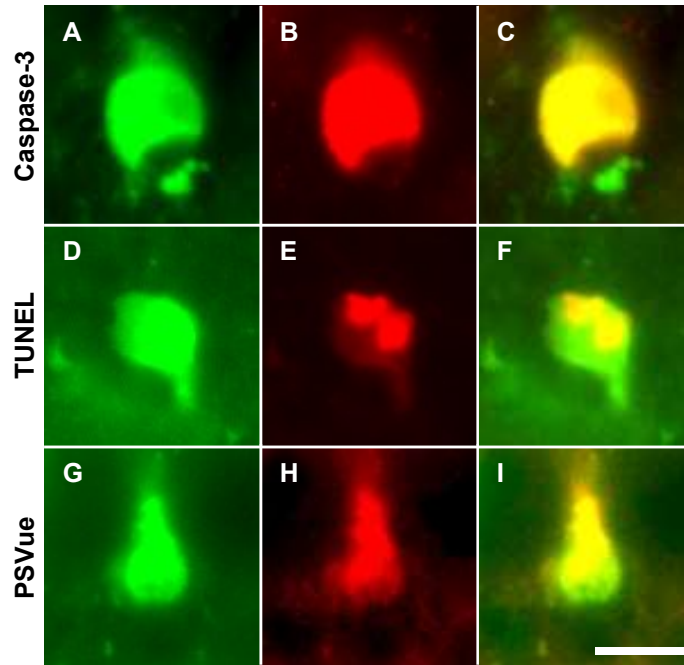
Supplementary Table 1

Supplementary Figures 1 to 5

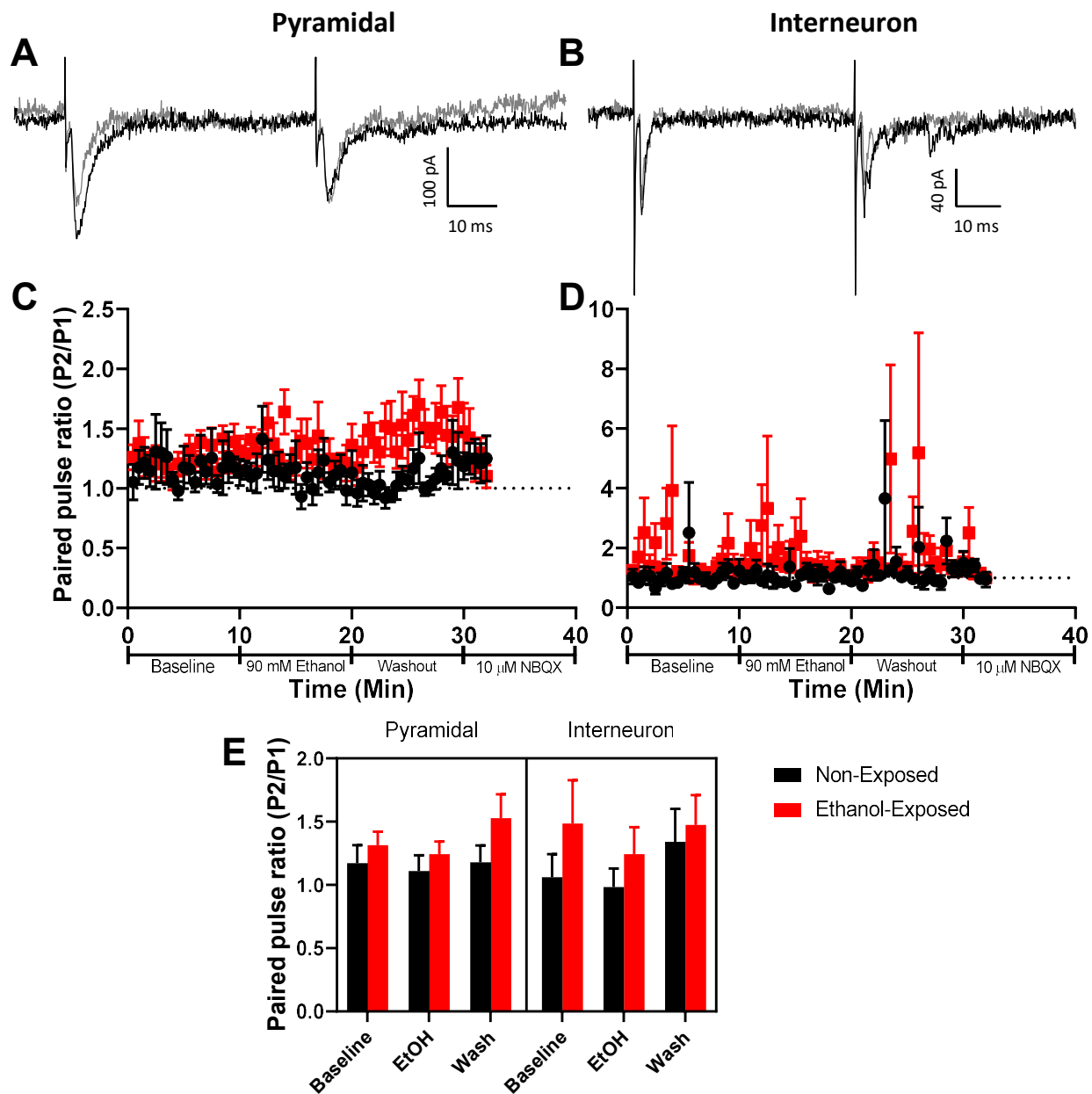
Supplemental Table 1. Comprehensive collection of all statistical analyses performed.

Figure or results section	Experiment	Test	Measure	df	Test statistic	p value	Effect size measure	Effect size	Notes	Passes SW normality test?	
Figure 2	Blood ethanol concentrations during and after vapor chamber exposure	One-way ANOVA	Effect of vapor chamber exposure on BECs	F(5,18)	40.029	<0.0001	Partial eta squared	0.917			
			0 h vs 2 h	t(18)	3.148	0.0278	Hedges' g	6.286	Bonferroni adjusted p-value		
		Multiple comparison: comparing all time points to 0 h (no-exposure) time point	0 h vs 4 h	t(18)	9.202	<0.0001	Hedges' g	5.152	Bonferroni adjusted p-value		Yes: residuals pass
			0 h vs 8 h	t(18)	10.140	<0.0001	Hedges' g	6.066	Bonferroni adjusted p-value		
			0 h vs 12 h	t(18)	7.019	<0.0001	Hedges' g	5.494	Bonferroni adjusted p-value		
			0 h vs 24 h	t(18)	0.154	>0.9999	Hedges' g	0.615	Bonferroni adjusted p-value		
Figure 3I	Caspase cell counts	Adjusted rank transform	Interaction (Layer X Time Point)	F(8,63)	5.185	<0.0001	Partial eta squared	0.397		No: residuals fail normality test	
			Scheirer-Ray-Hare Test	Layer	H(2)	21.760	<0.0001	Eta squared	0.123		
		Dunn's multiple comparison: comparing time points within layer	Time Point	H(4)	33.393	<0.0001	Eta squared	0.308			
			Layer 1 Control vs. 0 h	n/a	0.165	>0.9999	r	0.052	Bonferroni adjusted p-value		
			Layer 1 Control vs. 2 h	n/a	1.034	>0.9999	r	0.327	Bonferroni adjusted p-value		
			Layer 1 Control vs. 4 h	n/a	1.530	0.5043	r	0.484	Bonferroni adjusted p-value		
			Layer 1 Control vs. 8 h	n/a	1.835	0.2658	r	0.553	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 0 h	n/a	0.620	>0.9999	r	0.196	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 2 h	n/a	2.233	0.1023	r	0.706	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 4 h	n/a	3.266	0.0044	r	1.033	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 8 h	n/a	3.563	0.0015	r	1.074	Bonferroni adjusted p-value		
			Layer 5 Control vs. 0 h	n/a	1.240	0.8594	r	0.392	Bonferroni adjusted p-value		
			Layer 5 Control vs. 2 h	n/a	2.439	0.0589	r	0.771	Bonferroni adjusted p-value		
			Layer 5 Control vs. 4 h	n/a	2.729	0.0254	r	0.863	Bonferroni adjusted p-value		
			Layer 5 Control vs. 8 h	n/a	4.246	<0.0001	r	1.280	Bonferroni adjusted p-value		
Figure 3J	Caspase interneuron counts	Adjusted rank transform	Interaction (Layer X Time Point)	F(8,63)	3.880	0.0009	Partial eta squared	0.330		No: residuals fail normality test	
			Scheirer-Ray-Hare Test	Layer	H(2)	29.093	<0.0001	Eta squared	0.240		
		Dunn's multiple comparison: comparing time points within layer	Time Point	H(4)	21.704	0.0002	Eta squared	0.122			
			Layer 1 Control vs. 0 h	n/a	1.298	0.7769	r	0.410	Bonferroni adjusted p-value		
			Layer 1 Control vs. 2 h	n/a	0.085	>0.9999	r	0.027	Bonferroni adjusted p-value		
			Layer 1 Control vs. 4 h	n/a	0.830	>0.9999	r	0.262	Bonferroni adjusted p-value		
			Layer 1 Control vs. 8 h	n/a	2.312	0.0832	r	0.697	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 0 h	n/a	1.447	0.5915	r	0.458	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 2 h	n/a	2.357	0.0738	r	0.745	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 4 h	n/a	3.101	0.0077	r	0.981	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 8 h	n/a	2.879	0.0160	r	0.868	Bonferroni adjusted p-value		
			Layer 5 Control vs. 0 h	n/a	1.488	0.5466	r	0.471	Bonferroni adjusted p-value		
			Layer 5 Control vs. 2 h	n/a	3.018	0.0102	r	0.954	Bonferroni adjusted p-value		
			Layer 5 Control vs. 4 h	n/a	2.729	0.0254	r	0.863	Bonferroni adjusted p-value		
			Layer 5 Control vs. 8 h	n/a	3.527	0.0017	r	1.063	Bonferroni adjusted p-value		
Figure 4I	TUNEL cell counts	Adjusted rank transform	Interaction (Layer X Time Point)	F(2,30)	16.215	<0.0001	Partial eta squared	0.519		No: residuals fail normality test	
			Scheirer-Ray-Hare Test	Layer	H(2)	3.008	0.2222	Eta squared	<0.001		
		Dunn's multiple comparison: comparing time points within layer	Time Point	H(1)	22.249	<0.0001	Eta squared	0.575			
			Layer 1 Control vs. 8 h	U(n1=n2=6)	4	0.0780	r	0.647	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		
			Layer 5 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		
Figure 4J	TUNEL interneuron counts	Adjusted rank transform	Interaction (Layer X Time Point)	F(2,30)	5.149	0.0120	Partial eta squared	0.256		No: residuals fail normality test	
			Scheirer-Ray-Hare Test	Layer	H(2)	6.980	0.0305	Eta squared	0.066		
		Dunn's multiple comparison: comparing time points within layer	Time Point	H(1)	19.610	<0.0001	Eta squared	0.487			
			Layer 1 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		
			Layer 5 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		
Figure 5I	PSVue cell counts	Adjusted rank transform	Interaction (Layer X Time Point)	F(2,30)	44.306	<0.0001	Partial eta squared	0.747		No: residuals fail normality test	
			Scheirer-Ray-Hare Test	Layer	H(2)	17.935	0.0001	Eta squared	0.431		
		Dunn's multiple comparison: comparing time points within layer	Time Point	H(1)	13.616	0.0002	Eta squared	0.287			
			Layer 1 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		
			Layer 5 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		
Figure 5J	PSVue interneuron counts	Adjusted rank transform	Interaction (Layer X Time Point)	F(2,30)	19.027	<0.0001	Partial eta squared	0.559		No: residuals fail normality test	
			Scheirer-Ray-Hare Test	Layer	H(2)	20.406	<0.0001	Eta squared	0.514		
		Dunn's multiple comparison: comparing time points within layer	Time Point	H(1)	10.296	0.0013	Eta squared	0.177			
			Layer 1 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		
			Layers 2-4 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		
			Layer 5 Control vs. 8 h	U(n1=n2=6)	0	0.0066	r	0.832	Bonferroni adjusted p-value		

Figure or results section	Experiment	Test	Measure	df	Test statistic	p value	Effect size measure	Effect size	Notes	Passes SW normality test?
Figure 7D	Interneuron synaptic excitability	One sample t-test or Wilcoxon signed-rank test (% change vs. 0 for both tests)	% change after 90 mM ethanol	t(10)	5.189	0.0004	Hedges' g	1.294	One sample t-test compared to 0	Yes
			% change after 5uM AP5	t(10)	4.868	0.0007	Hedges' g	1.214	One sample t-test compared to 0	Yes
			% change after 50 nM NBQX	n/a	0.652	0.5147	r	0.206	Wilcoxon signed-rank test compared to 0	No
			% change after 5 uM AP5 + 50 nM NBQX	t(10)	7.456	<0.0001	Hedges' g	1.859	One sample t-test compared to 0	Yes
		Kruskal-Wallis test: % Change in excitability following drug application	Drug	$\chi^2(3)$	18.377	<0.0001	Partial eta squared	0.320	Is there a difference in how drugs affected AP#?	
			90 uM ethanol vs 5 uM AP5	n/a	0.331	>0.9999	r	0.071	Bonferroni adjusted p-value	
			90 uM ethanol vs 50 nM NBQX	n/a	2.886	0.0433	r	0.586	Bonferroni adjusted p-value	No: residuals fail normality test
			90 uM ethanol vs 5 uM AP5 + 50 nM NBQX	n/a	1.605	0.6507	r	0.342	Bonferroni adjusted p-value	
Dunn's multiple comparison: Comparing how drugs changed AP#	5 uM AP5 vs. 50 nM NBQX	n/a	2.363	0.1088	r	0.516	Bonferroni adjusted p-value			
	5 uM AP5 vs. 5 uM AP5 + 50 nM NBQX	n/a	1.936	0.3169	r	0.413	Bonferroni adjusted p-value			
	50 nM NBQX vs. 5 uM AP5 + 50 nM NBQX	n/a	4.253	0.0001	r	0.928	Bonferroni adjusted p-value			
	Interaction: Acute ethanol X cell type	F(1,20)	14.860	0.0010	Partial eta squared	0.426	Did 90 mM inhibit action potentials differently between cell types?	Yes: residuals pass		
Figure 7 C-D	Effect of ethanol on action potential number	Repeated measures two-way ANOVA	Main effect: Acute ethanol	F(1,20)	124.647	<0.0001	Partial eta squared	0.452		
			Main effect: Cell type	F(1,20)	14.860	0.0010	Partial eta squared	0.426		
			Interaction (exposure X acute effect)	F(2,28)	0.781	0.4678	Partial eta squared	0.053		
Supplemental figure 2C	AMPA PPR	Repeated measures two-way ANOVA: Effect of 90 mM ethanol application X vapor chamber exposure	Main effect: exposure	F(1,14)	1.663	0.2077	Partial eta squared	0.114		Yes: residuals pass
			Main effect: acute effect	F(2,28)	0.202	0.1136	Partial eta squared	0.106		
			Interaction (exposure X acute effect)	F(2,22)	0.904	0.4194	Partial eta squared	0.076		
Supplemental figure 2D	AMPA PPR	Repeated measures two-way ANOVA: Effect of 90 mM ethanol application X vapor chamber exposure	Main effect: exposure	F(1,11)	0.714	0.4162	Partial eta squared	0.061		Yes: residuals pass
			Main effect: acute effect	F(2,22)	3.757	0.0395	Partial eta squared	0.255		
			Interaction (phase X vapor chamber exposure X cell type)	F(2,50)	1.524	0.2278	Partial eta squared	0.057		
Supplemental Figure 2E	Effect of ethanol on AMPA PPR	Repeated measures three-way ANOVA	Interaction (phase X vapor chamber exposure)	F(2,50)	0.173	0.8414	Partial eta squared	0.007	Three way ANOVA: Phase(Baseline, Acute ethanol, Wash) X Vapor chamber exposure X cell type	Yes: residuals pass
			Interaction (phase X cell type)	F(2,50)	0.379	0.6864	Partial eta squared	0.015		
			Main effect: phase	F(2,50)	5.256	0.0085	Partial eta squared	0.174		
			Main effect: vapor chamber exposure	F(1,25)	2.041	0.1655	Partial eta squared	0.075		
		Multiple comparison	Main effect: cell type	F(1,25)	0.002	0.968	Partial eta squared	<0.001		
			Baseline vs. ethanol	t(50)	1.560	0.3754	Hedges' g	0.230	Bonferroni adjusted p-value	
			Baseline vs. Wash	t(50)	1.682	0.2965	Hedges' g	0.214	Bonferroni adjusted p-value	
			ethanol vs. Wash	t(50)	3.242	0.0064	Hedges' g	0.482	Bonferroni adjusted p-value	
Supplemental Figure 3	Effect of ethanol exposure on mini EPSC characteristics: pyramidal neurons	Unpaired t-test	mini EPSC amplitude	t(8,545)	1.935	0.0867	Hedges' g	0.865	Welch's correction for unequal variances	Yes
			mini EPSC frequency	t(7,454)	1.207	0.264	Hedges' g	0.601	Welch's correction for unequal variances	Yes
			mini EPSC decay tau	U(n1=7,n2=9)	9	0.0164	r	0.596	Ethanol-exposed fails normality test	
	Effect of ethanol exposure on mini EPSC characteristics: interneurons	Unpaired t-test	mini EPSC amplitude	t(13)	1.293	0.2185	Hedges' g	0.627		Yes
			mini EPSC frequency	U(n1=7,n2=8)	27	0.9551	r	0.030	Ethanol-exposed fails normality test	
			mini EPSC decay tau	U(n1=7,n2=8)	27	0.9551	r	0.030	Non-exposed fails normality test	
Supplemental Figure 4C	Effect of ethanol on Intrinsic excitability: Pyramidal neurons	Repeated measures two-way ANOVA	Interaction (phase X current injected)	F(1,629,13,030)	2.316	0.1437	Partial eta squared	0.224	Greenhouse-Geisser corrected p-value and F-ratio	Yes: residuals pass
			Main effect of phase (baseline, ethanol, wash)	F(1,237,9,895)	3.760	0.0757	Partial eta squared	0.320		
			Main effect of current injected (200,400,600 pA)	F(1,078,8,622)	135.4	<0.0001	Partial eta squared	0.944		
Supplemental Figure 4D	Effect of ethanol on Intrinsic excitability: Interneurons	Repeated measures two-way ANOVA	Interaction (phase X current injected)	F(1,423,8,539)	0.761	0.4530	Partial eta squared	0.113	Greenhouse-Geisser corrected p-value and F-ratio	Yes: residuals pass
			Main effect of phase (baseline, ethanol, wash)	F(1,889,11,340)	3.395	0.0534	Partial eta squared	0.394		
			Main effect of current injected (200,400,600 pA)	F(1,064,6,386)	340.6	<0.0001	Partial eta squared	0.983		
Supplemental Figure 5A	Mimicking 90 mM ethanol inhibition of NMDA currents with 5 uM AP5	Unpaired t-test	% inhibition: 90 mM ethanol vs. 5 uM AP5	t(14)	1.209	0.2468	Hedges' g	0.593		Yes
Supplemental Figure 5B	Mimicking 90 mM ethanol inhibition of non-NMDA currents with 50 nM NBQX	Unpaired t-test	% inhibition: 90 mM ethanol vs. 50 nM NBQX	t(11)	0.667	0.5183	Hedges' g	0.389		Yes

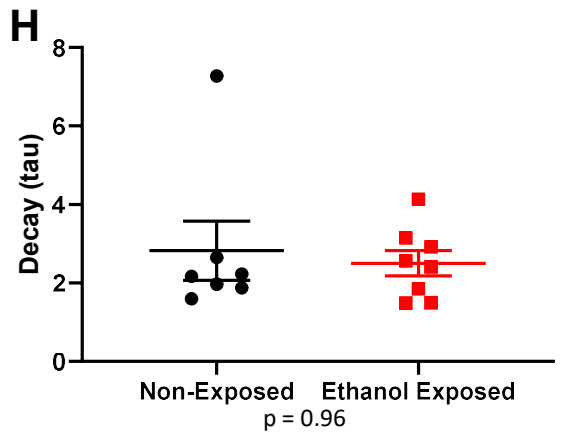
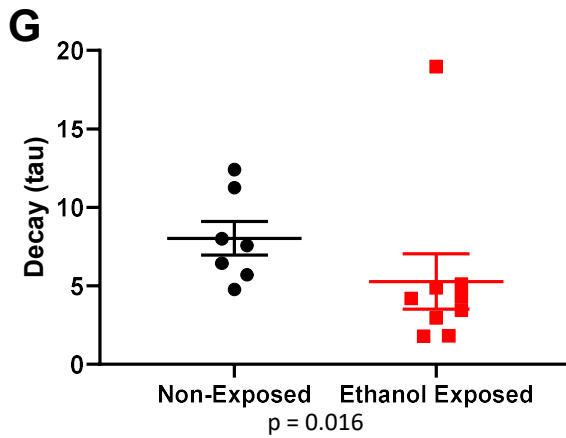
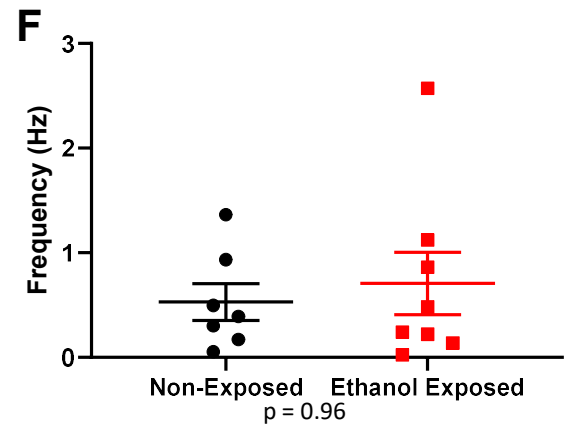
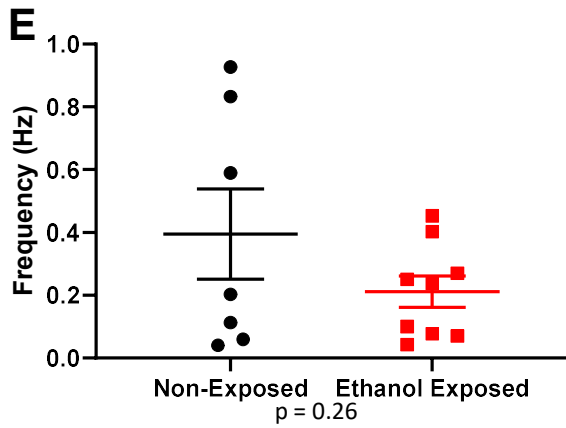
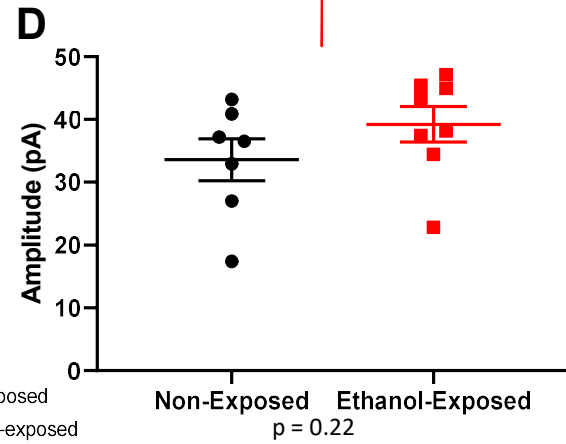
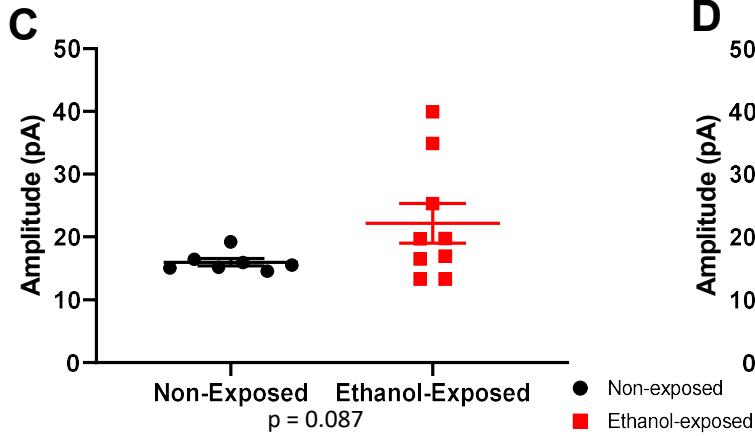
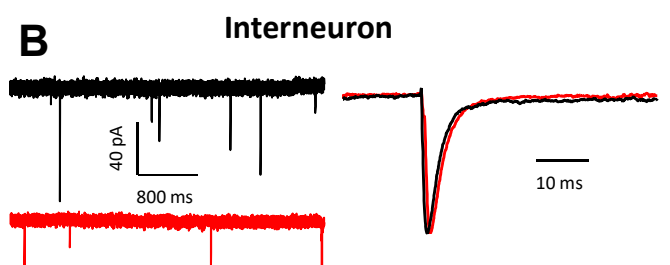
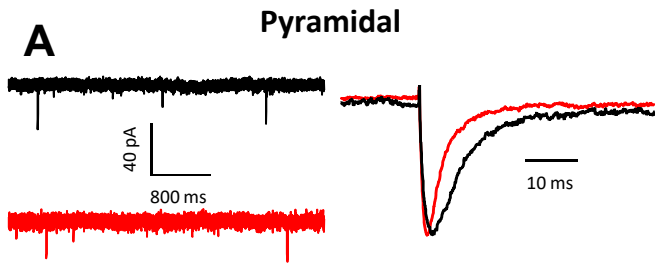


Supplemental Figure 1. High magnification images showing colocalization of apoptotic markers with Venus-positive interneurons. (40X objective, scale bar = 10 μ m) Co-localization of Venus fluorescence (panels A, D, and G) with cleaved caspase-3 (B), TUNEL (E) and PSVue (H) immunofluorescence. The corresponding merged images are shown in panels C, F, and I.

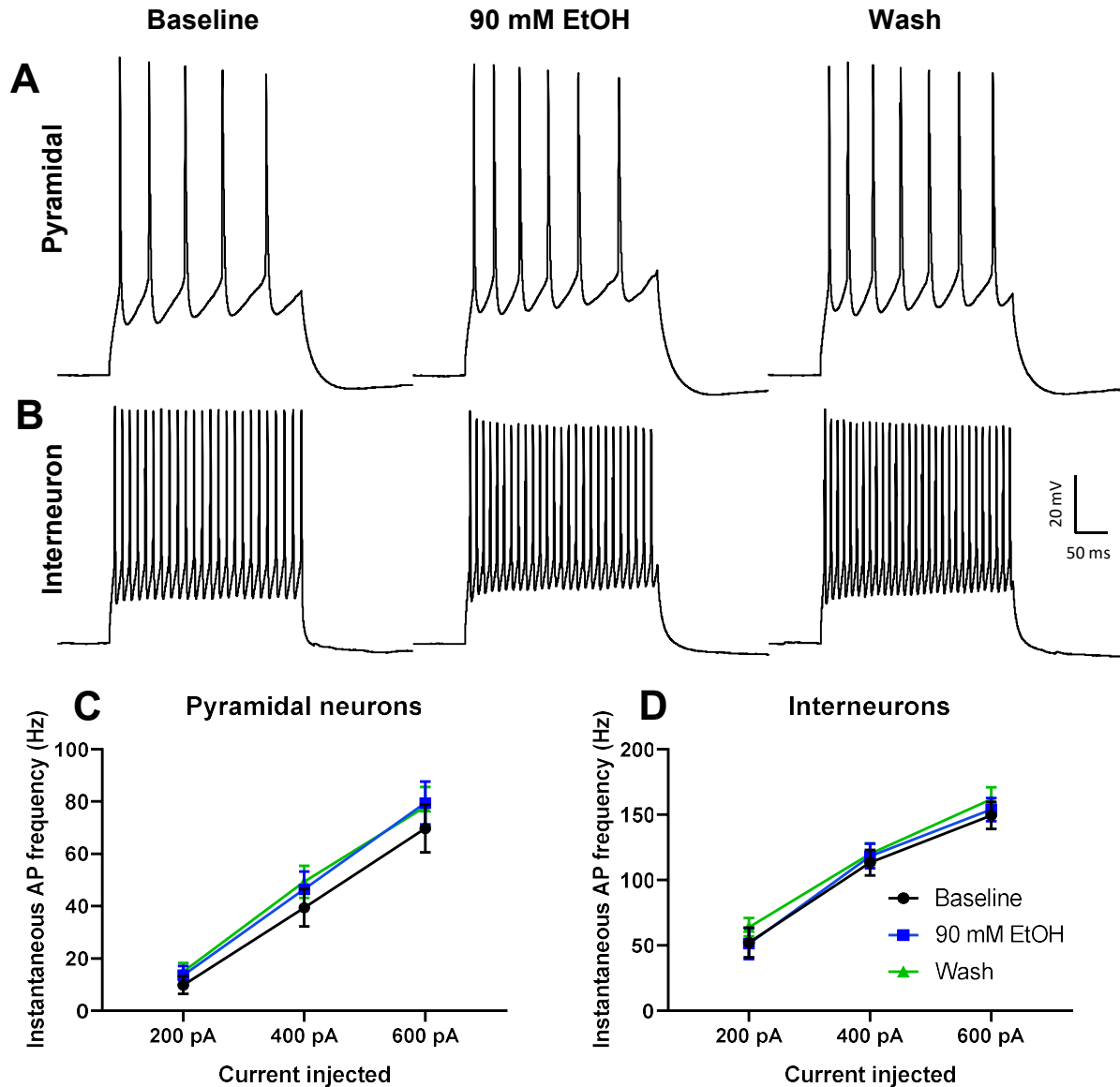


Supplemental Figure 2. Non-NMDA EPSC paired pulse ratios in pyramidal neurons and interneurons from control and ethanol exposed animals.

Representative evoked AMPA paired pulse traces are shown for (A) pyramidal neurons and (B) interneurons during baseline (black traces) and acute 90 mM ethanol bath application (grey traces) in animals not exposed to vaporized ethanol *in vivo*. Effect of 90 mM ethanol on paired pulse ratios in (C) pyramidal neurons and (D) interneurons from control animals (black data points) and 4-h ethanol vapor-exposed animals (red data points). E) Average paired pulse ratios from the last 3 min of each phase (baseline, 90 mM ethanol application, and washout) are shown for both pyramidal neurons and interneurons from non-exposed and 4-h ethanol vapor-exposed animals. Pyramidal neuron non-exposed and ethanol-exposed n = 8 animals (1 cell per animal, each from a different litter); interneuron non-exposed n = 6 animals (7 cells from 6 litters), ethanol-exposed n = 7 animals (1 cell per animal, each from a different litter). Data shown are mean \pm SEM.

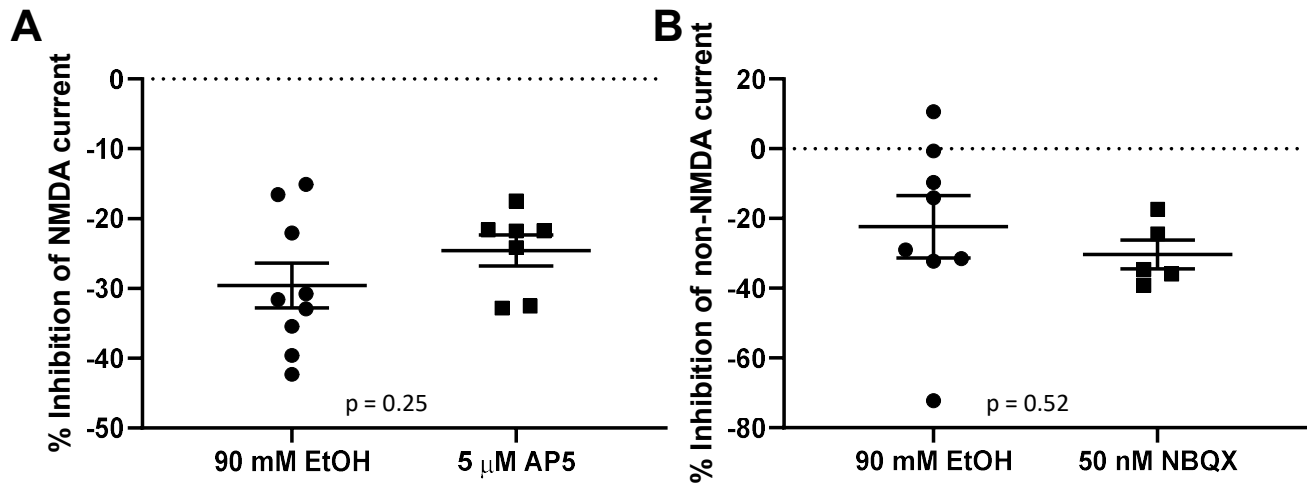


Supplemental Figure 3. mEPSC amplitude, frequency, and decay time constants in pyramidal neurons and interneurons from control and ethanol exposed animals. Representative mEPSC traces and average mEPSC waveforms (amplitudes normalized) from (A) pyramidal neurons and (B) interneurons from control (black traces) and ethanol exposed (red traces) animals. The average amplitude of mEPSCs in C) pyramidal neurons and D) interneurons is shown. E-F) The average frequency of mEPSCs in E) pyramidal neurons and F) interneurons is shown. The average decay constant (tau from a single-exponential curve fit) is shown for (G) pyramidal neurons and (H) interneurons. p-values shown are from appropriate parametric or non-parametric tests. For a detailed presentation of statistical tests performed please see Supplemental Table 1. Pyramidal non-exposed n = 7 animals (1 cell per animal, each from a different litter), ethanol-exposed n = 9 animals (1 cell per animal, each from a different litter); interneuron non-exposed n = 7 animals (1 cell per animal, each from a different litter), ethanol-exposed n = 8 animals (1 cell per animal, each from a different litter). Data shown are individual values from each cell along with the mean \pm SEM.



Supplemental Figure 4. Acute effect of 90 mM ethanol on action potential firing induced by current injection in pyramidal neurons and interneurons.

Shown are representative voltage traces recorded from (A) pyramidal neurons and (B) interneurons during a 400 pA, 300 ms current injection during baseline, acute application of 90 mM ethanol, and washout (all from animals not exposed to ethanol vapor). Also shown are plots illustrating that acute bath application of ethanol had little effect on the instantaneous action potential firing frequency in pyramidal neurons (C) and interneurons (D). For a detailed presentation of statistical tests performed please see Supplemental Table 1. Pyramidal neuron $n = 9$ cells (from 5 animals from 2 litters); interneuron $n = 7$ cells (from 5 animals from 2 litters). Data presented are mean \pm SEM.



Supplemental Figure 5. Mimicking the effects of acute bath application of 90 mM ethanol on NMDA and non-NMDA evoked EPSC amplitude with 5 μM DL-AP5 and 50 nM NBQX in pyramidal neurons, respectively. A) The percent inhibition on NMDA EPSC amplitude caused by 90 mM ethanol application (calculated from the data shown in Fig 6) and 5 μM DL-AP5 are compared. B) The percent inhibition on non-NMDA current amplitude caused by 90 mM ethanol application (calculated from the data shown in Fig 6) and 50 nM NBQX are compared. p-values presented are from unpaired t-tests. NMDA current 90 mM EtOH n = 9 cells (1 cell per animal, each from a different litter), 5 μM DL-AP5 n = 7 cells (1 cell per animal, each from a different litter). Non-NMDA current 90 mM EtOH n = 8 cells (1 cell per animal, each from a different litter), 50 nM NBQX n = 5 (1 cell per animal, each from a different litter). Data presented are individual values along with the mean ± SEM.