



Supplementary Information

OMEGA-3 FATTY ACIDS MITIGATE LONG-LASTING DISRUPTION OF THE ENDOCANNABINOID SYSTEM IN THE ADULT MOUSE HIPPOCAMPUS FOLLOWING ADOLESCENT BINGE DRINKING

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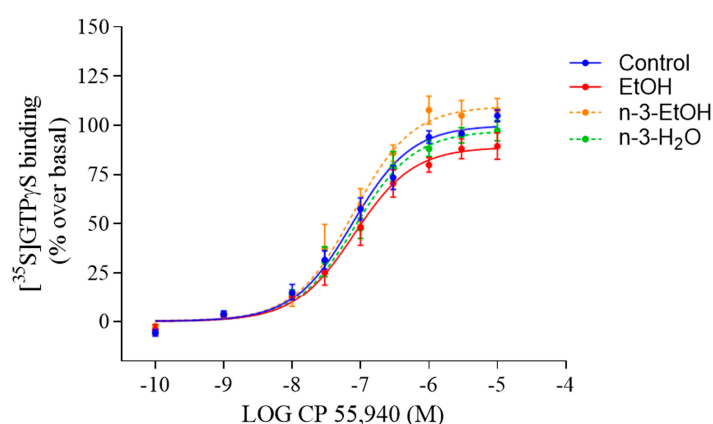
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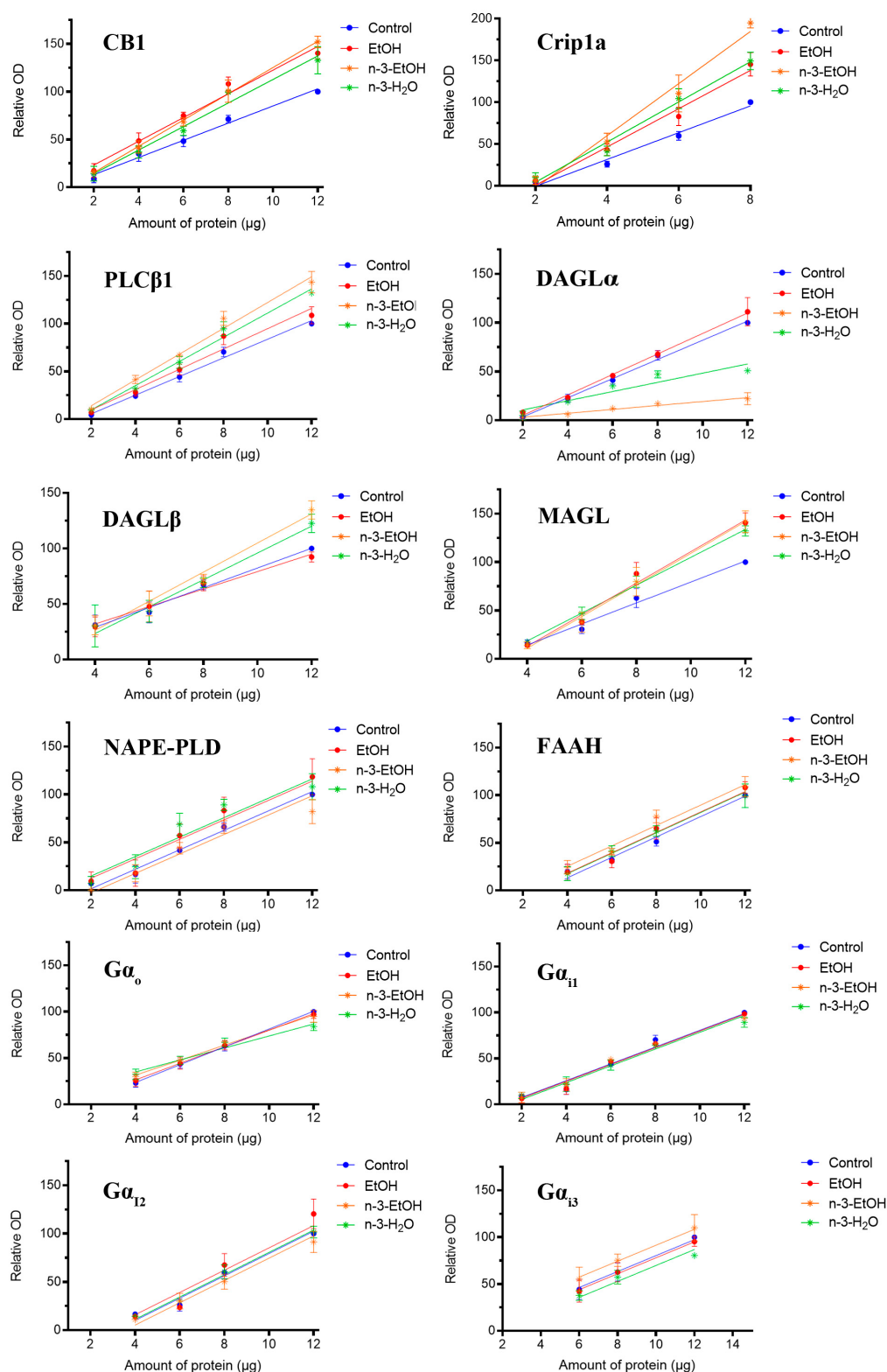
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Supplementary Figures

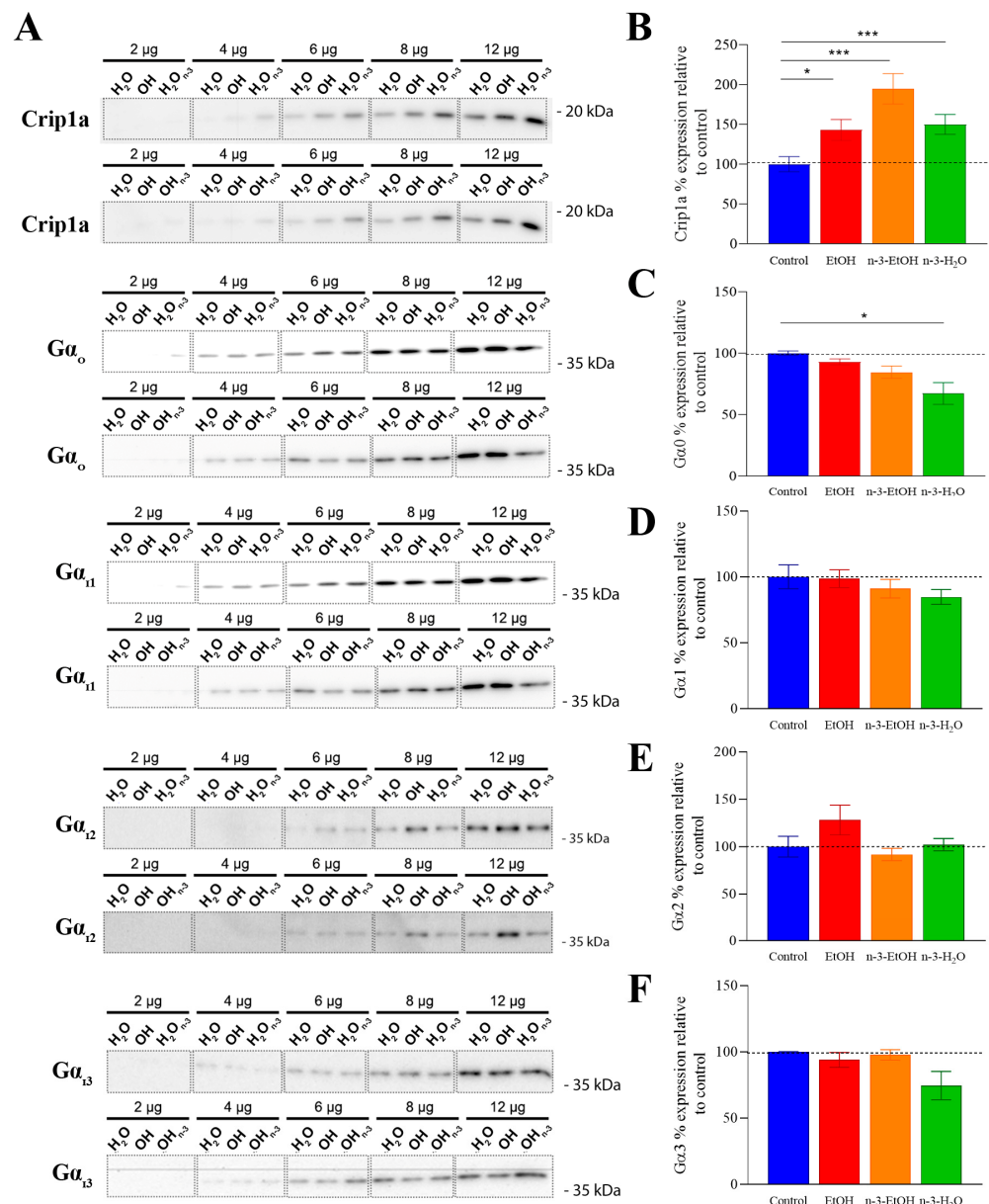


Supplementary Figure S1. CB1 receptor coupling to $G\alpha_{i/o}$ proteins in hippocampal synaptosomes from control, EtOH, n-3-EtOH and n-3-H₂O groups. Concentration–response curves for CP 55,940-stimulated [³⁵S]GTPγS binding. Data represents the means ± SE from three independent

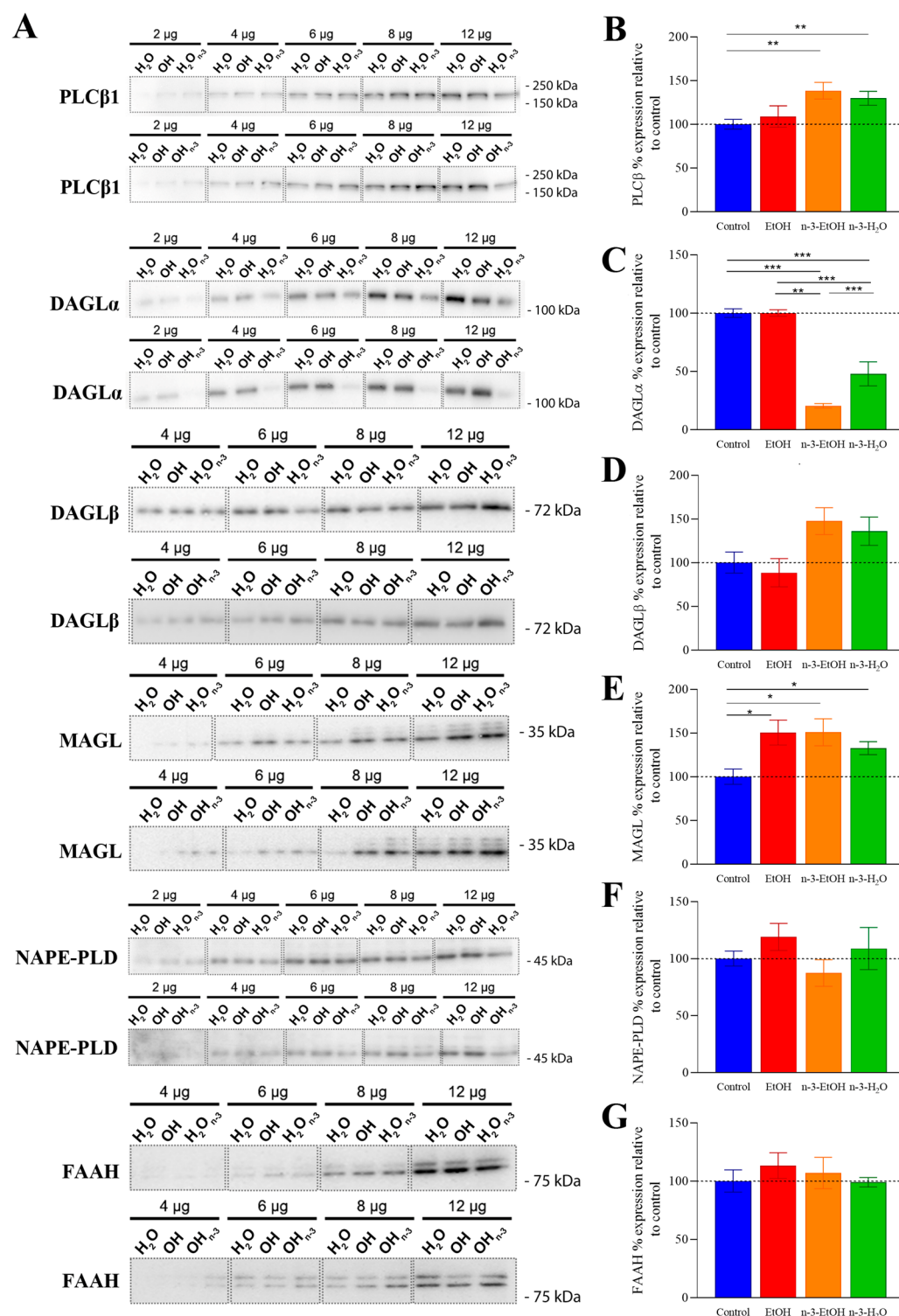
experiments, using synaptosomal membranes prepared through three fractionation procedures, with each fractionation including hippocampal samples pooled from at least six adult mice. Maximal response (E_{max}) and potency (pEC_{50}) values are expressed as % specific [35 S]GTP γ S bound of basal. E_{max} : Control 100.00 ± 2.57 ; EtOH $88.92 \pm 3.15^*$; n-3-EtOH $109.70^{\Phi\Phi\Phi} \pm 3.60$; n-3-H $_2$ O 97.27 ± 2.84 . pEC_{50} : Control 7.12 ± 0.06 ; EtOH 7.10 ± 0.09 ; n-3-EtOH 7.11 ± 0.09 ; n-3-H $_2$ O 7.08 ± 0.08 . The statistical significance between E_{max} and pEC_{50} parameters was determined using the extra-sum-of-squares F test (F) method. This method compares the goodness-of-fit of the experimental data for all groups combined (null hypothesis: E_{max} or pEC_{50} is the same across all datasets) against the goodness-of-fit for each group analyzed individually (alternative hypothesis: E_{max} or pEC_{50} differs across datasets).



Supplementary Figure S2. Regression analysis of immunoreactive signal values (integrated optical density) corresponding to increasing amounts of synaptosomal total protein. Regression analysis provided the equation of a simple linear regression model. The resulting slopes of the equations were subsequently used to determine the relative protein expression. Regression analysis data are presented as mean $\pm$ SE employing synaptosomal membranes obtained from three fractionation procedures and including hippocampal pools from at least six adult mice per fractionation procedure. CB1 receptor: control 8,98 $\pm$ 4,80; EtOH 12,41 $\pm$ 1,58; n-3-EtOH 13,731 $\pm$ 12,06; n-3-H₂O 12,31 $\pm$ 10,35; Crip1a: control 16,01 $\pm$ 32,52; EtOH 22,88 $\pm$ 45,06; n-3-EtOH 31,15 $\pm$ 64,66; n-3-H₂O 24,01 $\pm$ 43,74; PLC β 1: control 9,76 $\pm$ 13,94; EtOH 10,63 $\pm$ 11,54; n-3-EtOH 13,51 $\pm$ 12,94; n-3-H₂O 12,67 $\pm$ 15,56. DAGL α : control 9,82 $\pm$ 16,20; EtOH 10,49 $\pm$ 15,94; n-3-EtOH 2,00 $\pm$ 0,83; n-3-H₂O 4,70 $\pm$ 1,27; DAGL β : control 8,88 $\pm$ 5,41; EtOH 7,86 $\pm$ 0,61; n-3-EtOH 13,10 $\pm$ 26,07; n-3-H₂O 12,08 $\pm$ 24,80; MAGL: control 10,84 $\pm$ 28,95; EtOH 16,31 $\pm$ 42,18; n-3-EtOH 16,34 $\pm$ 54,29; n-3-H₂O 14,39 $\pm$ 38,95; NAPE-PLD: control 9,76 $\pm$ 16,25; EtOH 11,62 $\pm$ 17,14; n-3-EtOH 8,53 $\pm$ 12,64; n-3-H₂O 10,63 $\pm$ 8,478; FAAH: control 10,23 $\pm$ 25,93; EtOH 11,59 $\pm$ 31,29; n-3-EtOH 10,93 $\pm$ 19,11; n-3-H₂O 10,13 $\pm$ 20,90; G α _o: control 9,60 $\pm$ 14,71; EtOH 8,92 $\pm$ 9,20; n-3-EtOH 8,12 $\pm$ 0,94; n-3-H₂O 6,45 $\pm$ 9,23; G α _{i1}: control 9,77 $\pm$ 14,79; EtOH 9,63 $\pm$ 14,78; n-3-EtOH 8,27 $\pm$ 7,41; n-3-H₂O 8,89 $\pm$ 9,41; G α _{i2}: control 10,95 $\pm$ 31,61; EtOH 14,02 $\pm$ 48,74; n-3-EtOH 10,05 $\pm$ 29,61; n-3-H₂O 11,18 $\pm$ 32,18; G α _{i3}: control 9,29 $\pm$ 11,49; EtOH 8,72 $\pm$ 8,90; n-3-EtOH 9,07 $\pm$ 11,48; n-3-H₂O 6,92 $\pm$ 1,49. The statistical significance of differences between slopes was determined using the extra-sum-of-squares F test (F) method, analyzing the goodness of fit of the experimental data from each experimental groups as a whole (Null hypothesis = Slope same for all data sets) with the goodness of fit of the experimental data for each experimental group individually (Alternative hypothesis = Slope different for each data set).



Supplementary Figure S3. Relative expression of Crip1a and Gα_{i/o} subunits in hippocampal synaptosomes from control, EtOH, n-3-EtOH and n-3-H₂O groups. (A) Representative Western blot analysis with increasing amounts of synaptosomal protein (2, 4, 6, 8, and 12 μg/lane). Protein loading was validated using the Coomassie Brilliant Blue staining. Molecular weights of immunoreactive signals were determined using standard markers (indicated in the figure). Protein migration was consistent with the expected molecular masses (observed weights: Gα_o, 40.1 kDa; Gα_{i1}, 40.5 kDa; Gα_{i2}, 40.4 kDa; Gα_{i3}, 40.5 kDa; Crip1a, 18.6 kDa). H₂O, OH, H₂O_{n-3} and OH_{n-3} correspond to control, EtOH, n-3-H₂O and n-3-EtOH groups, respectively. (B–F) Histogram of relative expression of Crip1a (EtOH vs. Control $p=0.0356$; n-3-EtOH vs. Control $p=0.0006$; n-3-H₂O vs. Control $p=0.008$), Gα_o (n-3-H₂O vs. Control $p=0.0378$), Gα_{i1}, Gα_{i2}, Gα_{i3}. Linear regression analysis graphs for each protein are provided in Supplementary Figure 2 and were determined using the slope comparison method using an extra sum-of-squares F-test. Significance levels: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.



Supplementary Figure S4. Relative expression of key enzymes involved in AEA and 2-AG synthesis and degradation in hippocampal synaptosomes from control, EtOH, n-3-EtOH and n-3-H₂O groups. (A) Representative Western blots with increasing amounts of synaptosomal protein (2, 4, 6, 8, and 12 μg per lane). Protein loading was validated using the Coomassie Brilliant Blue gel staining. Molecular weights of the immunoreactive bands were determined with standard markers (as indicated in the figure) and matched the expected molecular masses: (PLCβ1: 138.3 kDa and 133.3 kDa, corresponding to the β1a and β1b isoforms, respectively; DAGLα: 115.3 kDa; DAGLβ: 73.9 kDa; MAGL: 33.3 kDa; NAPE-PLD: 45.8 kDa; FAAH: 63.2 kDa. H₂O, OH, H₂O_{n-3} and OH_{n-3} correspond to control, EtOH, n-3-H₂O and n-3-EtOH groups, respectively. (B–G) Histogram of relative expression PLCβ1 (n-3-EtOH vs. Control $p=0.0042$; n-3-H₂O vs. Control $p=0.009$), DAGLα (n-3-EtOH vs. Control $p=0.0006$; n-3-EtOH vs. EtOH $p=0.0024$; n-3-H₂O vs. Control $p=0.0006$; n-3-H₂O vs. EtOH $p=0.0006$; n-3-H₂O vs. n-3-EtOH $p=0.0024$), DAGLβ, MAGL (EtOH vs. Control $p=0.036$; n-3-EtOH vs. Control $p=0.049$; n-3-H₂O vs. Control $p=0.049$), NAPE-PLD and FAAH. Linear regression

analysis graphs for each protein are provided in Supplementary Figure 2 and were determined using the slope comparison method using an extra sum-of-squares F-test. Significance levels: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Supplementary tables

	Excitatory terminals		Inhibitory terminals		Astrocytic processes	
	% CB1+	Density	% CB1+	Density	% CB1+	Density
Control	25.75 ± 3.37	0.84 ± 0.03	91.28 ± 2.20	6.20 ± 0.26	34.17 ± 2.96	0.78 ± 0.03
EtOH	18.92 ± 2.75	0.80 ± 0.03	83.27 ± 4.64	7.58 ± 0.25**	33.57 ± 2.83	0.69 ± 0.03
n-3-EtOH	12.12 ± 2.10*	1.01 ± 0.06* $\phi\phi$	87.18 ± 1.9	9.07 ± 0.34*** $\phi\phi$	26.15 ± 2.20	0.70 ± 0.03
n-3- H₂O	18.33 ± 2.13	0.87 ± 0.04	90.78 ± 1.63	6.83 ± 0.26 $\dagger\dagger\dagger$	28.13 ± 2.33	0.66 ± 0.03
DID	p=0.0174*	ns	p=0.0486*	p<0.0001****	ns	ns
Diet	p=0.0099**	p=0.0011**	ns	p=0.0002***	p=0.0130*	ns
Interaction	ns	p=0.0170*	ns	ns	ns	ns

Supplementary Table S1. Density and percentage of CB1 receptor-positive excitatory and inhibitory terminals and GLAST-stained astrocytes in the middle third of the DG of control, EtOH, n-3-EtOH and n-3-H₂O groups (n= 3 mice per group). Values are expressed as mean ± SEM. The statistical significance was analyzed by two-way ANOVA and Tukey's multiple comparison test: * $p < 0.05$, ** $p < 0.01$, * $p < 0.0001$ compared to control; $\phi\phi$ $p < 0.01$ when compared to EtOH; and $\dagger\dagger\dagger$ $p < 0.0001$ compared to n-3-EtOH. Two way ANOVA examining the effects of DID, diet, and their interaction: ns= no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.**

	WIN 55,212-2	Capsaicin	Capsaicin +AMG9810
Control	65.71 ±0.61*		
EtOH	105.80 ±0.35		
n-3-EtOH	102.60 ±0.41	136.00 ±0.49****	105.20 ±0.91
n-3-H₂O	139.50 ±0.35*		

Supplementary Table S2. Excitatory synaptic transmission at MPP-granule cell synapses in the four experimental conditions. WIN 55,212-2 (5 μ M) (Control: response vs. baseline $p = 0.0373$; n-3-H₂O: response vs. baseline $p = 0.0361$), Capsaicin (1 μ M) (n-3-EtOH: response vs. baseline $p < 0.0001$), Capsaicin (1 μ M) + AMG9810 (3 μ M) were used. Data are expressed as mean ± SEM of at least three different mice. Parametric or non-parametric test (Paired t-test or Wilcoxon matched-pairs signed rank test, respectively) for drugs vs. baseline) were used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ vs. baseline.

AMG9810 (3 μM)	108.80 ±2.06**	THL (10 μM)	142.70 ± 0.83 ϕ
AM251 (4 μM)	131.80 ± 0.31 ϕ	LEI401 (10 μM)	109.00 ± 0.33**
Latrunculin A (500 μM)	133.10 ± 1.05 $\phi\phi$	AM404 (30 μM)	100.50 ± 1.98****
D-AP5 (50 μM)	102.00 ± 1.72****	URB597 (2 μM)	108.50 ± 0.85**
ω-conotoxin GVIA (1 μM)	139.70 ± 0.33 ϕ		

Supplementary Table S3. Excitatory synaptic plasticity at MPP-granule cell synapses in the four experimental conditions. Low-frequency stimulation (10 Hz for 10 minutes) was applied to investigate the mechanisms underlying MPP-LTP in the n-3-EtOH group, and fEPSPs were compared in the presence or absence of various compounds. Data are expressed as mean ± SEM of at least three different animals. Dunn's test was used ** $p < 0.01$, **** $p < 0.0001$ (response vs. LTP in

absence of compound) and paired t-test or Wilcoxon matched-pairs signed rank test were used. $^{\Phi}p<0.05$, $^{\Phi\Phi}p<0.01$ (response vs. baseline).

	H ₂ O	EtOH	n-3-EtOH	n-3-H ₂ O
DAY 1	68.89 ± 5.55	129.40 ± 13.35**	87.58 ± 11.45	93.00 ± 15.55
DAY 2	33.85 ± 4.12	72.62 ± 13.93	37.38 ± 7.78	63.10 ± 15.82
DAY 3	21.65 ± 3.33	62.18 ± 13.59*	26.10 ± 5.42	34.60 ± 9.07
DAY 4	19.83 ± 3.71	39.60 ± 13.12	24.85 ± 9.53	31.18 ± 8.89
DAY 5	11.78 ± 2.13	35.24 ± 11.45	14.85 ± 3.51	18.80 ± 6.49

Supplementary Table S4. Barnes Maze performance of the control (n=18), EtOH (n=17), n-3-EtOH (n=10) and n-3-H₂O (n=10) groups. Time required to locate the escape box over five days of testing (Day 1: EtOH vs. Control $p=0.0025$; Day 3: EtOH vs. Control $p=0.0414$). Data are presented as mean ± SEM and analyzed using two-way ANOVA followed by Tukey's multiple comparison test. Significance levels: * $p<0.05$, ** $p<0.01$ compared to control. Two way ANOVA revealed time effect *** $p<0.0001$ and experimental group effect * $p<0.05$, but no interaction between them $p>0.05$.

	H ₂ O	EtOH	n-3-EtOH	n-3-H ₂ O
DAY 1	15.49 ± 1.23	25.16 ± 2.66*	17.15 ± 2.89	16.58 ± 2.36
DAY 2	11.22 ± 1.42	19.13 ± 3.66	9.55 ± 2.38	11.83 ± 2.40
DAY 3	9.14 ± 1.25	17.93 ± 2.69*	9.45 ± 2.46	11.30 ± 2.83
DAY 4	8.46 ± 1.29	12.91 ± 2.91	8.73 ± 3.77	10.83 ± 3.29
DAY 5	4.50 ± 0.98	10.44 ± 2.68	4.65 ± 1.15	6.53 ± 2.37

Supplementary Table S5. Barnes Maze performance of the control (n=18), EtOH (n=17), n-3-EtOH (n=10) and n-3-H₂O (n=10) groups. Number of errors made to locate the escape box over five days of testing (Day 1: EtOH vs. Control $p=0.0217$; Day 3: EtOH vs. Control $p=0.0300$). Data are presented as mean ± SEM and analyzed using two-way ANOVA followed by Tukey's multiple comparison test. Significance levels: * $p<0.05$ compared to control. Two way ANOVA revealed time effect *** $p<0.0001$ and experimental group effect *** $p<0.0001$, but no interaction between them $p>0.05$.

Antigen	[μg/mL]	Species (clonality)	Immunogen	Source, Cat.
CB1	2	Guinea pig (polyclonal)	Mouse CB1, C-terminal 31 aa.	Nittobo Medical Co., CB1-GP-Af530
GLAST	0.3	Rabbit (polyclonal)	Mouse GLAST, C-terminal 41 aa	Nittobo Medical Co., GLAST-Rb-Af660
Guinea pig IgG	0.8	Goat (polyclonal). 1.4 nm gold-conjugated		Nanoprobes, #2055
Rabbit IgG	7.5	Goat (polyclonal). Biotinylated		Vector Labs, BA-1000

Supplementary Table S6. Primary and secondary antibodies used in immunoelectron microscopy.

	Control				EtOH				n-3-EtOH				n-3- H ₂ O			
	n 1	n 2	n 3	Total	n 1	n 2	n 3	Total	n 1	n 2	n 3	Total	n 1	n 2	n 3	Total
Excit. Ter.	289	247	395	931	394	288	323	1005	287	255	388	980	372	253	355	930
Inhib. Ter.	55	76	58	189	67	70	62	199	54	76	64	200	58	64	78	194
Astr. Proc.	365	305	213	883	374	216	269	859	384	322	348	921	363	264	294	1054

Supplementary Table S7. Counts of excitatory and inhibitory terminals and GLAST-stained astrocytic processes in the four experimental groups.

Antigen	Dilution	Species and clonality	Isotype	Immunogen	Source, Cat.
CB1	1:1000	Goat polyclonal	Serum	Mouse CB1, C-terminal 31 aa (NM007726)	Nittobo Medical Co., CB1-Go-Af450
NAPE-PLD	1:1000	Guinea pig polyclonal	Serum	Mouse N-terminal 1-41aa (AB112350)	Nittobo Medical Co., NAPE-PLD-GP-Af720
FAAH	1:1000	Rabbit polyclonal	Not specified	Synthetic peptide from the C-terminal region of rat FAAH	Cayman chemical, 101600
Crip1a	1:500	Rabbit polyclonal	IgG	Peptide mapping within an internal region of Crip1 of human origin	Santa Cruz Biotech., sc-137401
PLCβ1	1:2000	Sheep Polyclonal	IgG	Recombinant human PLC-β1, residues 27-245	Novus Biologicals, AF4466
DAGLα	1:1000	Rabbit polyclonal	Serum	Mouse DAGLα, C-terminal 42 aa (NM198114)	Nittobo Medical Co., DGLα-Rb-Af380
DAGLβ	1:2000	Rabbit monoclonal	IgG	Peptide corresponding to residues surrounding Leu505 of human DAGLβ	Cell signalling, 12574
MAGL	1:1000	Rabbit polyclonal	Serum	Mouse MAGL, residues 1-35	Nittobo Medical Co., MGL-Rb-Af200
Gα_o	1:200	Mouse monoclonal	IgG ₁ kappa light chain	Gα _o of bovine origin	Santa Cruz Biotech., sc-13532
Gα_{i1}	1:200	Mouse monoclonal	IgG _{2b} kappa light chain	Gα _{i1} of rat origin	Santa Cruz Biotech., sc-13533
Gα_{i2}	1:200	Mouse monoclonal	IgG _{2b} kappa light chain	Gα _{i2} of rat origin	Santa Cruz Biotech., sc-13534
Gα_{i3}	1:200	Mouse monoclonal	IgG ₃ kappa light chain	Epitope mapping between residues 339-354 of Gα _{i3} of rat origin	Santa Cruz Biotech., sc-365422

Supplementary Table S8. Primary antibodies used in Western blotting.