The Endocannabinoid 2-Arachidonoylglycerol Bidirectionally Modulates Acute and Protracted Effects of Predator Odor Exposure

Supplemental Information

Supplemental Methods

Animals and Drugs

Adult male and female outbred ICR (CD-1) mice were ordered from Envigo (Envigo, Indianapolis, IN) at 8-12 weeks, 30-44 grams. Mice were group-housed on a 12-hour light/12-hour dark cycle, with lights on at 06:00. All experiments were conducted during the light phase. Food and water were available *ad libitum*. Mice were given at least a one-week acclimation period in the mouse facilities before behavioral testing. All studies were carried out in accordance with the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* [1] and approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Drugs

All drugs were administered intraperitoneally (i.p.) 2 hours before initiation of behavioral testing. JZL184 (15mg/kg; Cayman Chemical, Ann Arbor, MI, USA) and PF3845 (1mg/kg; Cayman Chemical) was prepared in dimethylsulfoxide (DMSO; Sigma-Aldrich D8414, St. Louis, MO, USA) and injected at a volume of 1 µl/g bodyweight. DO34 (50 mg/kg; Glixx Laboratories Inc., Hopkinton, MA, USA) was prepared in a 1:1:18 mixture of ethanol, kolliphor (Sigma-Aldrich), and saline, and injected at a volume of 10 µl/g bodyweight. For co-administration experiments, JZL184 (15 mg/kg) was mixed with rimonabant (1 mg/kg; Cayman Chemical), or AM630 (5 mg/kg; Cayman Chemical). Vehicle-injected mice refers to mice just injected with DMSO.

Odor Exposure

All exposure experiments were performed in a Biological Safety Cabinet, Class II, Type B2, fume hood. 2-Methyl-2-thiazoline (2-MT; Tokyo Chemical Industry, Tokyo, Japan), butyric acid (Sigma-Aldrich, St. Louis, MO, USA) or water exposure occurred in an empty, novel cage (12in x 6in x 6in) and had a small square (1in x 1in) of black filter paper taped to the corner. 2-MT, butyric acid, or water was pipetted onto the filter paper at 40 µL (2MT or water) or 38.9µL (Fig. S2: butyric acid or water). Mice were placed individually into this cage. A plastic lid that contained holes for air flow was placed on the cage. Behavior was recorded for 10 minutes; a new cage was used for each mouse. All behavioral experiments were recorded and analyzed using AnyMaze Behavioral tracking software (Stoelting, Wood Dale, IL). 2-MT was stored in the cold room and kept on ice and covered with aluminum foil, to prevent light damage and degradation during behavioral testing.

Context Testing

All context testing was also performed in the same BSL-2 fume hood. Mice were placed in a novel cage (12in x 6in x 6in) that had a small square (1 in x 1 in) of black filter paper taped to the corner. Mice were placed individually into this cage and behavior was recorded for 10 minutes. A new cage was used for each mouse to prevent any lingering 2MT odor from causing behavioral effects. All behavioral experiments were recorded and analyzed using AnyMaze Behavioral tracking software.

Tissue Collection and Lipid Analysis

For brain tissue sample collection, mice were sacrificed via cervical dislocation and decapitation, followed by rapid removal of the brain. Brains were blocked around the PFC, AMY, and PAG (1mm thick section) and snap frozen by placing the blade and tissue on an aluminum block in dry ice. Punches (PFC = 1.5mm, AMY = 1mm, PAG = 1.5mm) were then taken as depicted in Figure 2, and stored at - 80°C for further processing. Importantly, brain punches do not differentiate between subregions/subnuclei, such as infralimbic vs. prelimbic cortex of PFC, or basolateral amygdala vs. central amygdala of AMY.

Endocannabinoid and related lipid concentrations in the collected tissues were determined by liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis in a manner as previously reported [2]. Briefly, brains were sonicated in 300 µL homogenization solution, placed in a bath sonicator for up to 2 minutes, and incubated at –20°C overnight. The next day, samples were centrifuged for 12 minutes at 4°C at 2060*g*. Supernatant was collected and dried under nitrogen. Samples were then reconstituted by an addition of 60 µL methanol followed by 30 µL distilled water and vortexed. Finally, samples were centrifuged at 2060*g* for 12 minutes at 4°C if they were cloudy or had visible particulate matter. LC-MS/MS analysis was performed with a Shimadzu Nexera X2 system in-line with SCIEX QTRAP 6500. Instrument control and data collection were performed using the Analyst software program. Data were normalized to tissue mass and presented as either pmol/g tissue or nmol/g tissue. Significance was analyzed using GraphPad PRISM. Analytes and internal standards were detected as previously described [3].

Slice Electrophysiology

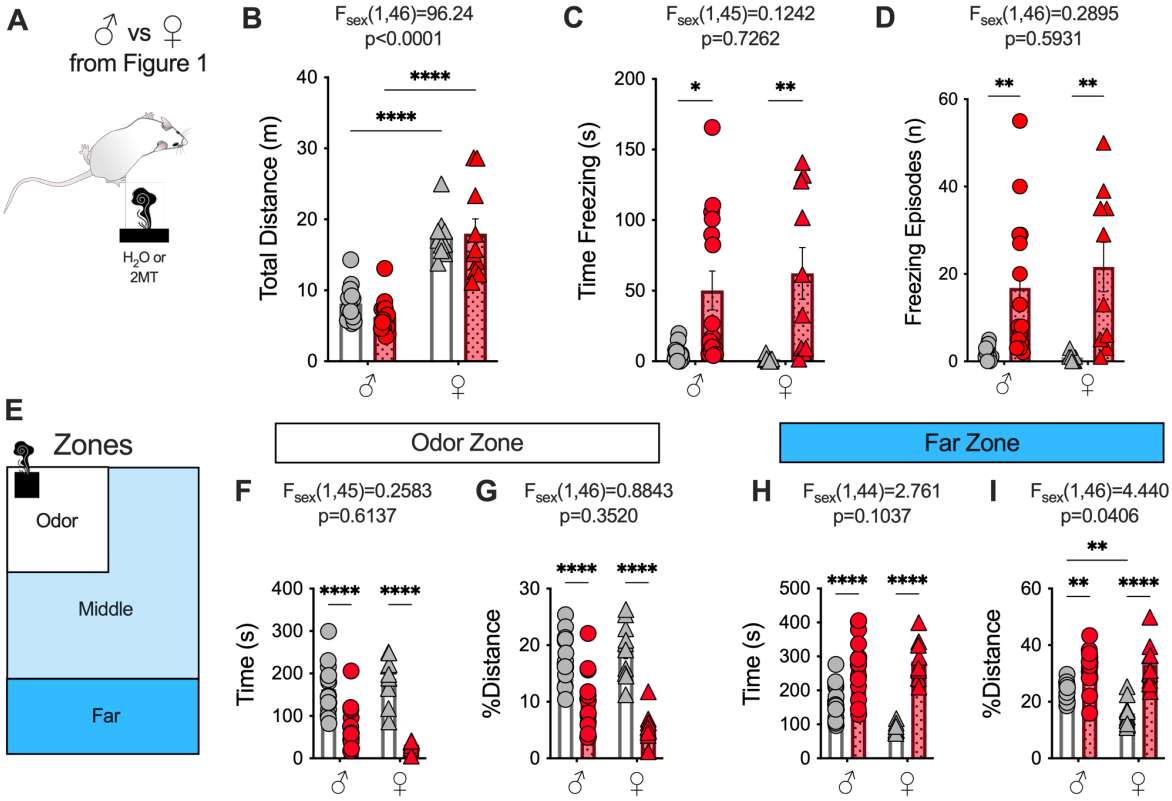
To assess changes in amygdalar activity of water (H_2O) and 2MT exposed mice, mice were exposed to water or 2MT, 70 minutes before slicing. To compare effects of *in vivo* JZL184 administration, mice were injected with JZL-184 (15 mg/kg) or DMSO 2 hours prior to water or 2MT exposure, and then brain slices of amygdala were obtained 70 minutes after exposure. Slice electrophysiology experiments were performed, as previously described [4]. Mice were deeply anesthetized with isoflurane and transcardially perfused with ice-cold oxygenated (95% v/v O2, 5% v/v CO2) N-methyl-D-glucamine (NMDG) based artificial cerebral spinal fluid (ACSF) containing (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na-ascorbate, 3 Na-pyruvate, 5 N-acetylcysteine, 0.5 $CaCl_2 \cdot 4H_2O$ and 10 $MgSO_4 \cdot 7H_2O$. The brain was quickly removed, a 3 mm coronal block containing the amygdala [central amygdala (CeA) and basolateral amygdala (BLA)] was cut using an ice-chilled coronal brain matrix, and 250 µm hemisected coronal slices of the amygdala were cut in ice cold NMDG solution using a Leica VT1000S vibratome (Leica Microsystems, Bannockburn, IL, USA). Slices were incubated for 10–15 min in 32 °C oxygenated NMDG-ACSF, and then moved into a HEPES-based ACSF containing (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 ascorbate, 3 Na-pyruvate, 5 N-acetylcysteine, 2 $CaCl_2 \cdot 4H_2O$ and 2 $MgSO_4 \cdot 7H_2O$. Slices were kept in this HEPES-ACSF until recording. Electrophysiological recordings were performed in a submerged recording chamber during continuous perfusion of oxygenated ACSF containing (in mM): 113 NaCl, 2.5 KCl, 1.2 $MgSO_4 \cdot 7H2O$, 2.5 $CaCl_2 \cdot 2H_2O$, 1 NaH_2PO_4, 26 $NaHCO_3$, 1 ascorbate, 3 Na-pyruvate and 20 glucose; at a flow rate of 2–3 mL/min. Slices were visualized using a Nikon microscope equipped with differential interference contrast video microscopy. Whole-cell voltage-clamp and current-clamp recordings from CeL or BLA cells were obtained under visual control using a 40x objective and 2–6 MΩ borosilicate glass pipettes.

For voltage-clamp recordings of synaptic transmission, pipettes were filled with a cesium solution containing (in mM): 120 CsOH, 120 D-gluconic acid, 2.8 NaCl, 5 TEA-Cl, 20 HEPES, 2.5 Mg-ATP, 0.25 Na-GTP. For spontaneous excitatory and inhibitory transmission, cells were held at -70mV and +10mV, respectively. For all voltage-clamp recordings, cells with an access resistance of >20 were excluded. A custom-made Clampfit template was used to analyze all spontaneous activity. In the BLA, cells that were smaller than 100 were presumed to be interneurons and also excluded. To assess neuronal excitability, current clamp recordings of somatic injection-induced AP firing was obtained with pipettes that were filled with a high [K^+] based solution containing (in mM): 125 K^+-gluconate, 4 NaCl, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, and 10 Na-phosphocreatine. All cells were held at -70mV and then 20pA steps were sequentially applied to depolarize the cell. Recordings were performed using a MultiClamp 700B amplifier (Molecular Devices), and Clampex software (version 10.2; Molecular Devices).

Statistical Analysis

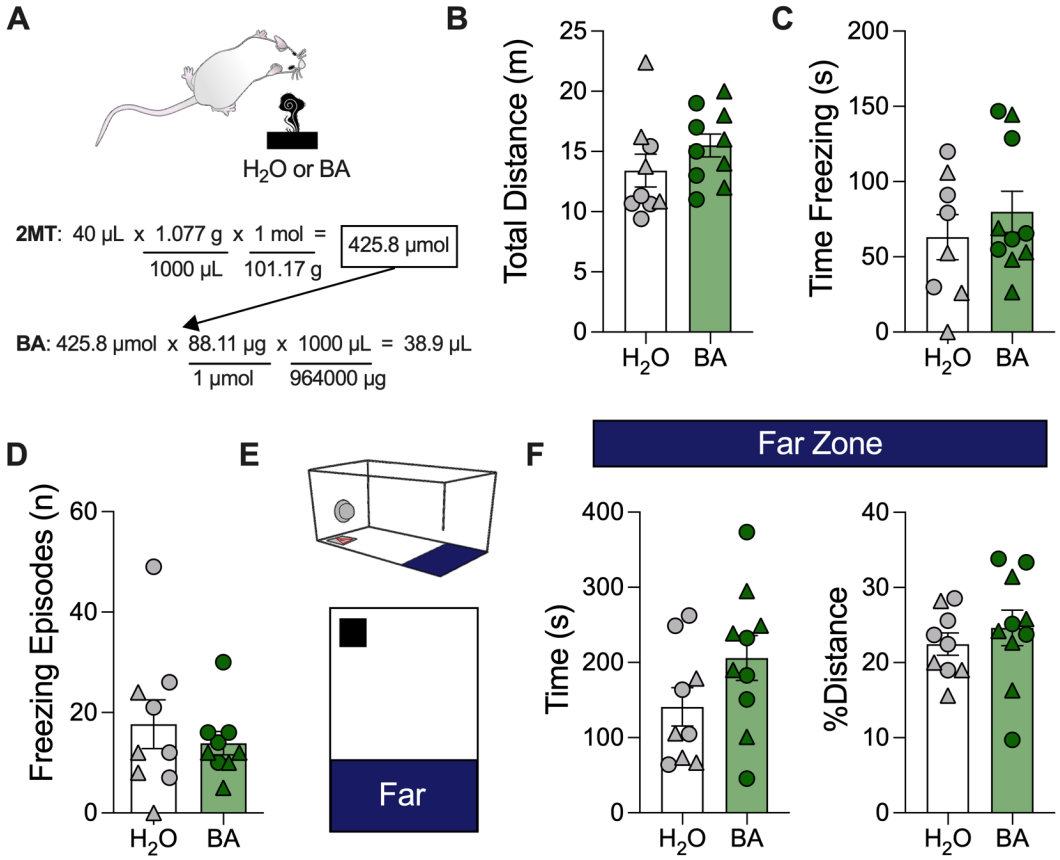
Statistical analyses were performed as outlined in the figure legends. A ROUT outliers test was performed on all data sets to exclude any outliers. For 2-Way ANOVA analysis, *post hoc* Holm-Sidak's multiple comparisons test was performed. A full list of statistical analysis is found in Supplemental Table 1. Significance is displayed as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$

Supplemental Figures



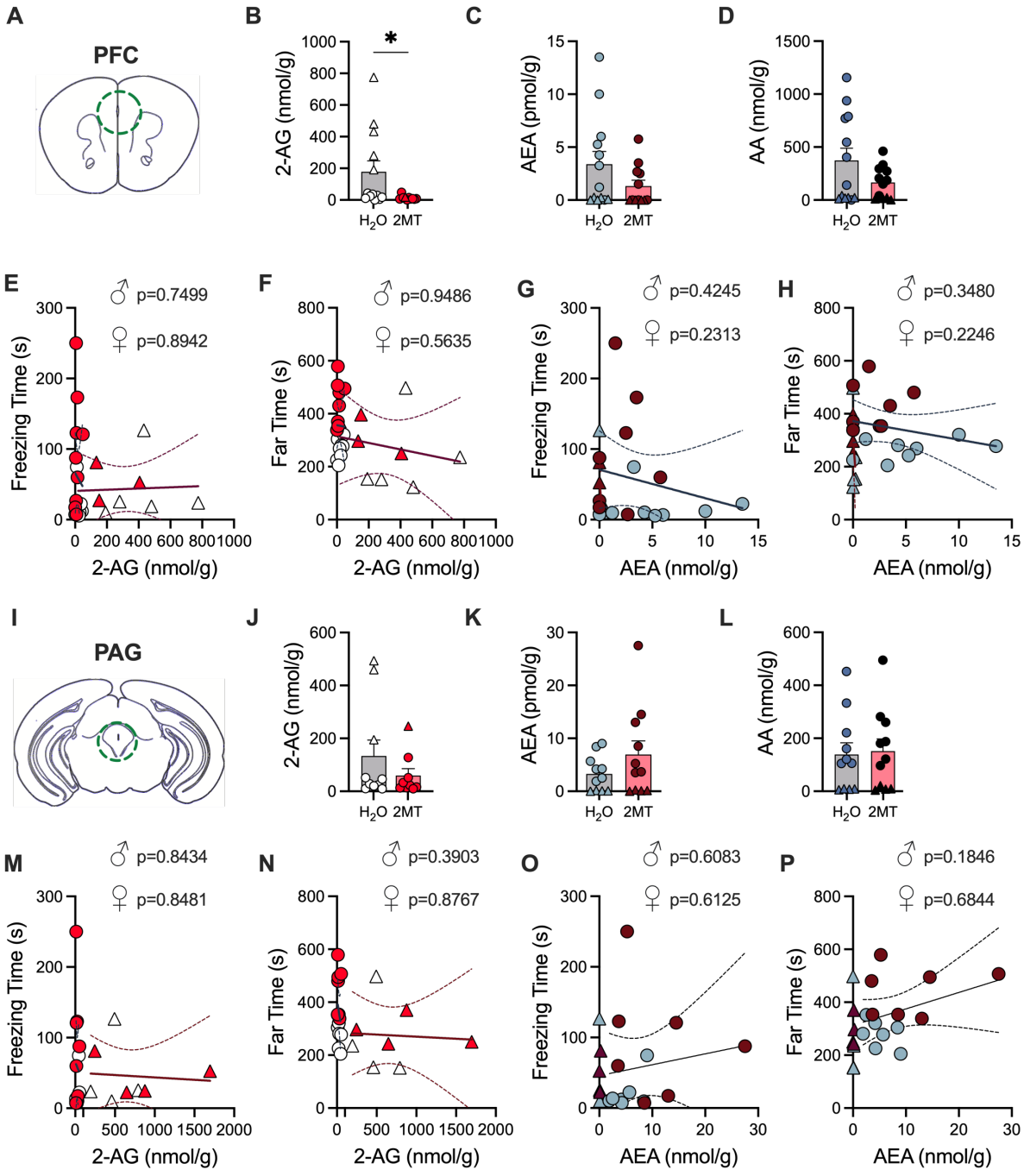
Suppl Fig 1 2MT causes similar fear behaviors in both males and females.

- **A)** Experimental design for splitting up data from Figure 1 into males and females. 2-Way ANOVA performed for all analysis and F values for main effect of sex shown above graph (C, E-F, H-K)
- **B)** Females show significantly increased distance travelled during exposure
- **C)** 2MT increases freezing time in male and female mice with no effect of sex
- **D)** There is no significant effect of sex on the number of 2MT-induced freezing episodes
- **E)** Zones used for quantitative analysis
- **F-G)** Odor Zone
- **F)** Sex has no effect on the time spent in the odor zone
- **G)** Sex has no effect on the percent distance travelled around the odor
- **H-I)** Far Zone
- **H)** Sex has no significant effect on the time spent in the far zone
- **I)** Female mice travel significantly less around the far zone, compared to male mice



Suppl Fig 2 Butyric acid has no effect on freezing or avoidance.

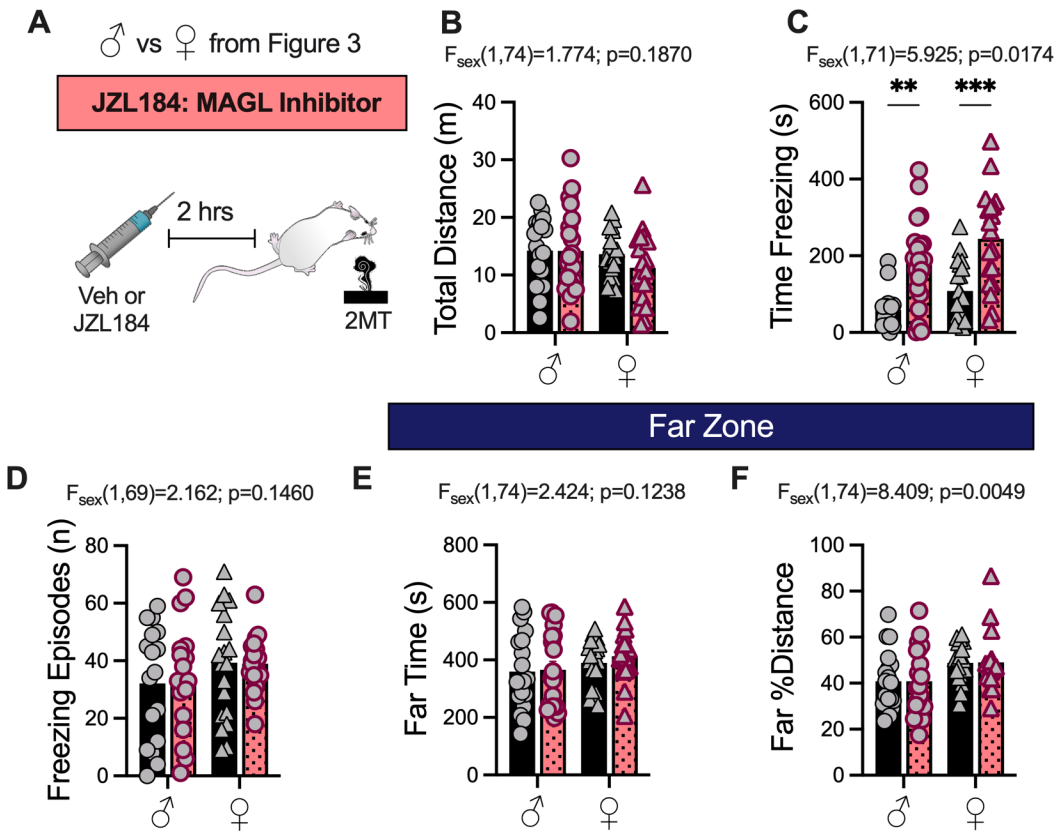
- **A)** Experimental design and calculations. The same number of moles of butyric acid were used as 2MT as control. Unpaired t-test performed for all analysis (H_2O: n=9; BA: n=10)
- **B)** BA has no effect on distance travelled
- **C)** Exposure to BA has no effect on freezing time
- **D)** BA has no effect on freezing episodes
- **E)** Schematic of far zone during odor presentation
- **F)** Mice exposed to BA show no significant differences in time (left) or %distance (right) spent in the far zone



Suppl Fig 3 PFC or PAG eCB levels do not correlate with freezing or avoidance

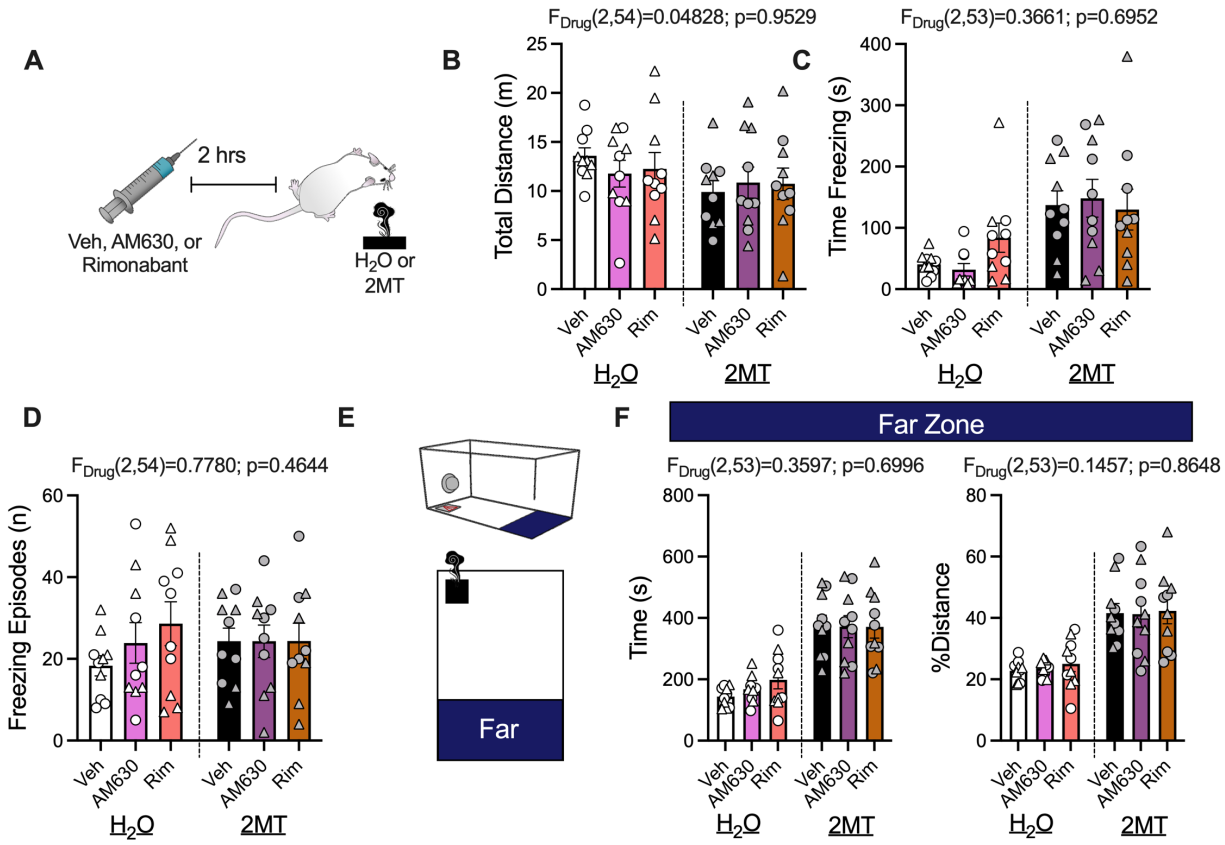
A. Schematic of PFC brain punches
B. 2MT decreases PFC 2-AG levels (H_2O: n=13; 2MT: n=10)
C. 2MT has no effect on AEA content in PFC (H_2O: n=13; 2MT: n=12)
D. 2MT has no effect on PFC AA levels (H_2O: n=13; 2MT: n=12)
E. There is no significant correlation between PFC 2-AG levels and time spent freezing in male or female mice

F. There is no significant correlation between 2-AG content in the PFC and time spent in the far zone in either sex
G. PFC AEA levels are not significantly associated with time spent freezing in male or female mice
H. There is no significant correlation between AEA content and time spent in the far zone in male or female mice
I. Schematic of PAG brain punches
J. 2MT has no significant effect on PAG 2-AG levels (H_2O: n=10; 2MT: n=9)
K. There is no significant change PAG AEA content by 2MT exposure (H_2O: n=11; 2MT: n=11)
L. 2MT has no effect on PAG AA content (H_2O: n=13; 2MT: n=12)
M. There is no significant correlation between PAG 2-AG content and freezing time during exposure in male or female mice
N. 2-AG content in the PAG is not significantly associated with time spent in the far zone in male or female mice
O. PAG AEA levels are not significantly correlated with time spent freezing during exposure in either sex
P. AEA levels in the PAG are not significantly associated with time spent in the far zone



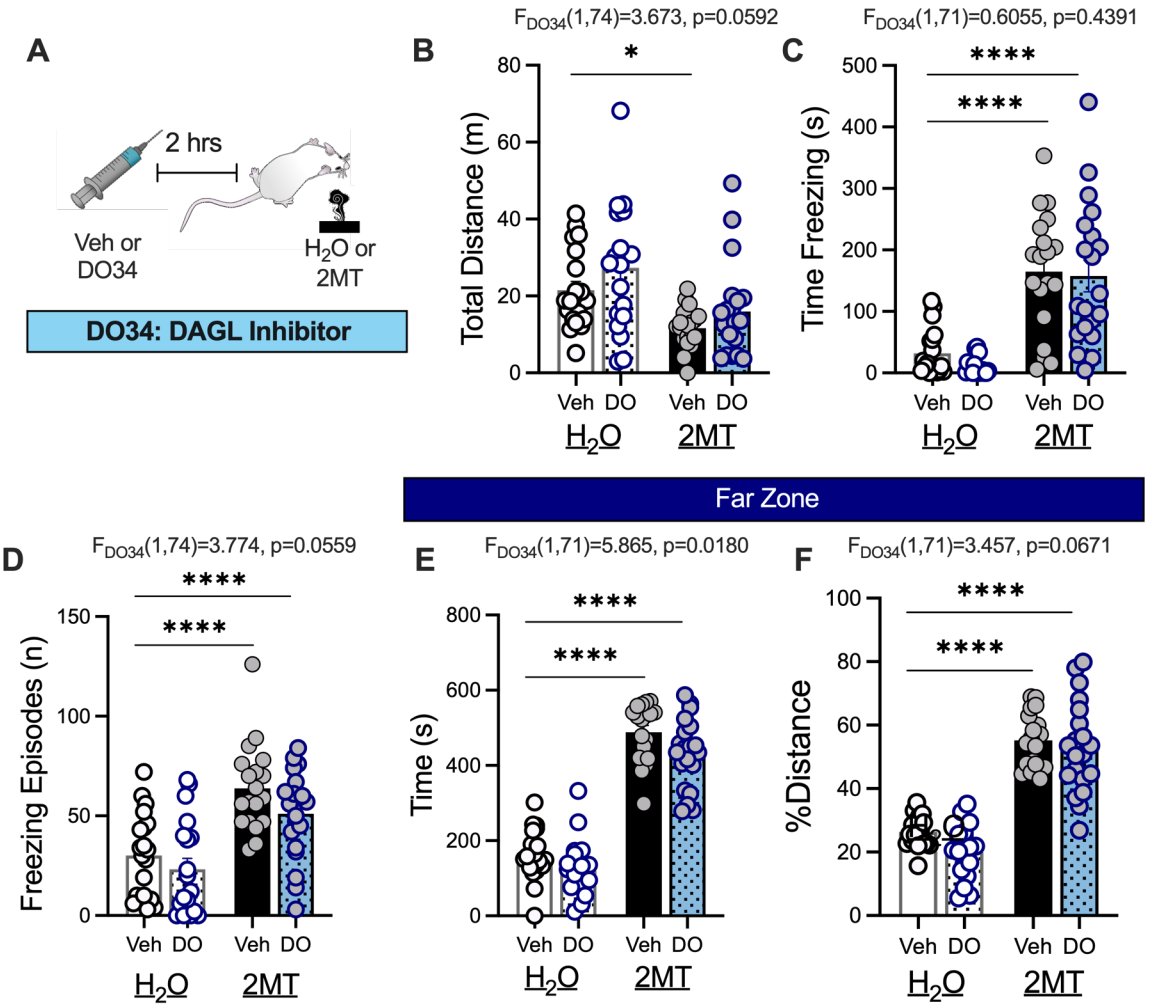
Suppl. Fig 4 JZL increases freezing in both male and female mice

A) Data from Figure 3 split up to show sex effects. 2-Way ANOVA performed and main effect of sex shown above each graph (B-F)
B) Sex has no significant effect on total distance travelled
C) While there was a significant effect of sex, JZL184 enhanced freezing in both male and female mice
D) Sex has no significant effect on freezing episodes
E) Sex has no effect on time spent in the far zone
F) While sex has a significant effect on %distance travelled in the far zone, there is no significant differences between males and females in the %distance travelled in the far zone



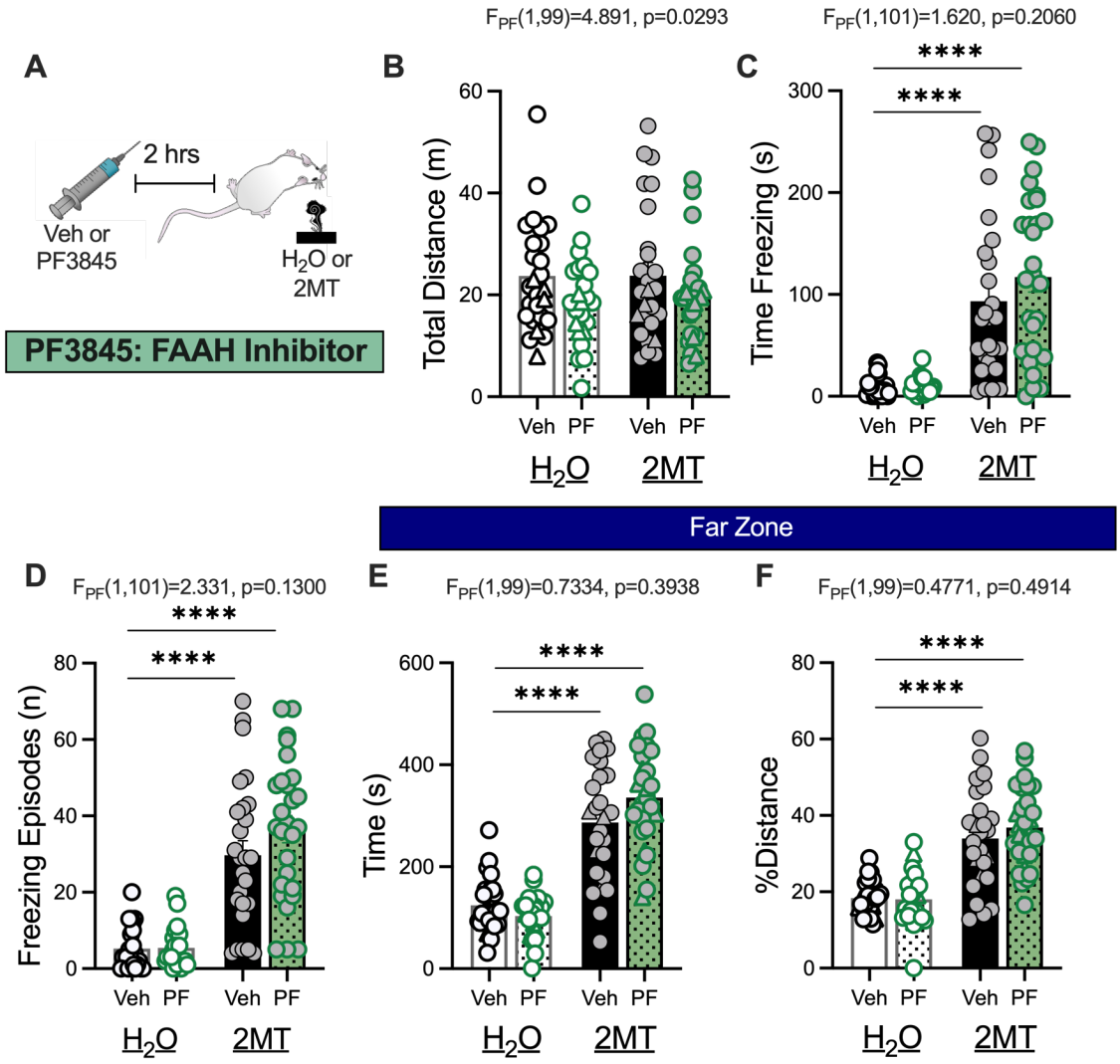
Suppl. Fig 5 CB1R and CB2R antagonists have no effect on freezing or avoidance during water or 2MT exposure

A) Schematic of experimental design. 2-Way ANOVA performed for all analysis and main effect of drug displayed (n=10/group)
B) There is no significant effect of drug injection on total distance travelled
C) Drug administration has no significant effect on freezing time
D) There is no significant effect of drug on the total number of freezing episodes
E) Schematic of far zone during water or 2MT exposure
F) Drug injection has no significant effect on time (left) or percent distance (right) spent in the far zone



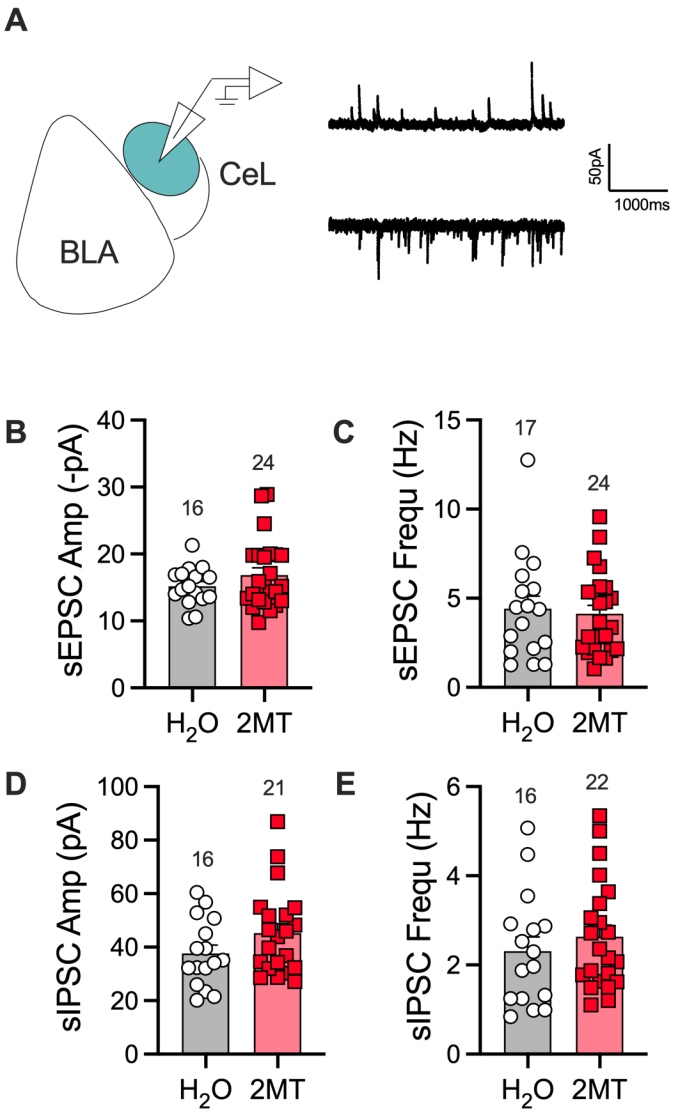
Suppl. Fig 6 DAGL inhibition has no effect on freezing to or avoidance of 2MT

A. Schematic of experimental design. Vehicle (DMSO) or DO34 (50mg/kg) was injected (i.p.), 2 hours before behavioral testing. 2-Way ANOVA analysis performed; DO34 main effect results displayed above each graph (H_2O, Veh: n=20; H_2O, DO34: n=19; 2MT, Veh: n=19; 2MT, DO34: n=20)
B. DO34 has no effect on total distance travelled
C. DO34 has no effect on freezing time, but 2MT increases the amount of time spent freezing
D. DO34 has no effect on freezing episodes
E. While DO34 has a significant effect on far zone time, post-hoc comparisons reveal no significance between vehicle or DO34 injected mice
F. DO34 has no significant effect on percent distance travelled in the far zone



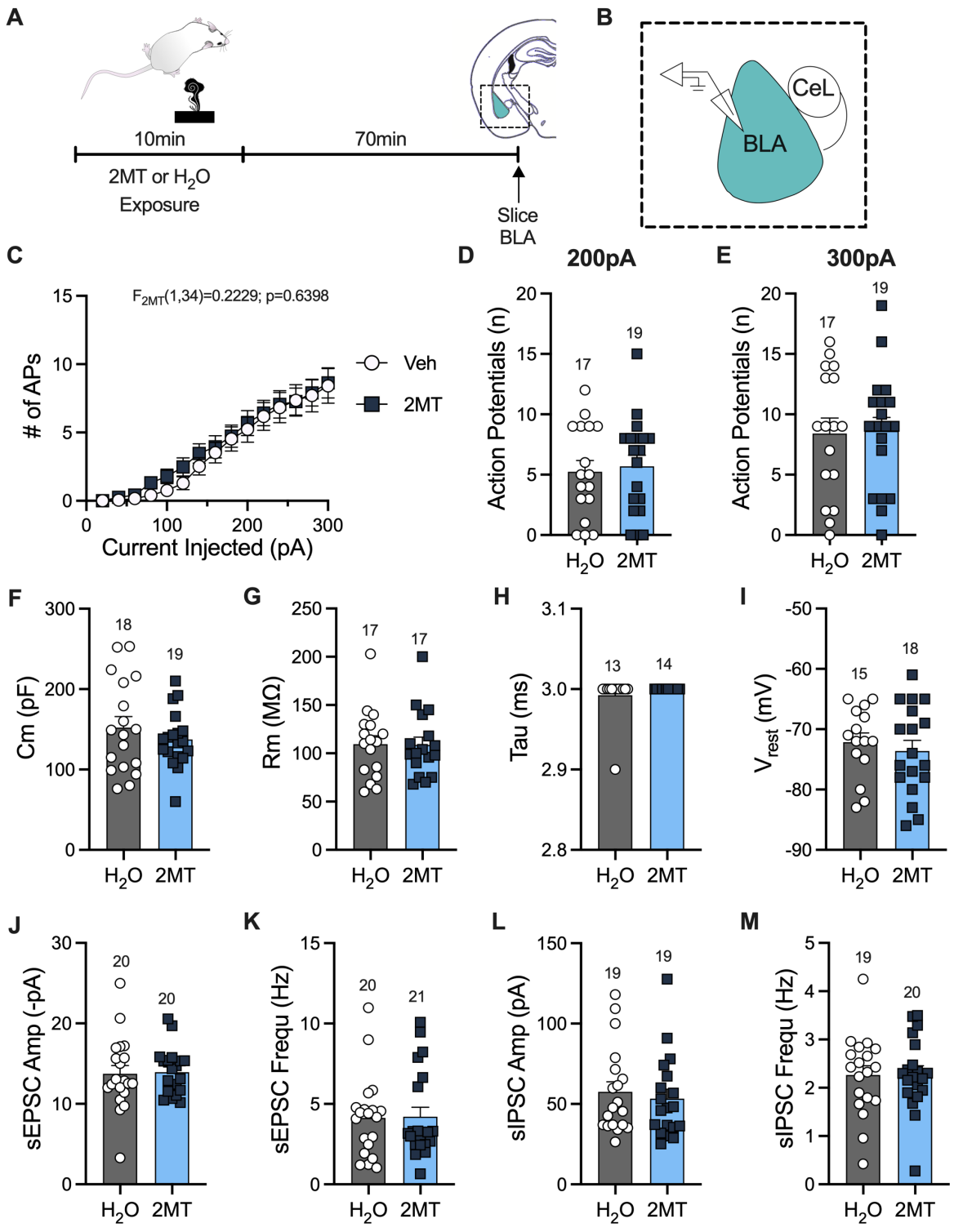
Suppl. Fig 7 Enhancing AEA levels has no effect on freezing during 2MT exposure

A. Schematic of experimental design. Mice were injected with DMSO (vehicle) or PF3845 (1 mg/kg) 2 hours prior to exposure. 2-Way ANOVA, PF3845 effect shown above each graph (H_2O, Veh: n=26; H_2O, PF3845: n=25; 2MT, Veh: n=26; 2MT, PF3845: n=28)
B. PF3845 has a significant effect on total distance travelled, but post-hoc comparisons reveal no significance between groups
C. PF3845 has no effect on freezing time
D. There is no significant effect of PF3845 on number of freezing episodes
E. PF3845 has no effect on time spent in the far zone
F. PF3845 has no effect on percent distance travelled in the far zone



Suppl. Fig 8 2MT has no effect on synaptic transmission in the CeL

A. Schematic of slice electrophysiology recordings in CeL showing spontaneous excitatory post-synaptic currents (sEPSCs) (bottom) and spontaneous inhibitory post-synaptic currents (sIPSCs) (top) recordings from the same cell. Unpaired, two-tailed t-test performed for each analysis. n, number of cells shown above each column, collected from the following number of mice: H_2O: n=5; 2MT: n=5
B. 2MT has no effect on sEPSC amplitude
C. 2MT has no significant effect on sEPSC frequency
D. 2MT does not affect sIPSC amplitude
E. sIPSC frequency is unaffected by 2MT exposure

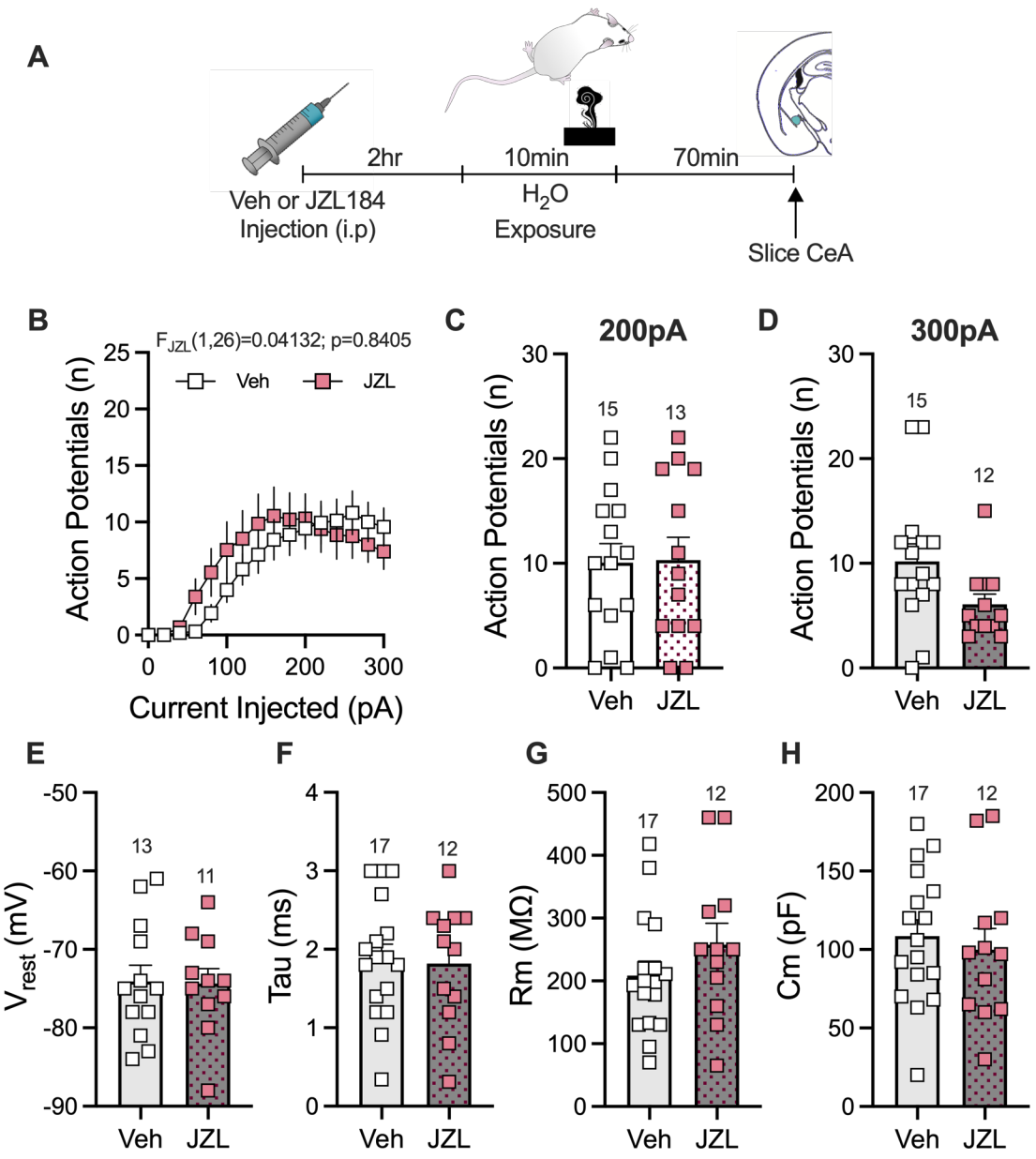


Suppl. Fig 9 2MT has no effect on intrinsic excitability or synaptic transmission in the BLA

A. Timeline. 2-Way ANOVA (2MT effect) shown (B) or unpaired, two-tailed t-test (D-N). n, number of cells shown above each column, collected from the following number of mice: H_2O: n=4; 2MT: n=4

B. Schematic of BLA slice electrophysiology recordings

C. 2MT has no effect on neuronal excitability
D. 2MT has no effect on the number of action potentials fired at 200pA current step
E. 2MT has no effect on the number of action potentials fired at 300pA current step
F. There is no effect of 2MT on cellular capacitance (Cm)
G. 2MT exposure has no effect on the membrane resistance (Rm) of cells
H. 2MT has no effect on the time constant (tau)
I. Resting membrane potential (Vrest) is unaffected by 2MT exposure
J. 2MT exposure has no effect on the amplitude of sEPSCs compared to water-exposed mice
K. Frequency of sEPSCs is unaffected by 2MT exposure
L. 2MT has no effect on the amplitude of sIPSCs
M. 2MT exposure does not alter the frequency of sIPSCs



Suppl. Fig 10 *In vivo* administration of JZL184 has no effect on CeL excitability in control mice exposed to H_2O

A) Schematic of experimental design. Mice were administered vehicle (DMSO) or JZL184, 2 hours prior to water exposure. 2-Way ANOVA performed and JZL effect shown (B) or unpaired t-test (C-H). n, number of cells shown above each column, collected from the following number of mice: Veh: n=5; JZL: n=4

B) JZL184 injected mice exposed to water show no significant change in intrinsic excitability compared to vehicle-injected mice

C) JZL184 has no effect on the number of action potentials fired at 200pA current step

D) JZL184 does not cause a change in the number of action potentials fired at 300pA current injection

E) JZL184 has no effect on the resting membrane potential (V_{rest})

F) Time constant (tau) is not significantly different between mice administered JZL184 or vehicle
G) JZL184 has no effect on membrane resistance (Rm)
H) Capacitance (Cm) is unaffected by JZL184 administration

Figure	Outliers Excluded (n)	Test Run	F(DFn, DFd) Value	P Values
1B	2	2-Way ANOVA	$F_{Inter}(9,405)=9.727$	<0.0001
			$F_{time}(9,405)=22.67$	<0.0001
			$F_{2MT}(1,45)=2.102$	0.1540
1C	2	Unpaired t-test		0.1150
1D	3	2-Way ANOVA	$F_{Inter}(9,405)=2.998$	0.0018
			$F_{time}(9,405)=3.302$	0.0007
			$F_{2MT}(1,45)=21.39$	<0.0001
1E	3	Unpaired t-test		<0.0001
1F	0	Unpaired t-test		<0.0001
1I	1	2-Way ANOVA	$F_{Inter}(2,141)=55.99$	<0.0001
			$F_{zone}(2,141)=55.97$	<0.0001
			$F_{2MT}(1,141)=0.1973$	0.6576
1J	0	2-Way ANOVA	$F_{Inter}(2,144)=25.41$	<0.0001
			$F_{zone}(2,144)=320.6$	<0.0001
			$F_{2MT}(1,144)=0.2576$	0.6125
2C	3	Unpaired t-test		0.0185
2D	3	Unpaired t-test		0.5525
2E	1	Unpaired t-test		0.6498
2F	2	Simple Linear Regression	$F_{Males}(1,20)=36.43$	<0.0001
			R squared=0.6456	
			$F_{Females}(1,8)=0.3946$	0.5474
			R squared =0.04701	
2G	2	Simple Linear Regression	$F_{Males}(1,20)=0.05245$	0.8212
			R squared=0.002616	
			$F_{Females}(1,8)=0.6503$	0.4433
			R squared=0.07517	
2H	1	Simple Linear Regression	$F_{Males}(1,21)=0.1834$	0.6729
			R squared=0.008656	
			$F_{Females}(1,8)=1.763$	0.2208
			R squared=0.1806	
2I	1	Simple Linear Regression	$F_{Males}(1,21)=15.52$	0.0008
			R squared=0.4250	
			$F_{Females}(1,8)=2.495$	0.1529
			R squared=0.2377	
3B	1	2-Way ANOVA	$F_{Inter}(1,114)=2.154$	0.1450
			$F_{JZL}(1,114)=6.278$	0.0136
			$F_{2MT}(1,114)=17.82$	<0.0001
3C	3	2-Way ANOVA	$F_{Inter}(1,112)=3.360$	0.0694
			$F_{JZL}(1,112)=17.33$	<0.0001
			$F_{2MT}(1,112)=49.18$	<0.0001
3D	0	2-Way ANOVA	$F_{Inter}(1,110)=0.1367$	0.7123
			$F_{JZL}(1,110)=0.3057$	0.5815
			$F_{2MT}(1,110)=34.40$	<0.0001

3F (left)	1	2-Way ANOVA	$F_{Inter}(1,114)=1.841$ $F_{JZL}(1,114)=0.3806$ $F_{2MT}(1,114)=178.7$	0.1775 0.5385 <0.0001
(right)	0		$F_{Inter}(1,115)=0.2268$ $F_{JZL}(1,115)=0.2771$ $F_{2MT}(1,115)=120.0$	0.6348 0.5996 <0.0001
3H	0	One-Way ANOVA	F(3,65)=7.224	0.0003
3I	0	One-Way ANOVA	F(3,65)=10.67	<0.0001
4B	0	2-Way ANOVA	$F_{Inter}(14,630)=1.976$ $F_{current}(14,630)=134.4$ $F_{2MT}(1,45)=5.362$	0.0174 <0.0001 0.0252
4C	1	Unpaired t-test		0.0141
4D	0	Unpaired t-test		0.0222
4E	0	Simple Linear Regression	F(1,14)=6.530 R squared=0.3181	0.0229
4G	0	Unpaired t-test		0.0225
4H	0	Unpaired t-test		0.8990
4I	0	Unpaired t-test		0.1015
4J	1	Unpaired t-test		0.6753
4K	0	Unpaired t-test		0.2117
4L	0	Unpaired t-test		0.0173
4M	1	Unpaired t-test		0.1441
4N	1	Unpaired t-test		0.0549
5B	1	2-Way ANOVA	$F_{Inter}(42,1162)=2.807$ $F_{current}(14,1162)=116.2$ $F_{drug}(3,83)=5.531$	<0.0001 <0.0001 0.0016
5C	7	One-Way ANOVA	F(3,76)=13.00	<0.0001
5D		One-Way ANOVA	F(3,84)=6.133	0.0008
5E	3	One-Way ANOVA	F(3,74)=13.63	<0.0001
5F	0	One-Way ANOVA	F(3,64)=1.109	0.3520
5G	1	One-Way ANOVA	F(3,80)=3.406	0.0215
5H	4	One-Way ANOVA	F(3,72)=1.414	0.2457
5I	0	One-Way ANOVA	F(3,71)=9.712	<0.0001
5J	0	One-Way ANOVA	F(3,81)=0.6308	0.5972
5K	1	One-Way ANOVA	F(3,82)=2.018	0.1178
5L	0	One-Way ANOVA	F(3,84)=0.9956	0.3991
6B	0	Unpaired t-test		0.0807
6C	1	Unpaired t-test		0.0066
6D	1	Unpaired t-test		0.0063
6F	0	Unpaired t-test		0.9341

6G	0	Unpaired t-test		0.2361
6I	0	Unpaired t-test		0.6670
6J	0	Unpaired t-test		0.5609
6K	0	Unpaired t-test		0.9150
6M	1	Unpaired t-test		0.0030
6N	0	Unpaired t-test		0.0382

Supplemental Figures				
S1B (top)	0	2-Way ANOVA	$F_{Inter}(9,243)=12.46$ $F_{time}(9,243)=28.44$ $F_{2MT}(1,27)=3.769$	<0.0001 <0.0001 0.0627
S1B (bottom)	0	2-Way ANOVA	$F_{Inter}(9,162)=2.894$ $F_{time}(9,162)=8.896$ $F_{2MT}(1,18)=0.03770$	0.0034 <0.0001 0.8482
S1C	0	2-Way ANOVA	$F_{Inter}(1,46)=1.148$ $F_{sex}(1,46)=96.24$ $F_{2MT}(1,46)=0.4299$	0.2895 <0.0001 0.5153
S1D (top)	1	2-Way ANOVA	$F_{Inter}(9,243)=2.946$ $F_{time}(9,243)=2.285$ $F_{2MT}(1,27)=10.91$	0.0024 0.0178 0.0027
S1D (bottom)	0	2-Way ANOVA	$F_{Inter}(9,162)=1.883$ $F_{time}(9,162)=2.103$ $F_{2MT}(1,18)=10.93$	0.0579 0.0320 0.0039
S1E	1	2-Way ANOVA	$F_{Inter}(1,45)=0.4883$ $F_{sex}(1,45)=0.1242$ $F_{2MT}(1,45)=21.00$	0.4883 0.7262 <0.0001
S1F	0	2-Way ANOVA	$F_{Inter}(1,46)=0.7361$ $F_{sex}(1,46)=0.2895$ $F_{2MT}(1,46)=26.65$	0.3954 0.5931 <0.0001
S1H	1	2-Way ANOVA	$F_{Inter}(1,45)=5.071$ $F_{sex}(1,45)=0.2583$ $F_{2MT}(1,45)=74.52$	0.0293 0.6137 <0.0001
S1I	0	2-Way ANOVA	$F_{Inter}(1,46)=4.812$ $F_{sex}(1,46)=0.8843$ $F_{2MT}(1,46)=70.47$	0.0333 0.3520 <0.0001
S1J	2	2-Way ANOVA	$F_{Inter}(1,44)=5.193$ $F_{sex}(1,44)=2.761$ $F_{2MT}(1,44)=64.99$	0.0276 0.1037 <0.0001
S1K	0	2-Way ANOVA	$F_{Inter}(1,46)=8.710$ $F_{sex}(1,46)=4.440$ $F_{2MT}(1,46)=57.09$	0.0050 0.0406 <0.0001
S2B	0	Unpaired t-test		0.2218
S2C	1	Unpaired t-test		0.4216
S2D	1	Unpaired t-test		0.4913
S2F (left)	0	Unpaired t-test		0.1200
(right)	0	Unpaired t-test		0.4603

S3B	3	Unpaired t-test		0.0460
S3C	1	Unpaired t-test		0.1445
S3D	1	Unpaired t-test		0.1188
S3E	0	Simple Linear Regression	$F_{Males}(1,15)=0.1054$ R squared=0.006979 $F_{Females}(1,7)=0.01900$ R squared=0.002708	0.7499 0.8942
S3F	0	Simple Linear Regression	$F_{Males}(1,15)=0.004291$ R squared=0.0002860 $F_{Females}(1,7)=0.3674$ R squared=0.04987	0.9486 0.5635
S3G	1	Simple Linear Regression	$F_{Males}(1,14)=0.6768$ R squared=0.04611 $F_{Females}(1,7)=1.718$ R squared=0.1970	0.4245 0.2313
S3H	1	Simple Linear Regression	$F_{Males}(1,14)=0.9431$ R squared=0.06311 $F_{Females}(1,7)=1.774$ R squared=0.2022	0.3480 0.2246
S3J	4	Unpaired t-test		0.2885
S3K	1	Unpaired t-test		0.2001
S3L	1	Unpaired t-test		0.8466
S3M	1	Simple Linear Regression	$F_{Males}(1,12)=0.04074$ R squared=0.003384 $F_{Females}(1,6)=0.03997$ R squared=0.006618	0.8434 0.8481
S3N	1	Simple Linear Regression	$F_{Males}(1,12)=0.7943$ R squared=0.06209 $F_{Females}(1,6)=0.02619$ R squared=0.004346	0.3903 0.8767
S3O	1	Simple Linear Regression	$F_{Males}(1,12)=0.2770$ R squared=0.02256 $F_{Females}(1,6)=0.2852$ R squared=0.04537	0.6083 0.6125
S3P	1	Simple Linear Regression	$F_{Males}(1,12)=1.981$ R squared=0.1417 $F_{Females}(1,6)=0.1821$ R squared=0.02946	0.1846 0.6844
S4B	0	2-Way ANOVA	$F_{Inter}(1,74)=0.7596$ $F_{sex}(1,74)=1.774$ $F_{2MT}(1,74)=0.7658$	0.3863 0.1870 0.3843
S4C	3	2-Way ANOVA	$F_{Inter}(1,71)=0.06806$ $F_{sex}(1,71)=5.925$ $F_{2MT}(1,71)=32.25$	0.7949 0.0174 <0.0001
S4D	0	2-Way ANOVA	$F_{Inter}(1,69)=0.07760$ $F_{sex}(1,69)=2.162$ $F_{2MT}(1,69)=0.03254$	0.7814 0.1460 0.8574
S4E	0	2-Way ANOVA	$F_{Inter}(1,74)=0.1206$ $F_{sex}(1,74)=2.424$	0.7293 0.1238

			$F_{2MT}(1,74)=0.3411$	0.5610
S4F	0	2-Way ANOVA	$F_{Inter}(1,74)=0.0009432$ $F_{sex}(1,74)=8.409$ $F_{2MT}(1,74)=0.001323$	0.9756 0.0049 0.9711
S5B	0	2-Way ANOVA	$F_{Inter}(2,54)=0.5384$ $F_{Drug}(2,54)=0.04828$ $F_{2MT}(1,54)=3.164$	0.5868 0.9529 0.0809
S5C	1	2-Way ANOVA	$F_{Inter}(2,53)=1.165$ $F_{Drug}(2,53)=0.3661$ $F_{2MT}(1,53)=19.56$	0.3198 0.6952 <0.0001
S5D	0	2-Way ANOVA	$F_{Inter}(2,54)=0.7493$ $F_{Drug}(2,54)=0.7780$ $F_{2MT}(1,54)=0.04633$	0.4775 0.4644 0.8304
S5F (left) (right)	1 1	2-Way ANOVA 2-Way ANOVA	$F_{Inter}(2,53)=0.5810$ $F_{Drug}(2,53)=0.3597$ $F_{2MT}(1,53)=75.35$ $F_{Inter}(2,53)=0.03508$ $F_{Drug}(2,53)=0.1457$ $F_{2MT}(1,53)=51.26$	0.5629 0.6996 <0.0001 0.9655 0.8648 <0.0001
S6B	0	2-Way ANOVA	$F_{Inter}(1,74)=0.07040$ $F_{DO34}(1,74)=3.673$ $F_{2MT}(1,74)=16.07$	0.7915 0.0592 =0.0001
S6C	3	2-Way ANOVA	$F_{Inter}(1,71)=0.1419$ $F_{DO34}(1,71)=0.6055$ $F_{2MT}(1,71)=57.71$	0.7075 0.4391 <0.0001
S6D	0	2-Way ANOVA	$F_{Inter}(1,74)=0.3423$ $F_{DO34}(1,74)=3.774$ $F_{2MT}(1,74)=37.44$	0.5603 0.0559 <0.0001
S6E	3	2-Way ANOVA	$F_{Inter}(1,71)=0.4169$ $F_{DO34}(1,71)=5.865$ $F_{2MT}(1,71)=292.4$	0.5206 0.0180 <0.0001
S6F	3	2-Way ANOVA	$F_{Inter}(1,71)=0.7460$ $F_{DO34}(1,71)=3.457$ $F_{2MT}(1,71)=182.3$	0.3907 0.0671 <0.0001
S7B	2	2-Way ANOVA	$F_{Inter}(1,99)=0.01646$ $F_{PF3845}(1,99)=4.891$ $F_{2MT}(1,99)=0.02117$	0.8982 0.0293 0.8846
S7C	0	2-Way ANOVA	$F_{Inter}(1,101)=0.5991$ $F_{PF3845}(1,101)=1.620$ $F_{2MT}(1,101)=53.14$	0.4407 0.2060 <0.0001
S7D	0	2-Way ANOVA	$F_{Inter}(1,101)=0.5146$ $F_{PF3845}(1,101)=2.331$ $F_{2MT}(1,101)=64.23$	0.4748 0.1300 <0.0001
S7E	2	2-Way ANOVA	$F_{Inter}(1,99)=4.586$ $F_{PF3845}(1,99)=0.7334$ $F_{2MT}(1,99)=147.7$	0.0347 0.3938 <0.0001
S7F	2	2-Way ANOVA	$F_{Inter}(1,99)=0.7780$ $F_{PF3845}(1,99)=0.4771$ $F_{2MT}(1,99)=87.60$	0.3799 0.4914 <0.0001
S8B	1	Unpaired t-test		0.2365

S8C	0	Unpaired t-test		0.7359
S8D	1	Unpaired t-test		0.1239
S8E	0	Unpaired t-test		0.4339
S9C	2	2-Way ANOVA	$F_{Inter}(14,476)=0.3448$ $F_{current}(14,476)=82.11$ $F_{2MT}(1,34)=0.2229$	0.9876 <0.0001 0.6398
S9D	1	Unpaired t-test		0.7348
S9E	0	Unpaired t-test		0.8966
S9F	0	Unpaired t-test		0.3450
S9G	3	Unpaired t-test		0.9381
S9H	10	Unpaired t-test		0.3086
S9I	0	Unpaired t-test		0.5374
S9J	1	Unpaired t-test		0.8713
S9K	0	Unpaired t-test		0.9235
S9L	1	Unpaired t-test		0.6135
S9M	0	Unpaired t-test		0.9222
S10B	0	2-Way ANOVA	$F_{Inter}(14,364)=2.133$ $F_{current}(14,364)=23.22$ $F_{JZL}(1,26)=0.04132$	0.0099 <0.0001 0.8405
S10C	0	Unpaired t-test		0.9325
S10D	1	Unpaired t-test		0.0560
S10E	0	Unpaired t-test		0.9207
S10F	0	Unpaired t-test		0.8331
S10G	0	Unpaired t-test		0.2260
S10H	0	Unpaired t-test		0.6050

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