

Supplementary Information for

Inhibitory neurotransmission drives endocannabinoid degradation to promote memory consolidation

Authors: Christophe J. Dubois, Jessica Fawcett-Patel, Paul A. Katzman and Siqiong June Liu

Affiliations: Department of Cell Biology and Anatomy, LSU Health Sciences Center, New Orleans, LA 70112 USA.

This PDF file includes:

- Supplementary Figures 1 – 6
- Supplementary Tables 1 – 4
- Reagents and resource table

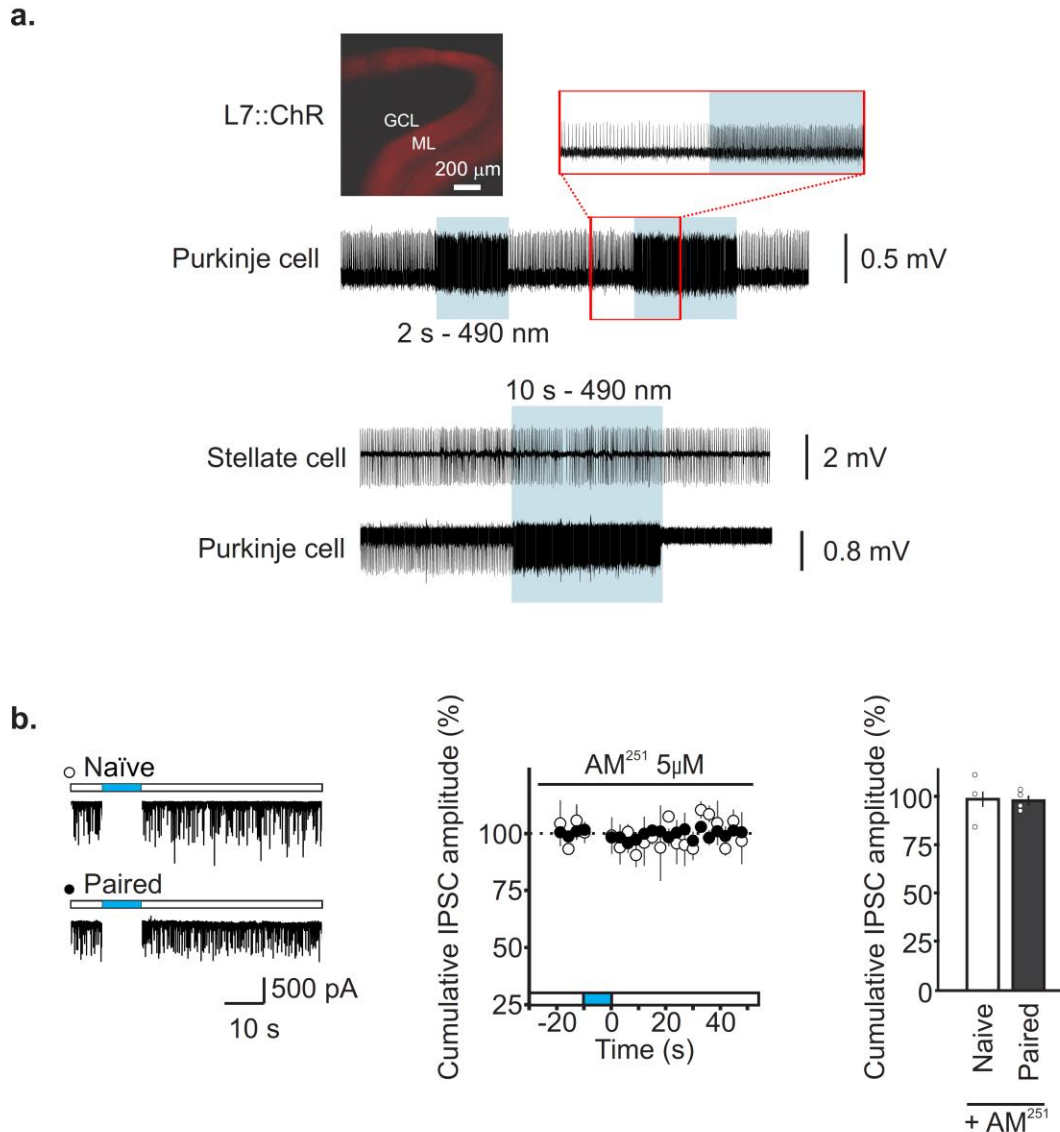


Figure S1. Related to figure 2. Optogenetic stimulation of Purkinje cells reduces spontaneous IPSCs in stellate cells via the activation of CB1Rs. (a) *Top left*, the expression of tomato-ChR in the cerebellar cortex of L7::ChR mice was restricted to Purkinje cells. *Middle*, action potential firing in a Purkinje cell was increased during photostimulation. *Bottom*, paired recording of action potentials in a stellate and a Purkinje cell from L7::ChR mouse. (b) Spontaneous IPSCs recorded in stellate cells from L7::ChR mouse. In the presence of the CB1R inverse agonist AM²⁵¹ photostimulation of Purkinje cells for 10s failed to reduce sIPSCs. Examples (left), averaged time courses (center) and peak depression (right) of cumulative IPSC amplitude from SCs of naïve mice (n=3 cells, two-sided paired t-test, P>0.05) and conditioned mice (n=4 cells, two-sided paired t-test, P>0.05). Data are presented as mean values $\pm$ SEM. Statistical analysis and P values can be found in the statistical table (Supplementary Table 4) and original data in the Source Data file. The representative experiment in (a) has been independently replicated more than 3 times.

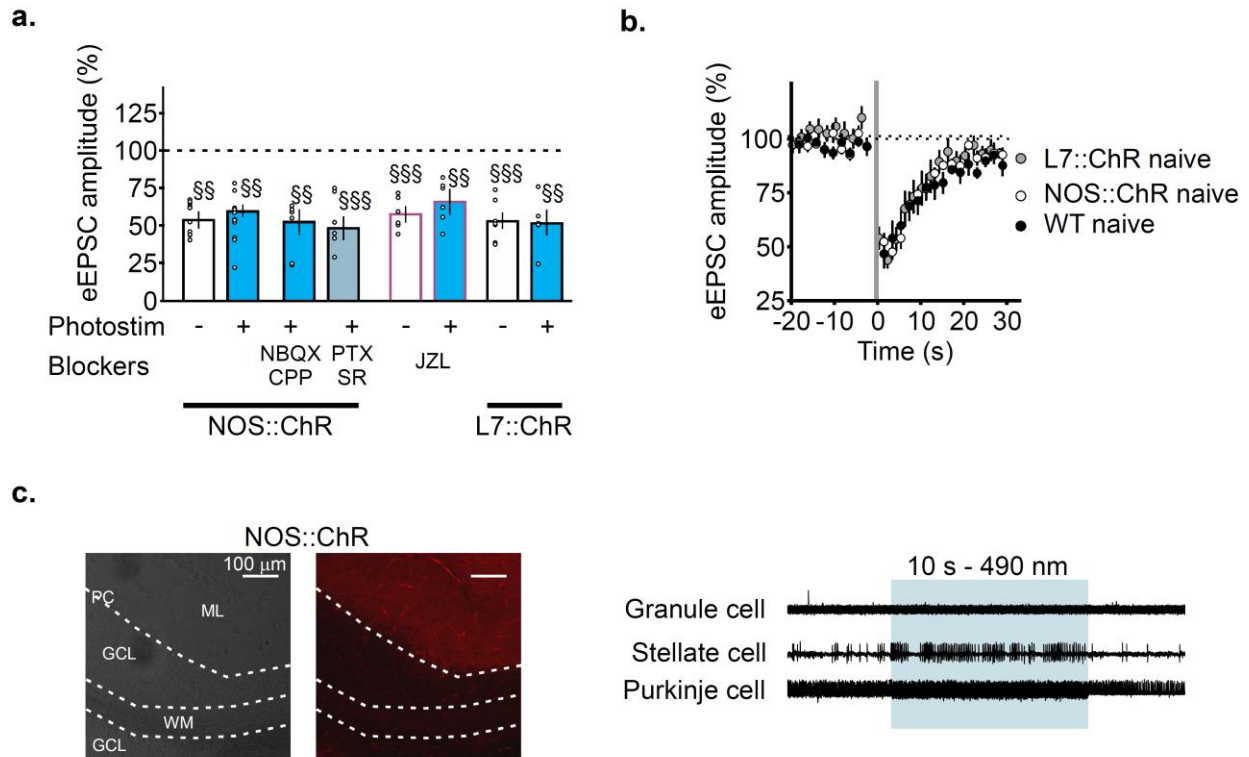


Figure S2. Related to figure 4. (a) Peak amplitude of DSE from experiments presented in Fig 4. **(b)** DSE in wild type naïve ($n = 11$ cells, black circles), non-stimulated NOS::ChR ($n = 6$ cells, open circles) and non-stimulated L7::ChR ($n = 7$ cells, grey circles) mice were not different. **(c)** Left, expression of tomato-ChR in the cerebellar cortex of NOS::ChR mice. Expression was restricted to GABAergic neurons. Right, typical extracellular recordings in granule, stellate and Purkinje cells showing an increased action potential firing in stellate and Purkinje cells, but not in granule cells, during photostimulation. Effect of each experimental condition on eEPSC amplitude after DSE in **(a)** was assessed with two-sided paired t-tests, §§, $P < 0.01$, §§§, $P < 0.001$. Data are presented as mean values $\pm$ SEM. Statistical analysis and P values can be found in the statistical table (Supplementary Table 4) and original data in the Source Data file. The representative experiment in **(c)** has been independently replicated more than 3 times.

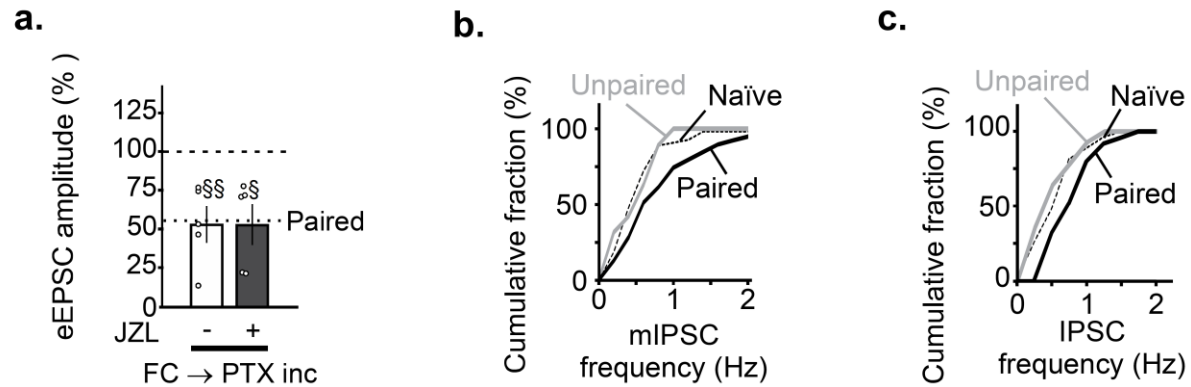


Figure S3. Related to figure 5. (a) Peak suppression of EPSCs from experiments presented in Fig 5. (b) mIPSCs were recorded in SCs. Cumulative fraction showing the distribution of mIPSC frequency (Naïve n=54 cells, UP n=19 cells, paired n=39 cells). (c) Cumulative fraction showing the distribution of spontaneous IPSC frequency (naïve n=27 cells, UP n=14 cells, paired n=25 cells). In (a) the effect of depolarization on eEPSC amplitude during DSE was assessed with two-sided paired t-tests §, $P < 0.05$, §§, $P < 0.01$. Data are presented as mean values $\pm$ SEM. Statistical analysis and P values can be found in the statistical table (Supplementary Table 4) and original data in the Source Data file.

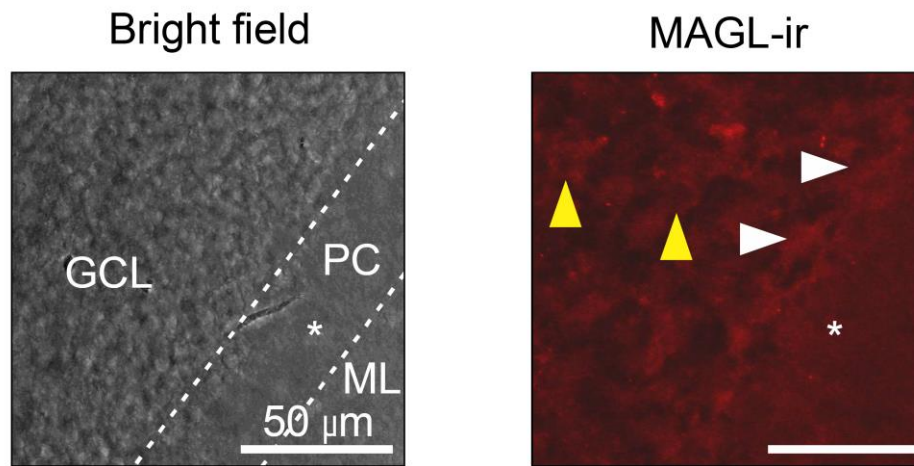


Figure S4. Related to figure 6. **MAGL expression in the cerebellar cortex.** Representative bright field and immunofluorescence for MAGL (MAGL-ir), independently replicated more than 3 times. MAGL-ir was found in the granule cell layer (GC) and molecular layers (ML), consistent with expression in granule cells (yellow arrows) and Bergman glial cells (white arrows) but not Purkinje cells (PC, *).

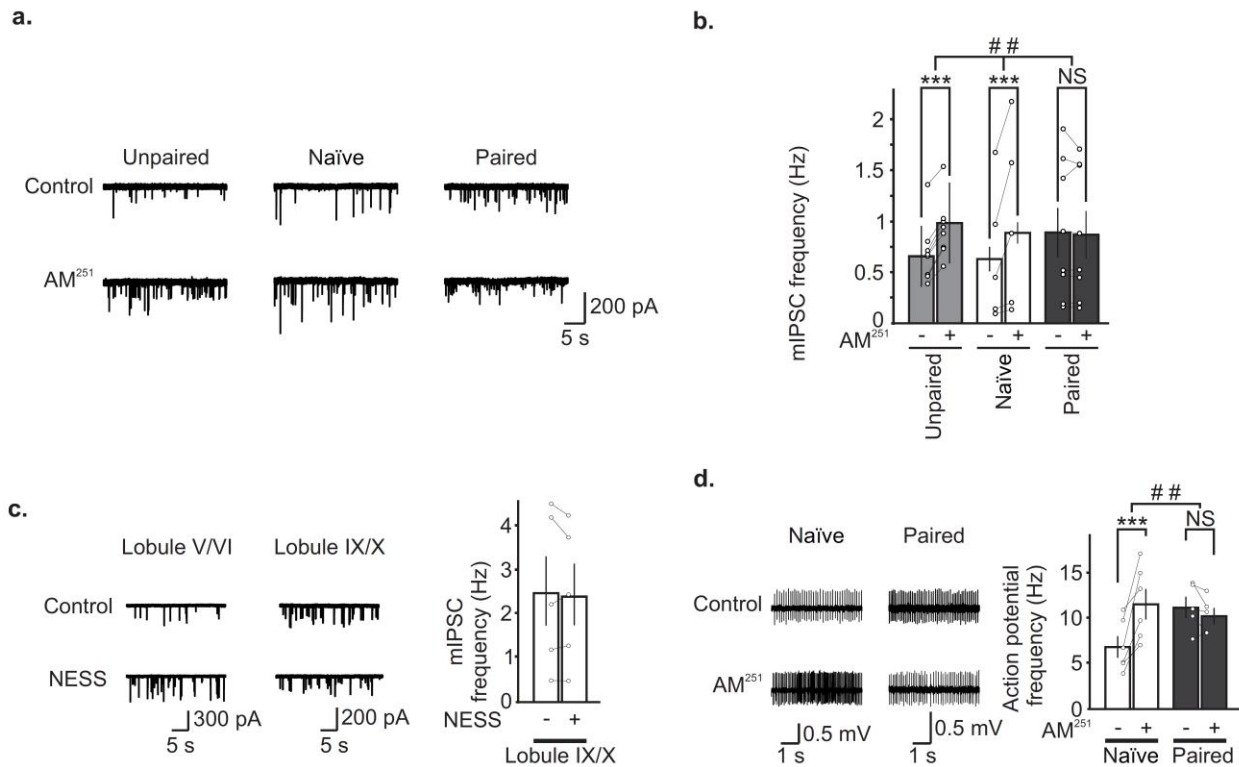
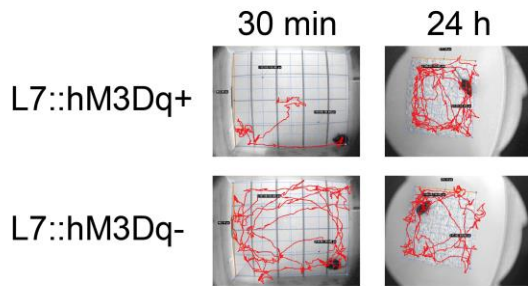


Figure S5. Related to figure 7. Fear conditioning abolishes the endogenous cannabinoid tone in cerebellar lobules V/VI. (a) Representative mIPSC recordings from stellate cells in lobules V/VI of naïve, unpaired control and conditioned mice before and during application of the CB1R inverse agonist AM²⁵¹. (b) Group data showing that AM²⁵¹ increased mIPSC frequency in naïve (n=8 cells) and unpaired controls (n=5 cells), but not after fear conditioning (n=8 cells), indicating that tonic eCB signaling is present in controls but absent after fear conditioning. (c) Lack of tonic eCB signaling in lobules IX/X of naïve mice. Left and center, representative recordings from stellate cells in lobules V/VI (left) or lobules IX/X (center) of naïve mice before and during application of NESS⁰³²⁷. Right, group data (n=5 cells). (d) The endogenous cannabinoid tone regulates action potential firing in stellate cells. Left, representative cell-attached recordings of action potential firing in stellate cells in the presence of inhibitory synaptic blockers with or without the CB1 inverse agonist AM²⁵¹. Right, Group data shows that AM²⁵¹ increased action potential frequency in stellate cells from naïve (n=5 cells) but not conditioned mice (n=5 cells). Group comparisons in (b) and (d) were assessed by two-way ANOVAs with repeated measures (##, $P=0.04$ for mIPSC frequency and $P=0.001$ for action potential frequency) followed by Tukey post-hoc tests for drug effect within groups (***, $P<0.001$). In (c) the effect of NESS on mIPSC frequency was tested using a 2 sided paired t-test ($P>0.05$, see 7d). Data are presented as mean values $\pm$ SEM. Statistical analysis and P and F values can be found in the statistical table (Supplementary Table 4) and original data in the Source Data file.

a.



b.

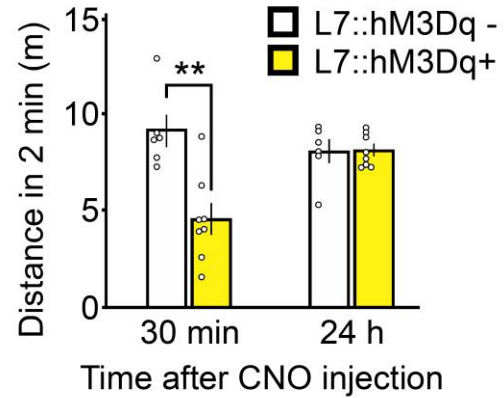


Figure S6. Related to figure 8. (a) Representative 2 min tracking of L7::hM3Dq+ (top) and L7::hM3Dq- (bottom) mice 30 min (left) and 24 hours (right) after CNO injection. (b) Group data of the average distance L7::hM3Dq+ (n=8 animals) and L7::hM3Dq- (n=6 animals) traveled during 2 min in a new environment, 30 min and 24 hours after CNO injection. The effect of CNO on the traveled distance was assessed with two-sided paired t-tests (**, $P=0.002$). Data are presented as mean values $\pm$ SEM. Statistical analysis and P values can be found in the statistical table (Supplementary Table 4) and original data in the Source Data file.

	L7::hM3Dq	L7::ChR	NOS::ChR
Cerebellar cortex	Purkinje cells	Purkinje cells	Purkinje cells Molecular Layer interneurons Blood vessels
Whole brain	Cerebellar Purkinje cells	NA	NA

Table S1. Summary of ChR-tomato or DREADD-mCitrine expression. For the L7::hM3Dq expression in the whole brain, both sagittal and coronal slices were examined. This table reflects a general observation and may not reflect scattered and rare ectopic expression as described by others ³¹.

Activation (%)	L7::hM3Dq	L7::ChR	NOS::ChR
Purkinje cells	100% (13/13)	100% (11/11)	100% (17/17)
Stellate cells	0% (0/4)	0% (0/13) wc 0% (0/22)	77% (10/13)
Basket cells		0% (0/5)	
Granule cells			0% (0/2)

Table S2. Summary of the pharmacogenetic and optogenetic activation per cell type in the cerebellum. Rate of successful activation in extracellular recordings (or whole cell voltage clamp recordings, wc) either with CNO (DREADD) or blue light (ChR) (Number of cells activated over the number of cells recorded).

L7::hM3Dq genotype		-	+	-	+	-	+
n		6	6	6	8	10	8
Drug injected		Saline	Saline	CNO	CNO	CNO + AM ⁴¹¹³	CNO + AM ⁴¹¹³
Fear conditioning	2 min habituation	3 ± 3	11 ± 5	5 ± 1	5 ± 2	2 ± 2	2 ± 2
	CS	43 ± 8	40 ± 2	70 ± 4	64 ± 3	66 ± 4	60 ± 6
Cued fear memory	2 min habituation	7 ± 4	10 ± 5	4 ± 1	1 ± 0	10 ± 6	14 ± 6
	CS	60 ± 9	66 ± 11	58 ± 5	34 ± 4	67 ± 5	76 ± 2
Contextual fear memory	2 min context	N/A	N/A	34 ± 6	40 ± 8	45 ± 6	53 ± 4

Table S3. Summary of percentage freezing of L7::hM3Dq- and L7::hM3Dq+ mice during fear conditioning and memory retention testing.

Fig	Parameter analyzed	Conditions	n	Analysis	F value	P value
1f	τ of DSE	(N vs FC) x (ctrl vs JZL)		2 WAY ANOVA	$F(1,30) = 4.936$	0.034
		N vs FC without JZL	11 - 12	Tukey		0.011
		N vs FC with JZL	5 - 6	Tukey		0.423
		UP vs FC without JZL	6 - 12	Unpaired t-test		<0.001
1g	Amplitude eEPSC	UP (pre vs post depolarization)	6	Paired t-test		<0.001
		N (pre vs post depolarization)	11	Paired t-test		<0.001
		FC (pre vs post depolarization)	12	Paired t-test		<0.001
		N + JZL (pre vs post depolarization)	5	Paired t-test		0.002
		FC + JZL (pre vs post depolarization)	6	Paired t-test		0.001
		N + NESS (pre vs post depolarization)	5	Paired t-test		0.808
		FC + NESS (pre vs post depolarization)	5	Paired t-test		0.889
		N vs FC	7 - 9	Unpaired t-test		<0.001
2c	τ of DSI	N (pre vs post depolarization)	7	Paired t-test		<0.001
		FC (pre vs post depolarization)	9	Paired t-test		0.006
		N vs FC (post depolarization)	7 - 9	Unpaired t-test		0.627
		N vs FC	13 - 7	Unpaired t-test		0.022
2f	cumulative amplitude IPSC	N (pre vs post stim)	13	Paired t-test		0.014
		FC (pre vs post stim)	7	Paired t-test		0.037
		N vs FC (post stim)	13 - 7	Unpaired t-test		0.364
		(N vs FC) x (pre vs WIN)		2 WAY RM ANOVA	$F(1,12) = 8.143$	0.15
3c	mIPSC frequency	N (pre vs WIN)	7	Tukey		0.028
		FC (pre vs WIN)	7	Tukey		<0.001
	mIPSC amplitude	(N vs FC) x (pre vs WIN)		2 WAY RM ANOVA	$F(1,12) = 1.206$	0.292
		N (pre vs WIN)	7	Tukey		0.56
3f	current amplitude	FC (pre vs WIN)	7	Tukey		0.081
		(N vs FC) x (voltage steps)		2 WAY RM ANOVA	$F(11,110) = 0.825$	0.615
		NOS::Chr (0x vs 8x Blue light) x (ctrl vs JZL)		2 WAY ANOVA	$F(1,32) = 5.018$	0.032
		NOS::Chr (0x vs 8x Blue light)	6 - 5	Unpaired t-test		0.026
4g	τ of DSE	NOS::Chr (NBQX/CPP vs PTX/SR)	6 - 6	Unpaired t-test		0.006
		NOS::Chr (8x No drug vs PTX/SR)	5 - 6	Unpaired t-test		0.007
		NOS::Chr in JZL (0x vs 8x Blue light)	6 - 6	Unpaired t-test		0.875
		L7::Chr (0x vs 8x Blue light)	7 - 5	Unpaired t-test		0.611
		Indicative: NOS::Chr 8x Blue light (No drug vs PTX/SR vs NBQX/CPP)		1 WAY ANOVA	$F(2,16) = 13.252$	<0.001
		N vs FC	9 - 12	Unpaired t-test		0.003
		UP vs FC	9 - 12	Unpaired t-test		0.006
		N vs UP	9 - 9	Unpaired t-test		0.453
5b	PPR	N vs FC	9 - 12	Unpaired t-test		0.002
		UP vs FC	9 - 12	Unpaired t-test		0.019
		N vs UP	9 - 9	Unpaired t-test		0.154
		N vs FC	54 - 39	Unpaired t-test		0.006
5d	mIPSC frequency	UP vs FC	19 - 39	Unpaired t-test		0.046
		N vs UP	54 - 19	Unpaired t-test		0.695
	mIPSC Amplitude	N vs FC	54 - 39	Unpaired t-test		0.095
		UP vs FC	19 - 39	Unpaired t-test		0.586
5f	IPSC frequency	N vs UP	54 - 19	Unpaired t-test		0.11
		N vs FC	27 - 25	Unpaired t-test		0.035
		UP vs FC	14 - 25	Unpaired t-test		0.019
		N vs UP	27 - 14	Unpaired t-test		0.445
	IPSC Amplitude	N vs FC	27 - 25	Unpaired t-test		0.298
		UP vs FC	14 - 25	Unpaired t-test		0.514
		N vs UP	27 - 14	Unpaired t-test		0.794
		(no PTX vs PTX) x (No JZL vs JZL)		2 WAY ANOVA	$F(1,29) = 16.883$	<0.001
5i	τ of DSE	FC vs FC+PTX	12 - 5	Tukey		<0.001
		FC+PTX vs FC+PTX+JZL	5 - 5	Tukey		0.804
		(UP vs FC) x (Lob V/VI vs Lob IX/X)		2 WAY RM ANOVA	$F(1,39) = 7.79$	0.021
		UP (Lob V/VI vs Lob IX/X)	10 - 10	Tukey		0.008
6d	MAGL-ir in the molecular layer	FC (Lob V/VI vs Lob IX/X)	10 - 10	Tukey		0.625
		Lob V/VI (UP vs FC)	10 - 10	Tukey		0.003
		Lob IX/X (UP vs FC)	10 - 10	Tukey		0.117
		(UP vs FC) x (Lob V/VI vs Lob IX/X)		2 WAY RM ANOVA	$F(1,39) = 0.407$	0.551
	MAGL-ir in the granule cell layer	UP (Lob V/VI vs Lob IX/X)	10 - 10	Tukey		0.117
		FC (Lob V/VI vs Lob IX/X)	10 - 10	Tukey		0.469
		Lob V/VI (UP vs FC)	10 - 10	Tukey		0.64
		Lob IX/X (UP vs FC)	10 - 10	Tukey		0.867
7a	2-AG measurement	N vs UP vs FC		1 WAY ANOVA	$F(2,13) = 8.574$	0.013
		N vs FC	5 - 5	Tukey		0.014
		UP vs FC	4 - 5	Tukey		0.045
		N vs UP	5 - 4	Tukey		0.851
7c	mIPSC frequency	(N vs FC vs UP) x (pre vs NESS)		2 WAY RM ANOVA	$F(2,39) = 4.144$	0.034
		N (pre vs NESS)	9	Paired t-test		<0.001
		UP (pre vs NESS)	6	Paired t-test		0.026
		FC (pre vs NESS)	5	Paired t-test		0.869
	mIPSC amplitude (not shown)	(N vs FC vs UP) x (pre vs NESS)		2 WAY RM ANOVA	$F(2,39) = 0.350$	0.709
		N (pre vs NESS)	9	Paired t-test		0.178
		UP (pre vs NESS)	6	Paired t-test		0.784
		FC (pre vs NESS)	5	Paired t-test		0.878
7d	mIPSC frequency	N in lob IX/X (pre vs NESS)	5	Paired t-test		0.539
		N (pre vs AM ²⁵¹)	9	Paired t-test		0.002
		N (pre vs AM ²⁵¹)	6	Paired t-test		0.044
		UP (pre vs AM ²⁵¹)	5	Paired t-test		0.048
		FC (pre vs AM ²⁵¹)	8	Paired t-test		0.687
	mIPSC amplitude (not shown)	N in lob IX/X (pre vs NESS)	5	Paired t-test		0.073
		N (pre vs AM ²⁵¹)	9	Paired t-test		0.182
		N (pre vs AM ²⁵¹)	6	Paired t-test		0.307
		UP (pre vs AM ²⁵¹)	5	Paired t-test		0.343
		FC (pre vs AM ²⁵¹)	8	Paired t-test		0.11

Fig	Parameter analyzed	Conditions	n	Analysis	F value	P value
7f	mIPSC frequency	N (pre vs JZL ^{184/195})	7	Paired t-test		0.142
		FC (pre vs JZL ^{184/195})	9	Paired t-test		0.008
		FC (JZL ^{184/195} vs JZL ^{184/195} +NESS ⁰³²⁷)	5	Paired t-test		0.039
	mIPSC amplitude (not shown)	N (pre vs JZL ^{184/195})	7	Paired t-test		0.397
		FC (pre vs JZL ^{184/195})	9	Paired t-test		0.169
		FC (JZL ^{184/195} vs JZL ^{184/195} +NESS ⁰³²⁷)	5	Paired t-test		0.39
8c	IPSC frequency	(pre vs 30 min after CNO) x (No NESS vs NESS)		2 WAY RM ANOVA	$F(1,17) = 10.005$	0.016
		FC (pre vs 30 min after CNO)	5	Tukey		0.002
		FC in NESS (pre vs 30 min after CNO)	4	Tukey		0.873
	IPSC amplitude (not shown)	(pre vs 30 min after CNO) x (No NESS vs NESS)		2 WAY RM ANOVA	$F(1,16) = 2.864$	0.142
		FC (pre vs 30 min after CNO)	5	Tukey		0.486
		FC in NESS (pre vs 30 min after CNO)	4	Tukey		0.169
8e	Freezing	Saline injection L7::hM3Dq - vs + (cued)	6 - 6	Unpaired t-test		0.638
		L7::hM3Dq - (cued) Saline vs CNO	6 - 6	Unpaired t-test		0.477
		L7::hM3Dq + (cued) Saline vs CNO	7 - 6	Unpaired t-test		0.024
		CNO injection L7::hM3Dq - vs + (cued)	6 - 8	Unpaired t-test		0.001
		CNO injection L7::hM3Dq - vs + (contextual)	6 - 8	Unpaired t-test		0.583
		L7::hM3Dq - vs + (cued)	12 - 9	Unpaired t-test		0.097
9b	Freezing	L7::hM3Dq - vs + (contextual)	12 - 9	Unpaired t-test		0.25
		L7::hM3Dq + (cued) CNO vs CNO+ AM	8 - 9	Unpaired t-test		<0.001
10c	MAGL-ir in the molecular layer	CNO injection L7::hM3Dq - vs + (cued)	6 - 10	Unpaired t-test		0.044
10e	IPSC frequency	L7::hM3Dq - vs + (No drug)	5 - 5	Unpaired t-test		0.044
		L7::hM3Dq + (No drug vs NESS)	5 - 4	Unpaired t-test		0.041
S1b	cumulative amplitude IPSC	N (pre vs post stim) in AM ²⁵¹	3	Paired t-test		0.765
		FC (pre vs post stim) in AM ²⁵¹	4	Paired t-test		0.245
		N vs FC (post stim) in AM ²⁵¹	3 - 4	Unpaired t-test		0.488
S2b	eEPSC Amplitude	NOS::Chr 0x BL (pre vs post depolarization)	6	Paired t-test		0.004
		NOS::Chr 8x BL (pre vs post depolarization)	5	Paired t-test		0.006
		NOS::Chr 8x BL NBQX/CPP (pre vs post depolarization)	6	Paired t-test		0.006
		NOS::Chr 8x BL in PTX/SR (pre vs post depolarization)	6	Paired t-test		<0.001
		L7::Chr 0x BL (pre vs post depolarization)	7	Paired t-test		<0.001
		L7::Chr 8x BL (pre vs post depolarization)	5	Paired t-test		0.009
S3a	eEPSC Amplitude	FC → PTX (pre vs post depolarization)	5	Paired t-test		0.006
		FC → PTX + JZL (pre vs post depolarization)	5	Paired t-test		0.024
		(N vs FC vs UP) x (pre vs AM ²⁵¹)		2 WAY RM ANOVA	$F(2,43) = 7.346$	0.004
S5b	mIPSC frequency	N (pre vs AM ²⁵¹)	9	Tukey		<0.001
		UP (pre vs AM ²⁵¹)	5	Tukey		<0.001
		FC (pre vs AM ²⁵¹)	8	Tukey		0.745
	mIPSC amplitude (not shown)	(N vs FC vs UP) x (pre vs AM ²⁵¹)		2 WAY RM ANOVA	$F(2,43) = 0.268$	0.768
		N (pre vs AM ²⁵¹)	9	Tukey		0.068
		UP (pre vs AM ²⁵¹)	5	Tukey		0.603
S5d	Action potential frequency	FC (pre vs AM ²⁵¹)	8	Tukey		0.18
		(pre vs AM ²⁵¹) x (N vs FC)		2 WAY RM ANOVA	$F(1,21) = 20.216$	0.001
		N (pre vs AM ²⁵¹)	5	Tukey		<0.001
S6b	Distance	FC (pre vs AM ²⁵¹)	5	Tukey		0.341
		L7::hM3Dq - vs + (30 min)	6 - 8	Unpaired t-test		0.002
		L7::hM3Dq - vs + (24h)	6 - 8	Unpaired t-test		0.922

Table S4. Statistical table. Bold *P* values indicate a significant effect ($P < 0.05$). N, Naive; UP, unpaired; FC, paired). RM ANOVA, repeated measures ANOVA.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat polyclonal anti-MAGL	Everest Biotech	EB08850
Donkey anti-goat Dylight 549	Jackson	#705-505-147
Donkey anti-goat Cy3	Immunoresearch	#705-165-147
Chemicals, Peptides, and Recombinant Proteins		
TTX (Tetrodotoxin)	Ascent Scientific	Asc-055
CPP (3-((R)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid)	AbCam	Ab120159
NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide)	AbCam	Ab120046
SR-95531 (6-Imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide)	AbCam	Ab120042
PTX (Picrotoxin)	AbCam	Ab 120315
AM²⁵¹ (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide)	Cayman chemical company	#71670
AM²⁸¹ (1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide)	Tocris	#1115
AM⁴¹¹³ 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide	Cayman chemical company	#20581
NESS⁰³²⁷ (8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydro-N-1-piperidinyl-benzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide)	Cayman chemical company	#10004184
WIN^{55,212-2} ((R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate)	Ascent Scientific	Asc-085
JZL¹⁸⁴ (4-[Bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester)	Enzo	BMLEI391-0010
JZL¹⁹⁵ (4-[(3-Phenoxyphenyl)methyl]-1-piperazinecarboxylic acid 4-nitrophenyl ester)	AbCam	Ab144653
CNO (8-Chloro-11-(4-methyl-4-oxido-1-piperazinyl)-5H-dibenzo[b,e][1,4] diazepine)	RTI international	CAS#34233-69-7 NH# C-929
Experimental Models: Organisms/Strains		
C57Bl/6J WT	The Jackson lab	stock 000664
NOS::CRE (B6.129-Nos1 ^{tm1(cre)} Mgmj>/J)	The Jackson lab	Stock 017526
L7::CRE (B6.129-Tg(Pcp2-cre)2Mpin/J)	The Jackson lab	stock 004146
floxed ChR (B6.Cg-Gt(ROSA)26Sortm27.1(CAG-COP4*H134R/tdTomato) Hze/J)	The Jackson lab	stock 012567
floxed Gq-DREADD (Gt(ROSA)26Sortm2 (CAG-CHRM3*, -mCitrine)Ute/J)	The Jackson lab	stock 026220
Software and Algorithms		
Frame by frame analysis software	Liu et al., 2010	

Resource table