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Supplemental information

Selective postnatal excitation of neocortical pyramidal neurons results in distinctive behavioral and circuit deficits in adulthood

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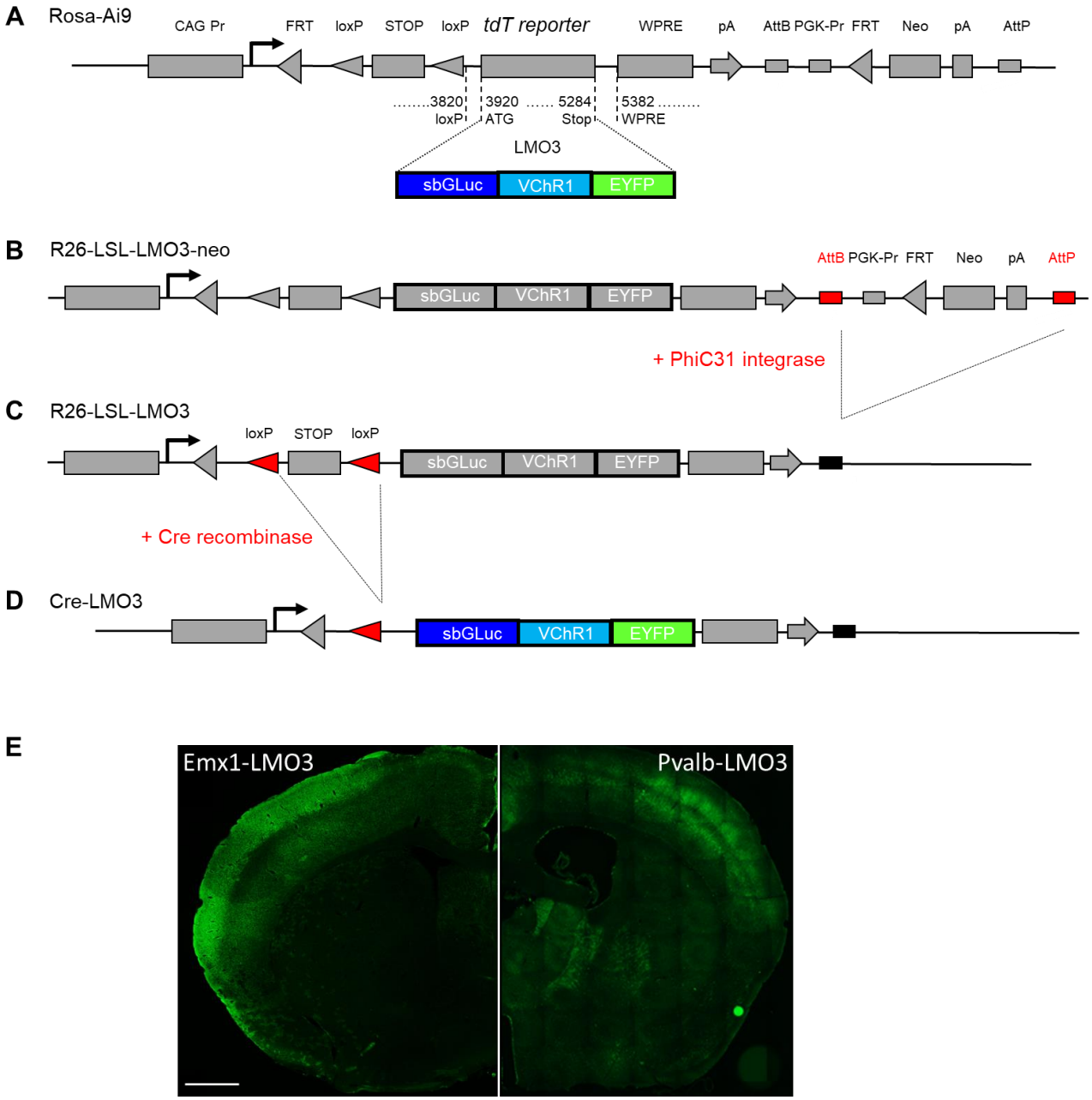


Figure S1. Conditional (lox-stop-lox) LMO3 mice, Related to Figure 1.
(A) Schematic of RosaAi9 targeting vector. The tdTomato reporter was replaced by the coding region for LMO3.
(B) Schematic of R26-LSL-LMO3-neo construct used for generating LSL-LMO3-neo embryonic stem cells and germline transmitting LSL-LMO3-neo mice.
(C) Schematic showing the modified Rosa26 locus after crossing LSL-LMO3-neo mice to PhiC31 integrase expressing mice, resulting in removal of the neo reporter in R26-LSL-LMO3 mice. These mice were crossed with the various Cre driver lines.
(D) Schematic of Rosa26 locus in cells co-expressing Cre recombinase, resulting in removal of the Stop sequence and expression of LMO3 in Cre-LMO3 mice.
(E) Fluorescence images of brain cross sections of Emx1-LMO3 (left) and Pvalb-LMO3 (right) mice. Scale bar: 1000 µm.

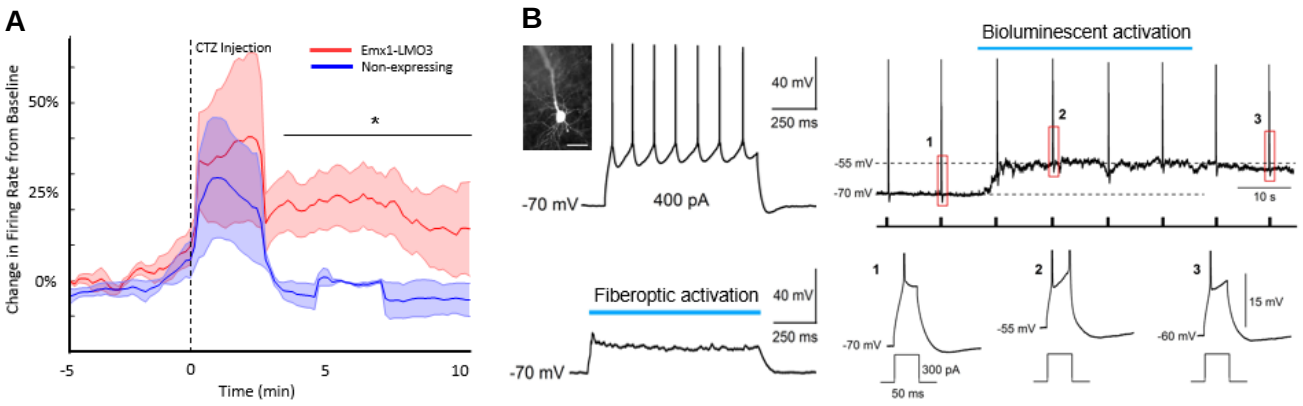
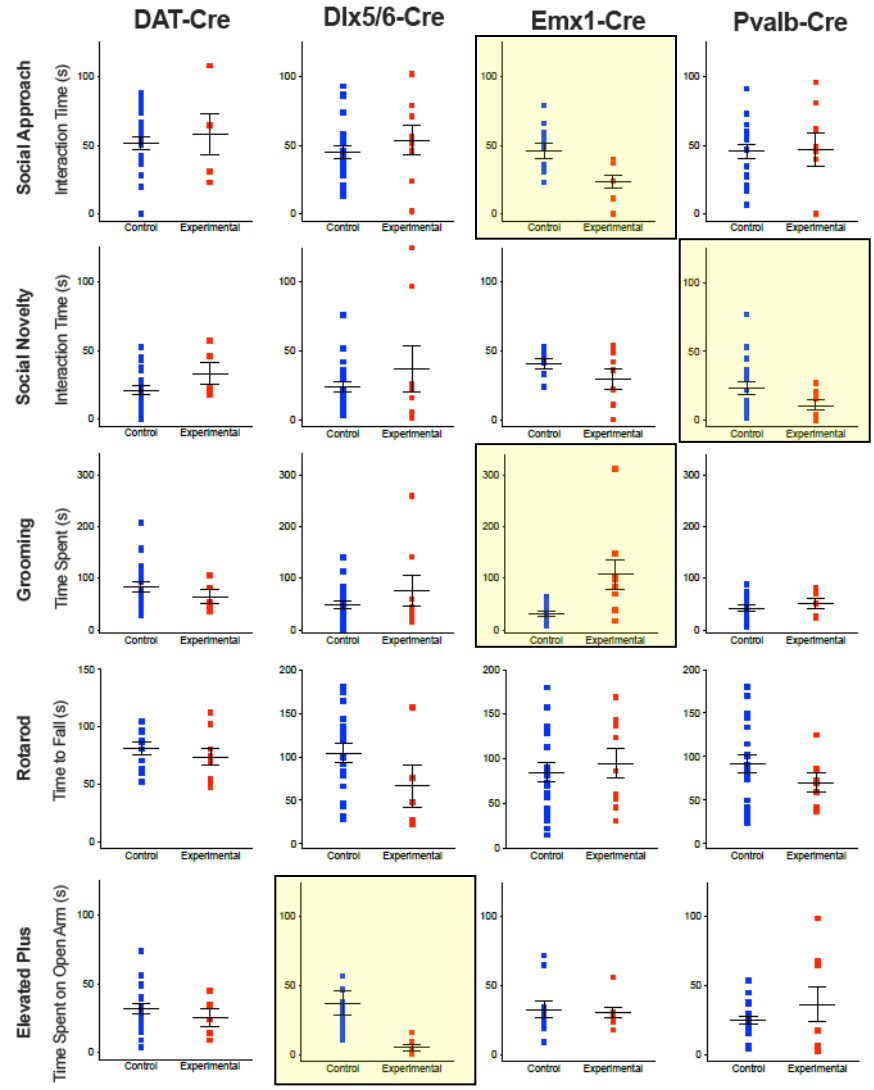
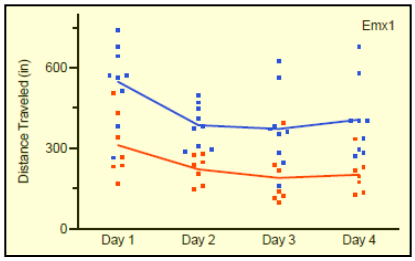
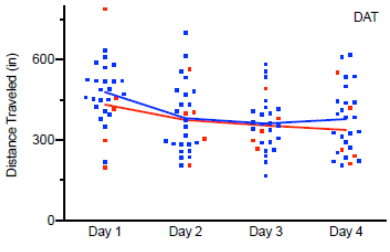
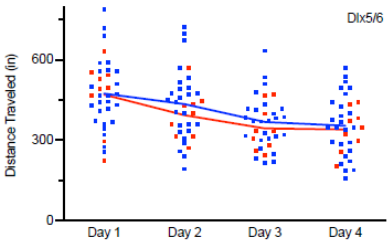


Figure S2. Bioluminescence emission causes changes in neural activity in postnatal pups *in vivo* and *ex vivo*, Related to Figure 1.
(A) Firing Rate Changes after CTZ application in P12-P14 pups. Laminar probes were inserted in the prefrontal cortex of pups aged P12 to P14 and expressing LMO3 in pyramidal neurons (Emx1-LMO3) or not. Intraperitoneal (ip) injection of CTZ during recording led to an initial non-specific increase in firing rate, after which activity in non-expressing mice went back to baseline (blue line, n=3), while Emx1-LMO3 mice showed a significant increase in firing rate (red line, n=3; $t(171) = 5.35$, $p = 2.8 \times 10^{-7}$). Shaded area represents mean ±SEM.
(B) Effect of CTZ on a prefrontal cortex layer 5 pyramidal cell expressing LMO3 at postnatal day 7. Pyramidal cell identity was confirmed through suprathreshold square current injection (800 ms, 400 pA) together with biocytin staining (left, upper panel; scale bar: 50 µm). LMO3-expression was confirmed through 480 nm light illumination delivered through the objective (30 mW/cm^2, 1 sec; left, lower panel). In these functionally immature cells action potential output required large amplitude current injections and exposure to 480 nm light evoked only subthreshold depolarization. The ability to drive excitation of young pyramidal neurons with BL-OG was tested during continuous membrane potential recording (-70 mV; maintained by continuously injecting negative DC current, <100 pA, in current clamp recording) while delivering periodic square current injections to evoke firing (0.1 Hz, 300 pA, 50 ms; right). Brief bath application of CTZ (300 µM) depolarized the membrane potential and increased the firing response to current stimulation. $*p < .05$.


DAT-Cre
Dlx5/6-Cre
Emx1-Cre
Pvalb-Cre
Social Approach
Interaction Time (s)
Social Novelty
Interaction Time (s)
Grooming
Time Spent (s)
Rotarod
Time to Fall (s)
Elevated Plus
Time Spent on Open Arm (s)
Control
Experimental


Emx1
Distance Traveled (in)
Day 1
Day 2
Day 3
Day 4


DAT
Distance Traveled (in)
Day 1
Day 2
Day 3
Day 4


Dlx5/6
Distance Traveled (in)
Day 1
Day 2
Day 3
Day 4

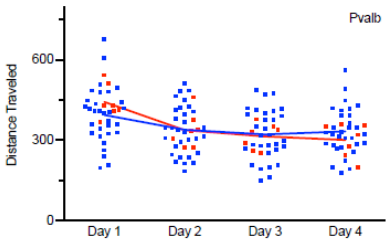

Pvalb
Distance Traveled
Day 1
Day 2
Day 3
Day 4

Figure S3. Behavioral tests, Related to Figure 1.
Results of behavioral tests showing measurements for control (non-expressing; blue color) and experimental (LMO3-expressing; red color) animals treated with CTZ during postnatal development (P4-14). These data are presented in Figure 1D as Cre (DAT, Dlx5/6, Emx1, Pvalb)-LMO3 mice normalized to their non-LMO3 expressing controls. Bars show mean ±SEM. Tests that showed significant differences are highlighted. For detailed statistics see Table S1.

Social Approach and Social Novelty: Developmentally hyperexcited Emx1-LMO3 mice show significantly reduced time interacting with the stationary mouse during the social approach test by nearly 50% compared to non-expressing littermates (students t-test: $t(18) = 3.07$, $p = 0.0067$). While Emx1-LMO3 mice display over 40% reduced time spent interacting with the novel mouse in the social novelty test compared to non-expressing littermates, these differences do not reach statistical significance (students t-test: $t(18) = 1.97$, $p = 0.0650$). In the social novelty test, Pvalb-LMO3 mice demonstrate over 50% reduced time spent interacting with the novel mouse compared to non-expressing littermates (students t-test: $t(21) = 2.19$, $p = 0.0398$, N = 8-15 per group).

Grooming: Emx1-LMO3 mice were the only group to exhibit evidence of repetitive behaviors. Emx1-LMO3 mice show significantly increased time spent grooming by 250% compared to non-expressing littermates (student's t-test: $t(18) = -2.92$, $p = 0.0091$).

Open Field: Emx1-LMO3 mice again were the only cohort to demonstrate altered exploration in open field. These mice show significantly reduced movements over time in open field (Two-way repeated measures ANOVA - Main effect between groups: $F(1,14) = 11.56$, $p = 0.0043$). All mice (expressing and non-expressing) show significant decline in movements over time, and Emx1-LMO3 show similar declines compared to non-expressing littermates (Main effect for Time: $F(11,154) = 31.30$, $p < 0.0001$; Interaction effect of Time x Group: $F(11,154) = 1.71$, $p = 0.0764$). When tested over 4 consecutive days, these results are maintained (Main effect between groups: $F(1,14) = 22.58$, $p = 0.0003$; Main effect for Time: $F(3,42) = 7.77$, $p = 0.0003$).

Table S1. Statistics, Related to Figures 1 – 3.

Figure	Group	Test	Finding	Significance
1D	Emx1-LMO3	Emx1-LMO3 vs non-expressing in social approach	reduced interaction times	students t-test: t(18) = 3.07, p = 0.0067; Two-way ANOVA: interaction effect between genotype and chamber: F(2,54) = 7.26, p = 0.0016
		Emx1-LMO3 vs non-expressing in social novelty	no significant differences versus littermate controls	students t-test: t(18) = 1.97, p = 0.0650
		Emx1-LMO3 vs non-expressing in grooming behavior	increased time spent grooming	student's t-test: t(18) = -2.92, p = 0.0091
		Emx1-LMO3 vs non-expressing in open field	reduced movements over time	Two-way repeated measures ANOVA - Main effect between groups: F(1,14) = 11.56, p = 0.0043
	Pvalb-LMO3	Pvalb-LMO3 vs non-expressing in social novelty	Reduced interaction times	Student's t-test: t(21) = 2.19, p = 0.0398
	Dlx6a-LMO3	Dlx6a-LMO3 vs non-expressing in elevated plus maze	reduced time on the open arms	log-transformed data, student's t-test: t(23) = 2.52, p = 0.0096
2B,C	Emx1-LMO3	event-related local field potentials (erLFP)	CTZ_{P4-14} mice vs VEH_{P4-14} mice: reduced amplitude during baseline	ANOVA: F(3,11752) = 51.28, p < 0.0001; Bonferroni post-hoc: p < .001
2D			CTZ_{P4-14} mice, no change in amplitude of erLFPs during light stimulus vs baseline	Bonferroni post-hoc: p = 0.913
			VEH_{P4-14} mice, increased amplitude of erLFPs during light stimulus vs baseline	Bonferroni post-hoc: p < 0.001
2E			Change in frequency of erLFPs during light stimulus vs baseline between CTZ_{P4-14} mice and VEH_{P4-14} mice	Mann-Whitney: z -1.964, p = 0.0496
			VEH_{P4-14} mice, increased frequency of erLFPs during light stimulus vs baseline	Mann-Whitney: z -1.964, p = 0.0496
2F		Power spectra	CTZ_{P4-14} vs VEH_{P4-14} mice, reduced power in the striatum	Theta: F(3,108) = 3.19, p = 0.0265; Alpha: F(3,108) = 3.48, p = 0.0184; Beta: F(3,108) = 3.40, p = 0.0204
2G - I			CTZ_{P4-14} mice, no change light stimulus vs baseline	Bonferroni post-hoc: p = 1.0
			VEH_{P4-14} mice, elevated power light stimulus vs baseline	Bonferroni post-hoc, Theta: p = 0.050; Alpha: p = 0.036; Beta: p = 0.042
2J		coherence between cortex and striatum	CTZ_{P4-14} vs VEH_{P4-14} mice, reduced coherence before and after light stimulus	Interaction of Time x Group: F(20,1128) = 1.87, p = 0.0117
3D	Emx1-LMO3	Cortical pyramidal neuron firing rate	CTZ_{P4-14} vs VEH_{P4-14} mice, larger increase in firing rate in response to light	Two-way Repeated Measures ANOVA, Main effect between groups: F(1,6) = 6.86, p = 0.0396;
			no differential effects between trials	(Main effect for trial: F(6,36) = 1.38, p = 0.2494)
			No differences in maximum firing rate between groups	Main effect between groups: F(1,6) = 1.30, p = 0.2977;
3F		pyramidal neuron firing rate	CTZ_{P4-14} vs VEH_{P4-14} mice, reduced firing rate at baseline	Students T-test: t(7) = -6.082, p = 0.0005;
			CTZ_{P4-14} vs VEH_{P4-14} mice, increased firing rate after light stimulus	t(7) = 2.541, p = 0.0386
		interneuron firing rate	CTZ_{P4-14} vs VEH_{P4-14} mice, reduced firing rate at baseline	Students T-test: t(7) = 2.526, p = 0.0395

			CTZ_{P4-14} vs VEH_{P4-14} mice, reduced firing rate after light stimulus	Students T-test: t(7) = -2.41, p = 0.0468
3G		Power spectra over time	CTZ_{P4-14} vs VEH_{P4-14} mice, increased Beta and Gamma	Beta range - Student's T-test: t(6) = 2.517, p = 0.0455; Gamma range - Student's T-test: (t(6) = 2.732, p = 0.0341
3H,I			CTZ_{P4-14} vs VEH_{P4-14} mice, reduced power in response to light stimulation in lower frequency	Two-way repeated measures – Interaction of time x group: Theta – F(20,1128) = 3.21, p < 0.0001; Alpha – F(20,1128) = 3.49, p < 0.0001; differences occurred within the first few seconds of light stimulation (Bonferroni post-hoc: Theta – p = 0.0203; Alpha – p = 0.0302)
3J			CTZ_{P4-14} vs VEH_{P4-14} mice, no difference in response to light stimulation in Beta frequency	F(20,1128) = 0.78, p = 0.7392;
3K			CTZ_{P4-14} vs VEH_{P4-14} mice, higher power in response to light stimulation in Gamma frequency	Two-way repeated measures – Interaction of time x group: F(20,1128) = 5.40, p < 0.0001;
4E	Emx1-LMO3	intrinsic firing properties	CTZ_{P4-14} vs VEH_{P4-14} mice, increased firing threshold	Students T-test: t(29) = -6.297, p < 0.0001
			CTZ_{P4-14} vs VEH_{P4-14} mice, increased rheobase current	Students T-test: t(25) = -3.494, p = 0.0017
			CTZ_{P4-14} vs VEH_{P4-14} mice, lower maximum firing frequency	Students T-test: t(25) = 5.386, p < 0.0001
			CTZ_{P4-14} vs VEH_{P4-14} mice, no effect on input resistance	Students T-test: t(25) = -0.461, p < 0.65
4G		mEPSCs	CTZ_{P4-14} vs VEH_{P4-14} mice, no change in frequency	Students T-test: t(29) = -1.167, p = 0.253
			CTZ_{P4-14} vs VEH_{P4-14} mice, no change in amplitude	Students T-test: t(29) = -0.841, p = 0.407
4I		mIPSCs	CTZ_{P4-14} vs VEH_{P4-14} mice, reduced frequency	Students T-test: t(29) = 2.414, p = 0.022
			CTZ_{P4-14} vs VEH_{P4-14} mice, reduced amplitude	Students T-test: t(29) = 2.219, p = 0.034
4J		E/I ratio	CTZ_{P4-14} mice, mPSC frequency – increased ratio	Students T-test: t(29) = -2.497, p = 0.018
			CTZ_{P4-14} mice, mPSC amplitude – increased ratio	Students T-test: t(29) = -3.457, p = 0.002
			CTZ_{P4-14} mice, synaptic drive – increased ratio	Students T-test: t(29) = -2.875, p = 0.007

TRANSPARENT METHODS

Animals

All experiments involving animals were carried out following the guidelines and protocols approved by the Institutional Animal Care and Use Committee at Central Michigan University and were in compliance with the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals.

Mice were group-housed in ventilated cages under 12-hour reverse light cycle, provided with tap water and standard chow and allowed to feed *ad libitum*.

Experimental animals were generated by crossing LSL-LMO3 mice (see below) with the following Cre driver lines: Emx1-Cre, JAX# 005628; Pvalb-Cre, JAX# 017320; Dlx5/6-Cre, JAX# 008199; DAT-Cre, JAX# 006660.

Generation of LSL-LMO3 mice

A ROSA26 targeting construct placing LMO3 (sbGLuc-VChR1-EYFP; Berglund *et al.*, 2016) under conditional (lox-stop-lox, LSL) control of the strong ubiquitous CAG promoter was generated by replacing the tdTomato gene in the Allen Brain Institute's Ai9 targeting vector (CAG-floxed tdTomato; Addgene plasmid 22799; contributed by Hongkui Zeng; Madisen et al. 2010). Embryonic stem cells were homologously targeted via electroporation of (129X1/SvJ x 129S1/Sv)F1-*Kitl*$^{+}$-derived R1 embryonic stem (ES) cells (Nagy et al., 1993), injected into blastocysts, and male chimeras were crossed with C57BL/6 females. Heterozygous LSL-LMO3 mice were further crossed to C57BL/6. The PGK-neo marker is flanked by a pair of PhiC31 recognition sites (*attB*/*attP*) and was deleted from the LSL-LMO3 line by crossing with PhiC31 deleter mice (Stock #007743, Jackson Laboratory; kindly provided by Dr. Hongkui Zeng, Allen Brain Institute). Routine genotyping for detecting the presence of the conditional alleles was done using forward primer 5'- ATGTCTGGATCCCCATCAAG, and reverse primer 5'- TCCGAAGCCAACCTTCACAGTAAC (Zhu et al., 2016). The LSL-LMO3 mouse line is available from The Jackson Laboratory (JAX#034853).

IVIS Imaging

Mouse pups aged post-natal day 4 were injected with CTZ intraperitoneally at a dose of 10µg/g. Pups were anesthetized on ice prior to being placed in the IVIS chamber. Images were taken in 5-minute bins over a 1-hour period and quantified using radiance (Perkin-Elmer, IVIS Lumina LT, Living Image Software).

Microscopy

Immunofluorescent images were captured on a Zeiss AxioCam M2 microscope using a 20x objective and digitized using the ZEN software (Carl Zeiss Inc., Thornwood, NY, USA).

Treatment of pups

The entire litter from heterozygous breeding pairs of Cre-mice and LSL-LMO3 received CTZ (water soluble native coelenterazine; Prolume Inc., cat# 3031) or vehicle (water soluble carrier without CTZ; Prolume Inc., cat# 3031C). CTZ or vehicle were injected intraperitoneally at a dose or volume equivalent of 10µg/g once per day during p4-14. Mice were then weaned, genotyped, and group-housed until used for behavioral and recording experiments.

Behavioral tests

All behavioral tests were performed with age-matched male and female littermates, starting at 2 – 3 months old and continuing over a 3-4 months period. We did not observe gender differences and thus male and female mice were pooled, generating groups of 8 – 22 animals. Mice were moved between holding room and behavioral suite, located within the same facility. Behavioral tests were carried out during the day in rooms under reverse light cycle. Testing was carried out by individuals blinded to experimental conditions. Scoring for each test was done by 2 independent individuals blinded to experimental conditions.

Three Chamber Test

Social behavior was tested using the 3-chamber test (Crawley, 2007; Yang et al., 2011). Animals were placed in a 27" x 14" chamber with 3 segments of equal size (Medendorp et al., 2018). Animals were allowed to explore the arena for 5 min to habituate to the apparatus. Mice were tested first for social approach, which has been demonstrated to relate to social deficits found in autism (Yang et al., 2011). Mice were additionally tested for social novelty immediately following the social approach test.

Social Approach: Two identical plastic cylinders were placed in either of the external chambers. These cylinders were clear, with regular holes to allow for visual and olfactory stimuli. A sex-matched, non-familiar mouse was placed in one of these cylinders. Experimental mice were placed in the middle, empty section of the 3-chamber apparatus and allowed to roam for 5 minutes. Time spent in the different chambers was measured as well as time spent interacting with the stationary mouse. Social interaction was defined by time spent within a 1-inch radius actually interacting with the stationary mouse.

Social Novelty: Following the social approach test, a novel, sex-matched, non-familiar mouse was placed in the previously empty cylinder. Experimental mice were again allowed to roam for 5 minutes. Social behavior was defined by time spent within a 1-inch radius actually interacting with the novel mouse compared to the previous mouse. The chamber was cleaned with 70% ethanol between testing mice.

Elevated Plus Maze

Mice were placed in the center of an elevated, plus-shaped apparatus (30.5" x 30.5" arms, 30.5" from the ground). Two of the external arms were covered, and 2 were open. Mice were allowed to roam the apparatus for 5 minutes. Mice were assessed for time spent on the open arms, with more time spent on the open arms indicative of less anxiety.

Open Field

Experimental mice were placed in a 17" x 9" cage and allowed to roam for 60 minutes. Movements were tracked by laser grid and analyzed using Hamilton-Kinder™ motor monitor software. Tests were repeated over 4 consecutive days using the same cage. Overall ambulation of the mice was assessed to establish normal exploratory behavior (Crawley, 1985).

Grooming Observation

Mice were observed in a 17" x 9" cage for a period of 10 minutes. Periods of grooming were noted and totaled over the 10-minute period.

Rotarod

Mice were placed on an accelerating rotarod and timed until they were unable to remain on the spinning rod. Mice were trained on the rotarod every day for 4 days prior to testing. The rotarod accelerated from 10-40 revolutions per minute (rpm) over the course of 30 seconds. At the end of the 30 seconds the

rotarod remained at 40 rpm until 180 seconds or until mice were unable to stay on the rotarod. Mice were tested for 3 trials of the rotarod and the scores averaged.

***In vivo* recording**

Mice were anesthetized with urethane at 1.5g/kg and mounted on a stereotax (Kopf Instruments). Laminar optoelectrodes were inserted in the prelimbic area of the medial prefrontal cortex (mPFC; 2mm anterior to bregma, 0.4mm lateral, and 2mm ventral). The striatal electrode (20^{o} lateral offset) was placed 0.7mm anterior to bregma, 3mm lateral, and 2.6mm ventral. Laminar probes consisted of single shank, 32-channel silicon probes with a fiber optic 50µm above the highest recording site (A1x32 Poly2-5mm-50s-177-OA32LP, Neuronexus Technologies; 0.15mm silver wire reference). Data was sampled at 30kHz and passed through a digital amplifier (Cereplex-µ, Blackrock Microsystems), and directed through HDMI to the Cereplex Direct data acquisition box (Blackrock Microsystems). Each mouse was assessed post-recording for electrode placement within both the mPFC as well as the medial dorsal striatum. Electrode placements were highly consistent between mice.

VChR1 photostimulation was carried out using a PlexBright optogenetic stimulation system (Plexon Inc.) with a blue LED module (465 nm). Mice were recorded for 20 minutes to establish a baseline. At 20 minutes, blue light was applied through the fiber optic at 300µA intensity for 10 seconds. Each light pulse was separated by 1 minute. Recordings were allowed to continue 1 hour after injection to assess response to stimulation. After recordings, brains were extracted and sectioned to confirm electrode placement.

Postnatal day 12-14 mice were anesthetized with urethane at 1g/kg and mounted on a stereotax (Kopf Instruments) via custom-made bars. Laminar electrodes (same as for adult mice) were inserted in the prelimbic area of the medial prefrontal cortex (mPFC; 0.5 mm anterior to bregma, 0.1-0.5 mm lateral to the midline; a silver wire was inserted into the cerebellum and served as ground and reference). Data was collected as above.

Coherence calculations were conducted in accordance with Kramer, 2013 in 3s bins to show the effects over time as light stimulation occurred. The Coherence calculation was started in the baseline period, where no significant differences in power were observed between CTZ- and VEH-treated mice in either the cortex or the striatum. This period also corresponds to the period where coherence is significantly different between VEH and CTZ groups. The coherence calculation encompasses the period of light stimulation, where differences in power are observed between VEH and CTZ groups; however, these periods result in no significant differences in coherence between groups.

Fast Fourier transforms were carried out using MATLAB (MATLAB FFT command). LFP waveform data were converted from time to frequency, producing power spectral density histograms. Data was pulled from a 12-second interval spanning 1second prior to light stimulus and 1 second after to encompass the entire 10 second light stimulation period. This was repeated for each light trial. Data was quantified from various frequency ranges including Delta (0-4Hz), Beta (4-8Hz), Theta (8-14Hz), and Gamma (30-100Hz).

Ex vivo recording

Acute brain slices were prepared from 4-6 week old Emx1-LMO3 mice that received daily CTZ or Vehicle injections between P4-14. A few P5-10 Emx1-LMO3 mice were included to confirm an excitatory effect of CTZ treatment during the early postnatal developmental period. Briefly, mice were anaesthetized via inhalation of isoflurane and, following decapitation, the brain was isolated and placed in ice-cold cutting solution containing (in mM): 92 NMDG, 2.5 KCl, 0.5 $CaCl_2$, 10 $MgSO_4$, 30 $NaHCO_3$, 1.25 NaH_2PO_4, 20 HEPES,

2 Thiourea, 5 Na-ascorbate, 3 Na-pyruvate and 25 D-glucose (310 mOsm/kg, pH 7.3-7.4). Coronal slices (300 µm) from prefrontal cortex were cut using a vibratome (VT1000s, Leica) and stored in a recovery solution containing (in mM): 92 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 30 $NaHCO_3$, 1.25 NaH_2PO_4, 20 HEPES, 2 Thiourea, 5 Na-ascorbate, 3 Na-pyruvate and 25 D-glucose (310 mOsm/kg, pH 7.3-7.4). After a one hour recovery period, a slice was transferred to a recording chamber mounted on an upright microscope (BX51WI, Olympus) and perfused with aCSF containing (in mM): 121 NaCl, 2.8 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$ and 15 D-glucose (310 mOsm/kg, pH 7.3-7.4) at a rate of 3 ml/min. All solutions were bubbled with a gas mixture of 95% O_2 and 5% CO_2. Whole-cell patch clamp recordings were performed using a Multiclamp 700b amplifier and Digidata 1440 digitizer together with the pClamp recording software (Molecular Devices). Borosilicate glass micropipettes were manufactured using a PC-100 puller (Narishige) and had resistances of 3–5 MΩ. Series resistance (Rs) was ≤15 MΩ in all cells after break-in and compensated by up to 70%. Rs was not allowed to fluctuate more than 25% from start to end of recording.

In current clamp recordings, pipettes were filled with intracellular solution containing (in mM): 130 K-gluconate, 10 KCl, 15 HEPES, 5 Na_2-phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP and 0.5% biocytin (310 mOsm/kg, pH 7.3). Intrinsic excitability was quantified using depolarizing square current injections (800 ms, 50 pA increments) and current ramps (1 s, -200 to +500 pA) from a membrane potential of -70 mV. The aCSF was supplemented with D-AP5 (50 µM), CNQX (15 µM) and picrotoxin (100 µM) to block fast glutamatergic and GABAergic synaptic transmission. Rs was compensated using bridge balance. In voltage clamp recordings the intracellular solution contained (in mM): 130 Cs-methanesulphonate, 2 NaCl, 15 HEPES, 0.6 EGTA, 5 Qx-314, 4 Mg-ATP, 0.4 Na-GTP and 0.5% biocytin (300 mOsm/kg, pH 7.25). Miniature AMPA- and $GABA_A$-receptor mediated synaptic currents (mEPSCs and mIPSCs) were recorded at -70 and +10 mV, respectively, in presence of 100 nM tetrodotoxin. Rs was continuously monitored using a -10 mV hyperpolarizing pulse present in the recording protocol. In all recordings, Emx1-LMO3-positive prefrontal L5 pyramidal neurons were visually targeted using epifluorescence microscopy together with a CMOS camera (ORCAFusion, Hamamatsu). A current/membrane potential response to blue light was confirmed for each cell before the recording started. Excitation light (480 nm, 1 s, 30 mW/cm^2) was delivered through a 40x water immersion objective using a 130 W metal halide light source (U-HGLGPS, Olympus) and GFP filter cube (Ex/Em: 480/550 nm, U-MNIBA3, Olympus). An electronic shutter (Lambda SC, Sutter Instruments) was used to control time window of illumination.

The total number of Vehicle and CTZ treated mice used in slice recordings were 9 and 10, respectively. Data was recorded from ≤ 2 cells per brain slice, ≤ 3 slices per animal. Total number of recorded cells are indicated in Figure 4. Data was sampled at 10 kHz, filtered at 3 kHz and analyzed in Igor Pro (WaveMetrics). Action potential threshold was defined as a dV/dt of >20 mV/ms and measured on the first action potential generated from a depolarizing current ramp protocol. In frequency–current plots, firing frequency was calculated from the total number of action potentials produced during depolarizing current injections (800 ms) ranging from 0 to 500 pA. Input resistance was calculated from hyperpolarizing current injections (800 ms, -200 pA) starting from -70 mV. Miniature postsynaptic currents were analyzed blinded to experimental group in MiniAnalysis (Synaptosoft), using 5 and 10 pA event detection thresholds, respectively. mEPSCs and mIPSCs were analyzed in 2 min segments and featured a minimum of 200 events. The liquid junction potential was not corrected for.

Post hoc staining

Neurons in brain slices were pipette-filled with biocytin (0.5% w/v, Sigma-Aldrich) and fixed overnight (4°C) in PBS (0.9% NaCl) containing 4% paraformaldehyde (pH 7.3-7.4). Slices were washed 3 x 10 min in PBS (pH 7.3-7.4) and incubated in 0.25% Triton X-100 together with 5% normal goat serum (Invitrogen) for 2 h at room temperature. Following a second wash the slices were incubated in streptavidin-CY5 (1:300, Invitrogen), 0.25% Triton X-100 and 5% normal goat serum for 2 h at room temperature. After a final wash, slices were mounted on slides and cover slipped with ProLong Gold mounting medium (Thermo Fisher). Streptavidin-CY5-labelled cells were imaged on an Axio Imager M2 microscope (Zeiss) using 10x, 20x and 40x air objectives.

Quantification and Statistical Analysis

All analysis was carried out using MATLAB and SPSS software. Data are displayed as mean±standard error of the mean (SEM). Behavior tests were tested using Student's t-test between expressing and non-expressing mice. The three-chamber tests were also analyzed with two-way ANOVA. Open field was tested using two-way repeated measures ANOVAs.

Electrophysiological *in vivo* data was high pass filtered at 250Hz to extract spikes and low pass filtered at 300Hz to extract LFPs. Spike data was thresholded at -63µV and sorted for each channel based on waveform characteristics using Principal Components Analysis (PCA). Spikes were binned to calculate frequency of firing over time. Differences between groups were assessed using two-way repeated measures ANOVAs (repeat trials per mouse). For *ex vivo* electrophysiological data, statistical significance between groups was determined using Student's T-test.

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