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Supplemental Information

Recombinase-Driver Rat Lines: Tools, Techniques, and Optogenetic Application to Dopamine-Mediated Reinforcement

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Supplemental Data

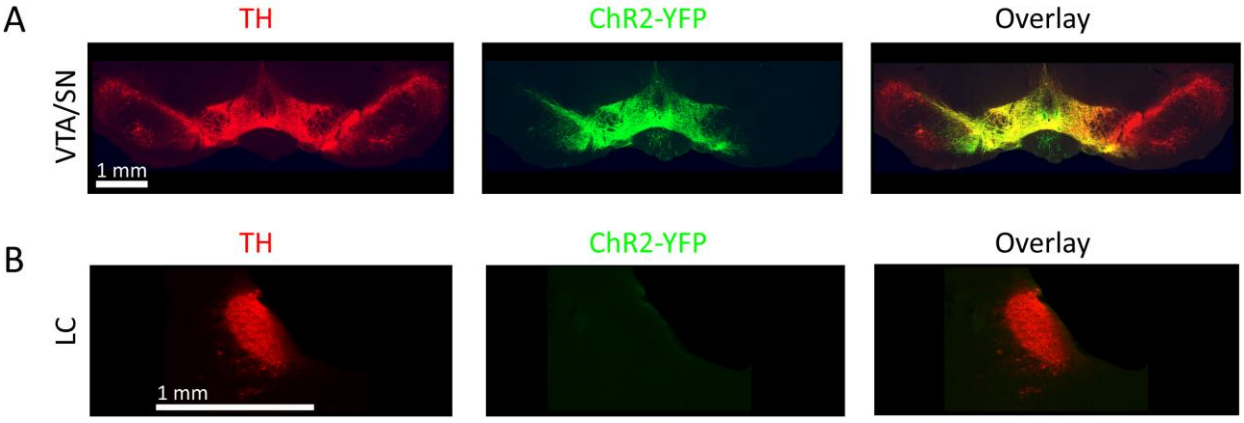

Figure S1

Locus coeruleus (LC) expression in TH::Cre rats injected with Cre-dependent ChR2-YFP virus in the VTA. **A.** Sections of the VTA/ SN demonstrating strong TH staining and ChR2-YFP expression. **B.** Section of the LC in the same rat demonstrating that virus does not spread to the LC. Red: TH antibody; Green: ChR2-YFP expression.

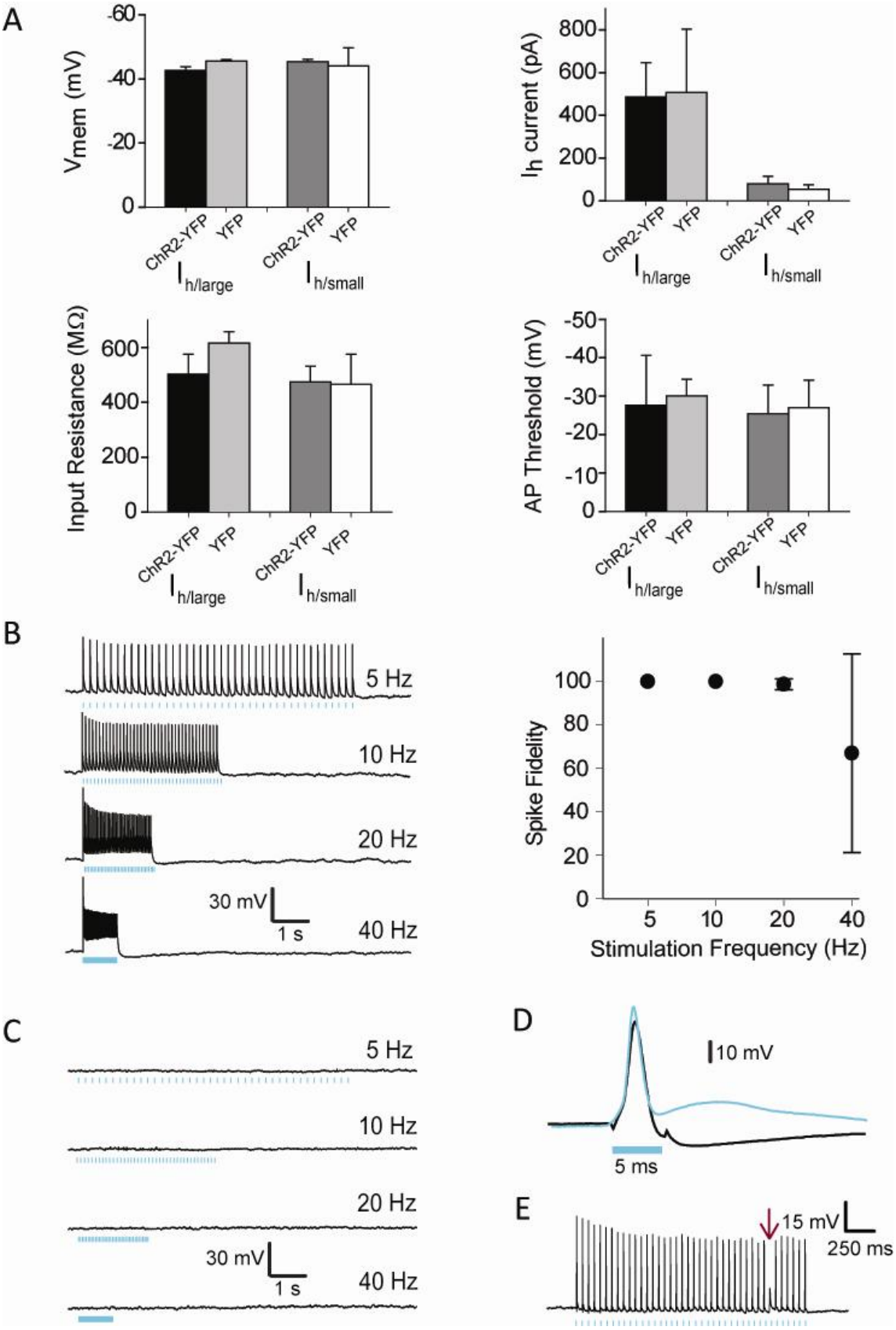
A
Vmem (mV)
-60
-40
-20
0
ChR2-YFP
YFP
ChR2-YFP
YFP
I h/large
I h/small
Ih current (pA)
800
600
400
200
0
Input Resistance (MΩ)
600
400
200
0
AP Threshold (mV)
-50
-40
-30
-20
-10
0
B
5 Hz
10 Hz
20 Hz
30 mV
1 s
40 Hz
Spike Fidelity
100
80
60
40
20
0
5
10
20
40
Stimulation Frequency (Hz)
C
5 Hz
10 Hz
20 Hz
30 mV
1 s
40 Hz
D
10 mV
5 ms
E
15 mV
250 ms

Figure S2

Intrinsic cellular and optogenetic properties of ChR2-YFP and YFP-only expressing neurons in the VTA of TH::Cre rats. **A.** Initial resting membrane potential (Vmem), magnitude of I_h current, input resistance, and action potential threshold (AP Threshold) for cells in the VTA of TH::Cre+ rats expressing ChR2-YFP or YFP-only. Both $I_{h/large}$ and $I_{h/small}$ cells were examined. Error bars represent SEM (ChR2 $I_{h/large}$ cells, *n*=7; YFP $I_{h/large}$ cells, n=3; ChR2 $I_{h/small}$ cells, n=4; YFP $I_{h/small}$ cells, n=5). There was no significant difference between any of these properties between ChR2-YFP and YFP-only expressing cells for either cell-type (2-tailed t-test, $p>0.05$) **B.** Representative traces showing light-evoked action potentials at a range of stimulation frequencies in an $I_{h/small}$ ChR2-YFP cell (left). Summary graph showing percent success rate for generating spikes at different light stimulation frequencies for all $I_{h/small}$ ChR2-YFP cells (right). Error bars represent SEM. **C.** Representative voltage traces in an example cell expressing YFP-only in response to light trains of various frequencies, confirming a lack of optical response. **D.** Comparison of light-evoked (blue) vs electrically-evoked (black) waveforms in a cell expressing ChR2-YFP; note prolonged depolarization linked to the expected off-kinetics of ChR2. **E.** Example of a single missed spike (arrow) in response to optical stimulation at 10 Hz. In all panels, duration of an optical or electrical stimulation pulse is 5 ms.

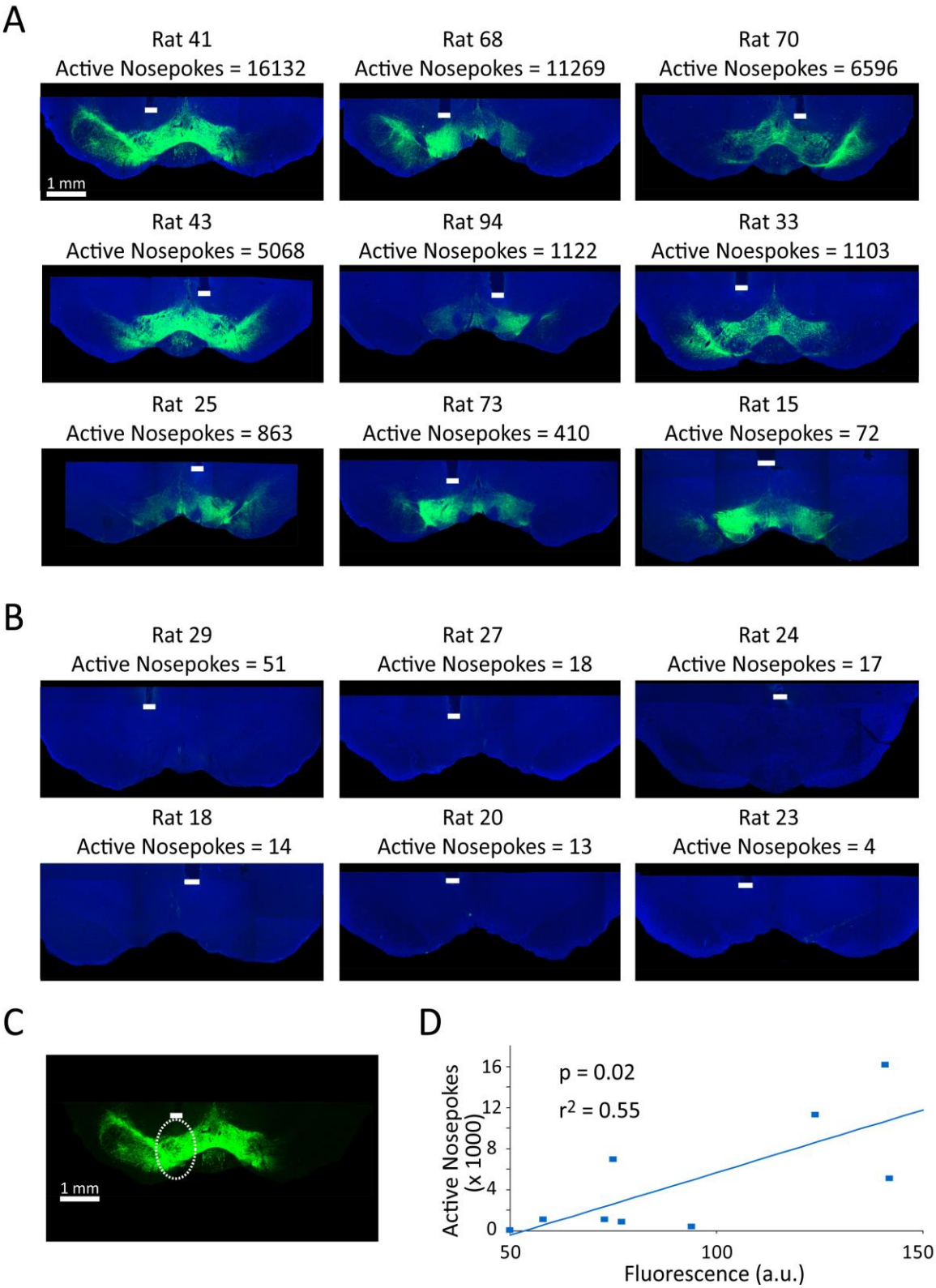
A
Rat 41
Active Nosepokes = 16132
Rat 68
Active Nosepokes = 11269
Rat 70
Active Nosepokes = 6596
1 mm
Rat 43
Active Nosepokes = 5068
Rat 94
Active Nosepokes = 1122
Rat 33
Active Noespokes = 1103
Rat 25
Active Nosepokes = 863
Rat 73
Active Nosepokes = 410
Rat 15
Active Nosepokes = 72
B
Rat 29
Active Nosepokes = 51
Rat 27
Active Nosepokes = 18
Rat 24
Active Nosepokes = 17
Rat 18
Active Nosepokes = 14
Rat 20
Active Nosepokes = 13
Rat 23
Active Nosepokes = 4
C
1 mm
D
p = 0.02
r2 = 0.55
Active Nosepokes (x 1000)
16
12
8
4
0
50
100
150
Fluorescence (a.u.)

Figure S3

Histology of all rats that were used in behavior. **A.** Sections of the VTA in TH::Cre+ rats, depicting fiber placement and ChR2-YFP expression, along with active nosepokes on Day 4 of FR1 training. Green: ChR2-YFP expression; Blue: DAPI nuclear counterstain; Horizontal white line: termination of fiber track. **B.** Same as **A**, but for TH::Cre- littermates. **C.** In each TH::Cre+ rat, YFP fluorescence was quantified within a selected region that was placed directly below the fiber tip (an oval that was 1mm x 1.5mm), as depicted in this example (Rat 41). Fluorescence was determined based on the average pixel intensity within the selected region using ImageJ software. **D.** A statistically significant relationship between fluorescence in the selected region and active nosepokes on day 4 of FR1 training was observed (*t*-test, p=0.02, r^2=0.55).

Table S1

Sample	Counts		Ratio	95% confidence (lower bound)	95% confidence (upper bound)	Estimated Copy number
preamp product	Cre (FAM)	GGT1 (Cy5)	Cre/GGT1			
Wildtype control	0	1373	0.000	0.631	0.750	0
TH::Cre Line 3.1+/-	943	1280	0.704	0.000	0.000	1
TH::Cre Line 3.1+/+	2041	1424	1.582	0.646	0.765	2
TH::Cre Line 3.5+/-	898	1245	0.688	1.478	1.695	1
TH::Cre Line 4.4+/-	1268	1758	0.670	0.623	0.720	1
ChAT::Cre Line 5.1+/-	3552	1450	3.888	3.654	4.145	6
ChAT::Cre Line 5.2+/-	3423	1392	3.767	3.536	4.019	6

Transgene copy number estimation for TH::Cre and ChAT::Cre rat lines. The number of copies of the Cre transgene in several TH::Cre and ChAT::Cre rat lines was estimated using digital PCR. All TH::Cre rat lines were found to have one transgene copy; the ChAT::Cre rat line was found to have 6 transgene copies.

Supplemental Experimental Procedures

Geneotyping for Cre Recombinase

In order to identify Cre-positive founders or their offspring, the following PCR program was used:

1. 94^{o}C for 3 min
2. 94^{o}C for 45 sec
3. 65^{o}C for 30 sec
4. 72^{o}C for 90 sec
5. Repeat Steps 2-4 29 more times.
6. 72^{o}C for 10 min
7. Hold at 4^{o}C

The primers were as follows: Cre-F AAGAACCTGATGGACATGTTCAGGGATCG, Cre-R CCACCGTCAGTACGTGAGATATCTTTAACC, and a positive band corresponded to 600 base pairs.

Virus construction and packaging

The Cre-inducible ChR2 viral construct used in this study has been described previously (Tsai et al., 2009); in some cases the H134R mutant was employed. With the exception of the LC image (Fig. 1D), recombinant AAV vector was serotyped with AAV5 coat proteins and packaged by the viral vector core at the University of North Carolina. Viral titers ranged from 1.5-8 x 10^{12} particles / mL. In the case of the LC, the same vector was serotyped with AAV10 coat proteins since AAV5 injection at the same coordinates failed to infect the LC.

Slice preparation for in vitro electrophysiology

Coronal slices (325µm) from adult rats (3 months or older) previously injected with virus were prepared after intracardial perfusion with ice-cold sucrose-containing artificial cerebrospinal fluid (ACSF; in mM: 85 NaCl, 75 sucrose, 2.5 KCl, 25 glucose, 1.25 NaH_2PO_4, 4 $MgCl_2$, 0.5 $CaCl_2$, and 24 $NaHCO_3$). Slices were incubated for 1 hr at 32-34ºC, and then at room temperature until transferred to an oxygenated standard ACSF solution (in mM: 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, and 10 glucose) for electrophysiological recordings performed at 32-34°C. Slices were visualized with an upright microscope equipped with a 40X water-immersion objective (DM-LFSA, Leica Microsystems) and infrared differential interference contrast (IR-DIC) optics. Individual neuron recordings were obtained after identifying fluorescent protein expression under constant ACSF perfusion. Filtered light from a broad-wavelength xenon lamp source (Sutter Instruments DG-4) was coupled to the fluorescence port of the microscope and used both to view fluorescence and deliver light pulses for opsin activation. Power density of the blue light was 4.3-5 mW/mm^2, measured with a power meter (ThorLabs). Whole-cell recordings were obtained with patch pipettes (3-5 MΩ) pulled from borosilicate glass capillaries (Sutter Instruments) with a horizontal puller (P-2000, Sutter Instruments) and contained the following internal solution (in mM: 135 K-gluconate, 5 KCl, 10 HEPES, 0.1 EGTA, 2 MgCl2, 2 MgATP, 0.2 NaGTP). Recordings were made using a MultiClamp700B amplifier (Molecular Devices). Signals were filtered at 1 kHz and digitized at 10-20 kHz with a Digidata 1440A analog–digital interface (Molecular Devices). pClamp10.3 software (Molecular Devices) was used to record and analyze data.

In vivo optrode recording

Simultaneous optical stimulation and extracellular electrical recording were performed in anesthetized rats as described previously (Gradinaru et al., 2007). Optrodes consisted of a tungsten electrode (1 MΩ; .005 in; parylene insulation) glued to an optical fiber (300 µm core diameter, 0.37 N.A.), with the tip of the electrode projecting beyond the fiber by 300-500 mm, and the optical fiber was coupled to a 473 nm laser. The light power was 1-3 mW at the fiber tip, which corresponds to a density at the tip of the electrode of about ~0.3-0.9mW/mm^2. Signals were amplified and band-pass filtered (300Hz low cut-off, 10 kHz high cut-off) before digitizing and recording to disk.

Fast-scan cyclic voltammetry

Coronal brain slices (300-400 µm) were prepared from adult rats with virus injected 5-7 weeks prior. Slices were maintained in artificial cerebral spinal fluid (ACSF) containing (in mM): 123 NaCl, 26 $NaHCO_3$, 3 KCl, 1.25 $NaH2PO_4*H_2O$, 1 $MgCl_2*6H_2O$, 2 $CaCl_2*2H_2O$, and 11 glucose. ACSF was bubbled with 95% O_2/5% CO_2 and slices were heated to 32-33ºC during recordings. A carbon fiber glass electrode (with the carbon fiber protruding beyond the glass tip by 200-300 um) was positioned in the NAc under fluorescent guidance. Voltammetric measurements were made every 100 ms by application of a triangular waveform (-0.4 V to +1.4 V, at 400 V/s) to the carbon fiber electrode vs. an Ag/AgCl reference electrode. Data was acquired and analyzed using custom software written in LabVIEW. Dopaminergic terminals were activated with an

optical fiber connected to a 473nm laser (20 Hz, 5ms pulse duration). Slices were allowed to recover 5 min between each stimulation train. To estimate changes in DA release, background current at the electrode was subtracted from the current measured immediately following optical stimulation. Background subtracted cyclic voltammogram showed peak oxidation and reduction currents at ~650 mV and -200 mV respectively, indicating the signals were due to the detection of evoked DA release, and consistent with previous results. Following slice recordings, electrodes were calibrated in 1 uM DA solution.

Histology

Rats were deeply anesthetized with sodium pentobarbital (390 mg／kg) and perfused transcardially with ice-cold 0.9% saline followed by 4% paraformaldehyde. Brains were removed, post-fixed in paraformaldehyde for up to 24 hours and cryoprotected in 25% sucrose for >48 hours. Brains were sectioned coronally at 60 um on a freezing microtome. Free-floating sections were washed in PBS and then incubated with bovine serum albumin (BSA) and Triton X-100 (each 0.2%) for 20 min. 10% normal donkey serum (NDS) was added for a further 30 min incubation. Primary antibody incubations were performed overnight at 4°C in PBS with BSA and Triton X-100 (each 0.2%). Concentrations and sources for primary antibodies were as follows: mouse anti-GFP (1:1500, Invitrogen), rabbit anti-TH (1:500, Fisher Scientific) and goat anti-ChAT (1:200, Millipore). Sections were then washed and incubated with 2% NDS in PBS for 10 minutes and secondary antibodies were added (1:200) conjugated to Alexa Fluor 488 or

594 dyes (Invitrogen) for 2 hours at room temperature. Sections were mounted onto microscope slides in phosphate-buffered water and coverslipped with Vectashield mounting medium that contained a DAPI nuclear counterstain (Fisher Scientific). Sections were then visualized on a confocal microscope to quantify virus expression, or determine fiber placements.

Copy number estimation

The method of Qin, Jones and Ramakrishnan (2008) was used to estimate the number of copies of the transgene in different samples, using digital PCR. Briefly, copy number variations were determined using Digital Array IFCs (Fluidigm Corporation), by comparing two genes, one of which was the gene of interest (Cre-recombinase) and the other a single copy reference gene GGT1 (Pawlak et al. 1988).

Primers and TaqMan probes were designed using the Beacon Designer (PREMIER Biosoft International) software package, and were ordered from Integrated DNA Technologies (IDT, Iowa). A preliminary specific target amplification (STA) step with primers for both the gene of interest as well as the reference gene was performed, for a limited number of thermal cycles, such that the copy number of both genes was proportionately increased.

STA was performed on an Eppendorf MasterCycler Gradient machine (Hamburg, Germany) in a 5-µl reaction containing 1× TaqMan PreAmp master mix (Life Technologies, Foster City, CA), 225 nM of primers for both the GGT1 and Cre gene, and about 50 ng DNA. Thermocycling conditions were 95˚C, 10-minute hot start and 7 cycles of 95˚C for 15 seconds and 60˚C for 2 minutes. The STA products were diluted

prior to the copy number analysis on the digital array chip based on their initial concentrations so that there would be about 200 or more GGT1 positive chambers per panel.

The copy number of the Cre gene was determined using the digital array chip as described (Qin et al. 2008). Each panel of an HD digital array chip (48.770) contains a total of 0.65 µl PCR reaction mix. A 4-µl reaction mix was prepared for each panel containing 1× TaqMan gene expression master mix (Life Technologies, Foster City, CA), 1× Cre FAM TaqMan assay, 1× GGT1 Cy5 assay (900 nM primers and 200 nM probe), 2× sample loading reagent (Fluidigm, South San Francisco, CA) and STA product. The reaction mix was uniformly partitioned into the 770 reaction chambers of each panel and six panels were used for each sample. The HD digital array chip was thermocycled on the BioMark system (Fluidigm, South San Francisco, CA). Thermocycling conditions included a 95˚C, 10 minute hot start followed by 40 cycles of two-step PCR: 15 seconds at 95˚C for denaturing and 1 minute at 60˚C for annealing and extension. Molecules of the two genes were independently amplified. FAM and Cy5 signals of all chambers were recorded at the end of each PCR cycle. After the reaction was completed, Digital PCR Analysis software (Fluidigm, South San Francisco, CA) was used to process the data and count the numbers of both FAM-positive chambers (Cre gene) and Cy5-positive chambers (GGT1 gene) in each panel. Standard analysis (Dube et al. 2008) was used to convert the numbers of positive chambers to the “true numbers” of both Cre and GGT1 molecules, and the ratio of these “true numbers” (Cre/GGT1) were calculated.

Given that there are 2 copies of GGT1 in the genome, a Cre/GGT1 ratio of 0.5 would indicate a copy number of 1 for Cre. However, the DNA was pre-amplified before the quantification (as described above), and during this step Cre amplifies slightly more efficiently than GGT1 (based on sequence differences as predicted by the Fluidigm Analysis Package, Qin et al. 2008). This difference generated a measured Cre/GGT1 ratio of ~0.7 which was slightly bigger than the predicted ratio of 0.5 for the single copy number lines (e.g. TH::Cre 3.1+/-). For all lines, the Cre/GGT1 ratio was a whole number multiple of 0.7, and that number was taken to be the estimated copy number.

Sequences:
GGT1: Forward Primer – CCACCCCTTCCCTACTCCTAC; Reverse Primer – GGCCACAGAGCTGGTTGTC; Probe: Cy5-CCGAGAAGCAGCCACAGCCATACCT-BHQ2
Cre: Forward Primer – TACCTGTTTTGCCGGGTCAG; Reverse Primer – CCTTAGCGCCGTAAATCAATCG; Probe: FAM-CGCCATCTGCCACCAGCCAGC-BHQ2

Supplemental References

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