Supplemental Figure 1.

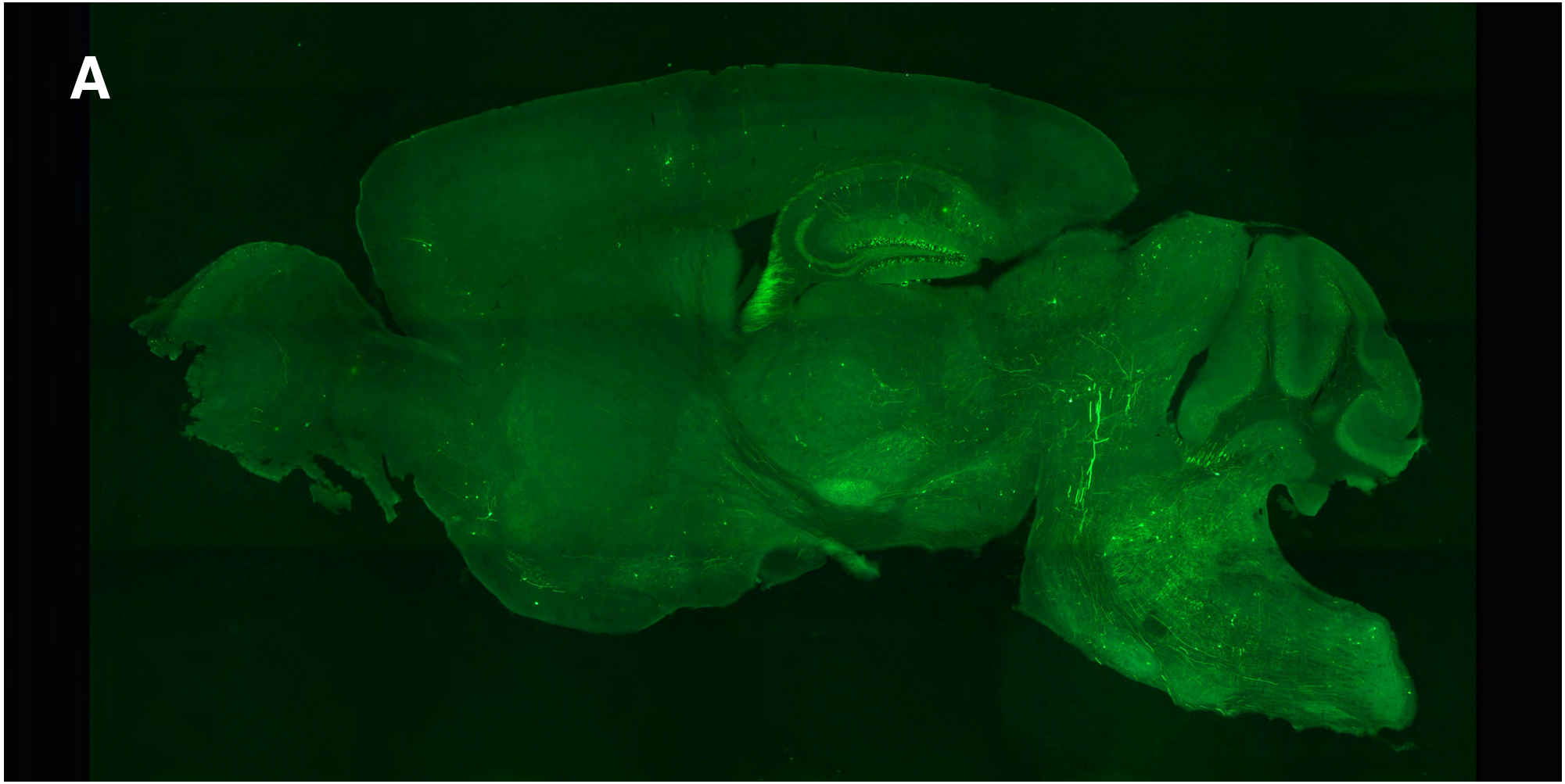
Comparison of YFP labeling in four SLICK-V mice.

YFP fluorescence was imaged in sagittal sections from SLICK-V transgenic mice.

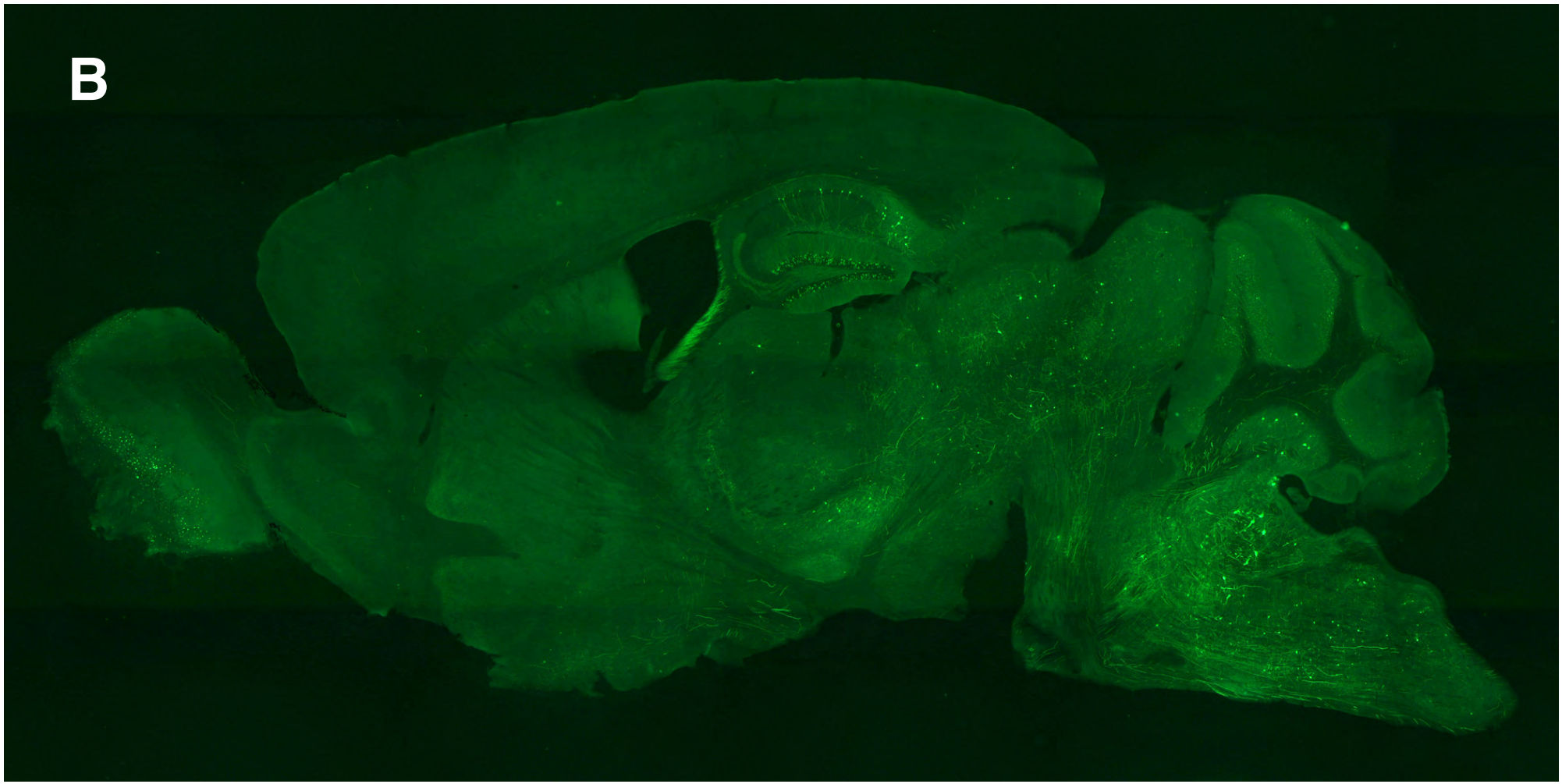
A and B. Images from two different mice of the same generation bred at Duke University. **C and D.** Images from two mice that had been bred independently for at least six generations in University College Cork. These mice exhibit the same expression pattern as those shown in A and B. While some differences in total number of labeled neurons are apparent between individuals, the pattern of YFP expression is extremely similar with labeled neurons found in the same brain regions in different animals.

Methods

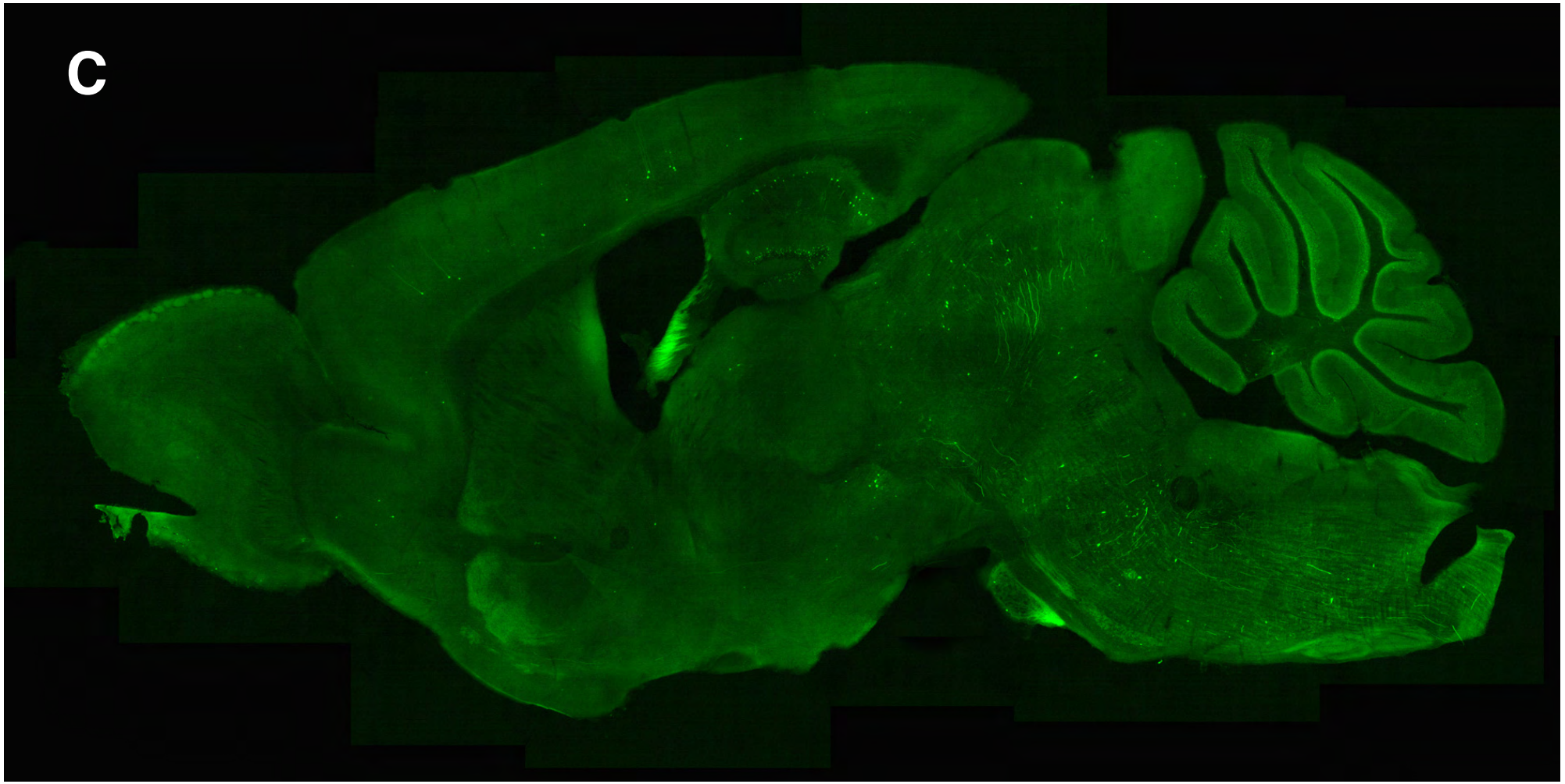
Mice were anesthetized and perfused through the heart, first with lactated Ringer's solution, then with 4% para-formaldehyde. For A and B, 50 μm vibrotome sections were mounted in 90% glycerol with 0.1% p-phenylenediamine and images acquired on a Zeiss Axioskop 2 fluorescent microscope using a 4X objective. For C and D, 66 μm vibrotome were dried onto gelatin coated slides, dehydrated in increasing concentrations of ethanol, allowed to dry, cleared twice in Xylene, mounted using Krystalon mounting medium (Merck) and imaged on an Olympus Provis AX 70 upright microscope using a 4x objective. Whole brain montages were generated using the Photoshop software package. Higher background fluorescence in C and D is due to the use of slightly different mounting and imaging methods.



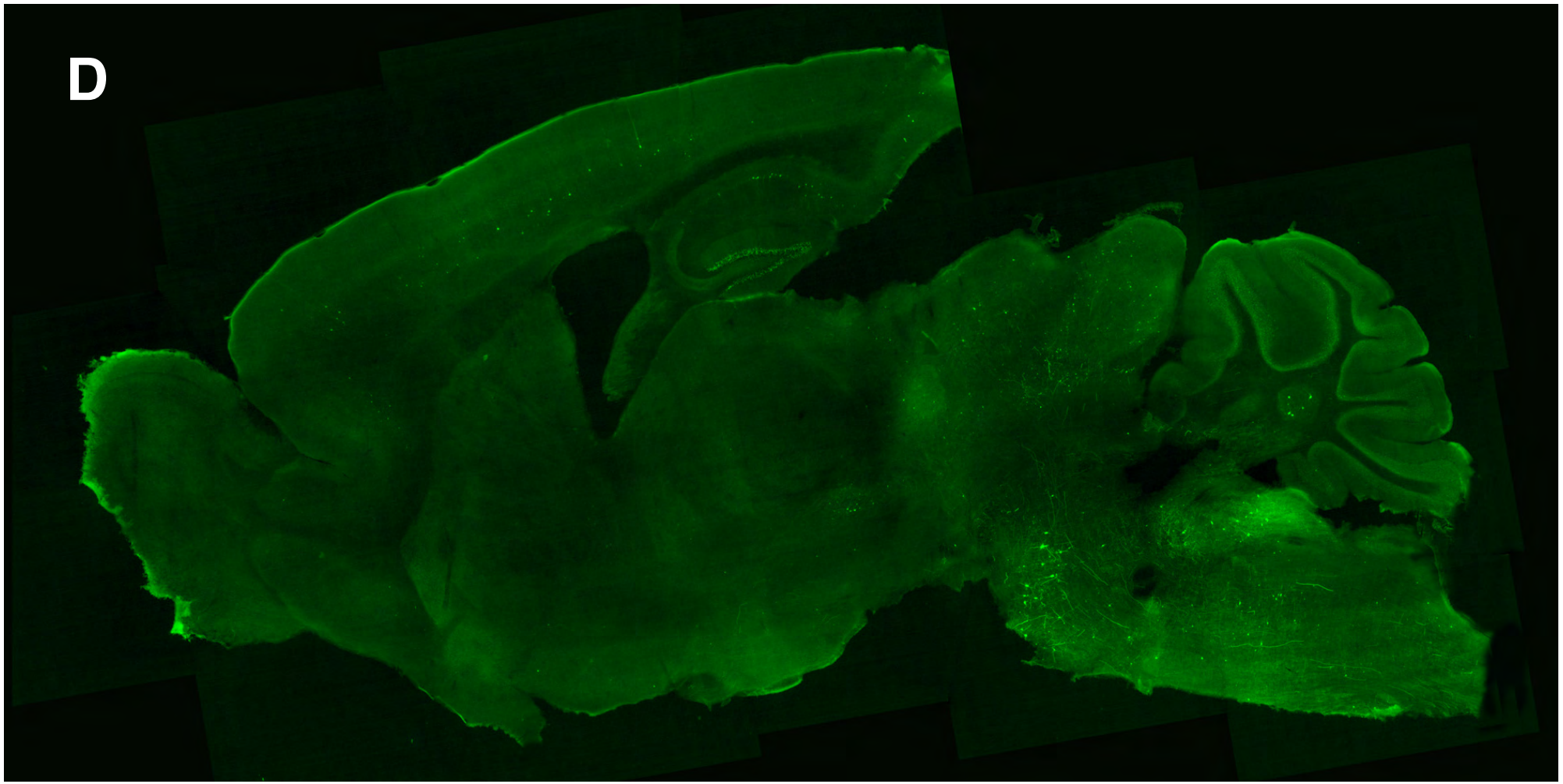
Young et al., Supplemental Fig. 1



Young et al., Supplemental Fig. 1



Young et al., Supplemental Fig. 1



Young et al., Supplemental Fig. 1

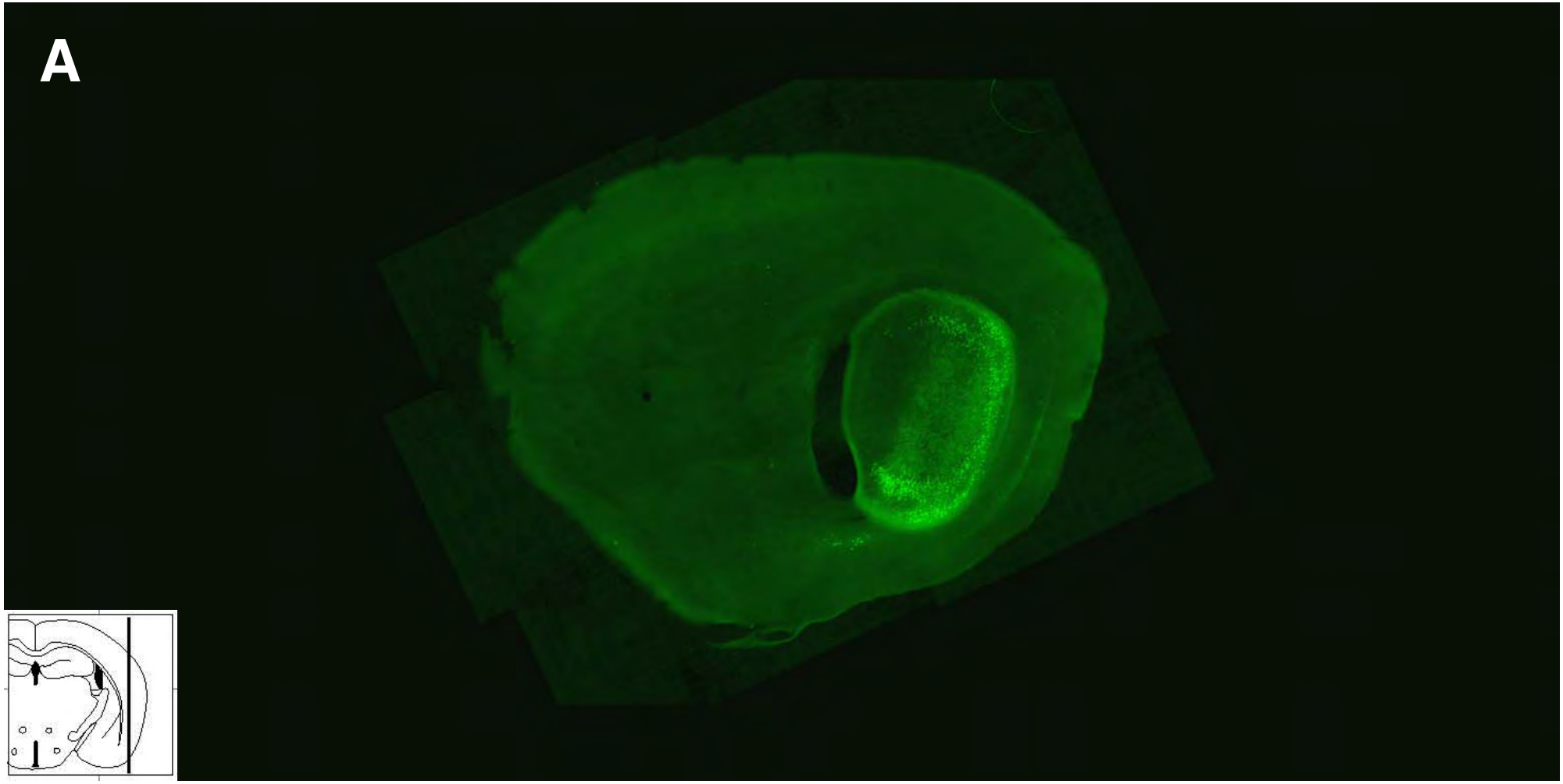
Supplemental Figure 2.

YFP fluorescence in sagittal brain sections from a SLICK-V transgenic mouse showing YFP labeling of subsets of neurons.

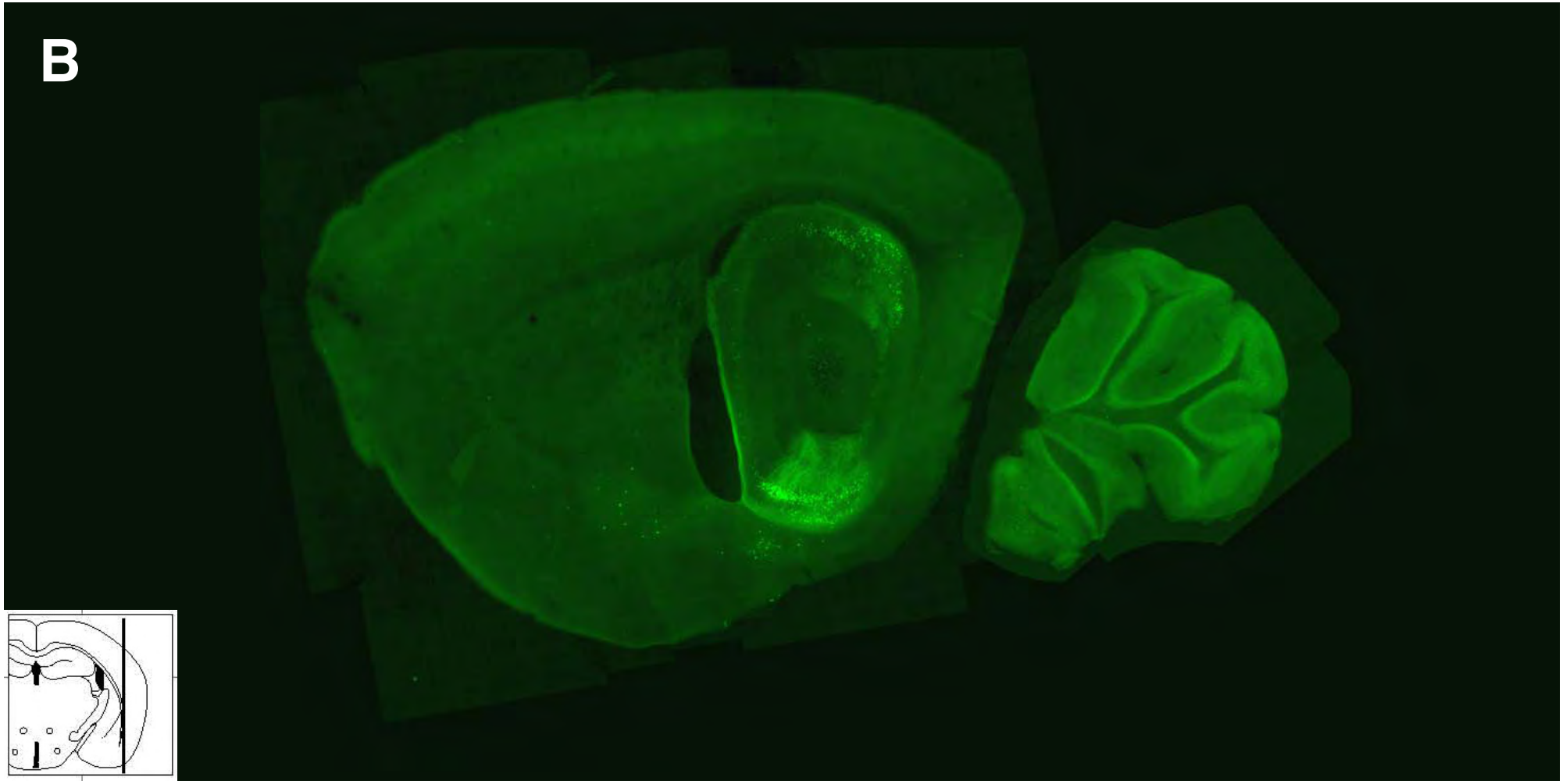
A-I. A series of eights sections spaced 300-500 μm apart. The lateral position of each section is indicated by the schematic in the lower left corner. Sections are from a female mouse aged 21 weeks with a C57/BL6 genetic background.

Methods

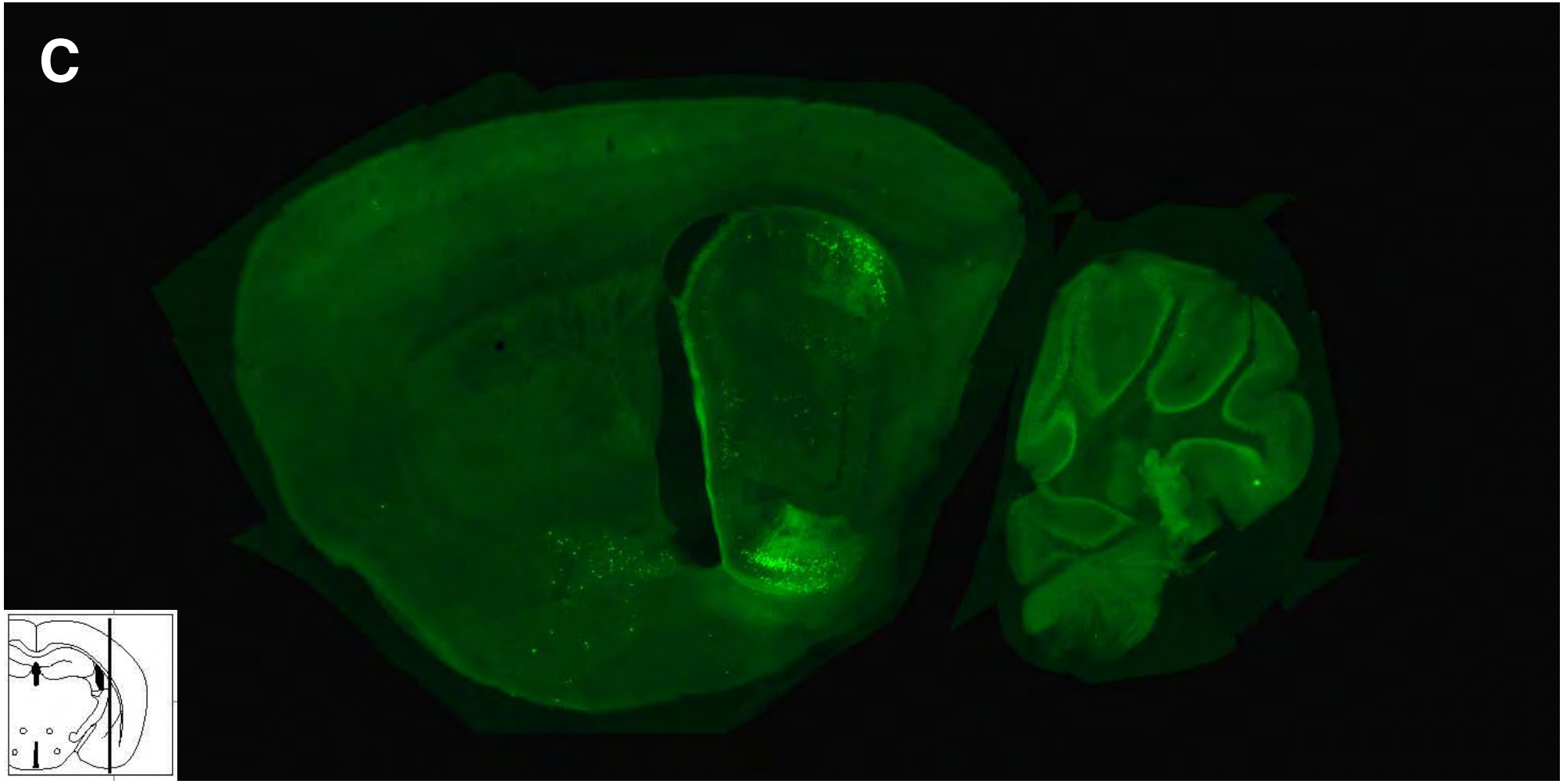
Mice were anesthetized and perfused through the heart, first with lactated Ringer's solution, then with 4% para-formaldehyde. 66 μm sections were cut using a vibrotome. Sections were dried onto gelatin coated slides, dehydrated in increasing concentrations of ethanol, allowed to dry, cleared twice in Xylene and mounted using Krystalon mounting medium (Merck). YFP fluorescence was imaged on an Olympus Provis AX 70 upright microscope using a 4x objective. Whole brain montages were generated using the Photomerge function in the Photoshop software package.



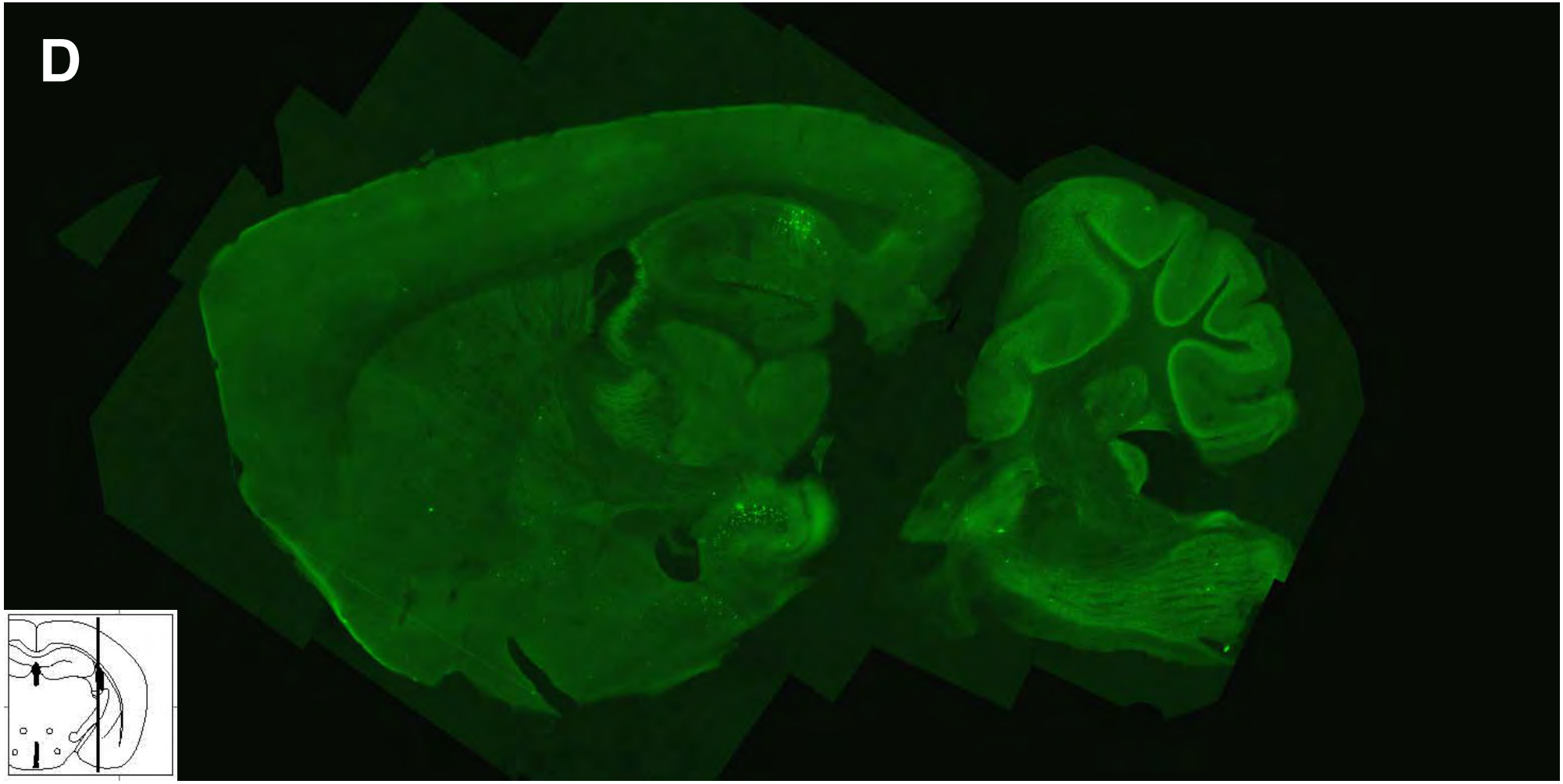
Young et al., Supplemental Fig. 2



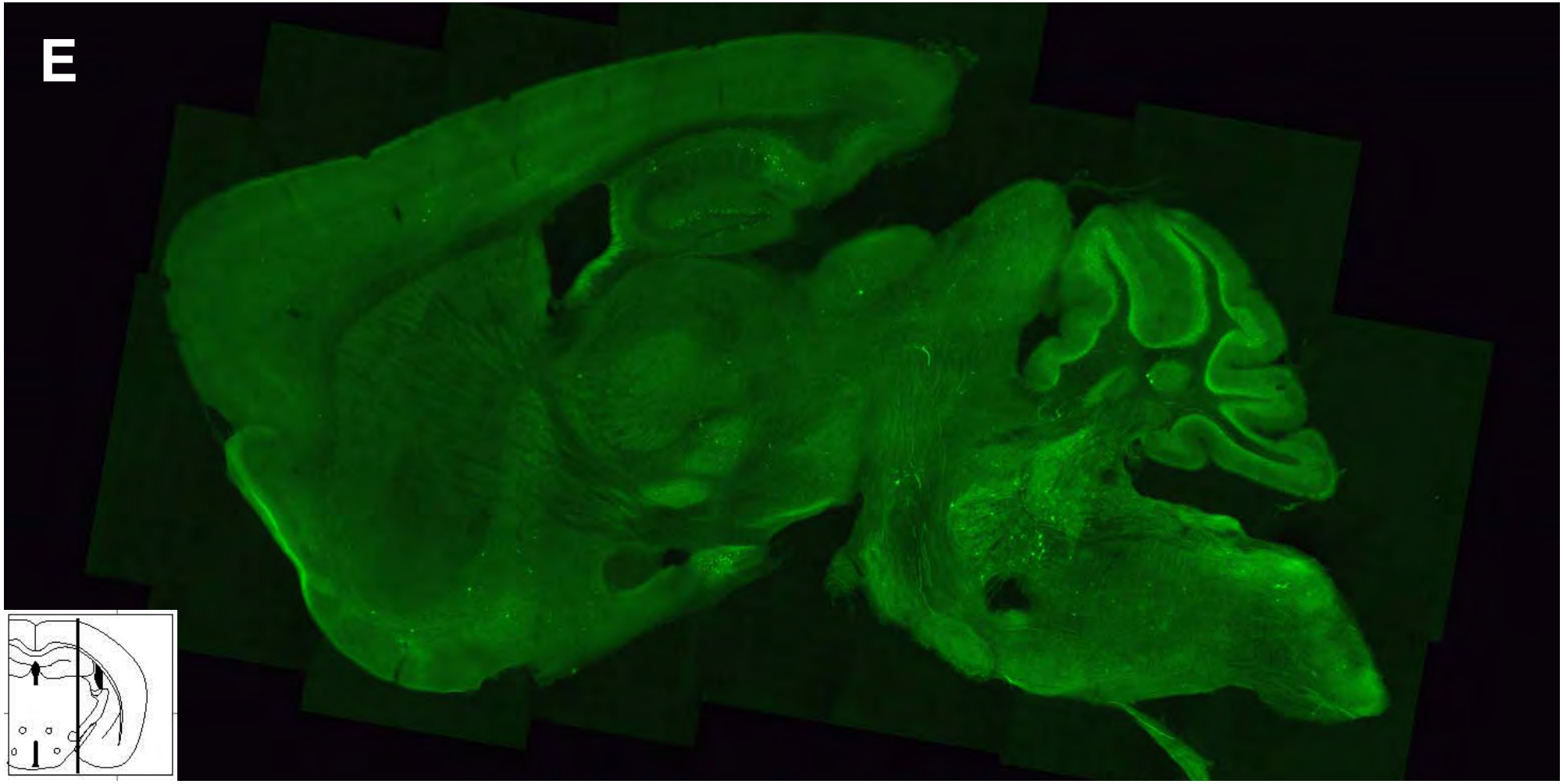
Young et al., Supplemental Fig. 2



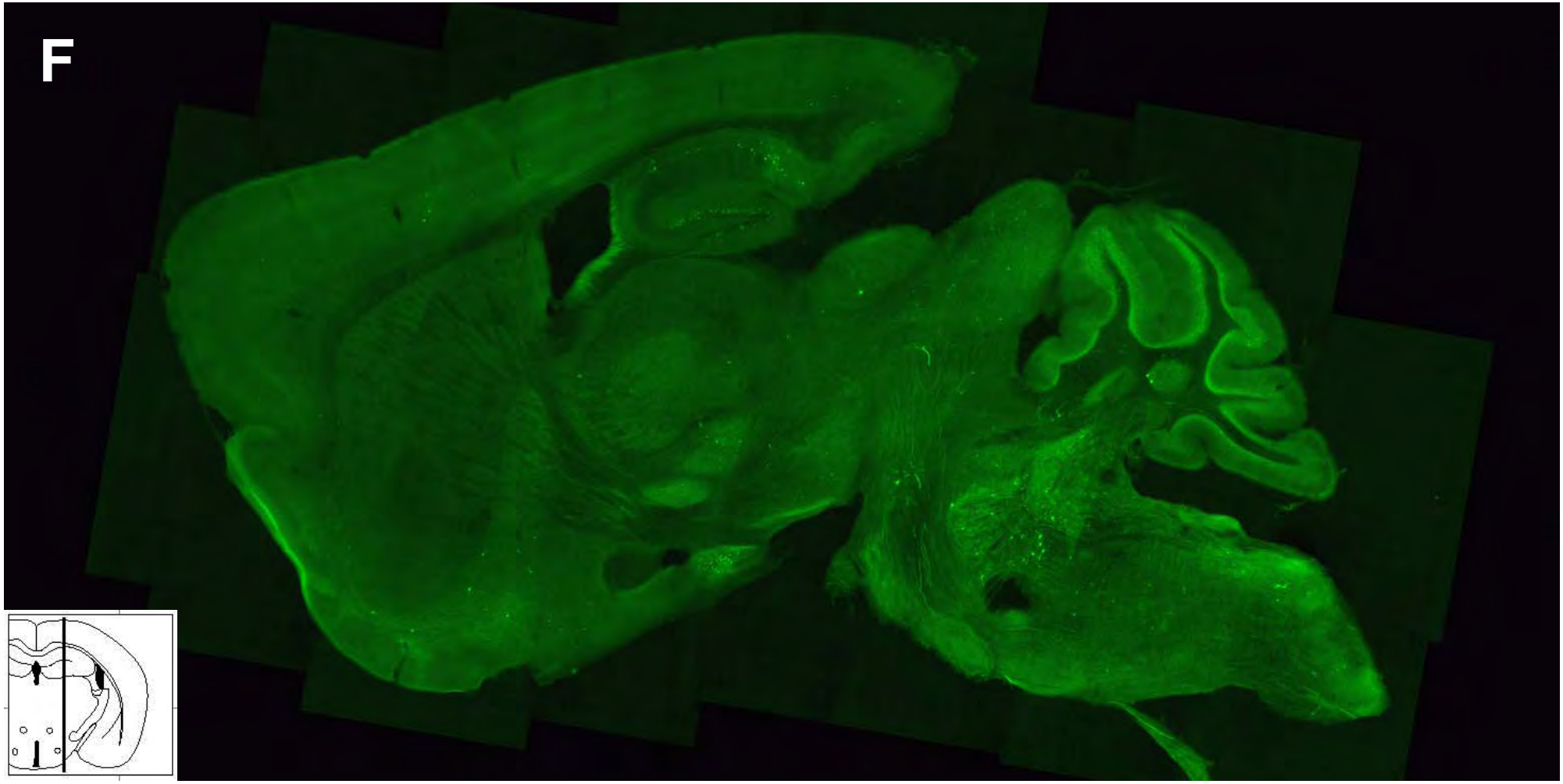
Young et al., Supplemental Fig. 2



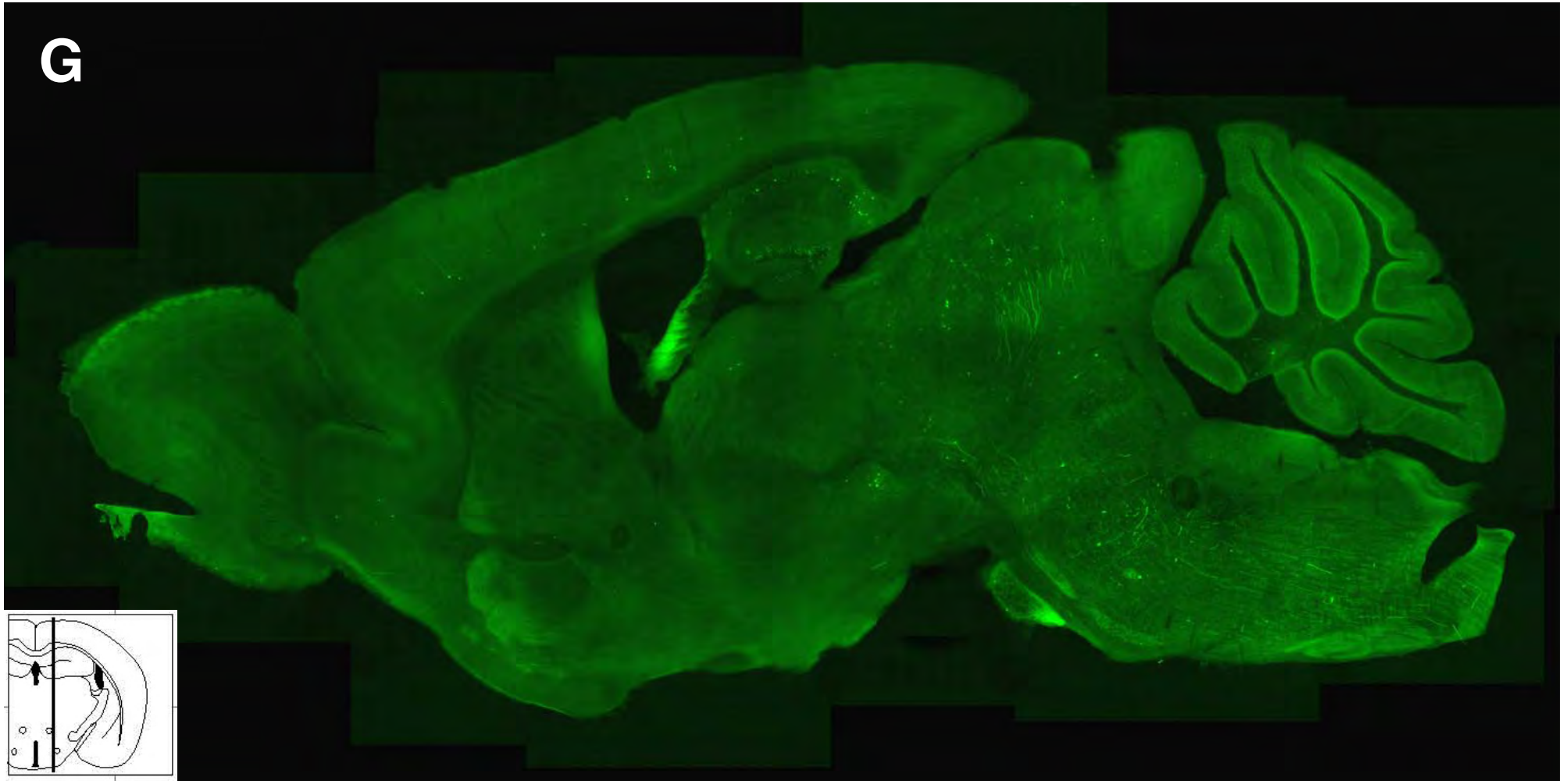
Young et al., Supplemental Fig. 2



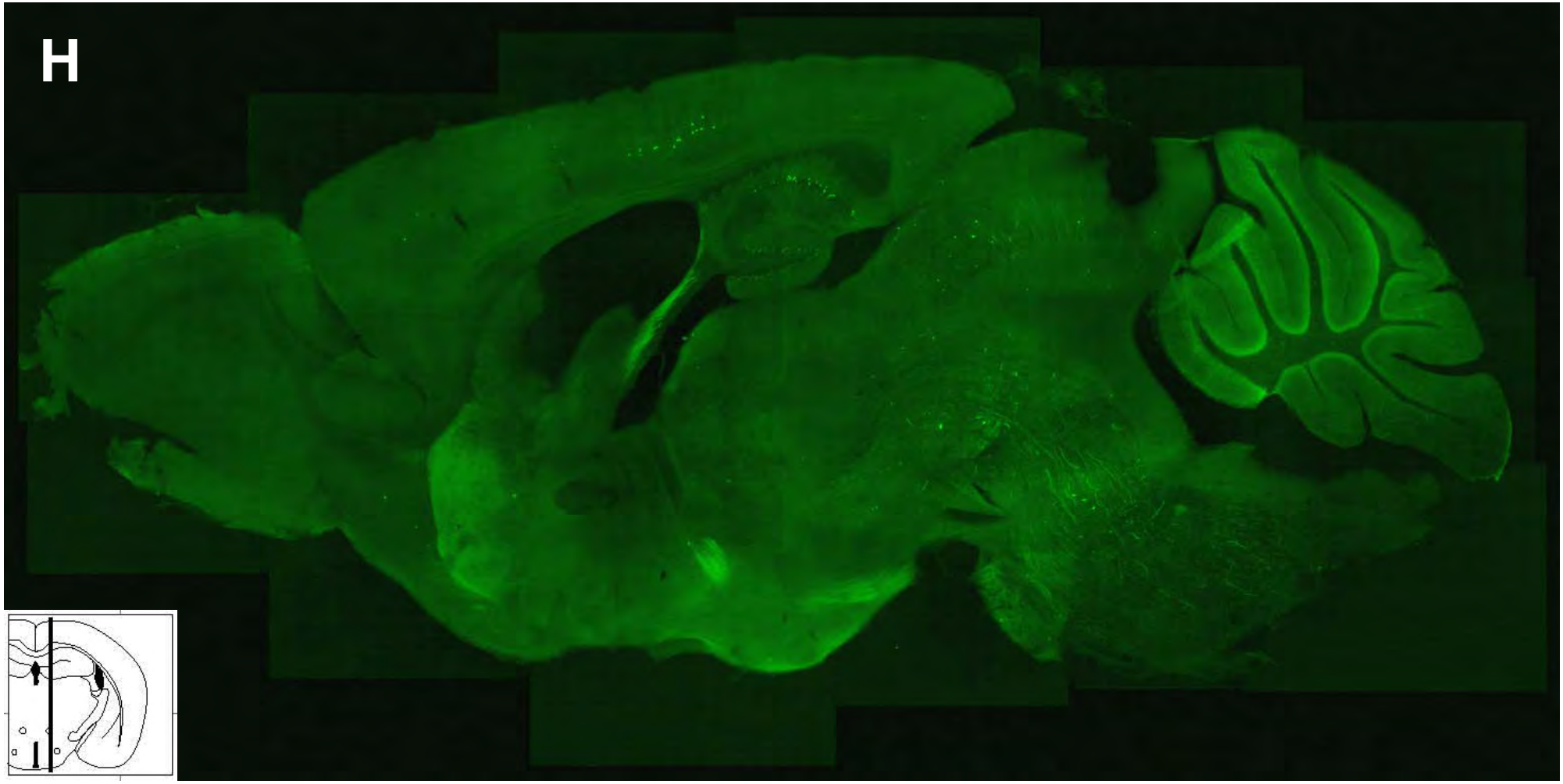
Young et al., Supplemental Fig. 2



Young et al., Supplemental Fig. 2



Young et al., Supplemental Fig. 2



Young et al., Supplemental Fig. 2

Supplemental Figure 3.

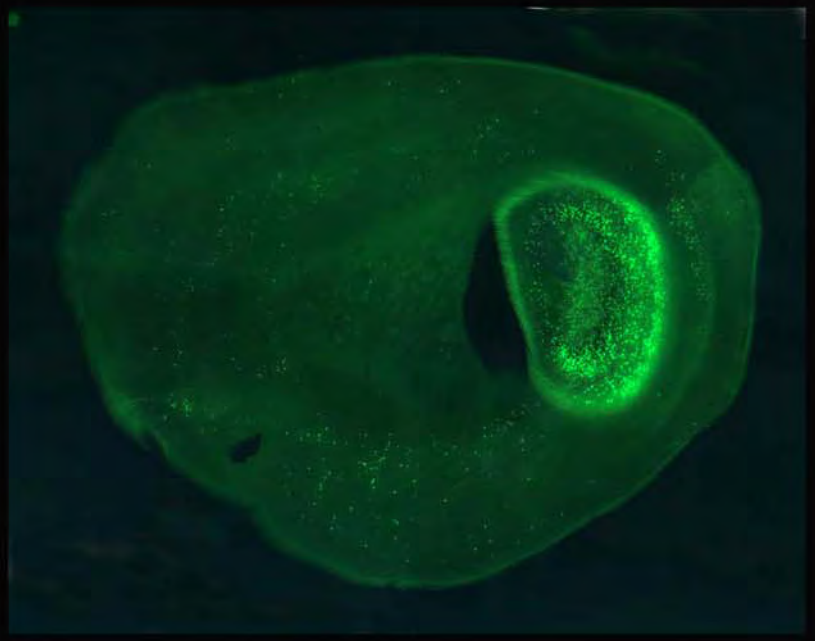
YFP fluorescence in sagittal brain sections from a SLICK-X transgenic mouse showing YFP labeling of subsets of neurons.

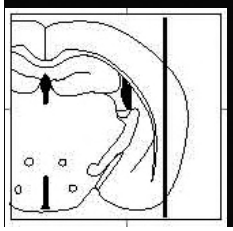
A-I. A series of eight sections spaced 300-500 um apart. The lateral position of each section is indicated by the schematic in the lower left corner. Sections are from a male mouse aged 24 weeks with a C57/BL6 genetic background.

Methods

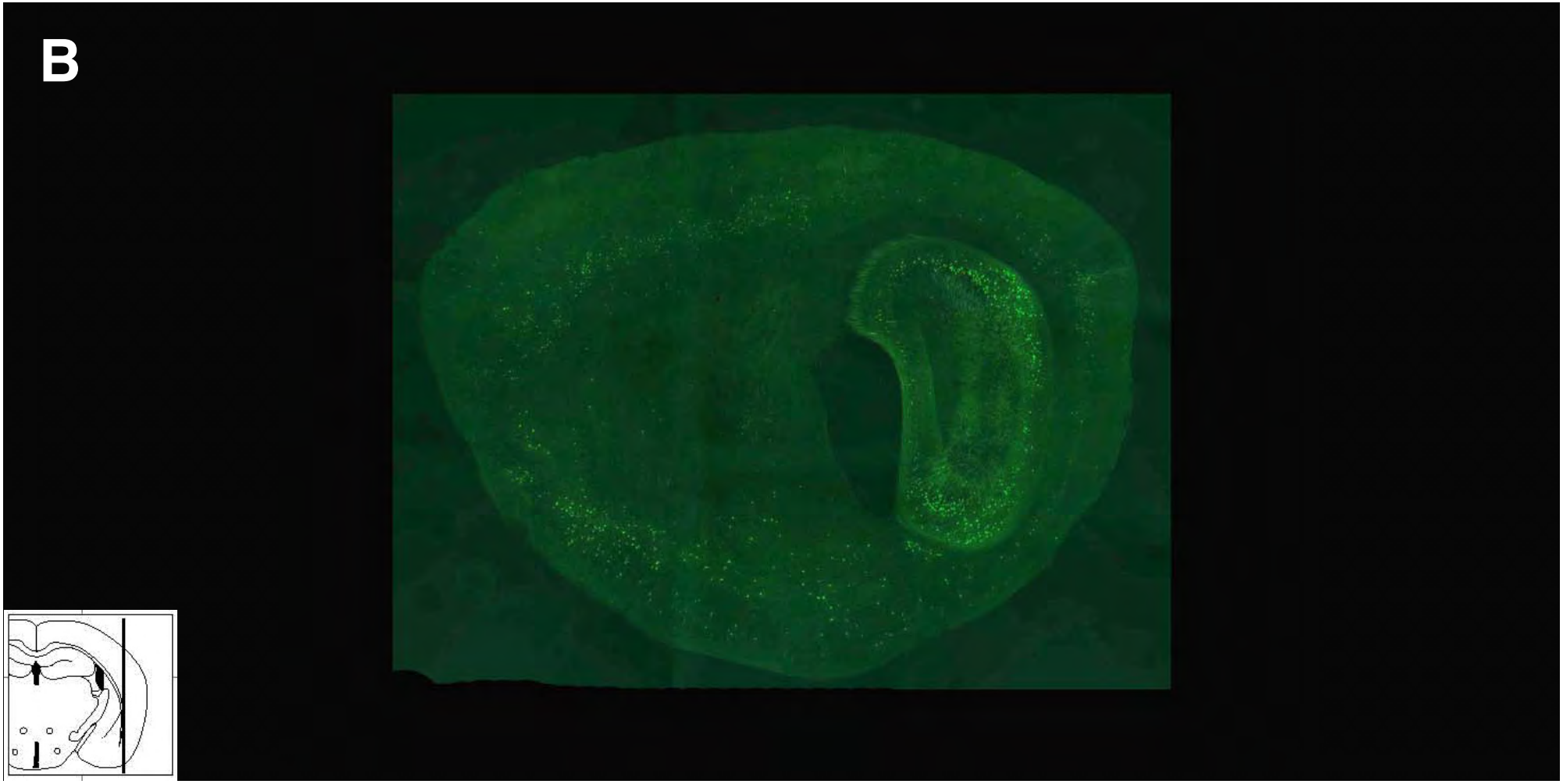
Mice were anesthetized and perfused through the heart, first with lactated Ringer's solution, then with 4% para-formaldehyde. 50 μm sections were cut using a vibrotome. Sections were mounted in 90% glycerol with 0.1% p-phenylenediamine. Images were acquired on a Zeiss Axioskop 2 fluorescent microscope using a 4X objective. Whole brain montages were generated using the Photoshop software package.

A

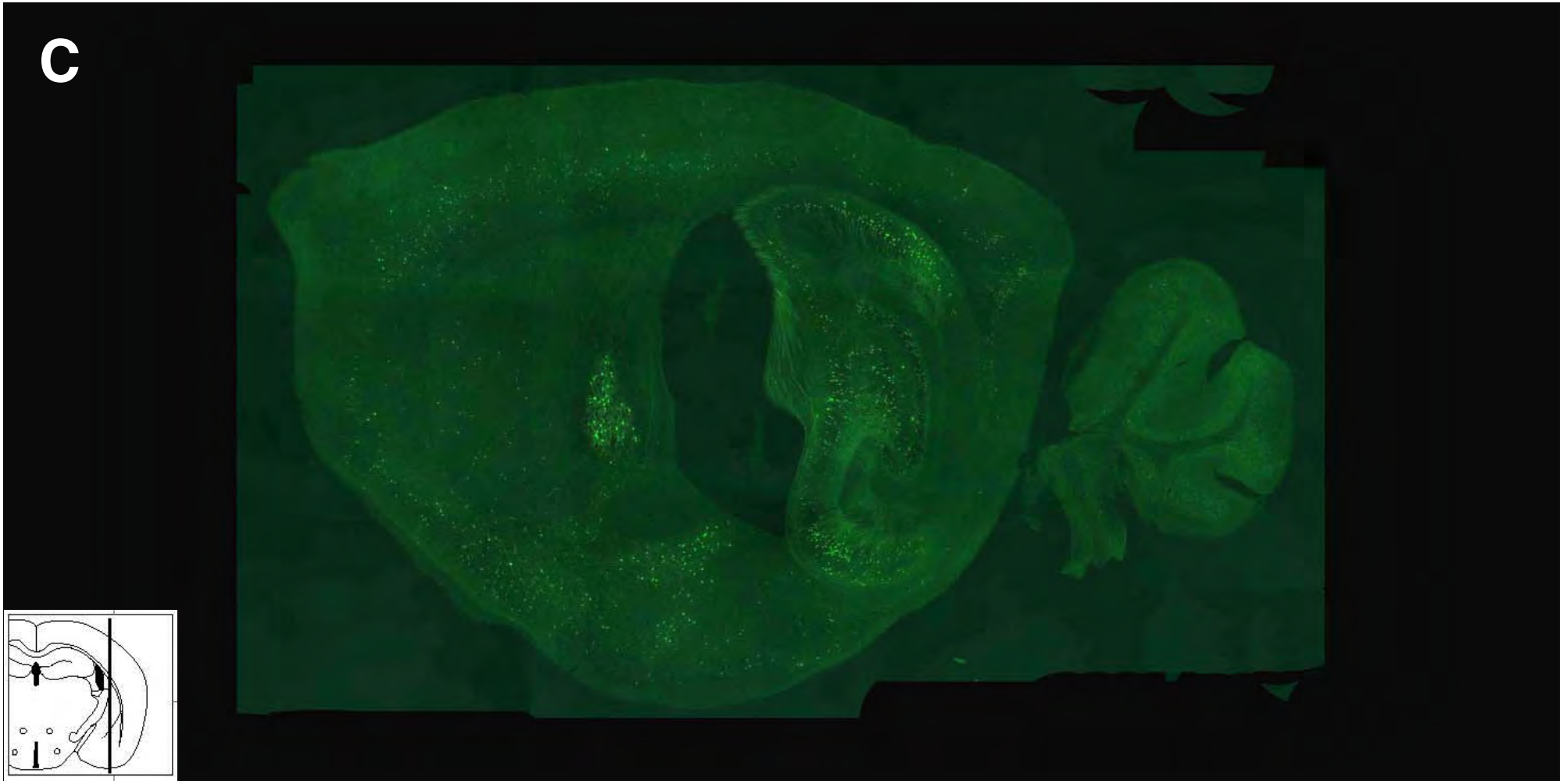




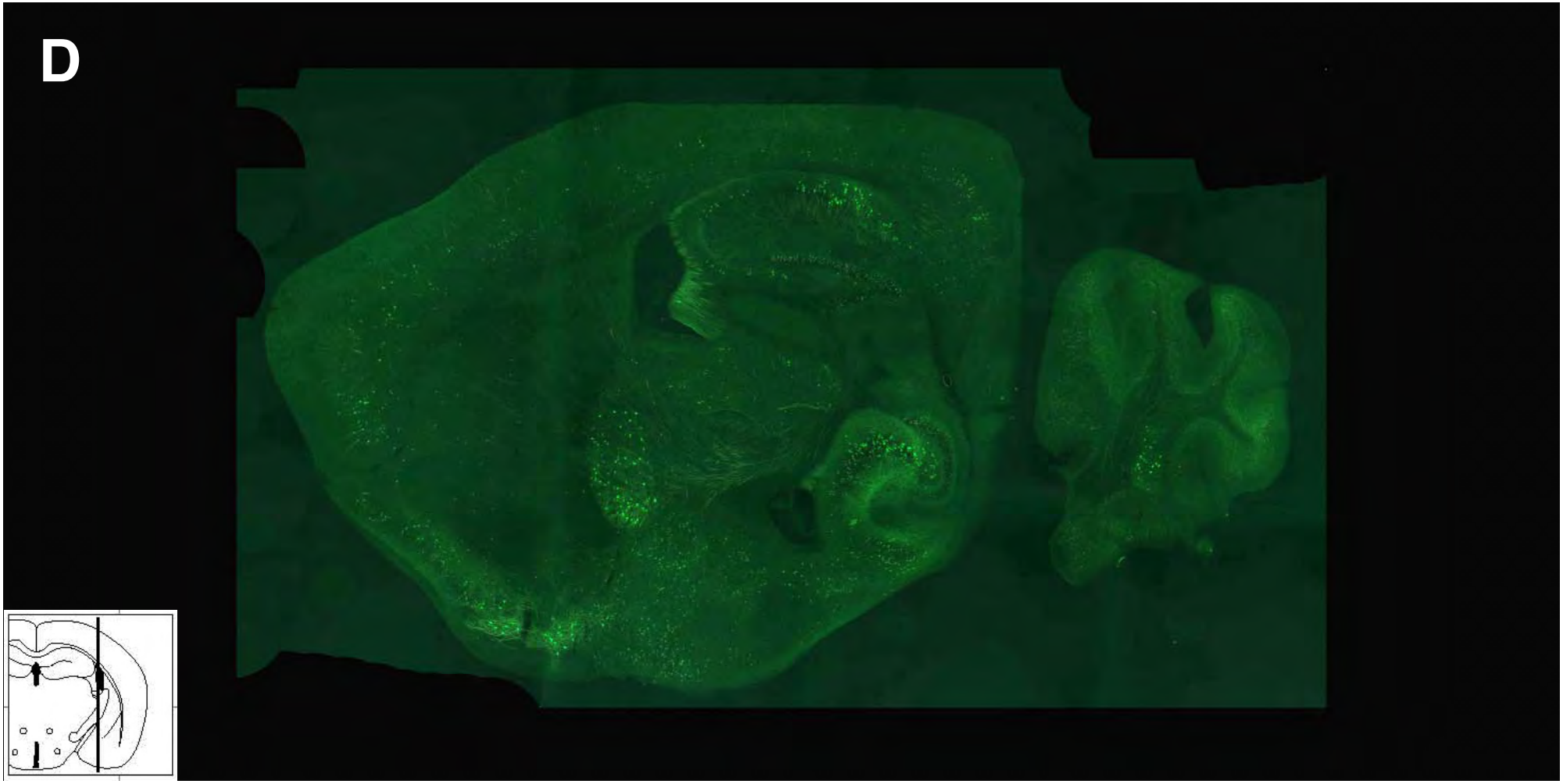
Young et al., Supplemental Fig. 3



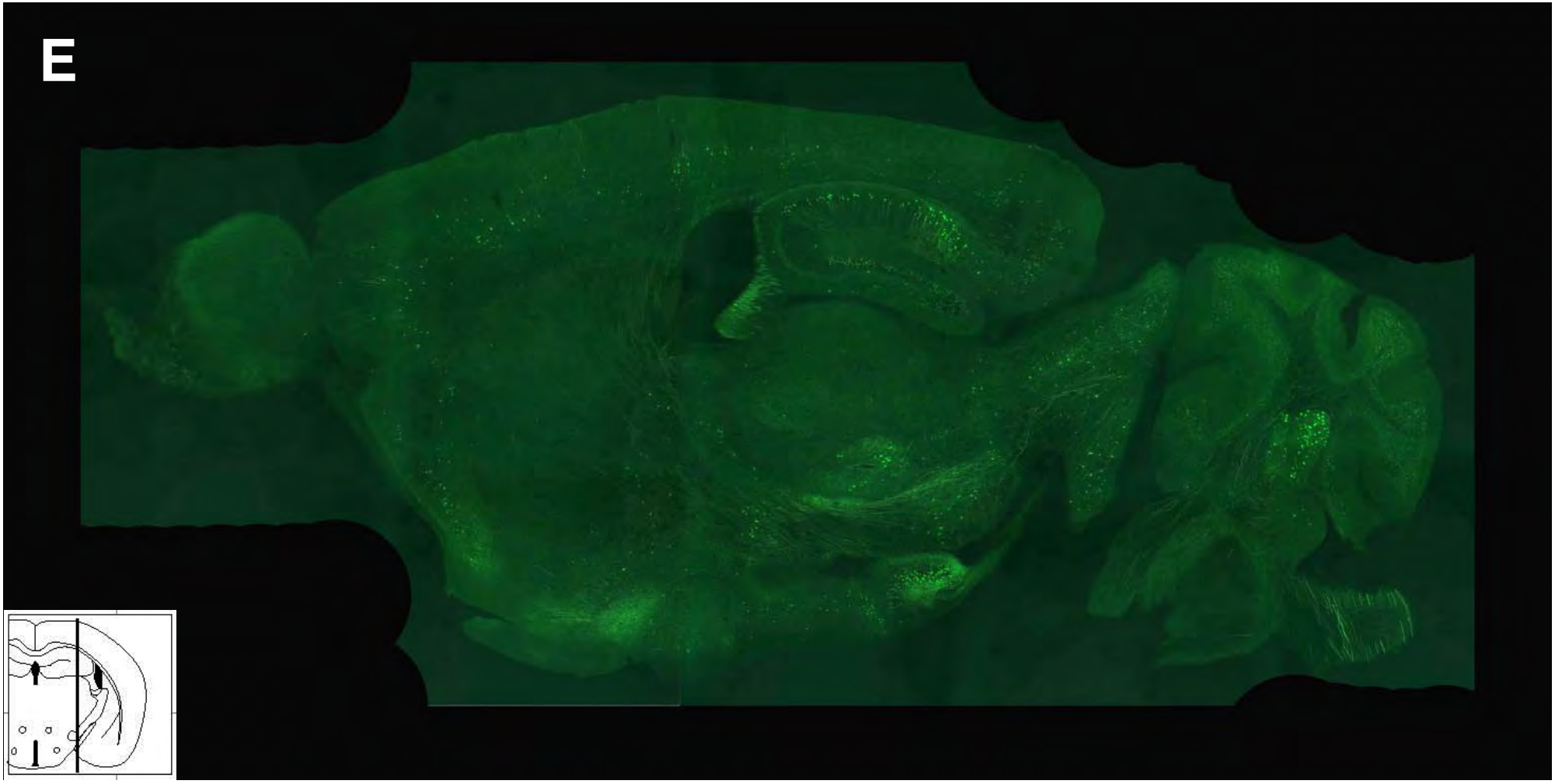
Young et al., Supplemental Fig. 3



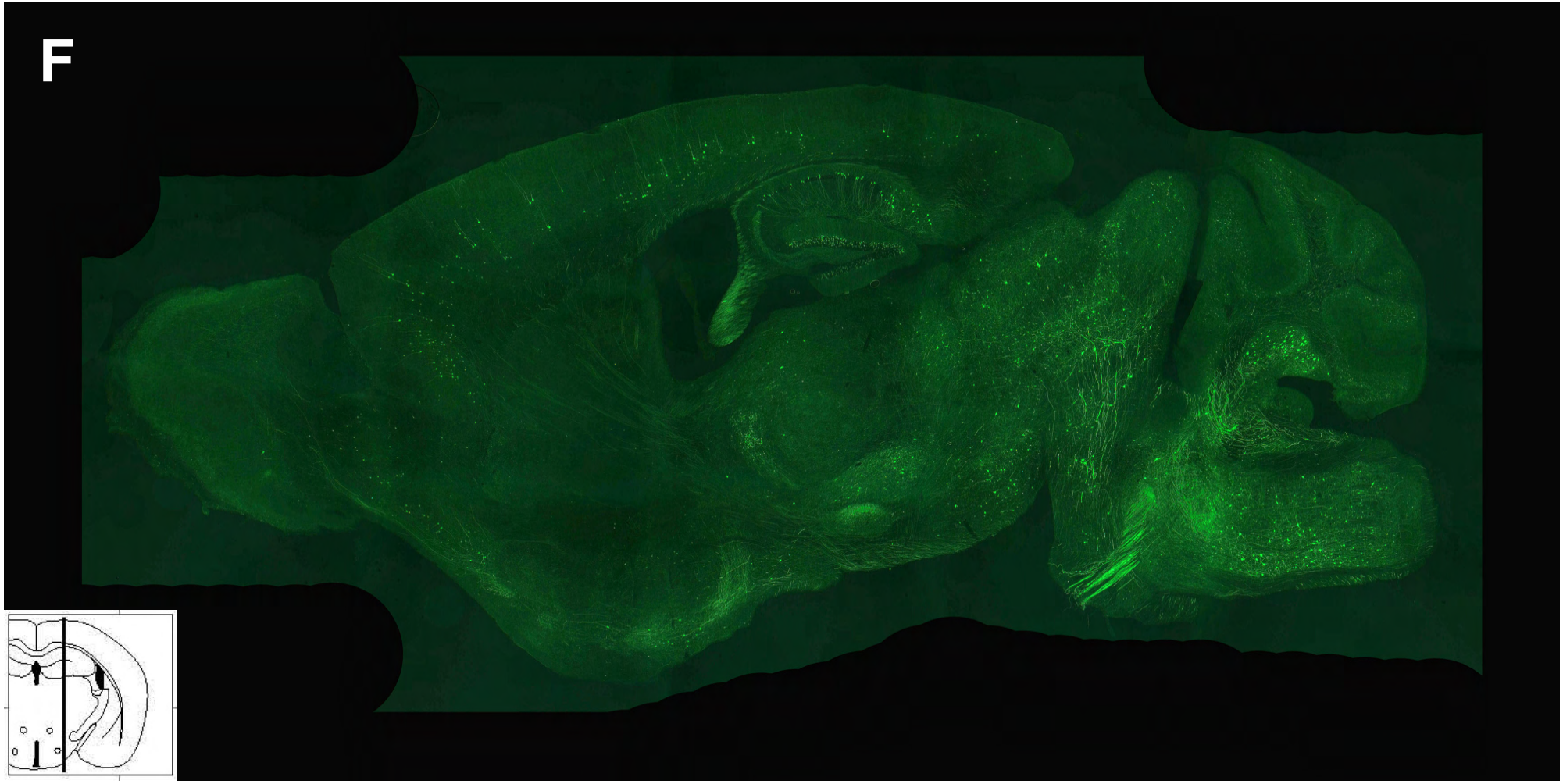
Young et al., Supplemental Fig. 3



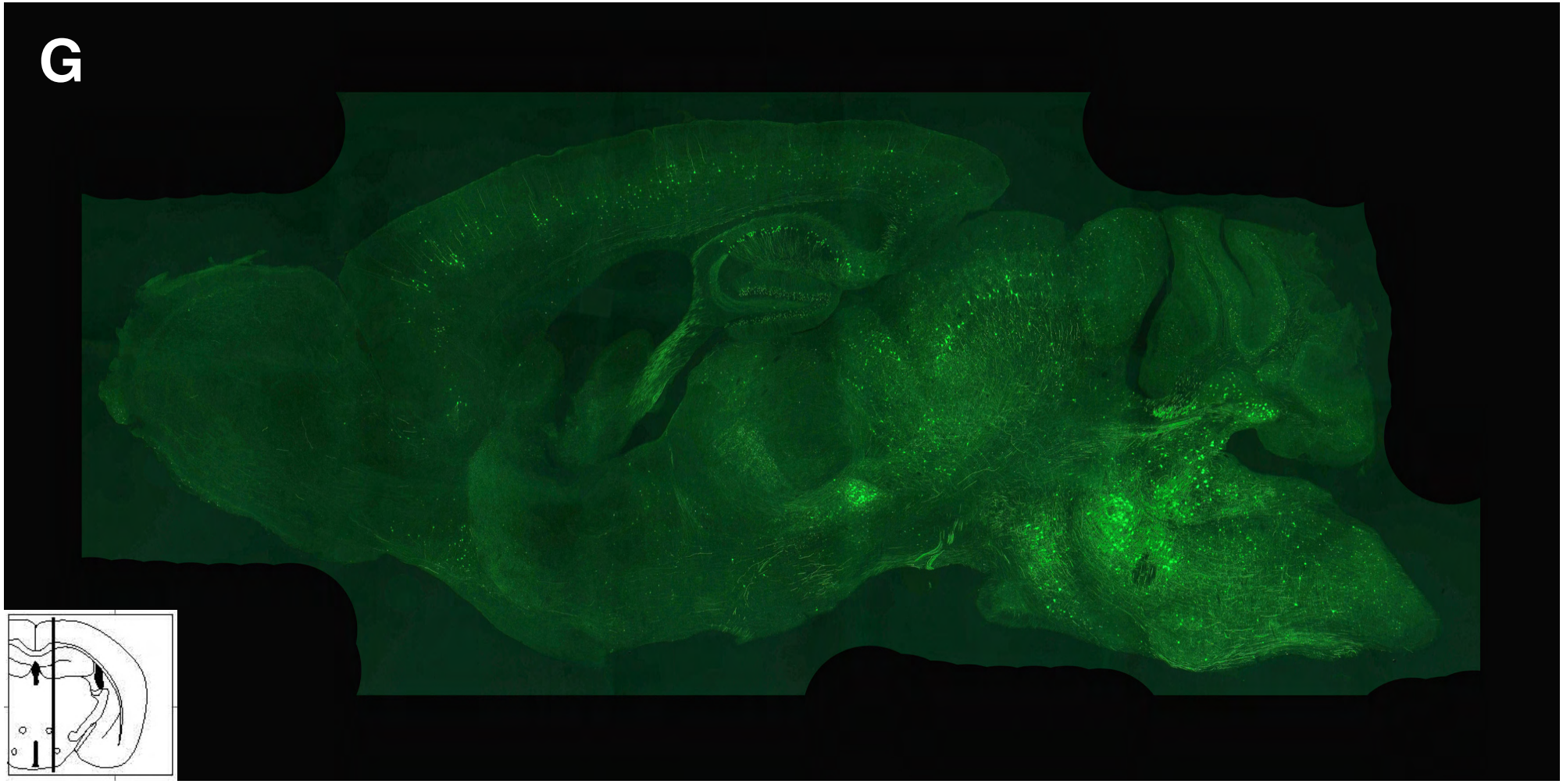
Young et al., Supplemental Fig. 3



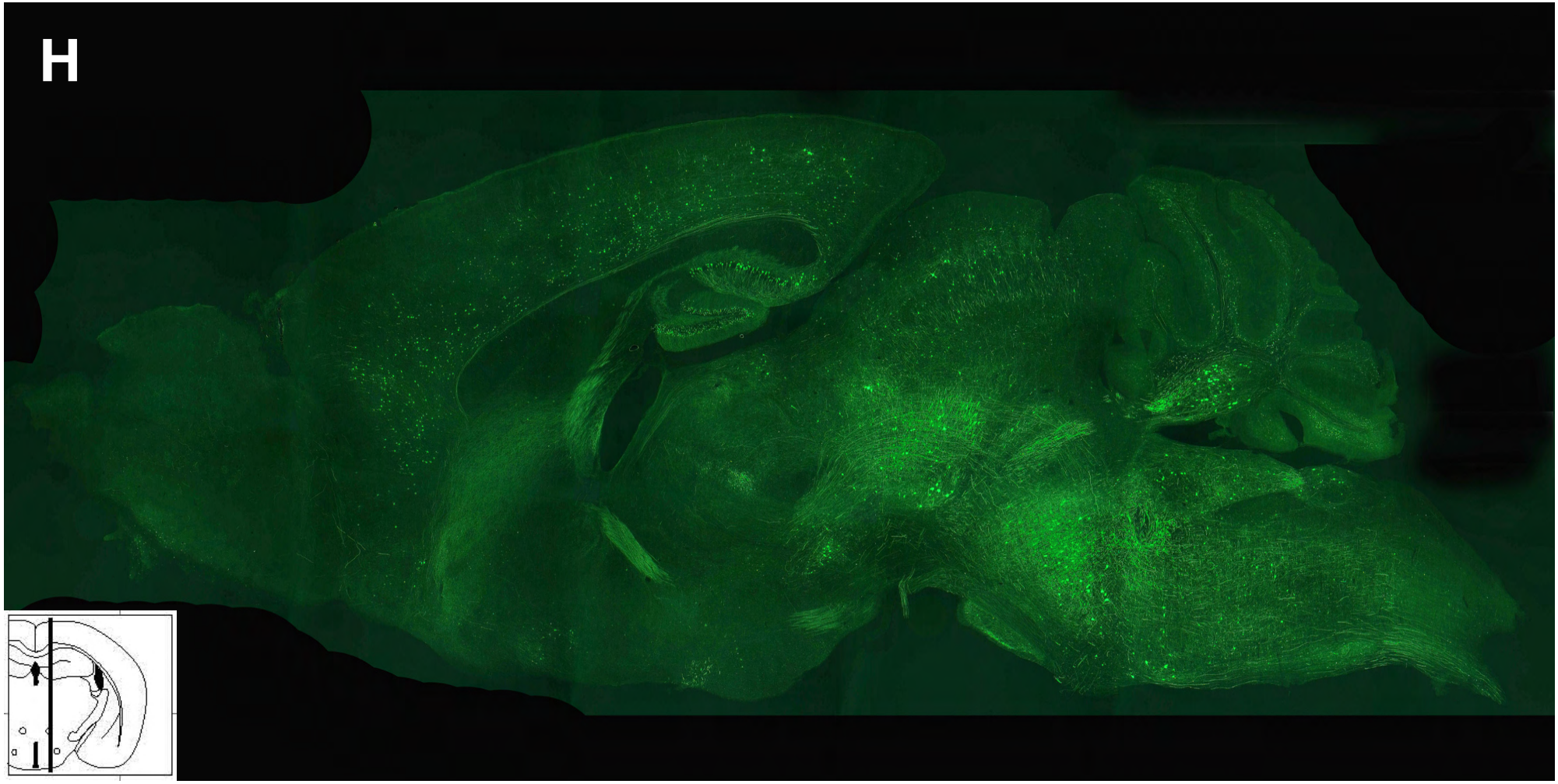
Young et al., Supplemental Fig. 3



Young et al., Supplemental Fig. 3



Young et al., Supplemental Fig. 3



Young et al., Supplemental Fig. 3

Supplemental Figure 4. Timing of recombination and reporter gene expression in SLICK / R26R mice.

To determine the time course of cre-mediated recombination in SLICK mice we used semi-quantitative PCR to detect the deletion of the floxed Neo/STOP cassette at the ROSA locus after a single dose of tamoxifen treatment. Recombination was detected 24 hours after tamoxifen treatment and had increased further by 48 hours (Supplementary Fig 4A). These results are in line with previous studies that reported activation of inducible forms of cre, and recombination, occuring within 24 hours following tamoxifen administration[1].

Since recombination occurs relatively rapidly the more important parameter to monitor when using the SLICK system is the length of time that it takes for the gene product of interest to dissappear following gene knockout or to be produced following conditional gene expression. For gene knockout this parameter will depend on mRNA and protein stability and will have to be determined individually for each gene product. For conditional gene expression using SLICK the rate of transgene production will be critical and will depend on the strength of the promoter being used.

We determined the timing of LacZ reporter gene expression from the ROSA locus in SLICK-V/R26R mice. LacZ expression was monitored by immunofluorescent microscopy 1,2, 5 and 10 days following a single dose of tamoxifen, in a "pulse-chase" type experiment. Significant expression of LacZ was not detected until 5 days following tamoxifen treatment and robust expression was seen by 10 days (Supplementary Fig 4B and 4C). Similar results were obtained with an X-gal based enzymatic assay for β-galactosidase, which is potentially more sensitive (not shown). Thus for the ROSA26 promoter there is a lag of several days following recombination before transgene expression was detectable. In addition, our data indicate that a single dose of tamoxifen is not sufficient to induce maximum recombination in adult SLICK mice (Supplementary Fig. 4B and 4C).

1. Hayashi, S. & McMahon, A.P. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* **244**, 305-18. (2002).

Supplemental Figure 4. Timing of recombination and reporter gene expression in SLICK / R26R mice.

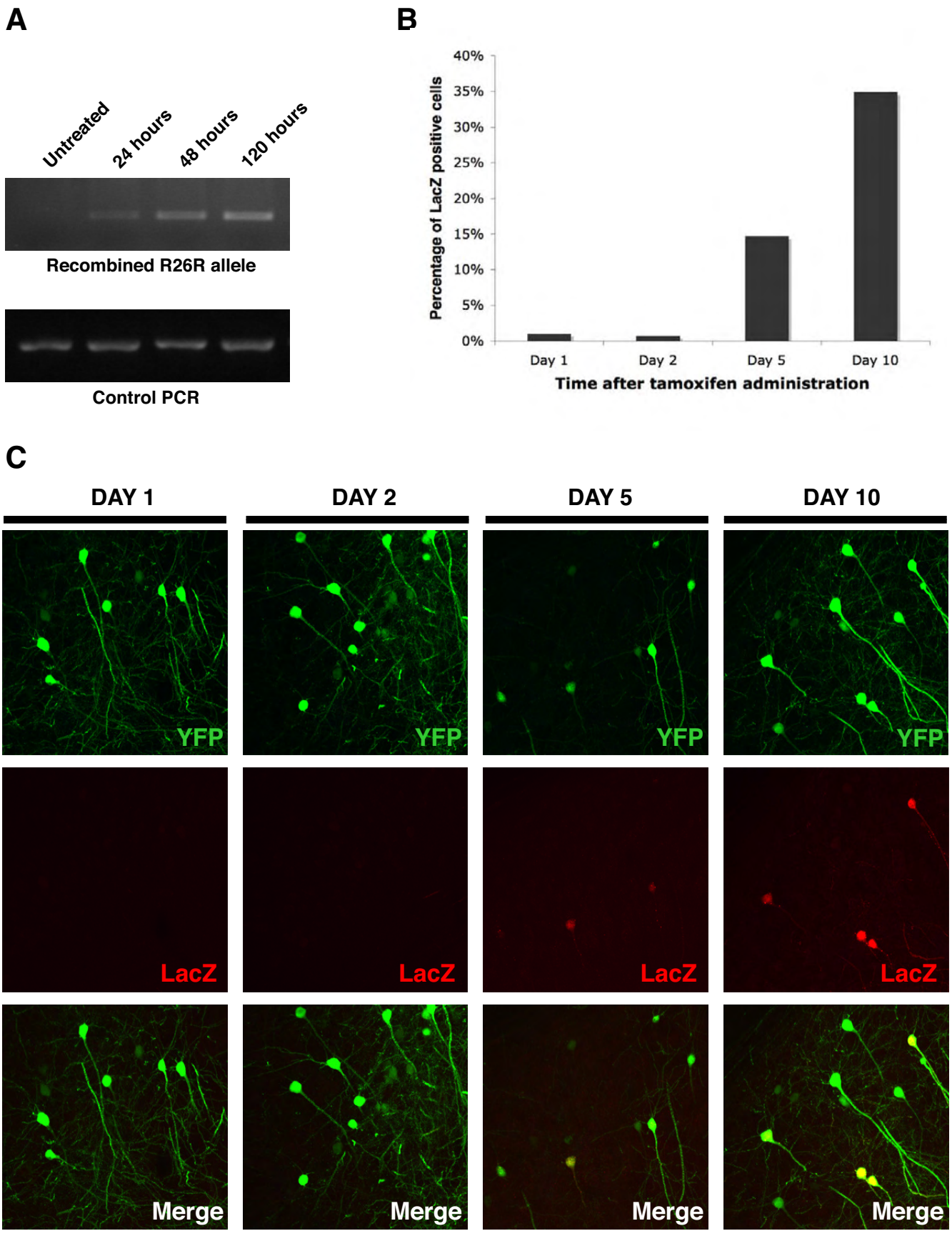
SLICK/R26R double heterozygous mice were treated with a single dose of tamoxifen and then killed and analyzed at the time points indicated.

A. Polymerase chain reaction (PCR) performed using forebrain genomic DNA as template and a primer pair that yields a 500 base pair product upon cre-mediated deletion of the Neo^R/STOP casette in R26R mice (top panel). Bottom panel shows a control PCR at a different genomic locus. Recombination appears to be largely complete by 48 hours in this semi quantitative assay.

B and C. Detection of LacZ reporter gene expression by immunofluorescent microscopy in the subiculum and CA1 hippocampal regions of SLICK-V / R26R mice. The percentage of YFP labeled neurons that are LacZ positive at each time point are quantified in (B). Representative images for each time point are shown in (C). Significant expression of LacZ was not detected until 5 days following treatment and robust expression was seen by 10 days. Note that the percentage of recombination observed is in line with what might be expected for a single dose of tamoxifen, given that 5 doses yield a recombination rate of 96% (Table 2). A similar time course of LacZ expression was observed in all other regions of the brain examined (data not shown). At least three brain sections from two mice were quantified for each time point. The number of YFP expressing neurons scored was n=196, 272, 68, 186 for days 1,2,5 and 10 respectively.

Methods

Mice were administered a single dose of 8mg tamoxifen by oral gavage. For PCR analysis SLICK-H / R26R mice were killed at different time points and genomic DNA isolated from forebrain tissue using the Nucleospin kit (Macherey Nagel, Düren, Germany). Semi quantitative PCR to detect recombination of the R26R reporter gene was performed using the primers R26R-oIMR0883 (AAAGTCGCTCTGAGTTGTTAT) and LacZ-R1 (CCCAGTCACGACGTTGTAAAACG). This primer pair yields a product of apporximately 500bp upon cre-mediated deletion of the / STOP casette (Neomycin resistance gene and multiple poly adenylation signal). For the control PCR primers to detect the wildtype ChAT locus were used (see genotyping methods in manuscript). Immunohistochemistry and imaging for 4B and 4C was performed as described in the manuscript.



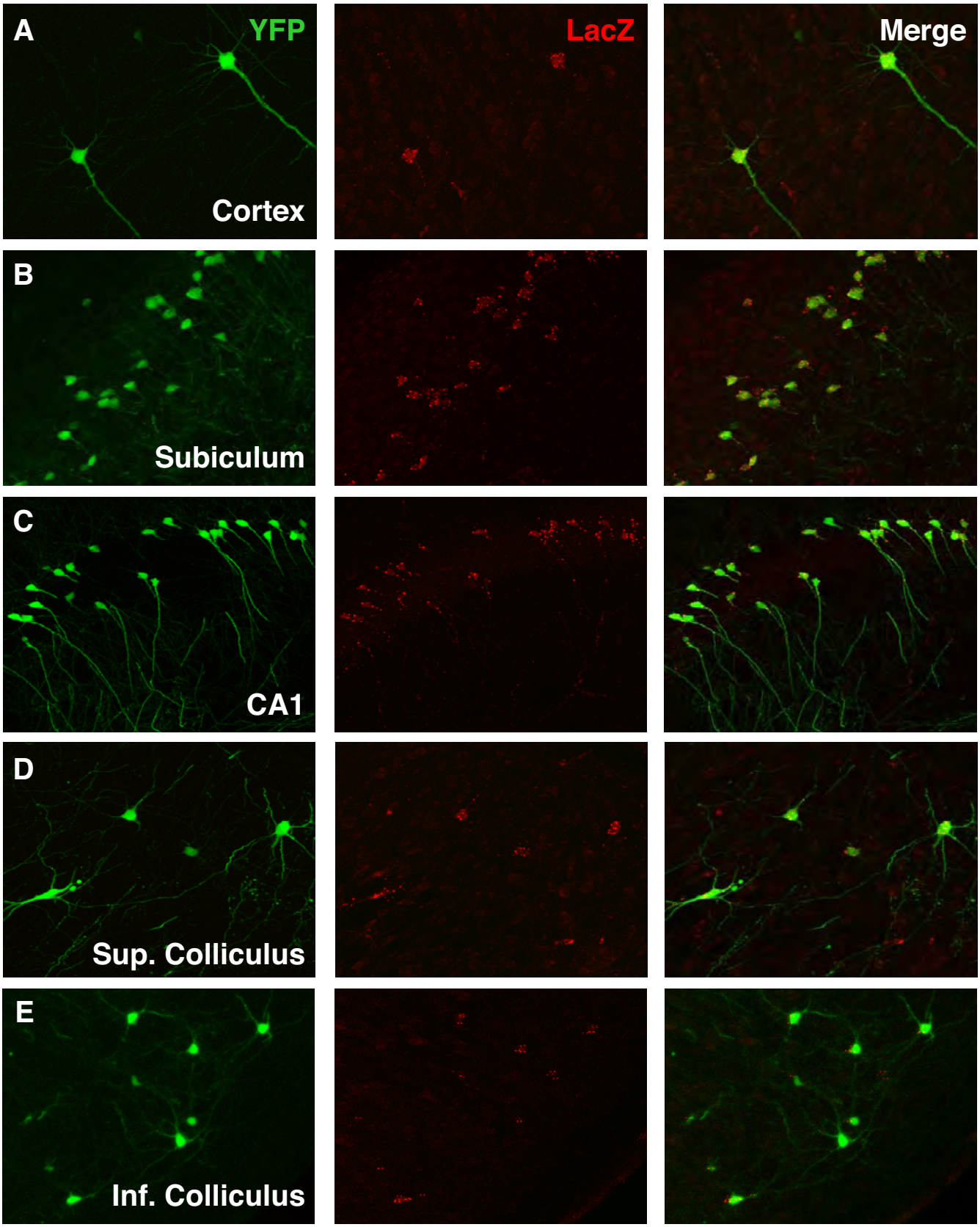
Young et al., Supplemental Fig. 4

Supplemental Figure 5. Genetic manipulation in SLICK-V transgenic mice

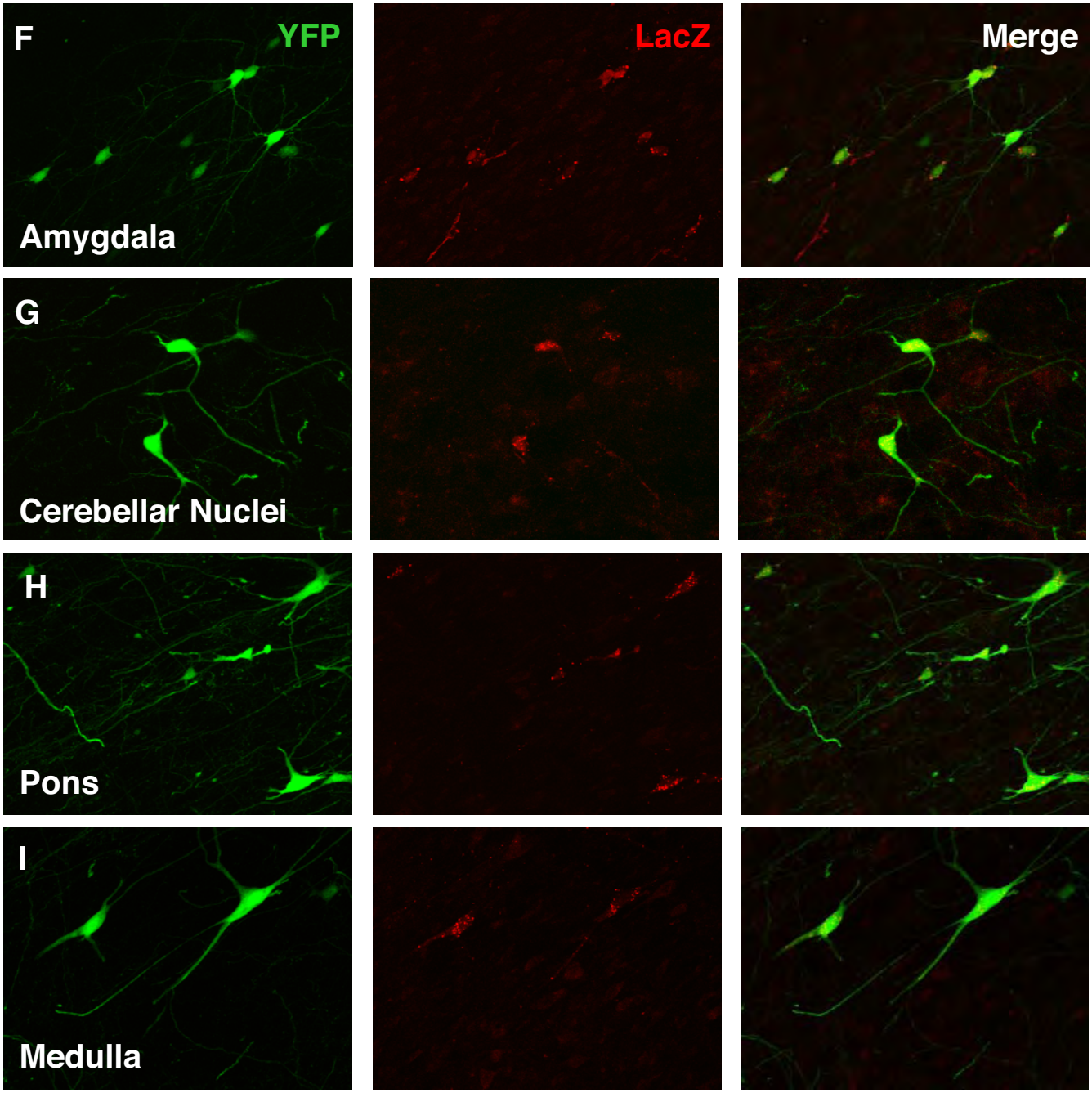
A-J Genetic manipulation as indicated by Lac-Z expression in fluorescently-labeled neurons from various brain regions of tamoxifen-treated SLICK-V/R26R mice. A = Cortex; B = Subiculum (97% n=95); C = CA1 hippocampal region; D = Superior Colliculus (91% n=44); E = Inferior Colliculus (85% n=71); F = Amygdala (84% n=32); G = Cerebellar Nuclei (100% n=10) H = Pons, I = Medulla. Recombination rates and number of cells quantified are shown in parentheses for those regions not detailed in Table 2.

Methods

Immunohistochemistry was performed as described in the manuscript. Images were acquired using a Zeiss LSM 510 confocal microscope.



Young et al., Supplemental Fig. 5, Part 1



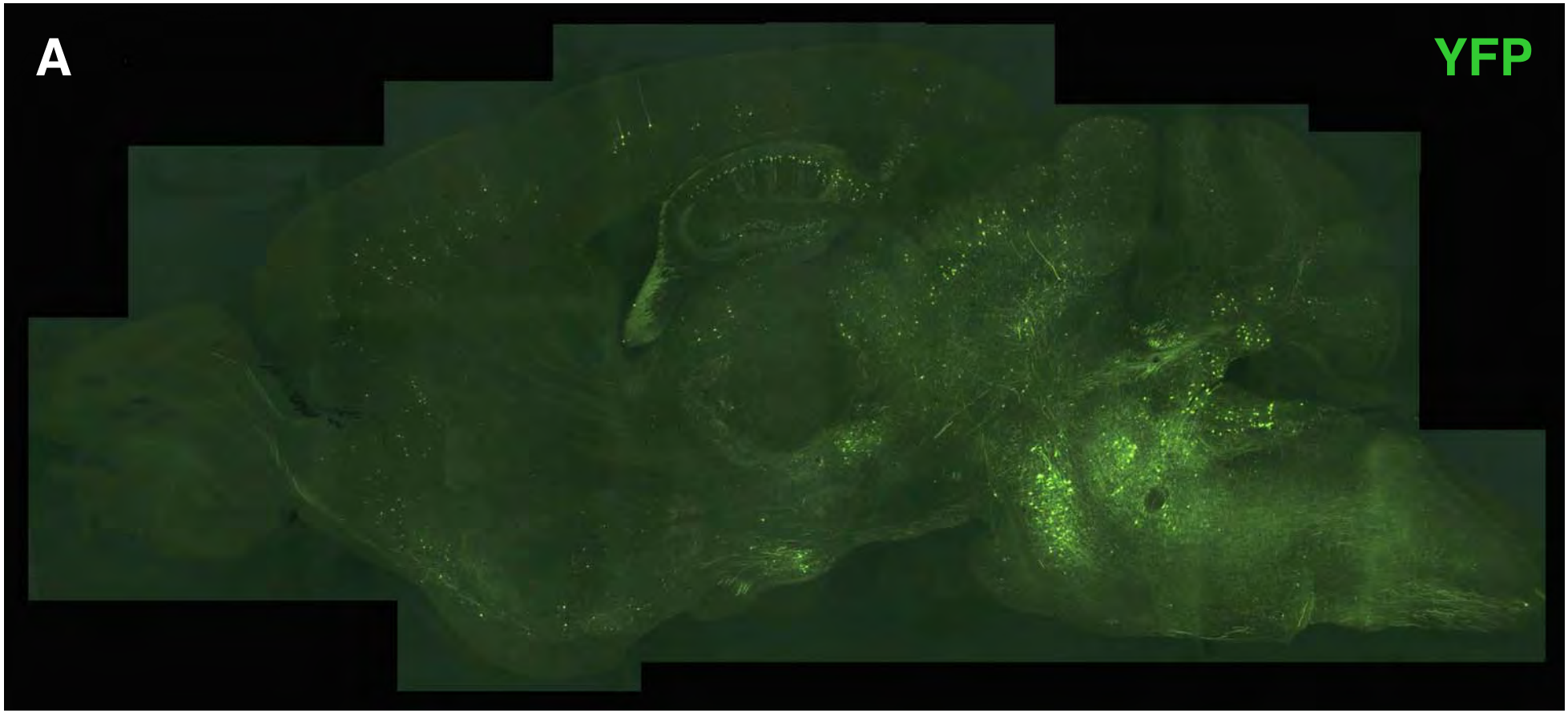
Young et al., Supplemental Fig. 5, Part 2

Supplemental Figure 6. Genetic manipulation in SLICK-X transgenic mice

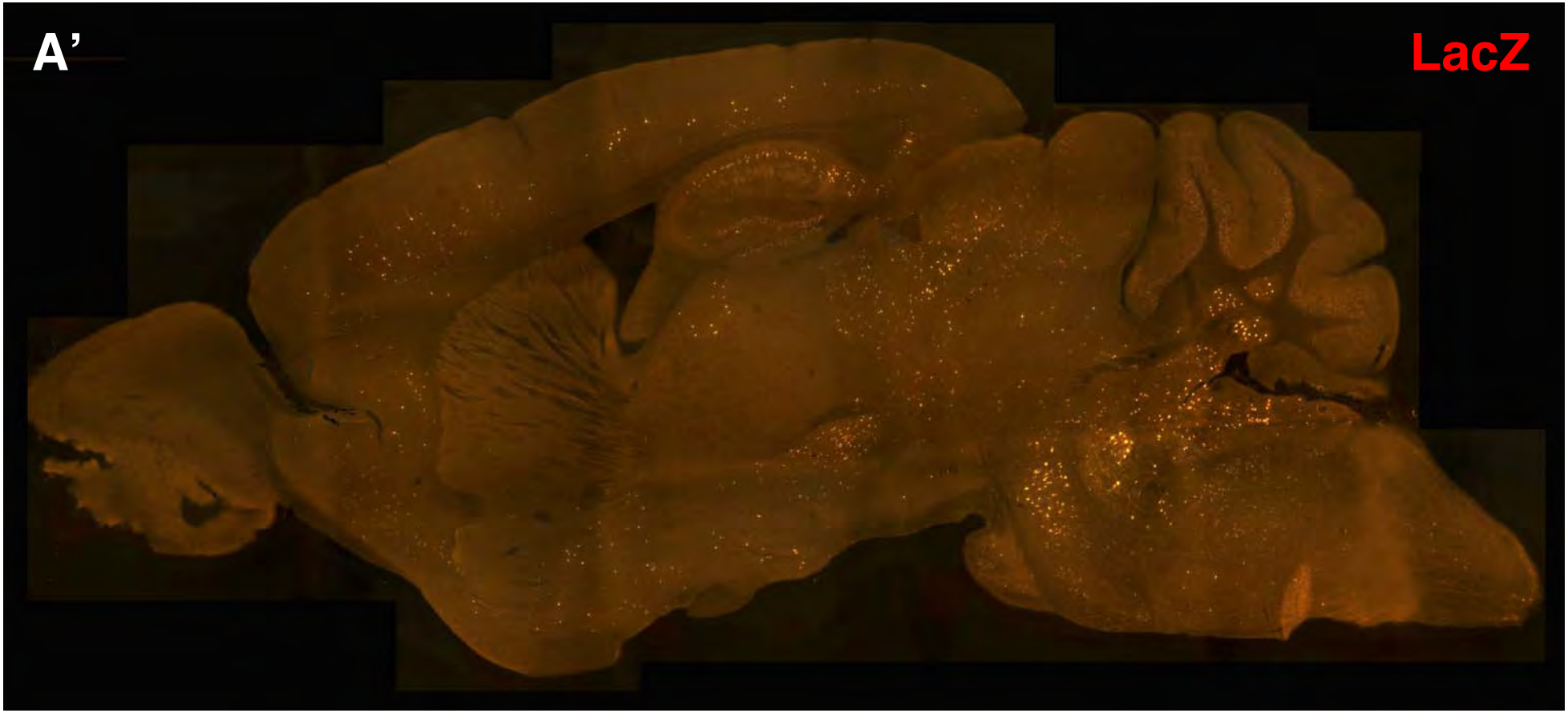
Genetic manipulation as indicated by Lac-Z expression in tamoxifen-treated SLICK-X/R26R mice. **A** Whole brain image showing YFP fluorescence in SLICK-X mice. **A'** Immunofluorescent staining of the same section showing LacZ expression. Efficient recombination in YFP positive cells is observed throughout the brain (see Table 2).

Methods

Immunohistochemistry was performed as described in the manuscript. Images were acquired with a Zeiss Axioskop 2 fluorescent microscope using a 4X objective. A whole brain montage was generated using Photoshop software.



Young et al., Supplemental Fig. 6, Part 1



Young et al., Supplemental Fig. 6, Part 2

SUPPLEMENTARY DISCUSSION

Explanation of YFP$^-$, ChAT$^-$ axons in ChAT / SLICK-A mice

In ChAT / SLICK-A mice treated with tamoxifen we observe efficient recombination of the floxed ChAT allele in YFP expressing cells as determined by immunostaining for ChAT. While ChAT is present in almost all YFP-negative axons and terminals in these animals we occasionally observe non-YFP-labeled axons and terminals in which ChAT is not detectable. These YFP$^-$, ChAT$^-$ axons are only observed in tamoxifen treated mice, suggesting that tamoxifen-induced ChAT knockout has occurred in these cells. It is likely that in these few cells there is a low level of transgene expression (both creER^{T2} and YFP). CreER^{T2} levels in the cell body would be sufficient to cause recombination upon tamoxifen administration but the levels of YFP expressed would not be high enough to label the distal portions of axons and nerve terminals.

Discussion of NMJ stability

While the mature mammalian NMJ is known to be an extremely stable structure, the molecular basis for this stability is not clear[1]. One possibility is that synaptic maintenance is passive and is simply due to inherent structural stability of the NMJ. Alternatively active signaling between nerve terminals and muscle cells may be required[1]. In support of this postsynaptic inhibition of protein synthesis leads to a relatively rapid retraction of presynaptic terminals, suggesting that active signaling between muscle and nerve are required for NMJ maintenance[2]. An active signal that could play a role in synaptic maintenance is neurotransmission itself. Previous studies using ChAT conditional knockout mice have established that while cholinergic neurotransmission is not absolutely required for NMJ formation it

strongly influences synaptic differentiation and competitive synapse elimination[3-5] . Additionally, chronic (2 weeks) blockade of neurotransmission using botulinum toxin disrupts NMJ structure and induces nerve sprouting in a subset of muscles in young adult mice[6]. This plasticity decreases with age and by 6 months of age NMJ maintenance is largely independent of ongoing neural activity. Our results provide further genetic evidence for the stability of mature NMJs when neurotransmission has been inhibited in a subset of motor axons for up to 8 weeks. The gross morphology of inactive terminals is not altered and we do not observe nerve sprouting under these experimental conditions. This indicates that acetylcholine-mediated neurotransmission is not required for the active maintenance of mature NMJs of adult mice, though we cannot exclude the possibility that other neurotransmitters such as ATP or neuropeptides might play such a role. Still, the more interesting result is that the inactive axons are not displaced from synaptic sites by active axons. Thus the activity-dependent competition that occurs between axons at earlier developmental stages is not reignited when a subset of axons are silenced in adult animals. This suggests that there are no signaling mechanisms whereby inactive terminals or muscle cells can recruit nearby active axons to innervate them.

References

1. Sanes, J.R. & Lichtman, J.W. Development of the vertebrate neuromuscular junction. *Annu Rev Neurosci* **22**, 389-442 (1999).
2. McCann, C.M., Nguyen, Q.T., Santo Neto, H. & Lichtman, J.W. Rapid synapse elimination after postsynaptic protein synthesis inhibition in vivo. *J Neurosci* **27**, 6064-7 (2007).
3. Brandon, E.P. et al. Aberrant patterning of neuromuscular synapses in choline acetyltransferase-deficient mice. *J Neurosci* **23**, 539-49 (2003).
4. Buffelli, M. et al. Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition. *Nature* **424**, 430-4. (2003).
5. Misgeld, T. et al. Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. *Neuron* **36**, 635-48 (2002).
6. Pun, S. et al. An intrinsic distinction in neuromuscular junction assembly and maintenance in different skeletal muscles. *Neuron* **34**, 357-70 (2002).

SUPPLEMENTARY METHODS

Genotyping of mice

Genotypes of SLICK mice were determined by PCR using the following primers: Thy1F1 (TCTGAGTGGCAAAGGACCTTAGG) from the thy1 promoter region and EYFPR1 (CGCTGAACTTGTGGCCGTTTACG) from the EYFP sequence. PCR reactions were performed in 25 μl total volume using TaqPro polymerase (Denville Scientific, Metuchen, NJ, USA) and 2 μl mouse tail DNA. Mouse tail DNA was prepared by digesting 5 mm of mouse tails overnight at 55^{o}C in 100 μl tail digestion buffer (50 mM Tris-HCl (pH 8.0), 1 mM CaCl2, 1% Tween-20) plus 10 μl proteinase K (10 mg/ml) (Roche, Indianapolis, IN, USA). Digested tails were boiled for 10 min to inactivate proteinase K. R26R mice were genotyped using the primers oIMR0883 (AAAGTCGCTCTGAGTTGTTAT) and oIMR0315 (GCGAAGAGTTTGTCCTCAACC). ChAT genotyping was performed using the primers F11 (TAACCAAACGTAATATATGTTTGTTGGAGC) and 4667 (TGGTTCTTTCCGCCTCAGGACTCTTCCTTT) for the knockout allele, F4 (CAACCGCCTGGCCCTGCCAGTCAACTCTAG) and R13 (GAGGATGAAATCCTGACAGATTCCAACAGG) for the wild type allele and F4 and 4667 for the floxed allele. The ChAT conditional knockout mice used in this study retain the neomycin resistance gene used for gene targeting.

Antibodies

Anti-digoxygenin and anti-fluorescein horseradish peroxidase conjugated antibodies used for *in situ* hybridization were from Roche (Indianapolis, IN, USA). The rabbit polyclonal β-galactosidase antibody was a gift from Joshua Sanes (Harvard University, USA). Goat anti-ChAT antibody was from Chemicon (catalog number

AB144P). Cy3 conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Tetramethyl rhodamine and Alexa-647 conjugated α-bungarotoxin was from Invitrogen-Molecular Probes (Carlsbad, CA, USA; catalog numbers T-1175 and B-35450). To reduce background staining, the anti- -galactosidase and ChAT antibodies were preabsorbed using appropriate mouse tissue. Tissue was homogenized in PBS with protease inhibitors, four volumes of cold acetone were added and the mixture incubated on ice for 30 minutes. The pellet was recovered by centrifugation at 10,000g, washed once, ground to a fine powder, air dried and stored at –80 ^{o}C. For preabsorption an aliquot of the pellet was rehydrated with PBS and incubated with the antibody in blocking solution for 4 to 6 hours. Following centrifugation the supernatant was used for immunofluorescent staining. The tissue used for preabsorption of anti- -galactosidase was wild type P21 mouse brain, while E18 ChAT$^{-/-}$ whole embryos were used for the anti-ChAT antibody.

Immunohistochemistry

Immunostaining for -galactosidase was performed on floating 50 μm sections. Tissues were fixed as described above, sections cut on a vibrotome, incubated in blocking solution (4% BSA, 5% normal goat serum and 0.2% Triton in PBS) for 1-2 hours and then incubated overnight with preabsorbed primary antibody at a 1:250 dilution in blocking solution. Sections were then washed 5 times over a period of 1-2 hours in PBS, incubated with the appropriate secondary antibodies for 2-6 hours, washed again and mounted in 90% glycerol with 0.1% p-phenylenediamine. Stained sections were imaged on a Nikon Eclipse confocal microscope using a 20X objective.

For ChAT immunohistochemistry, mice were anesthetized and perfused as described above but with 2% rather than 4% para-formaldehyde. Most muscles were prepared for cryostat sectioning by sinking successively in PBS with 15% sucrose and PBS with 30% sucrose prior to embedding in OCT compound. 40 μm longitudinal sections were then cut on a Leica CM 1850 cryostat. Sections were blocked with 2% BSA and 0.5% Triton in PBS for 6 hr and then incubated with primary antibody (1:25 dilution) overnight in blocking solution with 0.2% Triton. Sections were then washed 10 times over a period of 8 hours in PBS, incubated with Cy3 conjugated anti-goat antibody (1:1000 dilution) and Alexa647 conjugated α-bungarotoxin (1:100 dilution; Invitrogen) overnight, washed again and mounted in 90% glycerol with 0.1% p-phenylenediamine. For extraocular muscles whole-mount staining was performed as described above except that incubation times for primary and secondary antibodies were 48 hours and 24 hours respectively and each wash step was for 24 hours with numerous changes of PBS.