Full Experimental Procedures

All procedures were in accordance with protocols approved by the Janelia Research Campus and the University of California Berkeley Institutional Animal Care and Use Committees.

Library generation and viral production

Four previously generated virus libraries were used at the start of the directed evolution procedure: 1) a random mutagenesis library, generated by subjecting the AAV2 *cap* gene (encoding viral proteins VP1-3 and Assembly-activating protein, AAP) to error prone PCR (Maheshri et al., 2006); 2) a library of AAV2 *cap* gene variants containing 7-mer randomized peptide within a 10-mer insert of the form **LA**XXXXXXX**A** between N587 and R588 (Müller et al., 2003); 3) a library of AAV2 *cap* gene variants containing randomized loop regions (Koerber et al., 2009), and 4) a DNA shuffling library generated from wild-type AAV1, AAV2, AAV4, AAV5, AAV6, AAV8 and AAV9 *cap* gene sequences (Koerber et al., 2008). Each pool of mutant DNA had been originally sub-cloned into the replication-competent AAV packaging plasmid to create a viral plasmid library that, when packaged into AAV virions, can be selected for any new property or function. The replication-competent AAV system incorporates the mutant *cap* gene into the viral payload, and thus the genotype of each variant is linked to its phenotype. Capsid sequences of the desired property can then be recovered by DNA sequence analysis of the encapsulated AAV genome.

The four replication-competent AAV libraries were packaged by calcium phosphate transient transfection of HEK293-T cells followed by virus harvest, iodixanol gradient centrifugation, and Amicon filtration (Maheshri et al., 2006).

In vivo virus or tracer injections

For localized *in vivo* virus delivery, mice or rats were anaesthetized with isoflurane (~2% by volume in O_2; SurgiVet, Smiths Medical) and a small hole was drilled in the skull above the requisite injection site (see table below). For some injection sites, several injections were made at different depths (see table below). For viral injections, ~ 50–100 nl (mice) or 250–500 nl (rats) of virus containing solution was slowly injected at each depth into the tissue. For tracer injections, 50 nl of 5% Fluoro-Gold (Fluorochrome, Denver, CO) in 0.9% NaCl, or 100 nl of retro-beads (LumaFluor, Durham, NC) diluted 1:1 in 0.9% NaCl were injected at the same set of sites for each injection target. Injections were done with a pulled glass pipette (broken and beveled to 25–30 μm (outside diameter); Drummond Scientific,

Wiretrol II Capillary Microdispenser) back-filled with mineral oil. A fitted plunger was inserted into the pipette and advanced to displace the contents using a hydraulic manipulator (Narashige, MO-10). Retraction of the plunger was used to load the pipette with virus. The injection pipette was positioned with a Sutter MP-285 manipulator.

The following coordinates were used in this study:

Injection target	Injection coordinates, mm*
mouse substantia nigra pars reticulata	A/P: -3.5; M/L: 1.5; D/V: -4.0 and -4.5
mouse cerebellum	A/P: 7.1; M/L: 1.4; D/V: -0.8 and -0.5
mouse dorsomedial striatum	A/P: 0.6; M/L: 1.2; D/V -2.5
mouse dorsolateral striatum	A/P: 0.6; M/L: 2.25; D/V -2.6
mouse ventrolateral striatum	A/P: 0; M/L: 2.8; D/V -3.2
mouse pontine nucleus (BPN)	A/P: 0.40; M/L: 0.40; D/V: -5.50, -5.75, and -6.0
rat dorsomedial striatum	A/P 2.16; M/L 1.7 ; D/V -4.3
rat superior colliculus	A/P -6.0; M/L ±1.8; D/V -5.45

* All A/P coordinates are given with respect to Bregma; D/V coordinates are given with respect to the pia.

Library selection and evolution

The four mutant virus libraries were pooled and injected either into the substantia nigra pars reticulata or into the cerebellum of adult (6–8 week old) wild-type C57/Bl6J mice (either sex; Charles River). Three weeks after injection, striatal or hindbrain tissue was accordingly removed, DNA was extracted, and virions that had successfully reached the remote retrograde target tissue were PCR-amplified and recloned into rcAAV packaging plasmid to create a new replication competent AAV library for the next round of selection. After three selection steps, the rescued *cap* genes were randomly mutated by error prone PCR using 5'-ACGCGGAAGCTTCGATCAACTACGCAG-3' and 5'-AGACCAAAGTTCAACTGAAACGAATTAAACGG-3' as forward and reverse primers, respectively. Two additional *in vivo* selection rounds were then performed. Individual isolates were sequenced after rounds four and five to evaluate the degree of library enrichment (Table S1). Seventeen variants were chosen for secondary screening at the end of round five, and the corresponding *cap* gene sequences were recloned into an rAAV helper plasmid. Individual high-titer preps of the parental wild type AAV2 and of each of the seventeen chosen mutant variants carrying a *CMV*-EGFP payload were then performed by

Vector BioLabs, Inc (Philadelphia, PA). The *CMV* promoter is typically weak in neurons, and thus this secondary screen provided a stringent test of the efficiency of retrograde transport. The individual AAV variants were injected either into the cerebellum or into the globus pallidus. After three weeks, endogenous, unamplified EGFP fluorescence was visualized in the regions expected to be labeled if retrograde transport was efficient. Mutant r5H6 (insert **LA**DQDYTKT**A** + V708I + N382D) displayed the strongest retrograde transport in both circuits, and was thus chosen for further analysis and named rAAV2-retro.

The full gene sequence of the selected variant is as follows (changes from the wild type AAV2 *cap* gene sequence are indicated in red):

ATGGCTGCCGATGGTTATCTTCCAGATTGGCTCGAGGACACTCTCTCTGAAGGAATAAGACAGTGGTG
GAAGCTCAAACCTGGCCCACCACCACCAAAGCCCGCAGAGCGGCATAAGGACGACAGCAGGGGTCTT
GTGCTTCCTGGGTACAAGTACCTCGGACCCTTCAACGGACTCGACAAGGGAGAGCCGGTCAACGAGGC
AGACGCCGCGGCCCTCGAGCACGACAAAGCCTACGACCGGCAGCTCGACAGCGGAGACAACCCGTAC
CTCAAGTACAACCACGCCGACGCGGAGTTTCAGGAGCGCCTTAAAGAAGATACGTCTTTTGGGGGCAA
CCTCGGACGAGCAGTCTTCCAGGCGAAAAAGAGGGTTCTTGAACCTCTGGGCCTGGTTGAGGAACCTG
TTAAGACGGCTCCGGGAAAAAAGAGGCCGGTAGAGCACTCTCCTGTGGAGCCAGACTCCTCCTCGGG
AACCGGAAAGGCGGGCCAGCAGCCTGCAAGAAAAAGATTGAATTTTGGTCAGACTGGAGACGCAGAC
TCAGTACCTGACCCCCAGCCTCTCGGACAGCCACCAGCAGCCCCCTCTGGTCTGGGAACTAATACGAT
GGCTACAGGCAGTGGCGCACCAATGGCAGACAATAACGAGGGCGCCGACGGAGTGGGTAATTCCTCG
GGAAATTGGCATTGCGATTCCACATGGATGGGCGACAGAGTCATCACCACCAGCACCCGAACCTGGGC
CCTGCCCACCTACAACAACCACCTCTACAAACAAATTTCCAGCCAATCAGGAGCCTCGAACGACAATC
ACTACTTTGGCTACAGCACCCCTTGGGGGTATTTTGACTTCAACAGATTCCACTGCCACTTTTCACCAC
GTGACTGGCAAAGACTCATCAACAACAACTGGGGATTCCGACCCAAGAGACTCAACTTCAAGCTCTTT
AACATTCAAGTCAAAGAGGTCACGCAGAATGACGGTACGACGACGATTGCCAATAACCTTACCAGCA
CGGTTCAGGTGTTTACTGACTCGGAGTACCAGCTCCCGTACGTCCTCGGCTCGGCGCATCAAGGATGC
CTCCCGCCGTTCCCAGCAGACGTCTTCATGGTGCCACAGTATGGATACCTCACCCTGAACGACGGGAG
TCAGGCAGTAGGACGCTCTTCATTTTACTGCCTGGAGTACTTTCCTTCTCAGATGCTGCGTACCGGAAA
CAACTTTACCTTCAGCTACACTTTTGAGGACGTTCCTTTCCACAGCAGCTACGCTCACAGCCAGAGTCT
GGACCGTCTCATGAATCCTCTCATCGACCAGTACCTGTATTACTTGAGCAGAACAAACACTCCAAGTG
GAACCACCACGCAGTCAAGGCTTCAGTTTTCTCAGGCCGGAGCGAGTGACATTCGGGACCAGTCTAGG
AACTGGCTTCCTGGACCCTGTTACCGCCAGCAGCGAGTATCAAAGACATCTGCGGATAACAACAACAG
TGAATACTCGTGGACTGGAGCTACCAAGTACCACCTCAATGGCAGAGACTCTCTGGTGAATCCGGGCC
CGGCCATGGCAAGCCACAAGGACGATGAAGAAAAGTTTTTTCCTCAGAGCGGGGTTCTCATCTTTGGG
AAGCAAGGCTCAGAGAAAACAAATGTGGACATTGAAAAGGTCATGATTACAGACGAAGAGGAAATCA
GGACAACCAATCCCGTGGCTACGGAGCAGTATGGTTCTGTATCTACCAACCTCCAGAGAGGCAACCTA
GCAGACCAAGACTACACAAAAACTGCTAGGCAAGCAGCTACCGCAGATGTCAACACACAAGGCGTTC
TTCCAGGCATGGTCTGGCAGGACAGAGATGTGTACCTTCAGGGGCCCATCTGGGCAAAGATTCCACAC
ACGGACGGACATTTTCACCCCTCTCCCCTCATGGGTGGATTCGGACTTAAACACCCTCCTCCCCAGATT
CTCATCAAGAACACCCCGGTACCTGCGAATCCTTCGACCACCTTCAGTGCGGCAAAGTTTGCTTCCTTC
ATCACACAGTACTCCACGGGACAGGTCAGCGTGGAGATCGAGTGGGAGCTGCAGAAGGAAAACAGCA
AACGCTGGAATCCCGAAATTCAGTACACTTCCAACTACAACAAGTCTATTAATGTGGACTTTACTGTG
GACACTAATGGCGTGTATTCAGAGCCTCGCCCCATTGGCACCAGATACCTGACTCGTAATCTGTAA

Payloads and promoters used in the study

For all subsequent experiments, the *CMV* promoter was replaced with a promoter known to be more robust in adult neurons. Cre recombinase and the GCaMP6f calcium sensor were driven by the human *Synapsin-1* (*hSyn1*) promoter; all of the fluorophores were driven by the *CAG* promoter; and the color-flipping construct was driven by the *EF1α* promoter.

Virus production for the quantification of retrograde efficiency

hSyn-Cre payload was packaged using AAV1, AAV2, AAV5, AAV8, AAV9, DJ and AAV2-retro capsids at the Janelia Viral Shared Resource. All seven virus preparations were processed in parallel, and were titer matched before *in vivo* injections. All lots were diluted to the lowest measured titer (1.3E12 GC/ml), and each virus was injected into the right pontine nucleus of three adult *Rosa26*-Lox-STOP-Lox-H2B-EGFP mice (He et al., 2012).

Histology

Animals were sacrificed two to three weeks following virus injections, at which point the brains were harvested and the right hemisphere was sagittally sectioned at a thickness of 50 μm. In the triple labeling experiment in Figure 1B and Movie S1, the fluorophores were antibody-amplified; otherwise, unstained sections were used. The sections were mounted in VECTASHIELD Antifade Mounting Medium containing DAPI (Vector Laboratories), and imaged using a P-E Pannoramic slide scanner (3D Histech) using a 20x objective and FITC and DAPI filters.

When signal amplification was performed, the following antibody combinations were used: chicken anti-GFP (Rockland Immunochemicals) + goat anti-chicken IgY (H+L) Secondary Antibody Alexa Fluor® 488 (Invitrogen A11039); rabbit anti-RFP (Rockland Immunochemicals) + goat anti-rabbit IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor® 555 (Invitrogen); mouse anti-FLAG (Rockland Immunochemicals) + goat anti-mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 647 (Invitrogen). The sections were counterstained with Neurotrace (Invitrogen) and mounted in Aqua/Poly-Mount (Polysciences) before imaging.

Retrograde transport quantification

To quantify retrograde transport, the extent of corticopontine labeling was assessed in sagittal sections lateral to the injection tract (~1 mm lateral wrt midline) taken from *Rosa26*-Lox-STOP-Lox-H2B-EGFP animals injected in the basal pons with various AAV serotypes carrying Cre recombinase transgene. Images obtained with the Pannoramic slide scanner were stitched together and then analyzed using custom software written in Matlab (Mathworks) to detect the GFP labeled nuclei across the cortex.

For each chosen sagittal section, a region of interest (ROI) was manually drawn around the cortex to isolate the area in the image for automated cell counting. To enhance the detection of nuclei, the image was then convolved with a "Mexican Hat" kernel comprising the difference of two Gaussians (26.00 μm variance and 3.25 μm variance). Image noise was reduced using a median filter, and basic peak detection was then performed. Linear density was computed as the number of labeled nuclei per mm of the total length of a line drawn manually to trace the outline of cortical layer V.

Quantification of local infection efficiency at the injection site

For rAAV2, rAAV9 and rAAV2-retro, additional analysis of infection efficiency at the injection site was performed. A single sagittal section spanning the middle of the injection site—as judged by the presence of extensive labeling along the injection tract— was chosen for each of the injected brains. The green channel was convolved with the "Mexican Hat" kernel (see above), and peaks were then detected as local maxima on these threshold images using custom functions written in Matlab. An ROI was drawn over the midbrain area containing the basal pontine nuclei but excluding the injection tract, and the total number of labeled nuclei within the ROI was tabulated.

Analysis of the generality of retrograde transport

Rosa26-Lox-STOP-Lox-H2B-EGFP mice were injected with 25 nl of rAAV2-retro *hSyn1*-Cre (1.3E12 GC/ml) in the dorsal striatum. Three weeks after injection, coronally-sectioned brains were imaged using a Pannoramic Scanner to visualize DAPI stained nuclei and green fluorescence from H2B-GFP expressing nuclei. The green channel was similarly used for the detection of labeled nuclei (see above). The blue channel of each section was aligned to the Nissl images from the Allen Brain Institute's standardized mouse brain atlas using custom analysis routines written with the help of the Matlab Image Processing Toolbox. The annotated regions from the ABI's mouse brain atlas were then used to assign detected neurons in aligned sections to specific brain regions (Kuan et al., 2015).

We note that finite precision of the reference atlas together with anatomical variability of individual brains limit the robustness of this semi-automated process to prominent afferent inputs.

Heparin binding assay

The heparin affinity of rAAV2-retro and wild-type AAV2 were analyzed as previously described (Jang et al., 2011). Briefly, approximately 10^{11} purified genomic particles were loaded onto a 1 ml HiTrap heparin column (GE Healthcare Sciences, Piscataway, NJ) previously equilibrated with 150 mM NaCl and 50 mM Tris at pH 7.5. Elution was then performed by increasing the concentration of NaCl in steps of 50 mM up to a final concentration of 950 mM, followed by a wash with 1M NaCl. A small fraction of each elution was used to infect HEK293T cells, and the percentage of GFP positive cells was quantified 48 hours post-infection using a Guava EasyCyte 6HT flow cytometer (EMD/Millipore).

Imaging of neuronal population activity *in vivo* following retrograde delivery of GCaMP6f

Seven adult mice were anesthetized with isoflurane (2%) and placed in a stereotactic frame (Kopf Instruments; Tujunga, CA) on a 37°C heating pad. The scalp and periosteum over the skull were removed, a layer of UV-curing OptiBond adhesive (Kerr; Orange, CA) was applied, and a custom-made headpost (Osborne and Dudman, 2014) was affixed with dental cement. rAAV2-retro carrying a *hSyn1*-GCaMP6f (1.0E13 GC/ml) payload was injected into the basal pontine nuclei (BPN) (3.9 mm posterior and 0.4 mm lateral to Bregma, depths 5.8, 5.6 and 5.4 mm, 100 nl at each depth) using a Nanoliter 2010 injector (World Precision Instruments). A cranial window (one 170 μm-thick pane of laser-cut glass, 2 mm diameter) was placed over the primary motor cortex (centered on 0.7 mm anterior and 1.6 mm lateral to Bregma).

Following surgery, injections of ketoprofen (5 mg/kg) and buprenorphine (0.1 mg/kg; Henry Schein Animal Health; Melville, NY) were administered subcutaneously. Mice were allowed to recover for one-week following surgery and then were imaged briefly under a 2-photon microscope to assess virus expression. All animals had visually identified GCaMP6f expressing cells in layer V of M1 one-week post injection. Then the animals were habituated to head fixation in a custom-built apparatus and trained to retrieve a food pellet as previously described (Guo et al., 2015).

GCaMP6f was excited at 920 nm (typically 20–40 mW at the back aperture) with a Ti:Sapphire laser (Chameleon, Coherent) and imaged through a Nikon 16x, 0.8-N.A. objective. Emission light passed

through a 565 nm DCXR dichroic filter (Chroma Technology) and an ET525/70 nm m-2p filter (Chroma Technology) and was detected by a GaAsP photomultiplier tube (10770PB-40, Hamamatsu). Images (512 × 512 pixels) were acquired at ~30 Hz with resonating scanners using ScanImage software.

CRISPR/Cas9 genome editing

The *CMV* promoter in pAAV-*CMV*-SaCas9-empty (Slaymaker et al., 2016) was replaced with *hSyn1* to generate pAAV-*hSyn1*-SaCas9-empty. Oligonucleotides encoding sgRNA protospacer sequences were custom ordered, phosphorylated, hybridized and ligated into the *Bsa*I restriction sites of pAAV-*hSyn1*-SaCas9-empty to generate pAAV-*hSyn1*-SaCas9-tdTomato-1 to -10. Oligonucleotide sequences used were:

tdTomato sgRNA 1 Fwd

CACCGCAAGGGCGAGGAGGTCATCA

tdTomato sgRNA 1 Rev

AAACTGATGACCTCCTCGCCCTTGC

tdTomato sgRNA 2 Fwd

CACCGTGGAGGGCTCCATGAACGGCC

tdTomato sgRNA 2 Rev

AAACGGCCGTTCATGGAGCCCTCCAC

tdTomato sgRNA 3 Fwd

CACCGAGGACGGCGGCCACTACCTGG

tdTomato sgRNA 3 Rev

AAACCCAGGTAGTGGCCGCCGTCCTC

tdTomato sgRNA 4 Fwd

CACCGACAACAACATGGCCGTCATCA

tdTomato sgRNA 4 Rev

AAACTGATGACGGCCATGTTGTTGTC

tdTomato sgRNA 5 Fwd

CACCGAAGGACGGCGGCCACTACCTGG

tdTomato sgRNA 5 Rev

AAACCCAGGTAGTGGCCGCCGTCCTTC

tdTomato sgRNA 6 Fwd

CACCGACAACAACATGGCCGTCATCA

tdTomato sgRNA 6 Rev

AAACTGATGACGGCCATGTTGTTGTC

tdTomato sgRNA 7 Fwd

CACCGGTCACCTTCAGCTTGGCGGT

tdTomato sgRNA 7 Rev

AAACACCGCCAAGCTGAAGGTGACC

tdTomato sgRNA 8 Fwd

CACCGCCGTACATGAACTGGGGGGA

tdTomato sgRNA 8 Rev

AAACTCCCCCCAGTTCATGTACGG

tdTomato sgRNA 9 Fwd

CACCGTCTTGTAATCGGGGATGTCGG

tdTomato sgRNA 9 Rev

AAACCCGACATCCCCGATTACAAGAC

tdTomato sgRNA 10 Fwd

CACCGCCGTCCTGCAGGGAGGAGTC

tdTomato sgRNA 10 Rev

AAACGACTCCTCCCTGCAGGACGGC

The ability of each oligo to direct genome editing was first evaluated *in vitro*. Neuro2A cells were transfected with 800 ng of pAAV-*hSyn1*-SaCas9-tdTomato-1 to -10, 100 ng of pAAV-FLEX-*CAG*-tdTomato and 100 ng of pAAV-*CAG*-EGFP using polyethylenimine. At 72 hrs after transfection, cells were harvested and ~70,000 EGFP positive Neuro2A cells were isolated by fluorescence activated cell sorting (FACS) using a BD Influx Sorter (BD Biosciences). Genomic DNA was then extracted, and the frequency of tdTomato gene modification was evaluated by the Surveyor nuclease assay (Integrated DNA Technologies) as previously described (Cong et al., 2013). sgRNA 7—one of two that appeared to direct

two cleavage events within the tdTomato sequence—was packaged into AAV2-retro and used for *in vivo* genome editing.

~100 nl of rAAV2-retro-*hSyn1*-SaCas9-anti-tdTomato (5.0 E13 GC/ml) or rAAV2-retro-*hSyn1*-SaCas9-empty was then injected into the BPN of *Rbp4_KL100* Cre × tdTomato mice as described above. Six weeks after injections, brains were harvested and 40 μm-thick coronal sections were cut and stained against the HA-tagged Cas9 (anti-HA antibody C29F4 from Cell Signaling, diluted 1:1600; secondary antibody: donkey anti-mouse Alexa Fluor 488, from Jackson ImmunoResearch, diluted 1:250) and against the NeuN neuronal marker (anti-NeuN antibody A60 from Millipore, diluted 1:250; secondary antibody: donkey anti-rabbit Alexa Fluor 647 A-31573, from ThermoFisher, diluted 1:500). Following antibody staining, the sections were mounted onto slides with VECTASHIELD Antifade Mounting Medium containing DAPI (Vector Laboratories) and visualized using a Zeiss Axio Observer A1 inverted microscope (Zeiss). Quantification of immunostaining was performed using ImageJ analysis software (NIH).

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