Title: Scalable, cell type-selective *in vivo* AAV-based CRISPR screens in mice

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Abstract

CRISPR-based genetic screening directly in mammalian tissues *in vivo* is challenging due to the need for scalable, cell-type selective delivery and recovery of guide RNA libraries. We developed an *in vivo* adeno-associated virus-based and Cre recombinase-dependent workflow for cell type-selective CRISPR interference screening in mouse tissues. We demonstrate the power of this approach by identifying neuron-essential genes in the mouse brain using a library targeting over 2000 genes.

Main

CRISPR-based genetic screens are powerful tools for biological discovery. While such screens have been used extensively in cultured mammalian cells, they have been challenging to perform *in vivo* in tissues of live mammals. Most reported *in vivo* screens have used lentiviral delivery of pooled sgRNA library into the target tissue[1], which suffer from several significant limitations (Extended Data Fig. 1). First, available lentiviral envelope pseudotypes offer little control on tropism of different tissues and cell-types. Hence, in complex tissues with many distinct cell types, such as the brain, lentiviral screens cannot differentiate between genes affecting phenotypes in different cell types[2]. Second, lentivirus injected into a tissue has limited spread beyond the region of injection (Extended Data Fig. 2), therefore limiting the scope and scale of screens. Third, recovery of sgRNA information requires PCR amplification of sgRNA-encoding regions from genomic DNA, which becomes very costly when applied to whole tissues, limiting scalability.

Adeno-associated virus (AAV) has emerged as a robust, versatile way to deliver genes to different tissues, with several advantages over lentivirus for *in vivo* screening applications (Extended Data Fig. 1). AAV spreads broadly from the site of injection, and a growing number of AAV capsids have been optimized for targeting different cell types, along with options for intravenous delivery[3]. So far, only rare instances of AAV-based direct *in vivo* CRISPR screening (by which an sgRNA library is directly delivered into a target tissue) have been reported for mice[1]. AAVs rarely integrate into the host genome but are instead mostly maintained as circular episomes within the cell. Unlike lentivirus-based pooled CRISPR screening approaches that require amplification of sgRNAs from genomic DNA for screen analysis, AAV-based approaches could enable amplification of sgRNA sequences on isolated AAV episomes, vastly reducing the cost of analysis (Extended Data Fig. 1).

As TRIzol-chloroform extraction was previously used to capture AAV episomes for sequencing[4], we wanted to determine the feasibility of this approach to recover a library of sgRNAs from whole brain after AAV transduction (see Methods), as well to begin identifying neuron-essential genes based on sgRNAs that are significantly depleted compared to the input AAV library (Fig. 1a). We generated an AAV backbone (pAP210) and inserted a 12,350-element sgRNA library targeting 2,269 genes, including kinases and other druggable targets[5]; we packaged this AAV using the PHP.eB capsid, which can be delivered by intravenous injection and has strong CNS and neuronal tropism but can also transduce astrocytes and oligodendrocytes[6]. We intravenously delivered the pooled AAV sgRNA library into 9-week-old mice (n=4) that constitutively express CRISPR interference (CRISPRi) machinery, as well as into age-matched wild-type mice, followed by sequencing of sgRNAs from the recovered episomes after 4 weeks (Fig. 1a). The identified hits, knockdown of which decreases neuronal survival, only had a modest effect size and poor reproducibility between individual mice (Fig. 1c, Supplementary Table 1), despite full recovery of the sgRNA library from the brain with excellent correlation of sgRNA frequencies (Fig. 1c). We confirmed strong CRISPRi activity in cultured primary neurons isolated from this mouse line (Extended Data Fig. 3). Therefore, we still could not distinguish whether the recovered sgRNAs were from neurons, other cell types, or from non-transduced virions remaining in intravascular or interstitial spaces.

To overcome this limitation, we developed an AAV backbone (pAP215) for delivery of sgRNAs that additionally allows Cre recombinase-dependent cell type-selective sgRNA recovery (Fig. 1d). Specifically, along with the sgRNA and a fluorescent marker (nuclear localized mTagBFP2), this plasmid backbone contains a "handle" sequence flanked by specific Lox sequences that undergo predominantly unidirectional inversion upon Cre recombinase expression[4]. We packaged pAP215 containing the sgRNA library into AAV using the PHP.eB capsid. We delivered this AAV pool, along with AAV.PHP.eB-packaged Cre recombinase expressed under the neuron-specific hSyn1 promoter (hSyn1-Cre-NLS-GFP), by intracerebroventricular (ICV) injection into neonatal mice and harvested whole brains after three weeks to recover AAV episomes. We detected evidence of the inverted sequence only with expression of the Cre recombinase (Fig. 1e, Extended Data Fig. 4), confirming selective amplification of AAV episomes originating from Cre-expressing cells. Histologic examination of brains transduced with the sgRNA-containing pAP215 (BFP+) and hSyn1-driven Cre (GFP+) confirmed widespread distribution of these AAVs throughout the mouse brain and high specificity for hSyn1-Cre-GFP expression in neurons (Fig. 1f,g, Extended Data Fig. 5).

Next, we tested CRISPRi-mediated knockdown of genes by sgRNAs delivered via pAP215. We delivered an sgRNA targeting *Creb1*, a nuclear protein not essential for survival, or a non-targeting control sgRNA via ICV injection into mice with Cre-dependent conditional CRISPRi machinery[7], adding an additional level of cell-type specificity. Three weeks later, brain tissue

showed robust Creb1 knockdown only in neurons transduced with both sgRNA and Cre AAVs (Fig. 1h,i, Extended Data Fig. 6).

We moved the same 12,350-element sgRNA library described above into the pAP215 AAV backbone and delivered it by ICV injection into conditional CRISPRi neonates along with AAV.PHP.eB::hSyn1-Cre-NLS-GFP, followed by sgRNA recovery and sequencing at different time points, this time amplifying the sgRNAs using primers specific to the Cre-inverted handle in pAP215 (Fig. 2a).

Analysis of brains 12 weeks post-injection identified several strong hits knockdown of which decreased neuronal survival (Fig. 2b). Several genes belonged to biological categories including tRNA synthetases (*Wars, Hars, Sars*) or endolysosomal pathway (*Atp6v1c1*, *Rabggta*). Many of the neuron-essential genes were also identified in our previous screen in iPSC-derived neurons[8]; others were unique to the present *in vivo* screen. To determine if these hits are reproducible at different time points and are CRISPRi-dependent, we repeated the screen in both CRISPRi mice and non-transgenic wild-type mice and examined hits at 4, 6, and 12 weeks after injection. Most of the top hits were replicated across independent CRISPRi mice, whereas none were hits in wild-type mice, confirming high reproducibility of the top hits and CRISPRi dependency (Fig. 2c). The effect size was also stronger than seen by the non-selective screen of Fig. 1a,b, suggesting that Cre-dependent cell-type selectivity boosts the sensitivity of a screen.

We selected *Hspa5*, a top hit that was not previously identified as a hit in iPSC-derived neurons, for individual validation. In mouse embryonic fibroblasts expressing CRISPRi machinery, we confirmed that an sgRNA targeting *Hspa5* (sgHspa5) suppresses expression of the endogenous *Hspa5* transcript (Extended Data. Fig. 7). In primary neurons cultured from conditional CRISPRi mice, AAVs delivering sgHspa5 led to marked Cre-dependent neuronal death within 2 weeks of expression (Fig. 2d). Furthermore, injection of this sgRNA into neonatal mice led to a severe motor phenotype after approximately 2 weeks in mice co-expressing hSyn1-Cre, but not the guide alone (Supplementary Videos 1 and 2), and the brains from mice with sgHspa5 + hSyn1-Cre were markedly smaller in size relative to sgHspa5-only littermates (Fig. 2e). This confirms the predictive capability of our platform to define neuron-essential genes.

In summary, we have established an *in vivo* platform for cell-type selective CRISPR-based screening *in vivo* in mouse tissues with excellent scalability due to the amplification of sgRNA sequences from AAV episomes isolated from tissues of interest. Our pilot screen of >2,000 genes to uncover neuron-essential genes in the brain found high reproducibility of top hits between individual mice and identified *in vivo*-specific hits, supporting the importance of studying cells in their native niche. A limitation of the platform is that since AAV episomes are diluted during cell division, rapidly proliferating cell types may lose episomes over time, diluting screening power. Nonetheless, we envision that our approach can be applied to different cell types in the brain and other tissues, taking advantage of the growing repertoire of AAV capsids and cell type-specific promoters. Further, when combined with fluorescence activated cell sorting or single-cell RNA sequencing, our approach has the potential to not only identify essential genes, but to identify genes that control complex cellular phenotypes in the organismal context.

References

1. Kuhn, M., Santinha, A. J. & Platt, R. J. *Gene and Genome Editing* **2**, 100008 (2021).
2. Wertz, M. H. *et al. Neuron* **106**, 76-89.e8 (2020).
3. Chen, X. *et al. Neuron* **110**, 2242-2257.e6 (2022).
4. Deverman, B. E. *et al. Nat Biotechnol* **34**, 204–209 (2016).
5. Horlbeck, M. A. *et al. eLife* **5**, e19760 (2016).

6. Challis, R. C. et al. Nat Protoc 14, 379–414 (2019).
7. Gemberling, M. P. *et al. Nat Methods* **18**, 965–974 (2021).

Author contributions

BR, IVLR, AP and MK contributed to the study's overall conception, design, and interpretation and wrote the manuscript and created the figures, with input from the other authors. RT, KM and JP contributed preliminary experiments using lentiviral injection of mice. BR, IVLR, and AP conducted and analyzed all other experiments.

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Competing interests

BR, IVLR, AP and MK have filed a provisional patent application on in vivo screening methods. MK is an inventor on US Patent 11,254,933 related to CRISPRi and CRISPRa screening, serves on the Scientific Advisory Boards of Engine Biosciences, Casma Therapeutics, Cajal Neuroscience, Alector, and Montara Therapeutics, and is an advisor to Modulo Bio and Recursion Therapeutics.

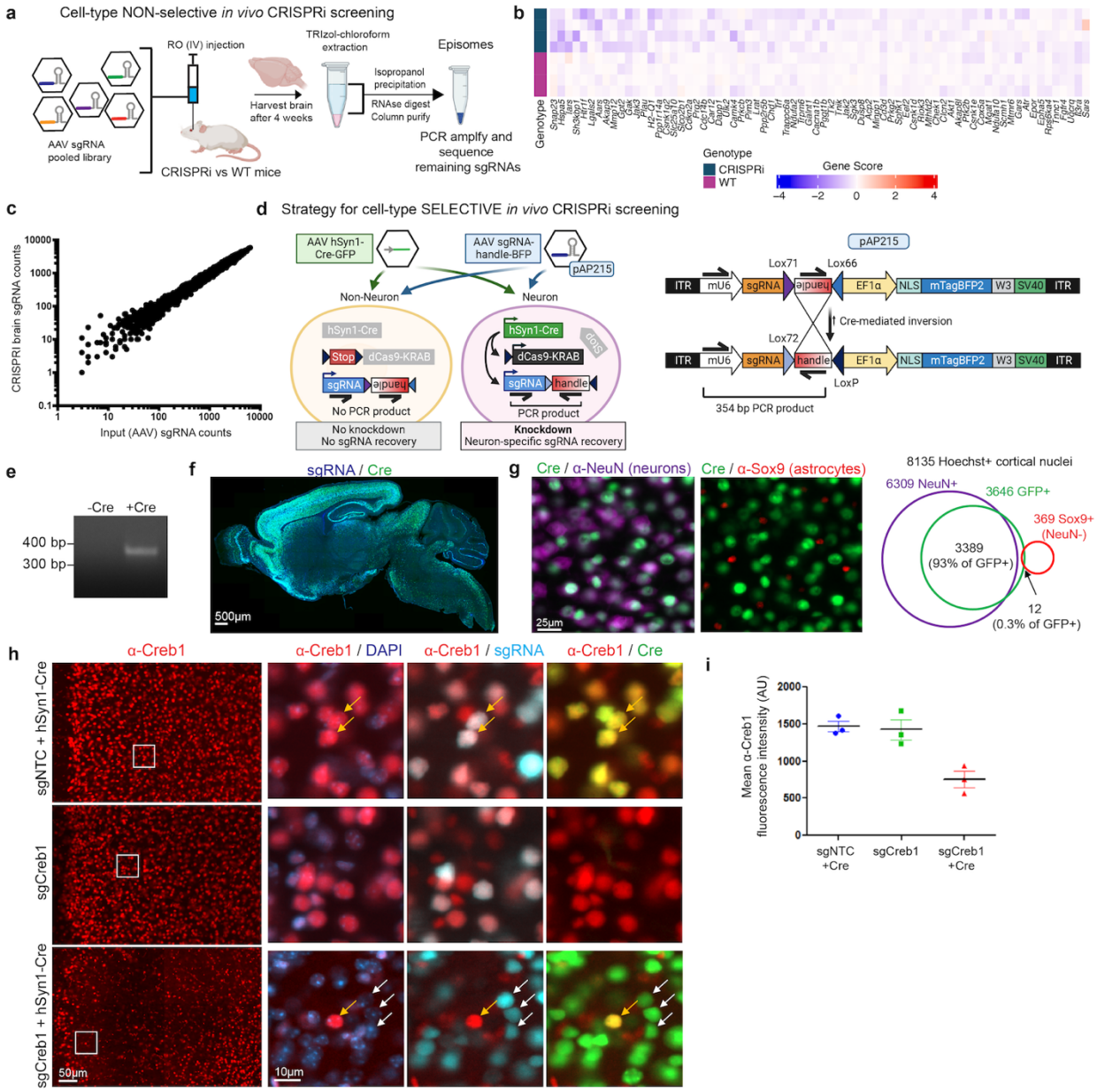


Fig. 1: AAV backbone for CRISPRi gene knockdown and Cre-dependent sgRNA recovery from neurons *in vivo*

a, Workflow for bulk tissue screening through AAV delivery of an sgRNA library to the brain of CRISPRi and wild-type (WT) mice. **b**, Results of the screen showing the hits with the most negative average Gene Score to identify neuron-essential genes for four CRISPRi and four WT mice (rows), FDR < 0.1. **c**, Representation of sgRNAs of the starting AAV library and a CRISPRi brain. **d**, Strategy for cell-type selective screening. The pAP215 AAV sgRNA backbone contains a Lox71/Lox66 flanked "handle" that undergoes inversion upon Cre recombinase expression, allowing for cell type-specific sgRNA recovery by PCR. **e**, PCR performed on episomes recovered from mouse brains expressing AAV pAP215 with or without hSyn1-Cre-NLS-GFP (Cre) using the primers diagrammed in (**d**) as black arrows. **f**, Expression of AAV-pAP215 (sgRNA) and AAV-hSyn1-Cre-NLS-GFP (Cre) in a mouse brain three weeks after neonatal intracerebroventricular (ICV) delivery. **g**, Immunofluorescent staining for neurons (marker:

NeuN) and astrocytes (marker: Sox9) in mouse cortex expressing AAV-hSyn1-Cre-NLS-GFP. **f**, Overlap of Cre expression with NeuN and Sox9 within a cortical region demonstrate high specificity for neurons and high coverage of neurons. **h**, sgRNAs targeting *Creb1* (sgCreb1) or a non-targeting control (sgNTC) in AAV-pAP215 were delivered with or without AAV hSyn1-Cre-NLS-GFP via ICV injection into neonatal mice with lox-stop-lox CRISPRi machinery. Brains were stained at 3 weeks with a representative area of frontal cortex shown. Right, higher magnification of the boxed regions in the left panels, with white arrows indicating examples of neurons having received both sgCreb1 and Cre, whereas yellow arrows indicate neurons that received Cre without sgCreb1. **i**, quantification of Creb1 levels in sgRNA-positive nuclei within a representative region of cortex (mean ± s.e.m, n = 3 independent mice).

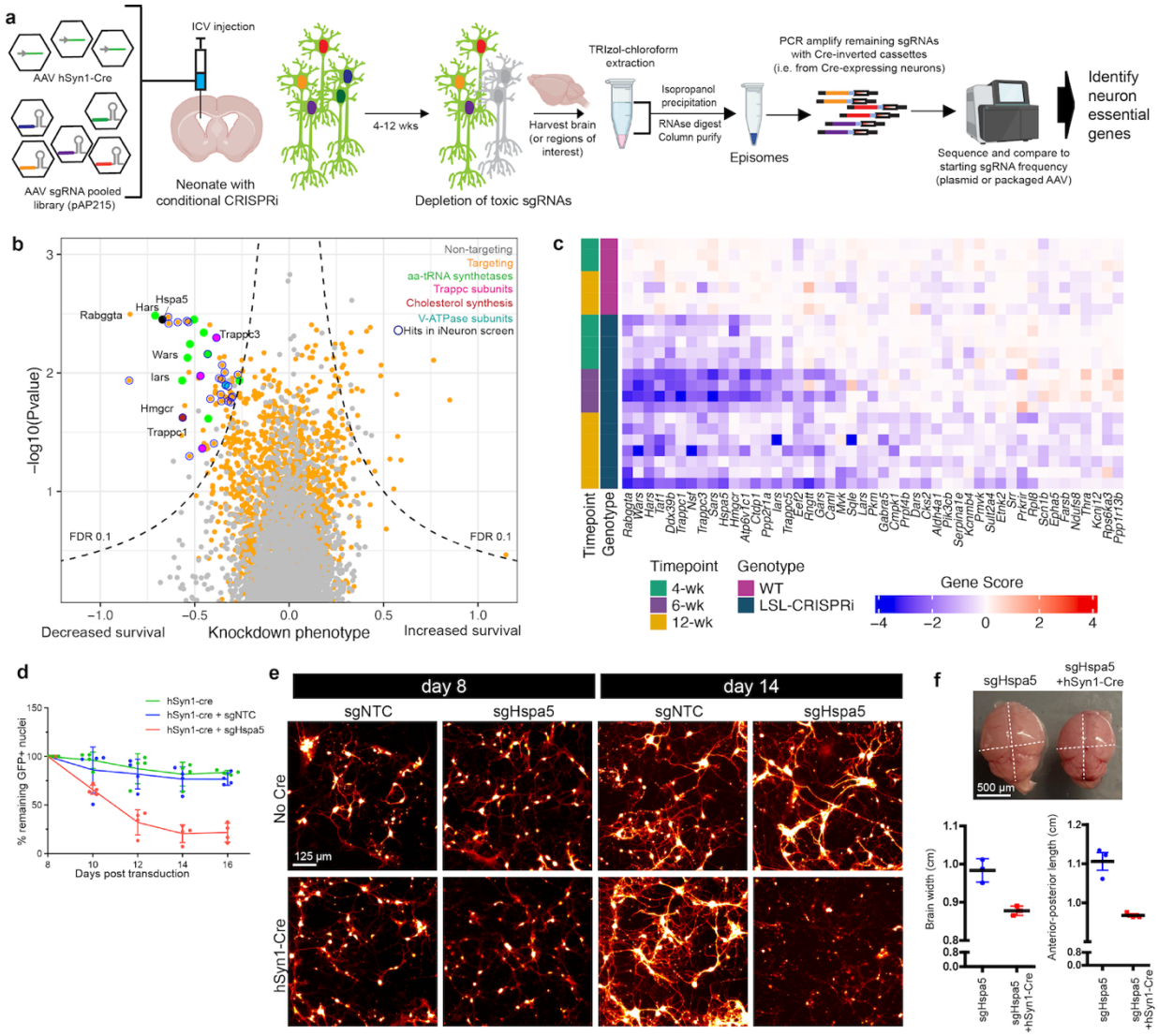
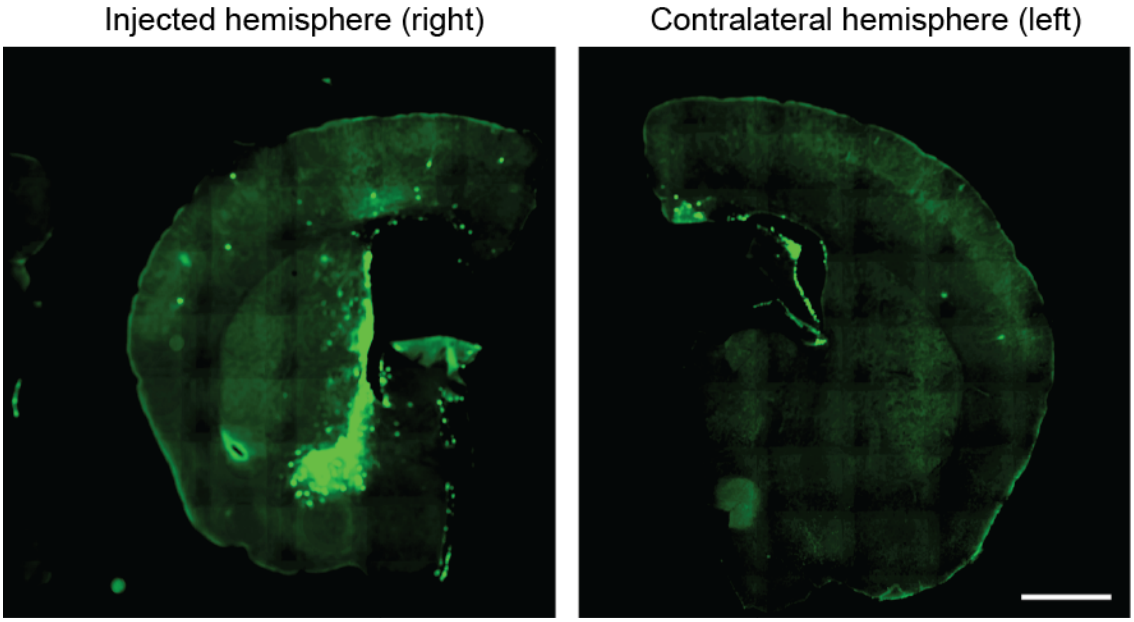


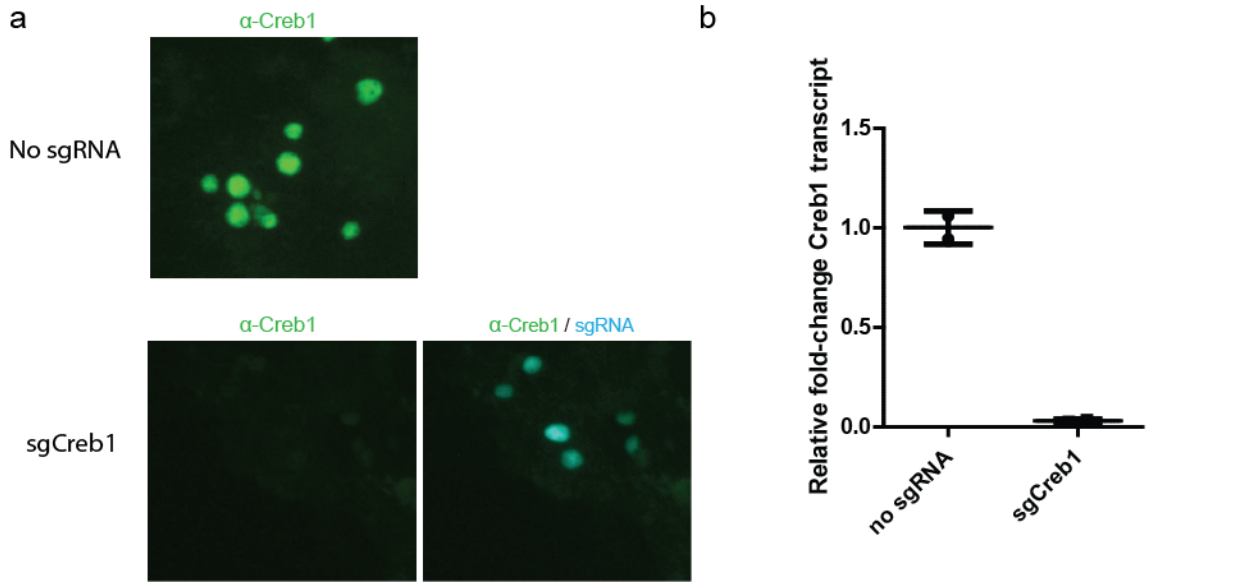
Fig. 2: CRISPRi screen to identify neuron-essential genes within mouse brains *in vivo*
a, Strategy for *in vivo* CRISPRi screening to identify neuron-essential genes. **b**, Knockdown phenotypes for 2,269 genes averaged across four mice at 12 weeks after ICV injection of an sgRNA library. Genes within enriched pathways are color-coded, and hits that overlap with essential genes in iPSC-derived neurons[8] are circled in blue. **c**, Phenotypes of neuron-essential genes from (**b**) with an FDR < 0.1 and negative Gene Score in individual mice (rows), and compared to conditional CRISPRi and wild-type mice at different time points; Pearson correlation is reported in Extended Data Fig. 7. **d**, Validation of the hit gene *Hspa5*, a gene not previously identified as neuron-essential in iPSC-derived neurons. Survival of primary neurons cultured from conditional CRISPRi mice following transduction with AAV-hSyn1-Cre-NLS-GFP alongside AAV-AP215 targeting *Hspa5* (sgHspa5) versus sgNTC (mean ± s.d., n = 4 wells). **e**, Representative images of primary neurons at 8- or 14-days post transduction with different AAVs. **f,** Representative brains of conditional CRISPRi mice 16 days after neonatal ICV injection of AAV-pAP215-sgHspa5 with or without hSyn1-Cre-NLS-GFP, and measurements (mean ± s.d., n = 3 independent mice). Gross motor phenotypes are shown in Supplementary Videos 1 and 2.

	Lentivirus	AAV
Biosafety level	BSL-2	BSL-1
Viral spread	Limited beyond site of injection	High spread throughout injected tissue
Capsid availability	Limited envelope pseudotypes (e.g. VSV-G)	> 16 AAV capsids and growing (e.g. AAV1-9, PHP.eB)
Integration	Integrating, gDNA needed to recover sgRNAs	Non-integrating, episomes needed to recover sgRNAs
PCR reaction volume estimated per brain	Up to 30,000µl to amplify sgRNA from gDNA	Less than 100µl to amplify sgRNAs from episomes

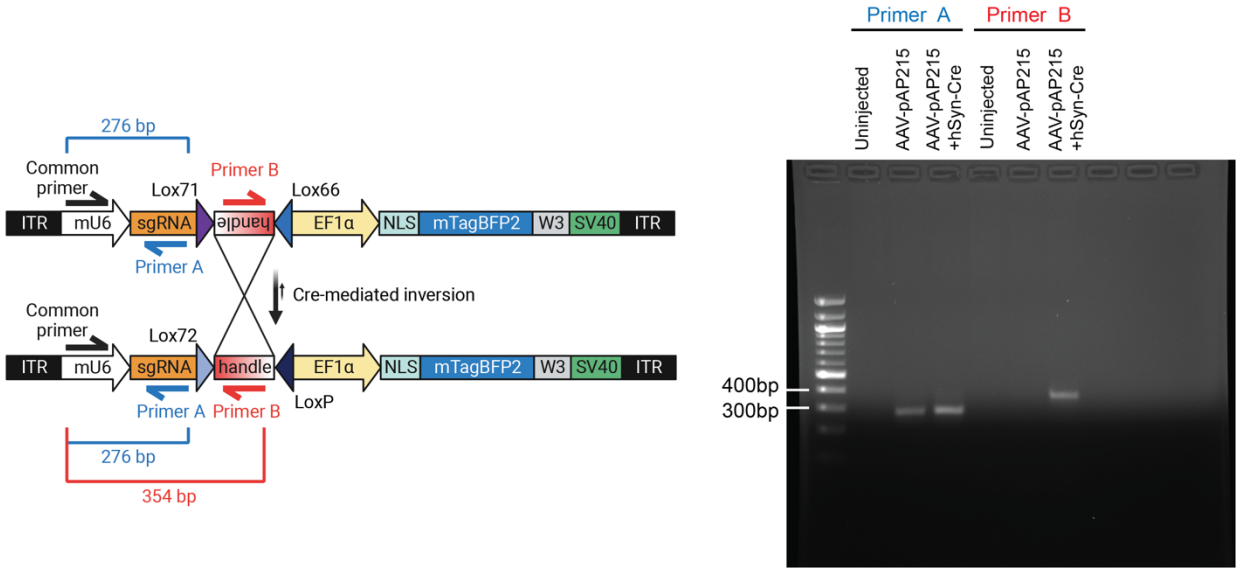
Extended Data Fig. 1: Contrasting lentivirus and AAV for direct *in vivo* CRISPR-based screening on mouse tissues



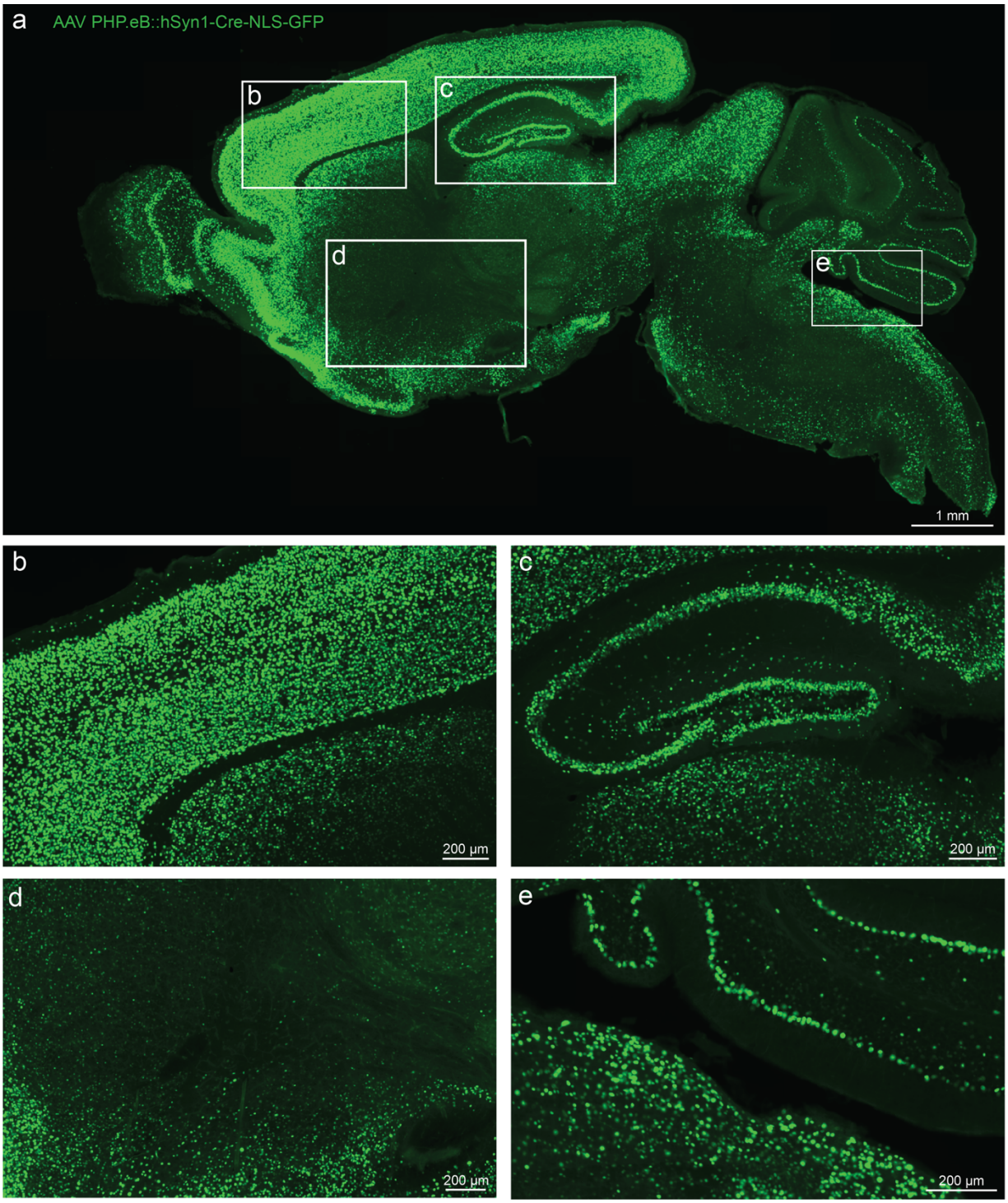
Extended Data Fig. 2: Distribution of lentivirus containing sgRNA in the mouse brain
Lentivirus containing pLG15[9] with a non-targeting sgRNA and a TagBFP marker was injected into the neonate mouse by ICV, and brains were extracted on day 14 and sectioned coronally. The slices were stained using an anti-TagBFP antibody (signal shown in green). Sections were from the same brain. Scale bar 1 mm.



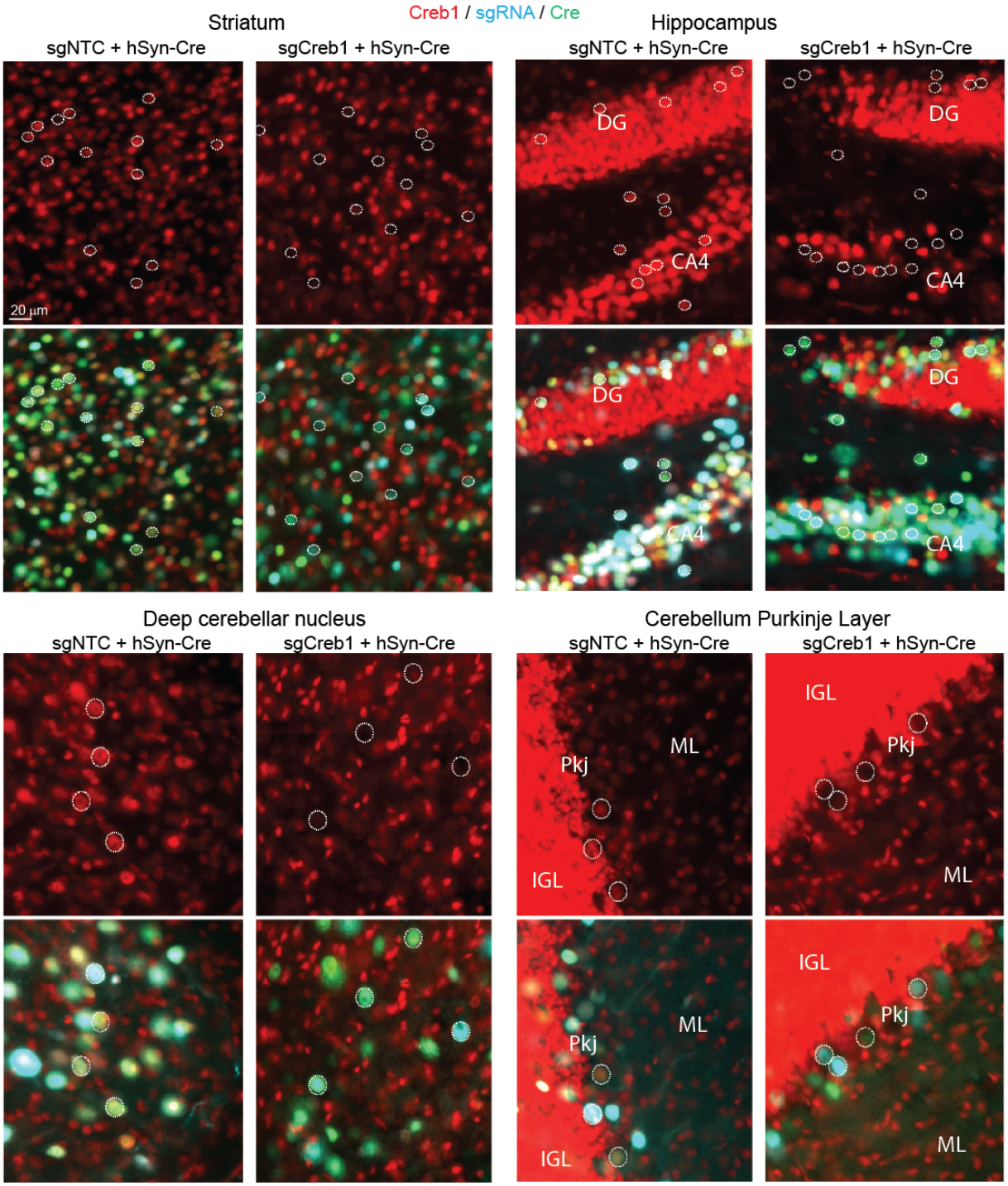
Extended Data Fig. 3: CRISPRi activity in cultured primary neurons of dCas9-KRAB mice. sgRNA targeting *Creb1* delivered by AAV into dCas9-KRAB primary neurons demonstrates marked knockdown of endogenous Creb1 by immunofluorescence (a) and by qRT-PCR (b, mean ± s.d., n = 2 technical replicates).



Extended Data Fig. 4: Cre-dependent inversion of the handle after ICV injection of AAV containing pAP215
PCR amplicons from brains of mice injected by ICV at P0 with pPA215 packaged into AAV PHP.eB, with or without hSyn1-Cre packaged into AAV PHP.eB. The first triplicate of uninjected brain, pAP215 alone, and pAP215 + hSyn1-Cre shows amplicons generated using Primer A, which captures presence of AAV episomes, but is not inversion specific. The second triplicate utilizes Primer B, where the reverse primer binds to the lox-flanked recombination site (handle) and will generate amplicon only in the presence of inversion driven by Cre.

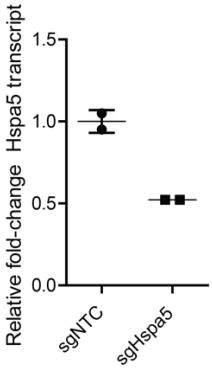


Extended Data Fig. 5: Distribution and expression of AAV in mouse brain. Sagittal section of mouse brain 3 weeks following ICV injection of AAV PHPe.B::hSyn1-Cre-NLS-GFP across the mouse brain (a), including cortex (b), hippocampus (c), striatum (d), and cerebellum (e). The same viral capsid, PHP.eB (ref [10]), was used for delivery of all AAVs in this study. Image intensity is consistent across panels (a), (c), (d), and (e), but is reduced in panel (b) so individual neuronal nuclei are more easily resolved.



Extended Data Fig. 6: Cre-dependent CRISPRi knockdown of Creb1 in different mouse brain regions

Immunofluorescent staining for Creb1 in mice injected with AAV sgNTC or sgCreb1 (blue) along with hSyn1-Cre-NLS-GFP (green), for the indicated brain regions. Representative nuclei containing both BFP and GFP are outlined with a dotted circle, with the same nuclei shown in the top and bottom panels of each brain region, highlighting markedly reduced Creb1 in double-positive nuclei with sgCreb1 as compared to sgNTC. DG: dentate gyrus; ML: molecular layer, Pkj: Purkinje cell layer (Pkj); IGL: internal granule layer.



Extended Data Fig. 7: sgHspa5 knocks down *Hspa5* mRNA level by qPCR in MEFs
Mouse embryonic fibroblasts (MEFs) isolated from constitutive dCas9-KRAB mice were cultured in vitro and transduced with lentivirus containing sgHspa5 (in pAP215). mRNA level was assayed by qPCR and compared between sgHspa5 and sgNTC control (mean ± s.d., n=2 technical replicates).

Methods

Animals

All mice were maintained according to the National Institutes of Health guidelines and all procedures used in this study were approved by the UCSF Institutional Animal Care and Use Committee. Mice were housed on a 12-h light/dark cycle at 22-25 °C, 50-60% humidity, and had food and water provided *ad libitum*. Mice were randomly assigned for the experimental groups at time of injection and both male and female mice were used. In accordance with approved protocol, mice were monitored post injection and if signs of distress appeared, mice were documented and euthanized promptly. The mice used in this study are LSL-dCas9-KRAB mice (B6;129S6-*Gt(ROSA)26Sor*$^{tm2(CAG\text{-}cas9^*/ZNF10^*)Gers}$/J, RRID: IMSR_JAX:033066)[7], dCas9-KRAB mice (B6.Cg-*Igs2*$^{tm1(CAG\text{-}mCherry,\text{-}cas9/ZNF10^*)Mtm}$/J, RRID: IMSR_JAX:030000), and B6 wild-type mice (C57BL/6J, RRID: IMSR_JAX:000664). The mice tested by lentiviral delivery were neonates from a cross between B6(SJL)-*Apoe*$^{tm1.1(APOE^*4)Adiuj}$/J (RRID:IMSR_JAX:027894) and *App*$^{NL\text{-}G\text{-}F}$ knock-in mice[11].

Plasmids

The screening vector pAP215, diagrammed in Figure 1b, was generated using the pAAV-U6-sgRNA-CMV-GFP plasmid as the starting backbone (a gift from Hetian Lei, Addgene plasmid # 85451, RRID:Addgene_85451)[12]. We replaced the hU6-sgRNA cassette with the mU6-sgRNA cassette from pMK1334 (ref. [8]) (Addgene plasmid #127965, RRID:Addgene_127965) by restriction cloning between sites MluI and XbaI. The mU6 was then replaced using Gibson Assembly with a gene block (gBlock, IDT technologies) containing a modified mU6 which combined the first 303 nucleotides from a separate mU6 sequence derived from pMU6-gRNA (gatccgacgccgccatctctaggcccgcgccggccccctcgcacagacttgtgggagaagctcggctactcccctgccccggttaat ttgcatataatatttcctagtaactatagaggcttaatgtgcgataaaagacagataatctgttctttttaatactagctacattttacatgata ggcttggatttctataagagatacaaatactaaattattattttaaaaaacagcacaaaaggaaactcaccctaactgtaaagtaattgt gtgttttgagactataaatatcccttggaga, sequence synthesized by referencing Addgene plasmid # 53187 (ref. [13]) with a BstXI restriction site (ccaccttgttg). The CMV-EGFP module was replaced by EF1a-2xmycNLS-tagBFP2 from pMK1334 by Gibson Assembly. The W3 terminator was cloned from Cbh_v5 AAV-saCBE C-terminal (a gift from David Liu, Addgene plasmid #137183, RRID:Addgene_137183)[14] and inserted at the EcoRI-XhoI multiple cloning site by Gibson

assembly. The hGH was replaced by the SV40 from pMK1334. The Lox66 and Lox71 sequences and their orientation was copied from the pFrt-invCAG-Luc (a gift from Ivo Huijbers, Addgene plasmid #63577, RRID:Addgene_63577)[15] and were inserted along with the 175-bp intervening spacer as a gBlock at the XbaI-KpnI multiple cloning site.

sgRNA cloning

To generate a functional mouse sgRNA pooled library of the pAP210 and pAP215 plasmids, we transferred the sgRNA sequences from the mCRISPRi-v2 M1 (Kinases, Phosphatases, and Drug Targets) gRNA pooled library (Addgene pooled library #83989)[5]. 20 µg of the library was digested with BstXI (Thermo Scientific, ER1021) and Bpu1102I (Thermo Scientific, ER0091). The guide-encoding inserts (84 bp) were resolved on a 4-20% Novex TBE gel (Invitrogen, EC62252BOX) and precipitated with GlycoBlue and sodium acetate. Inserts were washed with ethanol after precipitation and then eluted in DNase- and RNase-free water. The backbone vector, pAP215, was digested in parallel with BstXI and Bpu1102I, resolved on a 1% agarose gel, and purified from the gel (Zymo Research, D4001). The vectors and insert guides were annealed for 16 hrs overnight using T4 ligase (New England Biolabs, M0202L) at a 1:2 molar ratio of vector to insert, and then purified with sodium acetate and ethanol washing. After the final wash, the library product was transformed into chemically competent *E. coli* (Takara, 636763) and 10 colonies were picked at random to ensure that each colony was unique. Upon confirmation, the library product was electroporated into Mega-X competent cells (Invitrogen, C640003) and grown overnight, and a portion of the culture was plated to determine if a coverage of at least 250 colonies per guide was achieved, followed by growth of the remainder of the culture in 1 L of LB for 16 hrs, and purification of the library using ZymoPURE II Plasmid Gigaprep Kit (Zymo Research, D4204).

Individual sgRNAs were cloned in the pAP215 backbone digested with BstXI and Bpu1102I using annealed oligos. The protospacer sequences for the specific sgRNAs used in this study include: sgCreb1 (5'-GGCTGCGGCTCCTCAGTCGG-3'), sgHspa5 (5'-GAACACTGACCTGGACACTT 3'), and a non-targeting control (sgNTC) (5'-GGATGCATAGATGAACGGATG-3').

AAV packaging, purification, and titering

The pAP215-M1 library was packaged into AAV for transduction into neonatal mice as follows. Two 15-cm dishes were each seeded with 1.5×10^7 HEK 293T cells (ATCC, CRL-3216) in 25 ml DMEM complete medium: DMEM (Gibco, 11965-092) supplemented with 10% FBS (VWR, 89510, lot: 184B19), 1% penicillin-streptomycin (Gibco, 15140122), and 1% GlutaMAX (Gibco, 35050061). The next day, 20 µg of pAdDeltaF6 (a gift from James M. Wilson, Addgene plasmid # 112867, RRID:Addgene_112867), 7 µg of pAP215-M1 library plasmid, 7 µg of pUCmini-iCAP-PHP.eB (a gift from Viviana Gradinaru, Addgene plasmid #103005, RRID:Addgene_103005)[16], and 75 µl of 1 mg/ml polyethenylamine (PEI) (Linear, MW 25,000, Polysciences, 23966) were diluted into 4 ml of Opti-MEM (Gibco, 31985062), gently mixed, and incubated at room temperature for 10 min. The PEI/DNA transfection complex was then pipetted drop-wise onto the HEK 293T cells. After 24 hours, the media was replaced with 27 ml of fresh Opti-MEM.

AAV precipitation was performed as previously described[17], with modifications. Cold 5× AAV precipitation solution (40% polyethylene glycol (Sigma-Aldrich, 89510) and 2.5 M NaCl) was prepared. The cells and media were triturated and collected (~30 ml) into a 50 ml conical tube, followed by addition of 3 ml chloroform and vortexing for approximately 30 seconds. The homogenate was centrifuged at 3,000g for 5 min at room temperature, and the aqueous (top) phase was transferred to a new 50 ml conical tube and 5× AAV precipitation solution was added to a final 1× concentration, followed by incubation on ice for at least 1 hour. The solution was centrifuged at 3,000g for 30 min at 4°C. The supernatant was completely removed and the viral pellet was resuspended in 1 ml of 50 mM HEPES and 3 mM $MgCl_2$, and incubated with 1 µl DNase

I (New England Biolabs, M0303S) and 10 µl RNase A (Thermo Scientific, EN0531) at 37°C for 15 min. An equal volume of chloroform was added, followed by vortexing for 15 sec, and centrifuged at 16,000g for 5 min at RT. Using 400 µl at a time, the aqueous phase was passed through a 0.5-ml Amicon Ultra Centrifugal Filter with a 100 kDa cutoff (Millipore, UFC510024) by 3 min of centrifugation at 14,000g, followed by buffer exchange twice with 1× DPBS. This preparation yields 40 µl of AAV at a titer of approximately 2×10^{10} viral genomes per µl. Titering was performed by quantitative RT-PCR as previously described[18], using primers listed in Supplementary Table 3.

To prepare AAV for testing in primary neuronal cultures, HEK293T cells were seeded into a 6-well format containing 1.5 ml of DMEM complete media. The cells were transfected with 1 µg pAdDeltaF6, 350 ng pUCmini-iCAP-PHP.eB, and 350 ng of AAV transgene as above. Approximately 48 hours after transfection, the cells and media were collected in 2 ml microfuge tube, 200 µl of chloroform was added to each tube, vortexed for 15 sec, and centrifuged at 16,000g for 5 min at room temperature. The aqueous (top) phase was transferred to a new tube and AAV precipitation solution was added to 1× dilution, and incubated on ice for at least one hour. The precipitated AAV was centrifuged at 16,000g for 15 min at 4°C, the supernatant was removed, the pellet was resuspended in 100 µl of 1× PBS, and centrifuged again at 16,000g for 1 min to remove excess debris, and the supernatant (purified virus) was transferred to a new microfuge tube. 10 µl purified virus was used per well in primary neuronal cultures in a 24-well format.

Intracerebroventricular injection

Intracerebroventricular (ICV) injections were performed as previously described, with minor modifications[19]. Briefly, neonate mice were placed on a gauze-covered frozen cold pack and monitored for complete cryoanesthesia. The scalp was gently cleaned with an alcohol swab. 1 µl of each AAV with 0.1% trypan blue was loaded into 10 µl syringe (Hamilton, 1701) into a final volume of 2 µl for injection. The syringe was equipped with a 33-gauge beveled needle (Hamilton, 7803-05, 0.5 inches in length). The needle was inserted through the skull 2/5 of distance of the lambda suture to the eye and to a depth of 3 mm to target the left lateral ventricle. Following a one-time unilateral injection, the neonate was placed on a warming pad for recovery and returned to the parent cage.

Retroorbital injection

3 µl of purified AAV sgRNA library was diluted into 100 microliters of 1x PBS and loaded into a 29G x 0.5 inches 1cc insulin syringe. Mice were briefly anesthetized into a drop chamber containing gauze soaked in 0.5 ml isoflurane, followed by intravenous injection of the 100 µl of diluted AAV into the retroorbital space accessed medial to the mouse right globe. The mice were monitored for awakening returned to their cage for recovery. All eight mice injected were 9-week-old males.

sgRNA recovery, sequencing, and analysis

Animals were euthanized using CO_2, and their whole brains were removed and stored at -80°C. The sex of the mice was recorded prior to euthanasia. Each brain was placed in a PYREX 7 ml Dounce Homogenizer (Corning, 7722-7) with 2 ml of TRIzol (Invitrogen, 15596026) and thoroughly homogenized using the A pestle (0.0045 nominal clearance) for 10 or more strokes. The homogenate was divided into two 1.5 ml centrifuge tubes and 0.2 ml of chloroform was added to each tube, followed by centrifugation at 12,000g for 15 min at 4ºC. The aqueous phase was transferred to a new tube, and 0.5 ml of isopropanol was added and centrifuged at 12,000g for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml of 75% ethanol

in DNase/RNase-free water, then vortexed briefly, and then spun down at 7,500g for 5 min. The supernatant was then removed and the pellet was allowed to air dry for 10 mins, and then resuspended in 100 µl of DNase/RNase-free water and incubated with 1 µl of RNase A (Thermo Scientific, EN0531) at 37ºC overnight. The sample was then column purified by Zymo DNA Clean & Concentrator-25 kit (Zymo Research, D4033) and eluted in 50 µl of DNase/RNase-free water to yield recovered viral DNA.

The purified episomal DNA and the starting plasmid pooled library were PCR amplified with adapter sequences (Supplementary Table 3), sequenced on the Illumina HiSeq4000 using a custom sequencing primer (oMK734_HS4Kmirror_CRISPR_SP: CCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAAACTTGCTATGCTGT) at the UCSF Center for Advanced Technologies, and analyzed as previously described[8]. Phenotypes and adjusted *p*-values for each gene were generated using our previously established analysis pipeline (https://kampmannlab.ucsf.edu/mageck-inc)[8]. The PCR was performed using Q5 High-Fidelity 2X Master Mix (NEB, M0492L) with conditions described in Supplementary Table 3.

Lentivirus packaging, purification, and injection

The pLG15 vector containing a non-targeting control sgRNA was packaged into lentivirus as previously performed[9] by transfecting 10 µg of the transfer plasmid and 10 µg of lentiviral packaging plasmids (containing 1:1:1 pRSV, pMDL, pVSVG) into 1.0×10^7 HEK293T cells cultured in a 10-cm dish in DMEM complete medium. 48 hours after transfection, the virus was precipitated from the media supernatant using Lentivirus Precipitation Solution (Alstem, VC100) and resuspended in 50 µl of PBS. For ICV injection, 4 µl of virus was used for each neonatal mouse. Mouse brains were extracted on day 14 and sectioned coronally.

Mouse cortical neuron primary cultures and immunocytochemistry

Neonates were briefly sanitized with 70% EtOH and decapitated using sharp scissors, and the brains were removed and placed into cold HBSS (Gibco, 14175095). The meninges were removed under a dissecting microscope, and the cortices were transferred to a 15-ml conical tube containing 10 ml of 0.25% Trypsin-EDTA (Gibco, 25200056) and incubated at 37°C for 30 min. The trypsin was removed and the brains were gently rinsed twice in 5 ml of DMEM complete media, followed by trituration of brains in 5 ml of DMEM complete media using a glass Pasteur pipette (VWR, 14672-380). The triturated tissue was resuspended of DMEM complete media and filtered through a 40 µm nylon cell strainer (Corning, 352340). Approximately one brain was plated across each BioCoat Poly-D-Lysine 24-well TC-treated plate (Corning, 356414). The following day, day in vitro 1 (DIV1), the DMEM complete media was replaced with neuronal growth media composed of Neurobasal-A Medium (Gibco, 10888022), 1× B-27 Supplement minus vitamin A (Gibco, 12587010), GlutaMAX Supplement (Gibco, 35050079), and 1% penicillin-streptomycin (Gibco, 15140122). On DIV2, the cultures were further supplemented with cytarabine to a final concentration of 1 mM (Thermo Scientific Chemicals, 449561000). The primary neuronal cultures were transduced with AAV on DIV4 and imaged starting 4 days after transduction, every other day until day 16 post-transduction (DIV20).

Mouse embryonic fibroblast generation and lentiviral transduction

Mouse embryonic fibroblasts (MEFs) were harvested at approximately embryonic day 15 from a pregnant mouse containing homozygous constitutive dCas9-KRAB machinery. The heads and internal organs were removed and the embryos were dissected with sharp scissors into pieces ranging from 2-3 mm. The tissues were incubated with 10 ml 0.25% Trypsin-EDTA at 37°C for 30 mins. The trypsin was then carefully aspirated and replaced with 10 ml DMEM complete media, followed by vigorous pipetting, and filtering through a 40 µm nylon cell strainer (Falcon, 352340).

This cell suspension was then centrifuged at 200g for 5 mins, and resuspended in 10 ml DMEM complete media, and plated on a 10 cm tissue culture petri dish (Corning, 353003).

The sgRNA sequence targeting *Hspa5* was inserted into the pMK1334 lentiviral backbone[8] using the strategy described in the "sgRNA cloning" section above. 1 mg of plasmid was packaged into lentivirus using $1x10^6$ HEK293T cells plated in a 35 mm well, precipitated after 48 hours, and resuspended in 200 µL 1x PBS. 50 µL of the virus was used to transduce MEFs plated in a 12-well format, and the cells were selected with 2 µg/ml puromycin (Sigma-Aldrich, P9620) and harvested 5 days after transduction. The cells were then collected for RNA isolation and quantitative RT-PCR.

RNA isolation and quantitative RT-PCR

RNA was isolated with the Zymo Quick-RNA Microprep Kit (Zymo Research, R1050). Samples were prepared for qPCR in technical duplicates in 10-µl reaction volumes using SensiFAST SYBR Lo-ROX 2× Master Mix (Bioline, BIO-94005), custom qPCR primers from Integrated DNA Technologies used at a final concentration of 0.2 µM and cDNA diluted at 1:20 by volume. qPCR was performed on a Bio-Rad CFX96 Real Time System C1000 Touch Thermocycler. The following cycles were run (1) 98°C for 3 min; (2) 95°C for 15 s (denaturation); (3) 60°C for 20 s (annealing/extension); (4) repeat steps 2 and 3 for a total of 39 cycles; (5) 95°C for 1 s; (6) ramp 1.92°C s^{-1} from 60°C to 95°C to establish melting curve. Expression fold changes were calculated using the ΔΔCt method, normalizing to housekeeping gene *Gapdh.* RT-qPCR primers are listed in Supplementary Table 3.

Mouse brain immunohistochemistry

Whole brains were removed and fixed overnight at 4ºC in 4% paraformaldehyde (Electron Microscopy Sciences, 15710) diluted in 1× PBS. The following day, the fixative was replaced with 30% sucrose dissolved in 1× PBS for at least 48 hours. Fixed brains were blotting dry, cut down the midline with a razorblade, and mounting into a cryomold (Epredia, 2219) using OCT compound (Sakura Finetek, 4583). To snap freeze, cryomolds were partially submerged in a pool of 2-propanol cooled by a bed of dry ice. Brains were sectioned in the sagittal plane at 40 µm on a cryostat (Leica, CM1950) with a 34° MX35 Premier+ blade (Epredia, 3052835). The resulting brain sections were stored free-floating in 1× PBS + 0.05% NaN_3 at 4ºC. When ready for staining, representative brain sections were wasted three times in 1× PBS and incubated in a 24 well plate at room temperature for one hour in blocking buffer: 10% goat serum (Gibco, 16210064), 1% BSA (Sigma-Aldrich, A7906), and 0.3% Triton X-100 (Sigma-Aldrich, T8787) diluted in 1× PBS. The brain sections were incubated in primary antibodies diluted in blocking buffer overnight at 4ºC on a gentle shaker. The sections were washed three times in 1× PBS, then incubated in secondary antibodies for 2 hours at room temperature in the dark on a gentle shaker. Sections were washed three times in 1× PBS and moved to charged glass microscope slides (Fisher Scientific, 12-55015). After PBS was removed, Fluoromount-G with DAPI mountant (Invitrogen, 00-4959-52) was added, and a No. 1.5 coverslip (Globe Scientific, 1415-15) was applied. Slides were dried at room temperature in the dark overnight and sealed with nail polish. For experiments without DAPI, ProLong Gold Antifade mountant (Invitrogen, P10144) was used instead. For experiments with Hoechst instead of DAPI, sections were lastly incubated for 15 mins in Hoechst 33342 (BD Pharmingen, 561908) diluted 2 µg/ml in 1× PBS, then washed 3 times in 1× PBS before mounting using ProLong Gold mountant.

The following primary antibodies were used: rabbit anti-CREB (1:1,000 dilution, clone: 48H2, Cell Signaling Technologies, 9197), rabbit anti-SOX9 (1:2,000 dilution, polyclonal, EMD Millipore, AB5535), rabbit anti-Cre recombinase (1:500 dilution, clone: D7L7L, Cell Signaling Technologies, 15036), guinea pig anti-NeuN (1:500 dilution, polyclonal, Synaptic Systems, 266004), alpaca FluoTag-Q anti-TagFP nanobody (reacts to mTagBFP2 but not eGFP, 1:500 dilution, clone: 1H7,

Alexa647 pre-conjugated, NanoTag Biotechnologies, N0501-AF647-L). A different antibody for mTagBFP2 was used in Extended Data Fig. 2: rabbit anti-tRFP (1:1,000 dilution, polyclonal, Evrogen, AB233). The following secondary antibodies were used: goat anti-rabbit IgG Alexa Fluor 488 (1:1,000 dilution, Invitrogen A-11034), goat anti-rabbit IgG Alexa Fluor 568 (1:1,000 dilution, Invitrogen, A-11011), goat anti-rabbit IgG Alexa Fluor 647 (1:1,000 dilution, Invitrogen, A-21245), goat anti-guinea pig IgG Alexa Fluor 488 (1:1,000 dilution, Invitrogen, A-11073), goat anti-guinea pig IgG Alexa Fluor 647 (1:1,000 dilution, Invitrogen, A-21450). All secondary antibodies were highly cross-absorbed.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 10 min, washed briefly with 1x PBS, and incubated in blocking buffer (5% normal goat serum + 0.1% Triton X-100) for 15 min, all at room temp. Rabbit anti-CREB was applied at 1:2,000 dilution and incubated overnight at 4ºC. The cells were gently rinsed five times with 1x PBS, incubated for 1 hour with goat anti-rabbit IgG Alexa Fluor 488, rinsed five times with 1x PBS, and imaged.

Microscopy, image segmentation, and analysis

Slides containing brain sections were imaged using a Zeiss AxioScan.Z1 with a Zeiss Colibri 7 unit, ×20/0.8 NA objective lens, 5-30 ms exposure, 1×1 binning and 25-100% intensity using 425-nm, 495-nm, 570-nm and 655-nm lasers, running ZEN version 2.6 software. The images were imported into QuPath (version 0.4.2) for analysis. To identify overlap between BFP, NeuN, and Sox9, a representative region of the cortex was outlined and the nuclei were segmented on the DAPI channel using the 'Cell detection' module without expansion of the nuclei to develop virtual cell boundaries. Classifiers were created to distinguish BFP-, NeuN-, and Sox9-positive cells, and applied sequentially. Cells containing overlapping NeuN and Sox9 were considered to be neurons and only cells exclusively containing Sox9 were considered astrocytes.

To measure Creb1 levels, a 0.75 mm diameter circular region of the frontal cortex of each brain was selected, and the nuclei were segmented on the DAPI channel as above. The measurements for the segmented nuclei were exported. The mean fluorescence intensity for the anti-Creb1 channel was obtained selected by the top 200 nuclei of highest anti-mTagBFP2 fluorescence intensity.

For mouse primary neurons transduced with AAV, live imaging was performed every other day using an ImageXpress Micro Confocal HT.ai High-Content Imaging System (Molecular Devices). The imaging chamber was warmed to 37ºC and equilibrated with 5% CO_2. The system used an Andor Zyla 4.5 camera with a Plan Apo ×10/0.45NA objective lens, an 89 North LDI laser illumination unit, 10-500 ms exposure time, 1×1 binning, and 10% laser intensity using 405-nm, 475-nm, and 555-nm lasers, running MetaXpress (version 6.7.1.157). Resulting images were imported into Cell Profiler (version 4.2.1)[20] and analyzed using a custom pipeline. hSyn1-Cre-GFP+ nuclei were segmented using the 'IdentifyPrimaryObjects' module, with expected diameter 8-40 pixels, using an Adaptive threshold (size 50) and the Minimum Cross-Entropy method, with a 1.5 smoothing scale, 1.0 correction factor, and lower- and upper-bound threshold at 0.435 and 1, respectively. Segmented objects were exported, and counted in each field, then summed across all fields within a well to calculate the number of objects per well (n=29 fields per well, n=4 wells per condition), using a custom R script. This was repeated for each timepoint. Data was normalized to fluorescent intensity at day 8 (as before that day, fluorescence intensity increased linearly with time in all channels as cells manufactured fluorescent proteins) and percentage change was calculated for each well from day 8, for subsequent timepoints through day 16.

Statistics and Reproducibility

No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported in previous publications, as cited in the main text. Numbers of replicates are listed in each figure. No repeat measurements were made on the same samples. Data were assumed to be normally distributed except for instances within the MAGeCK-iNC pipeline where the two-sided Mann-Whitney U-test (no distributional assumptions) was used to determine statistical significance. The MAGeCK-iNC pipeline was used with FDR < 0.1 and controls for multiple comparisons. For cell culture experiments, randomization was not performed because treatment groups of cells were derived from the same parent population of cells. Data collection and analysis were not performed blinded to the conditions of the experiments. No animals or data points were excluded from the relevant analyses, with the exception of some 4-wk and 12-wk timepoint screens which were excluded due to poor technical quality (n= 7, of 30 total). Poor technical quality was defined as fewer than 1 million reads and/or had a poor correlation between the input (AAV) and output (brain) sgRNA counts ($R^2 < 0.9$). Major findings were validated using independent samples and orthogonal approaches.

Data availability

All Data are available from the corresponding author (MK), and will be made publicly available at the UCSF Dryad data repository opn publication. There are no restrictions on data availability.

Code availability

For pooled CRISPR screen analysis, we used MAGeck-iNC (version 5.2), which is available at https://kampmannlab.ucsf.edu/mageck-inc. The R notebooks for analysis are available at https://kampmannlab.ucsf.edu/article/scripts-vivo-screening-manuscript. The CellProfiler pipelines will be made available on request to the corresponding author (MK), and will also be submitted to the CellProfiler depository of published pipelines (https://cellprofiler.org/examples/published_pipelines.html) upon publication.

Supplementary Table legends

Supplementary Table 1: In vivo screening hits by individual mouse replicate
These tables provide combined epsilon, *p*-value, and gene scores (product value) per gene index for in vivo neuron-selective M1 library screens at 4-wk, 6-wk, and 12-wk timepoints, produced by MAGeCK-iNC analysis pipeline[21] for individual mice. Each tab contains an individual mouse, indicating its genotype ("CRISPRi" for LSL-dCas9-KRAB mice or "WT" for wild-type C57BL/6J mice), timepoint (4-wk, 6-wk, or 12-wk), sex, and replicate number.

Supplementary Table 2: In vivo screening hits by combined timepoint cohorts
These tables provide combined epsilon, *p*-value, and gene scores (product values) per gene index for combined cohorts of in vivo neuron-selective M1 screens at each timepoint. Each cohort consists of age-matched mice at 4-wk, 6-wk, or 12-wk timepoints pooled together bioinformatically prior to analysis by MAGeCK-iNC analysis pipeline[8]. Each tab is a specific timepoint (4-wk, 6-wk, or 12-wk) and genotype ("CRISPRi" for LSL-dCas9-KRAB mice or "WT" for wild-type C57BL/6J mice).

Supplementary Table 3: List of primers used for next generation sequencing and RT-qPCR and PCR cycling parameters
This table shows the primers and the corresponding Illumina indices for generating sgRNA amplicons for next generation sequencing for both pAP210 and pAP215 constructs, along with the custom sequencing primer. Separate tabs also list the PCR cycling parameters for library

generation and the primers used for RT-qPCR for *Hspa5*, *Creb1*, *Gapdh*, and ITRs for viral titering.

Supplementary Video legends

Supplementary Video 1: Gross motor phenotypes in sgHspa5 + hSyn1-Cre injected mice
LSL-dCas9-KRAB neonates were co-injected by ICV with AAV containing sgHspa5 (in pAP215) and AAV containing hSyn1-Cre. At 16 days post injection, mice displayed gross motor phenotypes, as recorded in the video. After video capture, mice were immediately euthanized as per IACUC protocol. Mice were littermates to those shown in Supplementary Video 2 and injected and recorded at the same timepoint.

Supplementary Video 2: Lack of gross motor phenotypes in sgHspa5 only injected mice
LSL-dCas9-KRAB neonates were co-injected by ICV with AAV containing sgHspa5 (in pAP215) and an equivalent volume of phosphate buffered saline. At 16 days post injection, mice displayed normal motor phenotypes, as recorded in the video. Mice were littermates to those shown in Supplementary Video 1 and injected and recorded at the same timepoint.

Supplementary Material References

8. Tian, R. *et al. Neuron* **104**, 239-255.e12 (2019).
9. Gilbert, L. A. *et al. Cell* **159**, 647–661 (2014).
10. Goertsen, D. et al. Nat Neurosci 25, 106–115 (2022).
11. Saito, T. *et al. Nat Neurosci* **17**, 661–663 (2014).
12. Duan, Y. *et al. J Biol Chem* **291**, 16339–16347 (2016).
13. Kabadi, A. M., Ousterout, D. G., Hilton, I. B. & Gersbach, C. A. *Nucleic Acids Res* **42**, e147 (2014).
14. Levy, J. M. *et al. Nat Biomed Eng* **4**, 97–110 (2020).
15. Huijbers, I. J. *et al. Nat Protoc* **10**, 1755–1785 (2015).
16. Chan, K. Y. *et al. Nat Neurosci* **20**, 1172–1179 (2017).
17. Negrini, M., Wang, G., Heuer, A., Björklund, T. & Davidsson, M. *Curr Protoc in Neurosci* **93**, e103 (2020).
18. Aurnhammer, C. *et al. Hum Gene Ther Methods* **23**, 18–28 (2012).
19. Kim, J.-Y., Grunke, S. D., Levites, Y., Golde, T. E. & Jankowsky, J. L. *JoVE* e51863 (2014).
20. Stirling, D. R. *et al. BMC Bioinformatics* **22**, 433 (2021).
21. Li, W. *et al. Genome Biol* **15**, 554 (2014).