

1 **Title:** CRISPR screening by AAV episome-sequencing (CrAAVe-seq) is a highly scalable cell
2 type-specific *in vivo* screening platform
3

4 **Authors:** Biswarathan Ramani^{*1,2}, Indigo V.L. Rose^{*1,3}, Noam Teyssier^{1,4}, Andrew Pan¹,
5 Spencer Danner-Bocks¹, Tanya Sanghal¹, Lin Yadanar¹, Ruilin Tian^{1,5}, Keran Ma^{6,7}, Jorge J.
6 Palop^{6,7}, Martin Kampmann^{1,8}
7

8 **Affiliations:**

- 9 1. Institute for Neurodegenerative Diseases; Weill Institute for Neurosciences, University of
10 California, San Francisco, San Francisco, CA, USA
- 11 2. Department of Pathology, University of California, San Francisco, San Francisco, CA,
12 USA
- 13 3. Neuroscience Graduate Program, University of California, San Francisco, San
14 Francisco, CA, USA
- 15 4. Biological and Medical Informatics Graduate Program, University of California, San
16 Francisco, San Francisco, CA, USA
- 17 5. Biophysics Graduate Program, University of California, San Francisco, San Francisco,
18 CA, USA
- 19 6. Gladstone Institute of Neurological Disease, San Francisco, CA, USA
- 20 7. Department of Neurology, University of California, San Francisco, San Francisco, CA,
21 USA
- 22 8. Department of Biochemistry and Biophysics, University of California, San Francisco, San
23 Francisco, CA, USA

24 * Equal contribution

25 Correspondence: martin.kampmann@ucsf.edu
26

27 **Abstract**

28 There is a significant need for scalable CRISPR-based genetic screening methods that can be
29 applied directly in mammalian tissues *in vivo* while enabling cell type-specific analysis. To address
30 this, we developed an adeno-associated virus (AAV)-based CRISPR screening platform, CrAAVe-
31 seq, that incorporates a Cre-sensitive sgRNA construct for pooled screening within targeted cell
32 populations in mouse tissues. We demonstrate the utility of this approach by screening two distinct
33 large sgRNA libraries, together targeting over 5,000 genes, in mouse brains to create a robust
34 profile of neuron-essential genes. We validate two genes as strongly neuron-essential in both
35 primary mouse neurons and *in vivo*, confirming the predictive power of our platform. By comparing
36 results from individual mice and across different cell populations, we highlight the reproducibility
37 and scalability of the platform and show that it is highly sensitive even for screening smaller
38 neuronal subpopulations. We systematically characterize the impact of sgRNA library size, mouse
39 cohort size, the size of the targeted cell population, viral titer, and multiplicity of infection on screen
40 performance to establish general guidelines for large-scale *in vivo* screens.
41

42 **Main**

43 CRISPR-based genetic screens are powerful tools for biological discovery since they enable the
44 massively parallel interrogation of gene function. Most CRISPR screens are carried out in cultured
45 cells. First applications in iPSC-derived brain cell types such as neurons^{1,2}, microglia³ and
46 astrocytes⁴ have uncovered cell type-specific mechanisms relevant to neuroscience and
47 neurological diseases. However, a major limitation of CRISPR-based screens in cultured cells is
48 that they do not fully recapitulate the physiological context of a multicellular organism, or tissue
49 states such as aging, inflammation, or disease. These limitations are particularly evident in
50 applications to biological questions related to complex organs like the brain, which involves
51

52 intricate spatial interactions between numerous distinct cell types and subtypes. Therefore, *in vivo* pooled CRISPR screens directly targeting endogenous cells in the brains of mice have the
53 potential to uncover insights that would be elusive in cell culture.
54

55 A small number of *in vivo* CRISPR screens targeting endogenous brain cells have previously been
56 reported^{5–10}, reviewed by Braun et al¹¹ and summarized in **Extended Data Fig. 1a**) Some of these
57 screens involved delivering single-guide RNA (sgRNA) libraries to the brain via lentivirus, but
58 there is increasing recognition that lentivirus-based approaches have several drawbacks
59 (summarized in **Extended Data Fig. 1b**). Such drawbacks include poor distribution of lentivirus
60 through the brain and the inability to differentiate between cell types in which specific sgRNAs
61 were expressed. To overcome some of these limitations, emerging studies have turned to adeno-
62 associated virus (AAV), which has more widespread brain transduction, and combined with
63 CRISPR perturbations and single-cell RNA sequencing (AAV-Perturb-Seq)^{9,10}. This powerful
64 approach provides crucial and granular details on transcriptional changes in specific cell types.
65 However, the current cost of scaling this strategy to study larger cellular populations in the mouse
66 brain and across multiple independent mice is prohibitive. Prior work using this approach has so
67 far not exceeded a library size of 65 sgRNAs (targeting 29 genes) and a sampling of 60,000 cells¹⁰
68 (**Extended Data Fig. 1a**). Screens at such a scale enable the phenotypic profiling of a small
69 number of preselected genes of interest, but not the unbiased discovery of unexpected genes
70 that have a phenotype of interest. As such, there is a great need for a substantially more scalable
71 *in vivo* CRISPR screening platform that retains the ability to discriminate between cell types.
72

73 We developed a strategy for screening in the mouse brain called "CRISPR screening by AAV
74 episome sequencing" (CrAAVe-seq). Incorporating a Cre recombinase-based genetic element
75 into the sgRNA library backbone enables the selective evaluation of phenotypes caused by
76 genetic perturbations only in cell types of interest. Furthermore, CrAAVe-seq exploits the
77 amplification of sgRNA sequences from AAV episomes^{12,13}, rather than genomic DNA, to
78 dramatically increase the scalability and reduce the cost of quantifying sgRNA frequencies from
79 whole brain homogenates. Using CrAAVe-seq, we profiled neuron-essential genes in the mouse
80 brain in different neuronal subpopulations, utilizing libraries containing ~12,000 and ~18,000
81 sgRNAs per brain and sampling at least 2.5 million neurons per brain. This approach yielded
82 highly reproducible top hits across independent mice. Therefore, CrAAVe-seq enables high-
83 throughput, cost-effective screening of the mammalian CNS, with immediate applicability to other
84 cell types and tissues.
85

86 Results

87

88 **CrAAVe-seq strategy for cell type-specific *in vivo* screening of neurons in the mouse brain**

89 We aimed to leverage the high CNS tropism and infectivity for neurons of AAV, particularly in
90 comparison to lentivirus, to develop an AAV-based system for pooled CRISPR perturbations of
91 endogenous neuronal genes in the mouse brain. However, because of the ability of AAV to
92 transduce many different cell types¹⁴, we sought a cell-type specific approach for CRISPR
93 screening. To address this, we designed pAP215, an AAV vector for single-guide RNA (sgRNA)
94 expression that contains an mU6-driven sgRNA sequence followed by a Lox71/Lox66-flanked
95 175 bp "handle" cassette that undergoes predominantly unidirectional¹⁵ inversion in cells
96 expressing Cre recombinase (**Fig. 1a**). The construct expresses a nuclear-localized blue
97 fluorescent protein (NLS-mTagBFP2) for visualization. A fully annotated map of the pAP215 is
98 provided (**Supplementary File 1**).
99

100

101 We devised a strategy using pAP215 to screen for essential neuronal genes in the mouse brain
102 *in vivo*, schematized in **Fig. 1b**. Along with uncovering key pathways involved in neuronal

103 homeostasis, neuronal survival screens can be readily applied to various mouse models of
104 neurological diseases, including neurodegenerative diseases, traumatic brain injury, and stroke,
105 to identify genetic drivers or modifiers of neuronal susceptibility. AAVs containing pAP215 and
106 promoter-driven Cre recombinase (e.g. a pan-neuronal hSyn1 promoter-driven Cre) are co-
107 injected into the brains of inducible CRISPR interference (Lox-Stop-Lox-dCas9-KRAB, or LSL-
108 CRISPRi) mice¹⁶. This leads to the activation of CRISPRi machinery and inversion of the handle
109 sequence in neurons. PCR amplification by priming against the inverted handle on AAV
110 episomes, followed by sequencing, enables the identification and quantification of sgRNAs in Cre-
111 expressing neurons.

112
113 We packaged our AAV plasmids into the PHP.eB capsid, which is known to enable widespread
114 transduction of brain cells¹⁷. We co-injected pAP215 (PHP.eB::pAP215) and an hSyn1 promoter-
115 driven Cre recombinase tagged with a nuclear GFP (PHP.eB::hSyn1-Cre, diagram of construct
116 shown in **Extended Data Fig. 1c**) by intracerebroventricular (ICV) injection into neonatal mice.
117 We saw broad distribution of both pAP215 and hSyn1-Cre reporters across the brain, particularly
118 in the cortex and hippocampus (**Fig. 1c**), consistent with the known distribution profile of the
119 PHP.eB capsid¹⁸. Contrasting this, we observe much more limited expression and spread of
120 lentivirus delivered by the same method and expressing the same nuclear mTagBFP reporter
121 (**Extended Data Fig. 2**).

122
123 CRISPR screens performed in cell culture using lentivirus, which integrate their DNA into the
124 host genome, require extraction of genomic DNA before PCR amplification of the integrated
125 sgRNAs. In contrast, recombinant AAV genomes are mostly maintained as circular or
126 concatenated DNA episomes, with a minor fraction integrating into the host genome¹⁹⁻²¹.
127 Consequently, a major potential advantage of AAV is that the viral genomes can be precipitated
128 and concentrated from the aqueous phase of a TRIzol-chloroform extraction as previously
129 demonstrated in the context of AAV capsid screening^{12,13}, which could vastly improve scalability
130 for a pooled CRISPR screen. For example, if PCR of sgRNAs from genomic DNA was
131 necessary, a whole mouse brain weighing approximately 500 mg is expected to yield up to 1500
132 µg of genomic DNA. Since PCR reactions have an upper limit for the amount of template DNA
133 (typically 10 µg per 100 µl reaction)^{22,23}, screening across a single brain would require up to
134 15,000 µl of PCR reaction volume. This imposes a severe restriction on scalability, as large
135 volumes of PCR reactions become economically and practically infeasible, especially for
136 screening libraries necessitating dozens of mice.

137
138 In comparison, we performed isopropanol precipitation of nucleic acids from the aqueous phase
139 of a TRIzol-chloroform extraction of an AAV-injected whole mouse brain. We resuspended the
140 nucleic acid pellet in 50 µL of water and treated it with RNase, which removes all RNA without
141 digesting the DNA of the AAV episomes. Using 1 µL of this “episome” fraction as a template, we
142 confirmed by PCR that pAP215 could be detected in the injected brains (**Fig. 1d**). Importantly, we
143 found that the Cre-inverted handle sequence was detectable only in mice co-injected with hSyn1-
144 Cre (**Fig. 1d**). With the episome fraction containing a nucleic acid concentration of less than 10
145 ng/µL, a single 50 to 100 µL PCR reaction volume could be used if desired.

146
147 We further confirmed that PHP.eB::hSyn1-Cre expresses in neurons by immunostaining for a
148 neuronal marker (NeuN) and an astrocyte marker (SOX9) (**Fig. 1e**). Image analysis showed that
149 93% of GFP⁺ co-localized with NeuN⁺ nuclei, while only 0.3% overlapped with SOX9⁺ nuclei. Of
150 note, manual inspection of the SOX9⁺ nuclei flagged as GFP⁺ showed GFP signal was emanating
151 from adjacent neurons that overlapped with SOX9⁺ nuclei. In effect, we did not observe any

152 SOX9⁺ nuclei with distinct GFP signal within their nucleus, confirming hSyn1-Cre's known
153 selective expression in neurons²⁴.

154
155 **CRISPRi knockdown *in vivo* using AAV**
156 To test that the pAP215 plasmid is effective for CRISPRi knockdown, we used an sgRNA targeting
157 Creb1 (sgCreb1), which encodes a ubiquitously expressed nuclear protein that is not essential
158 for neuronal survival²⁵. We also generated a non-targeting sgRNA control (sgNTC) in pAP215.
159 We co-injected PHP.eB::pAP215-sgCreb1 or PHP.eB::pAP215-sgNTC alongside
160 PHP.eB::hSyn1-Cre by ICV into neonatal LSL-CRISPRi mice, at approximately 1×10^{11} viral
161 particles of each virus per mouse. We also included a group of mice injected with sgCreb1 alone
162 to check for leakiness of the CRISPRi machinery. Three weeks after neonatal ICV injection,
163 immunofluorescence staining confirmed strong knockdown of endogenous CREB1 in all neurons
164 that received both sgCreb1 (BFP⁺ nuclei) and hSyn1-Cre (GFP⁺ nuclei) (**Fig. 2a-c, Extended**
165 **Data Fig. 3,4**). In contrast, neurons that received sgCreb1 alone did not show knockdown. We
166 also observed that the vast majority (>90%) of the cortex and hippocampus express both the Cre
167 and the sgRNA, with less co-infection in striatum and cerebellum (**Fig. 2d**). This indicated that co-
168 delivery of the sgRNA and Cre viruses can provide broad coverage and CRISPRi activity for a
169 broad distribution of brain regions. We noted that one of the sgCreb1+hSyn1-Cre mice showed
170 overall less viral transduction throughout the brain, most likely due to a technical issue during the
171 injection of the virus (mouse #1). However, even with sparse infection, there was still strong
172 knockdown of CREB1 in all BFP⁺/GFP⁺ nuclei at the level of individual cells, suggesting that
173 transduction by an AAV at low MOI (potentially a single sgRNA) is sufficient for knockdown.
174 Further supporting this conclusion, we found no correlation between BFP levels and the degree
175 of CREB1 knockdown in a brain transduced with greater amounts of sgCreb1+hSyn1-Cre (mouse
176 #3) (**Extended Data Fig. 4c**).
177

178 **Distribution and extent of multiple infections by AAV**

179 Given the high degree of co-infection between the sgRNA and Cre above in most mice, we next
180 aimed to obtain a semi-quantitative estimate of multiplicity of infection (MOI) by injecting AAVs at
181 multiple concentrations. We co-packaged equimolar concentrations of AAV plasmids encoding
182 three different nuclear-localized fluorescent proteins and performed neonatal ICV injections using
183 three different total viral particle amounts per mouse: 1×10^{10} , 1×10^9 , and 1×10^8 . Three weeks
184 post-injection, we imaged representative sagittal brain sections to evaluate the prevalence of
185 single-, double-, and triple-infected nuclei across different brain regions.
186

187 Even at the highest concentration of AAV injected, and in the most infection-prone regions, the
188 majority of nuclei expressed only one or two fluorescent proteins, with approximately 15% of
189 nuclei in the densely transduced forebrain showing infection with all three fluorescent proteins
190 (**Fig. 2e,f**). Regions with higher co-infection rates correlated directly with areas of enhanced viral
191 tropism, such as the middle to deeper layers of the cortex and the CA3 and CA2 sectors of the
192 hippocampus. At lower concentrations, there was a sharp decline in the total number of
193 transduced nuclei, accompanied by a higher fraction of nuclei expressing only one type of
194 fluorescent protein (**Fig. 2f, Extended Data Fig. 5**). In short, the results indicated that higher viral
195 concentrations maximized the number of transduced neurons, and that even at 1×10^{10} viral
196 particles per brain, a substantial number of cells in most brain regions express a single type of
197 fluorescent protein, indicating that most of these cells are transduced by a single virus (with
198 presumably a small fraction of these representing co-infection by two viruses expressing the same
199 fluorescent protein). Obtaining a precise MOI from these experiments is challenging given the
200 degree of variability of infection between different brain regions and neuronal types as dictated

201 by viral tropism. Despite this limitation, these findings help us broadly estimate that the MOI across
202 the whole brain injected with 1×10^{10} viral particles most likely ranges from 1 to 3, and could be
203 higher than 3 in focal areas of strong viral tropism.
204

205 CrAAVe-seq identifies neuron-essential genes

206 We generated two different sgRNA pooled libraries in the pAP215 AAV backbone by transferring
207 sgRNAs from our previously established mouse sgRNA libraries²⁶. This includes the 12,350-
208 element sgRNA “M1” library that contains sgRNAs targeting 2,269 genes, including kinases,
209 phosphatases, and other druggable targets, and the 14,975-element sgRNA “M3” library
210 containing sgRNAs targeting 2,800 proteostasis and stress genes²⁶. The M1 and M3 libraries
211 contain 250 and 290 non-targeting sgRNAs, respectively. We co-delivered PHP.eB::hSyn1-Cre
212 alongside either PHP.eB::pAP215-M1 library or PHP.eB::pAP215-M3 library by ICV injection into
213 LSL-CRISPRi mice. We injected n=13 mice with the M1 library and n=19 mice with the M3 library.
214 We collected the brains after 6 weeks, recovered episomes and amplified sgRNAs using primers
215 specific to the Cre-inverted handle in pAP215, followed by next-generation sequencing (**Fig.**
216 **3a**). We compared the sgRNA frequencies from the recovered episomes to the sgRNA
217 frequencies of the packaged and injected AAV sgRNA libraries to identify changes in sgRNA
218 frequency. We refer to this workflow as “CrAAVe-seq” throughout the rest of this manuscript. The
219 age, sex, and sgRNA read-depth for each mouse is provided in **Supplementary Table 1**.
220

221 Analysis of changes in sgRNA frequencies using an optimized computational pipeline (see
222 Methods for details) identified 147 genes with significant sgRNA depletion (false discovery rate
223 (FDR) < 0.1), indicating that knockdown of these genes promoted neuronal death (**Fig. 3b**). The
224 output knockdown phenotype of all screens is provided in **Supplementary Table 2**. Gene-set
225 enrichment analysis (with background correction for the genes in the library) further highlighted
226 that many hit genes belonged to distinct biological categories (**Fig. 3c**). Top categories included
227 members of the vacuolar ATPase complex, ESCRT pathways, COP-I and COP-II vesicle
228 trafficking, and members of the chaperonin/TRiC complex. Although not identified from gene
229 enrichment, we also noted some of the top hits to be members of the aminoacyl tRNA synthetase
230 families. Supporting the notion that the genes with a negative knockdown phenotype are neuron-
231 essential genes, we found that 91 of the 147 hit genes (62%) overlapped with known common
232 essential genes established by DepMap from cancer cell lines²⁷ (**Extended Data Fig. 6a**). We
233 similarly found substantial overlap with our prior screens for essential genes performed in induced
234 pluripotent stem cell-derived neurons² (**Extended Data Fig. 6b**). Even though the vast majority
235 of our hits are known essential genes in human cancer cell lines or human iPSC-derived neurons,
236 this method reveals some unique hits that have not been previously reported, including *Jtb*,
237 *Snx17*, *Pspn*, *Bag1*, and *Becln1*. We also note the presence of a few genes for which the sgRNAs
238 were weakly, but significantly enriched relative to input AAV, suggesting that knockdown of these
239 genes enhances neuronal survival.
240

241 To examine the robustness of our screening approach, we compared knockdown phenotypes for
242 top hits from each library in each mouse and found that phenotypes were highly reproducible
243 across individual mice (**Fig. 3d**), corroborating that hits obtained from analyzing the full cohort of
244 mice are not entirely driven by a subset of mice. We also compared two individual mice each from
245 the M1 and M3 sgRNA library cohorts, selecting the mice that called the highest number of hits.
246 We again observed a strong correlation in knockdown phenotypes, particularly for the strongest
247 hit genes (**Extended Data Fig. 7a**). The knockdown phenotypes also strongly correlated when
248 comparing male versus female mice from each cohort; no sex-specific neuron-essential genes
249 were observed (**Extended Data Fig. 7b**). We further confirmed that the knockdown phenotypes

250 of the top hit genes in the M1 library require the expression of hSyn1-Cre (**Extended Data Fig.**
251 **8a**). The knockdown phenotypes for all genes per individual mouse are provided in **Supplemental**
252 **Table 3**.

253
254 The amount of injected AAV library for the M1 and M3 screens above were 2×10^{10} and 5×10^{10}
255 viral particles, respectively, per mouse. Based on our MOI experiments (**Fig. 2d,e**), these
256 concentrations are expected to transduce neurons in several brain regions with an MOI of greater
257 than 1, leading to the expression of multiple different sgRNAs within some neurons. To investigate
258 the impact of MOI on screen quality, we injected a cohort of LSL-CRISPRi littermates with two
259 lower concentrations of the M1 AAV sgRNA library alongside a constant amount of hSyn1-Cre
260 (1×10^{11} viral particles per mouse). Evaluating n=2 mice injected with an intermediate amount
261 (7×10^9 viral particles) of the library showed a clear, strong correlation of the sgRNAs frequencies
262 recovered from mouse brains with those in the input AAV library (**Extended Data Fig. 8b**). In
263 contrast, n=4 mice injected with a 10-fold lower concentration (7×10^8 viral particles) showed much
264 poorer correlation and extensive sgRNA dropout. The intermediate concentration identified 20 hits
265 that overlap with hits of the M1 sgRNA library cohort from Fig. 3b, while the lower concentration
266 identified just 2 hits meeting these criteria (**Extended Data Fig. 8c**). In summary, although the
267 concentrations used for the original screens have an MOI greater than 1 in several brain regions,
268 injecting smaller amounts of virus dramatically reduces the power of the screen. We suggest that
269 the poor performance of lower viral concentrations is due to reducing coverage, indicated by
270 vastly increased sgRNA dropout in the lower concentration. Conversely, the infection of a
271 proportion of cells with more than one sgRNA expected at higher concentrations seems to have
272 a much less detrimental impact on the screen quality, likely because the majority of sgRNAs does
273 not cause a phenotype, and because each individual sgRNA is screened in many independent
274 neurons.

275
276 **Screening for essential genes in CaMKII⁺ neurons *in vivo***

277 To determine if a different neuronal Cre driver targeting a large neuronal subpopulation produces
278 similar findings, we tested a CaMKII promoter that has traditionally been used to target
279 predominantly forebrain excitatory neurons²⁸. We first co-injected PHP.eB::CaMKII-Cre, a LoxP-
280 dependent GFP reporter (FLEX-GFP), and PHP.eB::pAP215-sgCrb1 into an LSL-CRISPRi
281 mice. We observed widespread FLEX-GFP expression throughout the brain, particularly strong
282 in the cortex (**Fig. 4a**). This broad promoter activity is consistent with recent reports of AAV-
283 delivered CaMKII-driven reporters in the mouse brain^{29,30}. In areas of the forebrain with high GFP
284 expression, we observed strong knockdown of CREB1 in BFP⁺ nuclei (**Fig. 4a,b**). We performed
285 CrAAVe-seq in cohort of 12 mice injected with PHP.eB::pAP215-M1 library and CaMKII-Cre,
286 revealing a robust profile of neuron-essential genes (**Fig. 4c**). The knockdown phenotypes of the
287 top hits of this CaMKII-Cre cohort correlated very strongly with the hits from the hSyn1-Cre cohort
288 of Fig. 3 (**Fig. 4d**). This further supported that CrAAVe-seq reproducibly detects neuron-essential
289 genes *in vivo*.

290
291 **Screening on small neuronal subpopulations and requirement of the Cre-inverted handle**
292 We next wanted to evaluate whether CrAAVe-seq is sensitive enough to identify neuron-essential
293 genes from smaller neuronal subpopulations. We selected a recently developed AAV plasmid that
294 utilizes enhancer elements designed to express Cre recombinase in forebrain GABAergic
295 neurons (CN1851-rAAV-hl56i-minBglobin-iCre-4X2C-WPRE3-BGHpA, which we refer to here as
296 “hl56i-Cre”)³¹. Co-injection of this Cre alongside PHP.eB::pAP215-sgCrb1 and PHP.eB::FLEX-
297 GFP in LSL-CRISPRi mice showed its distribution to be much more restricted than that of hSyn1-
298 Cre and CaMKII-Cre, with a large degree of transduction within the olfactory bulb (**Fig. 5a**). In

299 areas of the cortex with sparse GFP expression where individual non-overlapping nuclei could be
300 confidently examined, GFP⁺ nuclei showed reduced levels of CREB1, again confirming Cre-
301 dependent CRISPRi knockdown (**Fig. 5b**).
302

303 We performed CrAAVe-seq on a cohort of 11 mice at 6 weeks after neonatal ICV injection of the
304 PHP.eB::pAP215-M1 library alongside hI56i-Cre. This screen again revealed a profile of essential
305 neuronal genes (**Fig. 5c**), with hits that almost fully overlapped with the hSyn1-Cre and CaMKII-
306 Cre screens (**Extended Data Fig. 9a**). One exception was *Rpia*, which was uniquely identified as
307 a hit in this hI56i-Cre cohort, but we also noted that its effect was driven by one sgRNA out of the
308 5 targeting the gene; all other hit genes resulted from at least 2 significant differentially abundant
309 sgRNAs for that gene. Nonetheless, these screens established that CrAAVe-seq sensitively
310 detects neuron-essential genes on a smaller neuronal population. Top hits showed consistently
311 reproducible knockdown phenotypes in individual mice (**Extended Data Fig. 9b**).
312

313 To determine if the Cre-dependent recovery of sgRNA sequences is required for screening *in*
314 *vivo*, we examined cohorts of mice injected with the PHP.eB::pAP215-M1 library together with
315 either PHP.eB::hSyn1-Cre or PHP.eB::hI56i-Cre. 6 weeks after ICV injection, we performed
316 episome isolation as above but used half of the episome preparation for PCR amplification of all
317 sgRNAs, and the other half for PCR amplification of only those that underwent Cre-dependent
318 inversion of the handle (**Fig. 5d**, primer pairs as schematized in Fig. 1a). We found that for screens
319 conducted in large neuronal populations accessed with hSyn1-Cre expression, analyzing all
320 sgRNAs provides similar knockdown phenotypes of the top hit genes, but also demonstrates a
321 noticeable increase in the knockdown phenotypes for most genes when analyzing only sgRNAs
322 with the Cre-inverted handle (**Fig. 5d**). This nevertheless indicated that hSyn1-Cre drives sgRNA
323 activity in a sufficiently large number of neurons to permit identification of hit genes when
324 evaluating all recovered sgRNAs, but that the presence of non-transduced or transduction in non-
325 neuronal cells could partially mask the effects of active hits. More importantly and in contrast,
326 screens in a small neuronal subpopulation accessed by hI56i-Cre showed that knockdown
327 phenotypes of the top known hits required amplification with the Cre-inverted handle (**Fig. 5d**).
328 Indeed, hit genes were significantly different compared to non-targeting controls when examining
329 sgRNAs with the Cre-inverted handle ($p < 10^{-46}$), but not when examining all sgRNAs ($p = 0.72$)
330 (Kolmogorov-Smirnov test, **Extended Data Fig. 9c**). This supports that sgRNAs expressed in
331 Cre-negative neurons can fully obscure the signal of active sgRNAs, making the Cre-inverting
332 handle in pAP215 essential for screening smaller subpopulations of a broadly transduced
333 population.
334

335 To further quantify the proportion of the total sgRNAs and Cre-inverted sgRNAs, as well as to
336 determine their relative proportions after inversion with different Cres, we conducted PCR with
337 different primer pairs. Episomes were recovered from mice injected PHP.eB::pAP215-M1 library
338 alone, or with either PHP.eB::hSyn1-Cre or PHP.eB::hI56i-Cre. Standard PCR with examination
339 of the products by gel electrophoresis showed that with primers targeting total sgRNAs, there is
340 qualitatively an approximately equal intensity of the PCR products. When using primers targeting
341 the Cre-inverted handle, there was a strong band in mice injected with hSyn1-Cre, no detectable
342 product in mice without Cre, and a weak band with hI56i-Cre (**Fig. 5e**).
343

344
345 To determine the absolute number of template molecules between conditions, we performed
346 digital PCR using the same primer pairs and the same samples. We first determined the total
347 number of sgRNA molecules and found that $15\text{--}30 \times 10^6$ episomal DNA molecules can be

348 recovered from each brain, and within similar ranges in both Cre conditions (**Fig. 5f**). We then
349 found that the number of sgRNAs containing the Cre-inverted handle in the hSyn1-Cre condition
350 were generally a majority fraction of the total, with $5.4\text{--}13 \times 10^6$ molecules detected corresponding
351 to 57% of total sgRNAs on average (**Fig. 5f,g**). In contrast, with hl56i-Cre expression we detected
352 far fewer molecules, $0.8\text{--}3.8 \times 10^6$ molecules containing the inverted handle, corresponding to
353 12% of the total sgRNAs (**Fig. 5f,g**), consistent with findings on the DNA gel in **Fig. 5e**. In
354 comparison, the no-Cre controls generated around 5% signal for inverted PCR primer pair, which
355 we interpret as technical background, as no Cre was present within these mice to invert the handle
356 sequence.

357
358 Overall, these results indicate that CrAAVe-seq is extremely sensitive and is critical for screening
359 on neuronal subpopulations. Moreover, the number of molecules detected also demonstrate the
360 substantially large numbers of neurons being sampled per mouse. Assuming at minimum, when
361 performing CrAAVe-seq using hSyn1-Cre, we estimate from the above digital PCR experiments
362 that 5 million Cre-inverted sgRNA molecules are captured and sequenced from a whole brain.
363 Based on the frequency of the multiple infections observed in Fig. 2, we estimate that most of the
364 transduced neurons express between 1 to 3 transgenes. Therefore, we estimate at least 1.67
365 million neurons are screened per brain. Importantly, this number can be dramatically boosted by
366 increasing the number of mice for a screen at minimal additional cost. For example, the M3 library-
367 injected cohort of 19 mice represents screening of at least 30 million neurons.

368
369 **Estimating number of mice required for screening in differing cell populations**

370 To investigate the impact of cohort size on hit recovery under different Cre conditions, we
371 performed a bootstrap analysis on the CaMKII-Cre screen cohort (from Fig. 4c) and hl56i-Cre
372 screen cohort (from Fig. 5c), which were all injected with the same preparation and concentration
373 of PHP.eB::pAP215-M1 library. Briefly, we randomly subsampled mice without replacement at
374 incrementing cohort sizes 50 times for each cohort. We measured the overlaps of hits identified
375 in the subset of mice at an FDR cutoff of 0.1 with the set of hit genes identified from the full
376 corresponding cohort (i.e. n=12 CaMKII-Cre mice or n=11 hl56i-Cre mice) (**Fig. 6a**).

377
378 Using the cohort of mice from the CaMKII-Cre screen, we observed that the complete set of hits
379 was recovered with fewer than the complete set of mice (n = 11); 90% of the hits were recovered
380 with just three mice (**Fig. 6a**). When examining each of the 61 hits identified from the full CaMKII-
381 Cre cohort, the majority of genes were called hits even in individual mice. (**Fig. 6b, top**). When
382 bootstrapping by randomly sampling n=6 mice from the cohort, nearly all hit genes were recovered
383 in the vast majority of bootstraps. (**Fig. 6b, bottom**).

384
385 In contrast, the hl56i-Cre screen cohort overall showed fewer hits, further supporting that targeting
386 a neuronal subpopulation reduces the power of the screen (**Fig. 6a**). Moreover, while few hits
387 were robustly recovered in individual mice (**Fig. 6c, top**), there was a much stronger benefit of
388 additional mice, since most hits were recovered robustly when bootstrapping on n=6 mice. (**Fig.**
389 **6c, bottom**).

390
391 Together, these findings confirm that relatively few mice are required when screening on large
392 neuronal populations, while more mice are required to effectively power screens when screening
393 on neuronal subpopulations. In future applications, this bootstrapping approach can be
394 implemented to empirically establish whether an *in vivo* screen is powered for the given neuronal
395 population and library size.

396
397 **Screening on essential chaperone genes with a focused sgRNA library**

398 To test the impact of using a smaller sgRNA library on screening performance by CrAAVe-seq,
399 we designed a library of 2,172 sgRNAs targeting 354 known molecular chaperone and co-
400 chaperone genes and cloned it into the pAP215 vector (pAP215-chap library). Given the
401 importance of protein homeostasis in neurons, this would also further establish the chaperones
402 that are critical for neuronal survival. We co-injected PHP.eB::pAP215-Chap library and
403 PHP.eB::hSyn1-Cre into a cohort of 4 neonatal LSL-CRISPRi mice, followed by CrAAVe-seq at
404 6 weeks. The screen revealed 32 hit genes, 26 of which had a negative knockdown phenotype
405 indicating them to be neuron-essential chaperones (**Fig. 7a**). The hits included several genes that
406 were identified from the M1 and M3 library screens in Fig. 3 (e.g. *Hspa5* and subunits of the
407 chaperonin complex). Notably, contrasting the M3 library screen, the chaperonin subunits were
408 even more strongly represented and exhibited a lower FDR, supporting that a reduced library size
409 increased the power of this screen. The high power and sensitivity of this screen with a focused
410 library is further corroborated by the finding that more than half of the 32 hits could be captured
411 from one mouse, and this number substantially increases with using 2 mice (**Fig. 7b**).
412

413 **Confirmation of *Hspa5* and *Rabggta* as essential genes in mouse neurons**

414 We selected two top hits from our *in vivo* screens, *Hspa5* and *Rabggta*, for individual validation
415 to establish that screening for sgRNA depletion from recovered episomes reflects capture of
416 neuron-essential genes. *Hspa5* and *Rabggta* were also consistently among the strongest hits in
417 individual mice and highlight two distinct biological pathways. *Hspa5* encodes an ER-associated
418 Hsp70 chaperone, helping facilitate the folding of newly synthesized proteins. Prior work has
419 shown that knockdown or knockout of *Hspa5* in select neuronal populations *in vivo* leads to their
420 apoptotic cell death^{32,33}. *Rabggta* encodes a geranylgeranyl transferase that regulates GTPase
421 activity of Rab proteins, which are involved in intracellular vesicle trafficking. The impact of
422 knocking down these genes broadly in neurons had not been previously examined.
423

424 In primary neurons cultured from mice with constitutive CRISPRi machinery, we confirmed that
425 sgRNAs targeting *Hspa5* (sg*Hspa5*) and *Rabggta* (sg*Rabggta*) suppress expression of their
426 respective endogenous transcripts (**Fig. 8a, f**). Furthermore, injection of PHP.eB::pAP215-
427 sg*Hspa5* and PHP.eB::hSyn1-Cre into neonatal LSL-CRISPRi mice led to a severe motor
428 phenotype after approximately 2 weeks in mice, but not the sgRNA alone (**Supplementary**
429 **Videos 1 and 2**), requiring prompt euthanasia. The brains from mice co-injected with sg*Hspa5*
430 and hSyn1-Cre were markedly smaller in size relative to littermates with sg*Hspa5* alone (**Fig.**
431 **8b,c**). In primary neurons cultured from LSL-CRISPRi mice, AAVs delivering sg*Hspa5* led to
432 marked Cre-dependent neuronal death within 2 weeks of expression (**Fig. 8d,e**).
433

434 Similarly, neonatal LSL-CRISPRi mice co-injected with sg*Rabggta* and hSyn1-Cre showed a
435 severe motor phenotype at 24 days after injection. Following euthanasia, the brains of these mice
436 weighed less and measured smaller in comparison to littermates injected with sg*Rabggta* alone
437 (**Fig. 8g,h**). Primary neurons transduced with sg*Rabggta* and hSyn1-Cre showed increased
438 neuronal death beginning at approximately 14 days after transduction, with near complete death
439 at 26 days (**Fig. 8i,j**). These findings confirm that *in vivo* CRISPR screening using CrAAVe-seq
440 identifies essential genes that validate even when sgRNAs are introduced into matured primary
441 neuronal cultures postnatally.
442

443 **Discussion**

444 Here we established CrAAVe-seq, a platform for cell type-specific CRISPR-based screening *in*
445 *vivo* in mouse tissues with very high scalability due to the amplification of sgRNA sequences from
446 AAV-derived episomes. Our pan-neuronal CRISPRi screen targeting over one quarter of the
447 protein-coding genes in the mouse genome uncovered neuron-essential genes in the brain with

448 high reproducibility of top hits between individual mice. Many of these genes have been previously
449 recognized as common essential genes by DepMap²⁷ or in our prior screens in iPSC neurons².
450 Some hits were unique to the *in vivo* screen, and include *Jtb* (involved in cytokinesis), as well as
451 *Snx17* and *Snx20*, members of the sorting nexin family. Therefore, our screens already begin to
452 uncover neuronal vulnerability genes in the mouse brain that have not been previously
453 documented. Conversely, some hits were strong essential genes in our screens in mono-cultured
454 human iPSC-derived neurons, such as *SOD1* and genes involved in the exocyst complex
455 (*EXOC3*, *EXOC7*), possibly because they cause vulnerabilities that are buffered in the context of
456 the brain. Our screens also uncovered a few genes whose knockdown produced a positive
457 phenotype, and include *Fbxl16*, *Gaa*, *Dstn*, and *Kdelc16*. It is unclear whether these gene
458 knockdowns increase resiliency from death or from inducing proliferation of a transduced cell
459 population; they warrant further investigation.
460

461 Our Cre-dependent sgRNA recovery strategy enables highly sensitive screens in smaller neuronal
462 subpopulations, providing an avenue for CRISPR screening on molecularly defined cell types that
463 can be accessed through specific Cre-drivers, or brain regions that can be physically dissected.
464 One area for ongoing optimization is increasing sgRNA recovery and coverage across cell
465 populations. Our current proof-of-concept studies rely on co-injecting AAVs for the sgRNA libraries
466 and Cre recombinase, but using transgenic Cre mouse lines could maximize active sgRNA
467 expression without relying on co-infection. Additionally, temporal control over gene perturbation
468 can be achieved by delivering sgRNA libraries later, via intravenous delivery, or by crossing LSL-
469 CRISPRi mice to Cre^{ERT2} lines for tamoxifen-inducible activation. Furthermore, the Lox71/Lox66
470 system used for unidirectional handle inversion may not be the maximum possible efficient, so
471 we are developing a FLEX-based AAV sgRNA backbone for more efficient Cre-dependent handle
472 switching. So far, our screens using different Cre drivers showed strong correlation between
473 independent screens, but no clear evidence of genes essential only in specific neuronal
474 populations. This could be due to the genes included in the M1 library, which may not be optimally
475 suited to detect population-specific vulnerabilities. It may also require longer screening durations
476 to identify genes that impart differential susceptibility.
477

478 When applying CrAAVe-seq to new applications, factors such as sgRNA library size and viral
479 tropism must be considered. Unlike CRISPR screens in cultured cell-based systems, where low
480 MOI ensures that each cell mostly receives only one sgRNA, the complexity of CNS architecture
481 and viral tropism in different brain regions makes controlling global MOI challenging, as each
482 region will have a local MOI. In our studies, the MOI across the brain was generally 1 to 2, with a
483 few areas exceeding 3. Despite this variability, the large screening library and large number of
484 transduced neurons greatly buffers the potential for complex sgRNA interaction within the same
485 cell, ensuring discernible phenotypes even when randomly paired with other sgRNAs. We found
486 that reducing AAV library concentration significantly diminishes screen performance, indicating
487 that transducing a large number of neurons is more critical than maintaining a low MOI. We also
488 observed, through bootstrapping analyses, that a screen on smaller neuronal populations requires
489 a larger number of mice. Future CNS screens must balance library size, target population(s) with
490 consideration of dissected brain regions, and desired phenotypes, and we recommend using
491 digital PCR and bootstrap analyses in optimizing for *in vivo* screens.
492

493 This platform can be immediately applied to existing neurodegeneration mouse models to
494 systematically screen for modifiers of neuronal vulnerability in large brain regions like the cortex
495 or focused populations, such as Purkinje neurons, where the abundance of internal granule cells
496 might mask biological hits. Moreover, the persistent expression of AAV episomes in non-dividing
497 cells³⁴ makes CrAAVe-seq well-suited for aging studies. While our screen mainly identified gene
498 perturbations reducing neuronal survival, future studies in neurodegenerative models could

499 identify perturbations that rescue neuronal survival to reveal potential therapeutic targets. Beyond
500 neurodegeneration, we conceive possible applications to study genetic factors that modify cell
501 survival or growth/proliferation after traumatic brain injury and stroke.
502

503 Further, while our interest is in the brain, we believe this strategy will be useful for screening
504 specific subpopulations of cells throughout the body. The growing toolkit of AAV capsids and Cre
505 drivers allows for immediate application of this approach to study a wide array of different cell
506 types. A minor limitation of using non-integrating AAVs is the gradual loss of AAV episomes during
507 cell division, reducing the fraction of cells from which phenotypic data can be collected, particularly
508 in proliferating tissues. Consequently, this approach would not be best suited to screen for
509 phenotypes related to increased cellular proliferation. This is not a major issue for neurons, as
510 they are postmitotic, and this still permits screening for genes that prevent neuronal death in
511 disease models. For other proliferating cell types, integrating a transposase system into the host
512 genome could help maintain sgRNA expression, as demonstrated previously³⁵. While this would
513 partly negate the advantage of recovering sgRNAs from episomes, the other advantages afforded
514 by AAV (**Extended Data Fig. 1a**), including superior biodistribution and safety profile remain
515 valuable.
516

517 CrAAVe-seq complements orthogonal *in vivo* CRISPR screening techniques but is especially well
518 suited for probing specific cell populations with far larger sgRNA libraries for unbiased biological
519 discovery. By taking advantage of widespread AAV transduction in the brain, CrAAVe-seq allows
520 screening across millions of neurons per mouse, which was not feasible with prior options, and
521 this can be further scaled by using multiple mice per screen. While CrAAVe-seq does not provide
522 the same cell-type resolution as scRNA-seq-based Perturb-seq, CrAAVe-seq offers a practical
523 and far less expensive approach for large-scale initial screens using broader libraries (>1,000s of
524 sgRNAs vs. ~10s of sgRNAs) and larger targeted cell populations (~millions vs. 10,000s of cells).
525 We are currently developing a genome-wide library in the pAP215 backbone for future CrAAVe-
526 seq applications. While our current work demonstrates the use of CrAAVe-seq to uncover
527 modifiers of cell survival, the strategy can be expanded to other relevant phenotypes in the future.
528 For example, delivering an AAV library *in utero* could be used to comprehensively profile the
529 genes involved in the migration and differentiation of different cell types in development. As for
530 previous screens in cultured cells, the use of reporters and fluorescent read-out in conjunction
531 with flow cytometry will enable the identification of modifiers of a plethora of cellular phenotypes.
532 Thus, CrAAVe-seq has the potential to accelerate the rate of biological and therapeutic discovery
533 in relevant animal models while minimizing cost and animal use.
534

535 References

- 536 1. Tian, R. *et al.* CRISPR Interference-Based Platform for Multimodal Genetic Screens in
537 Human iPSC-Derived Neurons. *Neuron* **104**, 239–255.e12 (2019).
- 538 2. Tian, R. *et al.* Genome-wide CRISPRi/a screens in human neurons link lysosomal failure to
539 ferroptosis. *Nat Neurosci* **24**, 1020–1034 (2021).
- 540 3. Dräger, N. M. *et al.* A CRISPRi/a platform in human iPSC-derived microglia uncovers
541 regulators of disease states. *Nat Neurosci* **25**, 1149–1162 (2022).
- 542 4. Leng, K. *et al.* CRISPRi screens in human iPSC-derived astrocytes elucidate regulators of
543 distinct inflammatory reactive states. *Nat Neurosci* **25**, 1528–1542 (2022).
- 544 5. Chow, R. D. *et al.* AAV-mediated direct *in vivo* CRISPR screen identifies functional
545 suppressors in glioblastoma. *Nat Neurosci* **20**, 1329–1341 (2017).
- 546 6. Wertz, M. H. *et al.* Genome-wide *In Vivo* CNS Screening Identifies Genes that Modify CNS
547 Neuronal Survival and mHTT Toxicity. *Neuron* **106**, 76–89.e8 (2020).
- 548 7. Jin, X. *et al.* *In vivo* Perturb-Seq reveals neuronal and glial abnormalities associated with
549 autism risk genes. *Science* **370**, eaaz6063 (2020).

- 550 8. Ruetz, T. J. *et al.* CRISPR-Cas9 screens reveal regulators of ageing in neural stem cells.
551 *Nature* (2024) doi:10.1038/s41586-024-07972-2.
- 552 9. Zheng, X. *et al.* Massively parallel in vivo Perturb-seq reveals cell-type-specific transcriptional
553 networks in cortical development. *Cell* **187**, 3236-3248.e21 (2024).
- 554 10. Santinha, A. J. *et al.* Transcriptional linkage analysis with in vivo AAV-Perturb-seq. *Nature*
555 **622**, 367–375 (2023).
- 556 11. Braun, C. J., Adames, A. C., Saur, D. & Rad, R. Tutorial: design and execution of CRISPR in
557 vivo screens. *Nat Protoc* **17**, 1903–1925 (2022).
- 558 12. Deverman, B. E. *et al.* Cre-dependent selection yields AAV variants for widespread gene
559 transfer to the adult brain. *Nat Biotechnol* **34**, 204–209 (2016).
- 560 13. Goertsen, D. *et al.* AAV capsid variants with brain-wide transgene expression and
561 decreased liver targeting after intravenous delivery in mouse and marmoset. *Nat Neurosci*
562 **25**, 106–115 (2022).
- 563 14. Brown, D. *et al.* Deep Parallel Characterization of AAV Tropism and AAV-Mediated
564 Transcriptional Changes via Single-Cell RNA Sequencing. *Front Immunol* **12**, 730825
565 (2021).
- 566 15. Zhang, Z. & Lutz, B. Cre recombinase-mediated inversion using lox66 and lox71: method to
567 introduce conditional point mutations into the CREB-binding protein. *Nucleic Acids Res* **30**,
568 e90 (2002).
- 569 16. Gemberling, M. *et al.* Transgenic mice for in vivo epigenome editing with CRISPR-based
570 systems. 2021.03.08.434430 Preprint at <https://doi.org/10.1101/2021.03.08.434430> (2021).
- 571 17. Chan, K. Y. *et al.* Engineered AAVs for efficient noninvasive gene delivery to the central and
572 peripheral nervous systems. *Nat Neurosci* **20**, 1172–1179 (2017).
- 573 18. Mathiesen, S. N., Lock, J. L., Schoderboeck, L., Abraham, W. C. & Hughes, S. M. CNS
574 Transduction Benefits of AAV-PHP.eB over AAV9 Are Dependent on Administration Route
575 and Mouse Strain. *Mol Ther Methods Clin Dev* **19**, 447–458 (2020).
- 576 19. Schnepf, B. C., Jensen, R. L., Chen, C.-L., Johnson, P. R. & Clark, K. R. Characterization
577 of Adeno-Associated Virus Genomes Isolated from Human Tissues. *Journal of Virology* **79**,
578 14793–14803 (2005).
- 579 20. Penaud-Budloo, M. *et al.* Adeno-Associated Virus Vector Genomes Persist as Episomal
580 Chromatin in Primate Muscle. *Journal of Virology* **82**, 7875–7885 (2008).
- 581 21. Wang, J.-H., Gessler, D. J., Zhan, W., Gallagher, T. L. & Gao, G. Adeno-associated virus as
582 a delivery vector for gene therapy of human diseases. *Sig Transduct Target Ther* **9**, 1–33
583 (2024).
- 584 22. Yau, E. H. & Rana, T. M. Next-Generation Sequencing of Genome-Wide CRISPR Screens.
585 *Methods Mol Biol* **1712**, 203–216 (2018).
- 586 23. Mathiowitz, A. J., Roberts, M. A., Morgens, D. W., Olzmann, J. A. & Li, Z. Protocol for
587 performing pooled CRISPR-Cas9 loss-of-function screens. *STAR Protoc* **4**, 102201 (2023).
- 588 24. Rincon, M. Y. *et al.* Widespread transduction of astrocytes and neurons in the mouse central
589 nervous system after systemic delivery of a self-complementary AAV-PHP.B vector. *Gene
590 Ther* **25**, 83–92 (2018).
- 591 25. Mantamadiotis, T. *et al.* Disruption of CREB function in brain leads to neurodegeneration.
592 *Nat Genet* **31**, 47–54 (2002).
- 593 26. Horlbeck, M. A. *et al.* Compact and highly active next-generation libraries for CRISPR-
594 mediated gene repression and activation. *eLife* **5**, e19760 (2016).
- 595 27. Tsherniak, A. *et al.* Defining a Cancer Dependency Map. *Cell* **170**, 564-576.e16 (2017).
- 596 28. Madisen, L. *et al.* A robust and high-throughput Cre reporting and characterization system
597 for the whole mouse brain. *Nat Neurosci* **13**, 133–140 (2010).
- 598 29. Radhiyanti, P. T., Konno, A., Matsuzaki, Y. & Hirai, H. Comparative study of neuron-specific
599 promoters in mouse brain transduced by intravenously administered AAV-PHP.eB. *Neurosci
600 Lett* **756**, 135956 (2021).

- 601 30. Veres, J. M., Andras, T., Nagy-Pal, P. & Hajos, N. CaMKIIα Promoter-Controlled Circuit
602 Manipulations Target Both Pyramidal Cells and Inhibitory Interneurons in Cortical Networks.
603 *eNeuro* **10**, ENEURO.0070-23.2023 (2023).
- 604 31. Graybuck, L. T. *et al.* Enhancer viruses for combinatorial cell-subclass-specific labeling.
605 *Neuron* **109**, 1449-1464.e13 (2021).
- 606 32. Wang, M. *et al.* Essential role of the unfolded protein response regulator GRP78/BiP in
607 protection from neuronal apoptosis. *Cell Death Differ* **17**, 488–498 (2010).
- 608 33. Kawaguchi, Y. *et al.* Endoplasmic reticulum chaperone BiP/GRP78 knockdown leads to
609 autophagy and cell death of arginine vasopressin neurons in mice. *Sci Rep* **10**, 19730
610 (2020).
- 611 34. Forrest, A. R. R. *et al.* A promoter-level mammalian expression atlas. *Nature* **507**, 462–470
612 (2014).
- 613 35. Ye, L. *et al.* In vivo CRISPR screening in CD8 T cells with AAV-Sleeping Beauty hybrid
614 vectors identifies membrane targets for improving immunotherapy for glioblastoma. *Nat
615 Biotechnol* **37**, 1302–1313 (2019).
- 616
- 617

618 **Author contributions**

619 BR, IVLR, NT, AP and MK contributed to the study's overall conception, design, and interpretation,
620 with input from the other authors. BR, IVLR and NT created the figures and BR, IVLR and MK
621 wrote the manuscript, with NT and AP contributing further in Materials and Methods. RT, KM and
622 JP contributed preliminary experiments using lentiviral injection of mice. BR, IVLR, AP, SDB, TS
623 and LY conducted and analyzed all other experiments. NT performed bootstrapping analysis and
624 wrote the CRISPR screen analysis pipeline.

625 **Acknowledgements**

626 The authors would like to thank other members of the Kampmann Lab for discussions on data
627 analysis (Olivia Teter, Steven Boggess, Emmy Li, and Reet Mishra). We also thank Abby Oehler
628 and Rigo Roman-Albarran for help with imaging and discussing protocols. We thank Charlie
629 Gersbach and lab for providing the LSL-dCas9-KRAB mouse line. For assistance and advice with
630 sequencing, we would like to thank Lea Kiefer from the Daniele Canzio's lab, Eric Chow and team
631 at the UCSF Center for Advanced Technologies, and Angela Detweiler at the Chan Zuckerberg
632 Biohub Network. For assistance and advice with imaging, we would like to thank Caroline Mrejen
633 and team at the Innovation Core at the Weill Institute for Neurosciences at UCSF. We would also
634 like to thank Alex Pollen, Mercedes Paredes, Daniele Canzio, Eric Huang, Dan Mordes, and
635 Cathryn Cadwell and their labs for the use of numerous items of shared molecular biology and
636 histology equipment.

637

638 This work was supported by the Ben Barres Early Career Acceleration Award from the Chan
639 Zuckerberg Neurodegeneration Challenge Network (MK); a Tau Consortium Investigator Award
640 (MK); NIH/NIA grant R01 AG082141 (MK); the UCSF-CIRM Scholars Training Program, CIRM
641 grant EDUC4-12812 (IVLR); UCSF Hillblom/BARI Graduate Fellowship Award (IVLR);
642 NIH/NINDS grant T32NS115706 (IVLR); NIH/NINDS grant R25NS070680 (BR) and
643 1K08NS133300 (BR); NIH/NIA grant RF1AG062234 (JJP); and NIH/NIA grant K99AG062776
644 (KM).

645 **Competing interests**

646 BR, IVLR, AP and MK have filed a patent application on in vivo screening methods. MK is an
647 inventor on US Patent 11,254,933 related to CRISPRi and CRISPRa screening, a co-scientific
648 founder of Montara Therapeutics and serves on the Scientific Advisory Boards of Engine

651 Biosciences, Alector, and Montara Therapeutics, and is an advisor to Modulo Bio and Recursion
652 Therapeutics.

653

654 Methods

655

656 Animals

657

All mice were maintained according to the National Institutes of Health guidelines and all
658 procedures used in this study were approved by the UCSF Institutional Animal Care and Use
659 Committee. Mice were housed on a 12-h light/dark cycle at 22-25 °C, 50-60% humidity, and had
660 food and water provided *ad libitum*. Mice were randomly assigned for the experimental groups at
661 time of injection and both male and female mice were used. In accordance with approved protocol,
662 mice were monitored post injection and if signs of distress appeared, mice were documented and
663 euthanized promptly. The mice used in this study are LSL-dCas9-KRAB (LSL-CRISPRi) mice
664 (B6;129S6-Gt(ROSA)26Sor^{tm2(CAG-cas9*ZNF10*)Gers}/J, RRID: IMSR_JAX:033066)¹⁶ and dCas9-KRAB
665 mice (B6.Cg-Igs2^{tm1(CAG-mCherry-cas9/ZNF10*)Mtm}/J, RRID: IMSR_JAX:030000). A summary of the
666 individual mice used for CRISPR screening and select *in vivo* experiments is provided in
667 **Supplementary Table 1**.

668

669 Plasmids

670

The screening vector pAP215 (**Fig. 1a**, fully annotated map in **Supplemental File 1**) was
671 generated using the pAAV-U6-sgRNA-CMV-GFP plasmid as the starting backbone (Addgene
672 plasmid # 85451, a gift from Hetian Lei)³⁶. We replaced the sgRNA scaffold sequence with one
673 from pMK1334 (Ref. ¹) and the mU6 using a gene block (gBlock, IDT technologies) using a
674 modified mU6 sequence as reported in Addgene plasmid #53187 (Ref. ³⁷). The CMV-EGFP
675 module was replaced with EF1a-2xmycNLS-tagBFP2 from pMK1334 by Gibson Assembly. The
676 W3 terminator was cloned from Cbh_v5 AAV-saCBE C-terminal (Addgene plasmid # 137183, a
677 gift from David Liu)³⁸. The hGH was replaced by the SV40 from pMK1334. The Lox66 and Lox71
678 sequences and their orientation were copied from the pFrt-invCAG-Luc (Addgene plasmid #
679 63577, a gift from Ivo Huijbers)³⁹ and were inserted along with the 175-bp intervening spacer as
680 a gBlock.

681

682 Additional plasmids in this study include pENN-AAV.hSyn.HI.eGFP-Cre.WPRE.SV40 (Addgene #
683 105540, a gift from James M. Wilson), pENN-AAV.CamKII.HI.GFP-Cre.WPRE.SV40 (Addgene #
684 105551, a gift from James M. Wilson), CN1851-rAAV-hl56i-minBglobin-iCre-4X2C-WPRE3-
685 BGhpA (Addgene # 164450, a gift from The Allen Institute for Brain Science & Jonathan Ting)
686 (PMID: 33789083), and pAAV-FLEX-GFP (Addgene plasmid # 28304, a gift from Edward
687 Boyden). The NLS-mScarlet and NLS-mNeonGreen AAV plasmids were generated by restriction
688 cloning, replacing the GFP sequence in plasmid CAG-NLS-GFP (Addgene # 104061, a gift from
689 Viviana Grdinaru)¹⁷, and replacing the NLS sequence with one from the pMK1334 plasmid.

690

691 sgRNA cloning

692

We transferred the sgRNA sequences from our pooled mCRISPRi-v2 sgRNAs, subpools M1-top
693 5 (targeting Kinases, Phosphatases, and Drug Targets) and M3-top 5 (targeting the proteostasis
694 network)²⁶ into the pAP215 plasmid backbone. 20 µg of the library was digested with BstXI
695 (Thermo Scientific, FD1024) and Bpu1102I (Thermo Scientific, FD0094). The guide-encoding
696 inserts (84 bp) were resolved on a 4-20% Novex TBE gel (Invitrogen, EC62252BOX) and
697 precipitated with GlycoBlue and sodium acetate. Inserts were washed with ethanol after
698 precipitation and then eluted in DNase- and RNase-free water. 20 µg of the backbone vector,
699 pAP215, was digested in parallel with BstXI and Bpu1102I, resolved on a 1% agarose gel, and
700 purified from the gel (Zymo Research, D4001). The vectors and insert guides were annealed for
701 16 hrs overnight using T4 ligase (New England Biolabs, M0202L) at a 1:2 molar ratio of vector to

702 insert, and then purified with sodium acetate and ethanol washing. After the final wash, a portion
703 of the ligated library product was transformed into chemically competent *E. coli* (Takara, 636763)
704 and 10 colonies were picked at random to ensure that each colony contained a unique sgRNA
705 sequence. The remainder of the library product was electroporated into Mega-X competent cells
706 (Invitrogen, C640003) and grown overnight, and a portion of the culture was plated to determine
707 if a coverage of at least 250 colonies per guide was achieved, followed by growth of the remainder
708 of the culture in 1 L of LB for 16 hrs and purification of the library using ZymoPURE II Plasmid
709 Gigaprep Kit (Zymo Research, D4204).

710
711 The mouse chaperone targeting library was designed by selecting the mouse orthologs of a
712 human chaperone targeting library that we previously developed⁴⁰ and included 350 non-targeting
713 control sgRNAs. Oligonucleotide pools were synthesized by Agilent, amplified by PCR, and
714 cloned into the pAP215 backbone as above.

715
716 Individual sgRNAs were cloned in the pAP215 backbone digested with BstXI and Bpu1102I using
717 annealed oligonucleotides with compatible overhangs. The protospacer sequences for the
718 specific sgRNAs used in this study include sgCreb1 (GGCTGCGGCTCCTCAGTCGG), sgHspa5
719 (GAACACTGACCTGGACACTT'), sgRabgta (GCGGCGAACTCACCTGCTCA), and a non-
720 targeting control (sgNTC) (GGATGCATAGATAACGGATG).

721
722 **AAV packaging, purification, and titering**

723 The pAP215-M1 library was packaged into AAV for transduction into neonatal mice as follows.
724 Two 15-cm dishes were each seeded with 1.5×10^7 HEK 293T cells (ATCC, CRL-3216) in 25 ml
725 DMEM complete medium: DMEM (Gibco, 11965-092) supplemented with 10% FBS (VWR,
726 89510, lot: 184B19), 1% penicillin-streptomycin (Gibco, 15140122), and 1% GlutaMAX (Gibco,
727 35050061). The next day, 20 µg of pAdDeltaF6 (Addgene # 112867, a gift from James M. Wilson),
728 7 µg of pAP215-M1 library plasmid, 7 µg of pUCmini-iCAP-PHP.eB (Addgene # 103005, a gift
729 from Viviana Grdinaru)¹⁷, and 75 µl of 1 mg/ml polyethenylamine (PEI) (Linear, MW 25,000,
730 Polysciences, 23966) were diluted into 4 ml of Opti-MEM (Gibco, 31985062), gently mixed, and
731 incubated at room temperature for 10 min. The PEI/DNA transfection complex was then pipetted
732 drop-wise onto the HEK 293T cells. After 24 hours, the media was replaced with 27 ml of fresh
733 Opti-MEM.

734
735 72 hours after transfection, AAV precipitation was performed as previously described⁴¹, with
736 modifications. Cold 5× AAV precipitation solution (40% polyethylene glycol (Sigma-Aldrich,
737 89510) and 2.5 M NaCl) was prepared. The cells and media were triturated and collected (~30
738 ml) into a 50 ml conical tube, followed by addition of 3 ml chloroform and vortexing for
739 approximately 30 seconds. The homogenate was centrifuged at 3,000g for 5 min at room
740 temperature, and the aqueous (top) phase was transferred to a new 50 ml conical tube and 5×
741 AAV precipitation solution was added to a final 1× concentration, followed by incubation on ice for
742 at least 1 hour. The solution was centrifuged at $3,000 \times g$ for 30 min at 4°C. The supernatant was
743 completely removed and the viral pellet was resuspended in 1 ml of 50 mM HEPES and 3 mM
744 MgCl₂, and incubated with 1 µl DNase I (New England Biolabs, M0303S) and 10 µl RNase A
745 (Thermo Scientific, EN0531) at 37°C for 15 min. An equal volume of chloroform was added,
746 followed by vortexing for 15 sec, and centrifuged at 16,000g for 5 min at RT; this step was repeated
747 once. Using 400 µl at a time, the aqueous phase was passed through a 0.5-ml Amicon Ultra
748 Centrifugal Filter with a 100 kDa cutoff (Millipore, UFC510024) by 3 min of centrifugation at
749 14,000g, followed by buffer exchange twice with 1× DPBS. Titering was performed by quantitative
750 RT-PCR as previously described⁴² using primers listed in **Supplementary Table 4**.

751

752 To prepare AAV for testing in primary neuronal cultures (for longitudinal imaging and qRT-PCR),
753 HEK293T cells were seeded into a 6-well format containing 1.5 ml of DMEM complete media. The
754 cells were transfected with 1 µg pAdDeltaF6, 350 ng pUCmini-iCAP-PHP.eB, and 350 ng of AAV
755 transgene using PEI as above. Approximately 48 hours after transfection, the cells and media
756 were collected in 2 ml microfuge tube, 200 µl of chloroform was added to each tube, vortexed for
757 15 sec, and centrifuged at 16,000 × g for 5 min at room temperature. The aqueous (top) phase
758 was transferred to a new tube and AAV precipitation solution was added to 1× dilution, and
759 incubated on ice for at least one hour. The precipitated AAV was centrifuged at 16,000 × g for 15
760 min at 4°C, the supernatant was removed, the pellet was resuspended in 100 µl of 1× PBS, and
761 centrifuged again at 16,000 × g for 1 min to remove excess debris, and the supernatant (purified
762 virus) was transferred to a new microfuge tube. 10 µl purified virus was used per well in primary
763 neuronal cultures in a 24-well format.
764

765 **Intracerebroventricular injection**

766 Intracerebroventricular (ICV) injections were performed as previously described, with minor
767 modifications⁴³. Briefly, neonatal mice were placed on a gauze-covered frozen cold pack and
768 monitored for complete cryoanesthesia. The scalp was gently cleaned with an alcohol swab. AAVs
769 were diluted in 1× PBS with 0.1% trypan blue into a 2 µl final volume per mouse and loaded into
770 10 µl syringe (Hamilton, 1701). The syringe was equipped with a 33-gauge beveled needle
771 (Hamilton, 7803-05, 0.5 inches in length). The needle was inserted through the skull 2/5 of
772 distance of the lambda suture to the eye and to a depth of 3 mm to target the left lateral ventricle.
773 Following a one-time unilateral injection, the neonate was placed on a warming pad for recovery
774 and returned to the parent cage. The number of viral particles injected in each mouse is listed in
775 **Supplementary Table 1**.

776 *Initial protocol for sgRNA recovery*

777 **sgRNA recovery and sequencing for CrAAVe-seq**
778 Animals were euthanized using CO₂, and their whole brains were removed and stored at -80°C.
779 The sex of the mice was recorded prior to euthanasia.

780 *Initial protocol for sgRNA recovery*

781 This protocol for episome recovery was used in the following figures: Fig. 1d, M1 library screen in
782 Fig 3, the hSyn1-Cre screen in Fig 5d, the hSyn1-Cre versus no Cre screens in Extended Data
783 Fig. 6, and the screens in Extended Data Fig. 7. Each brain was placed in a PYREX 7 ml Dounce
784 Homogenizer (Corning, 7722-7) with 2 ml of TRIzol (Invitrogen, 15596026) and thoroughly
785 homogenized using the A pestle (0.0045 nominal clearance) for 10 or more strokes. 0.4 ml of
786 chloroform was added, vigorously shaken for 30 seconds, and centrifuged at 12,000g for 15 min
787 at 4°C. The aqueous phase (top) was collected and nucleic acids precipitated using 1 ml
788 isopropanol, incubated on ice for 10 min, and centrifuged at 12,000g for 10 min at 4°C. The
789 supernatant was discarded and the pellet was washed in 2 ml of 75% ethanol in DNase/RNase-
790 free water and spun down at 7,500g for 5 min. The supernatant was then removed and the pellet
791 was allowed to air dry for 10 mins, and then resuspended in 100 µl of DNase/RNase-free water
792 and incubated with 1 µl of RNase A (Thermo Scientific, EN0531) at 37°C overnight. The sample
793 was then column purified by Zymo DNA Clean & Concentrator-25 kit (Zymo Research, D4033)
794 and eluted in 50 µl of DNase/RNase-free water to yield recovered viral DNA. The remaining
795 RNAse-treated samples were considered recovered episomes for use in PCR below.

796 *Optimized protocol for sgRNA recovery*

797 An optimized protocol for episomal sgRNA recovery was used in the following figures: the M3
798 library screen in Fig. 3, the CaMKII-Cre screens in Fig 4., the hi56i-Cre screens in Fig. 5, and for
799 the dPCR experiments in Fig 5. All steps in this protocol are the same as the above initial protocol
800 except for two modifications. First, each brain was homogenized in 4 ml of TriZOL, phase

803 separated using 0.4 ml chloroform, and the aqueous phase precipitated with 2 ml isopropanol,
804 before resuspending in 100 µL of DNase/RNase-free water. Second, following overnight RNase
805 A treatment as above, the sample was directly transferred to -20 °C without column purification.
806

807 *PCR-amplification of sgRNAs and sequencing*

808 The PCR was performed using Q5 High-Fidelity 2× Master Mix (NEB, M0492L). Each reaction
809 contained 100 µL of recovered episomes, 110 µL of Q5 2× master mix, and 5.5 µL of each primer.
810 For amplification of the AAV sgRNA libraries, the purified AAV was diluted 10-fold into H2O, and
811 1 µL of the diluted AAV was used as a template in a 100 µL PCR reaction. The reaction was
812 distributed into PCR tubes at the maximum volume allowed by the PCR equipment.

813 The following PCR cycling conditions were used: 98°C 30s, (98°C 30s, 60°C 15s, 72°C 15s) × 23
814 cycles, 72°C 10min.
815

816 100 µL of each PCR reaction was purified using 1.1× SPRI beads (SPRIselect Beckman Coulter,
817 B23317) and resuspended in 25 µL elution buffer (Machery Nagel, 740306). The purified products
818 were pooled and sequenced on the Illumina HiSeq4000 at the UCSF Center for Advanced
819 Technologies or the Illumina NextSeq2000. The amplification primers (with adapters) and custom
820 sequencing primers are listed in **Supplementary Table 4**.
821

822 **Lentivirus packaging, purification, and injection**

823 The pLG15 vector containing a non-targeting control sgRNA was packaged into lentivirus as
824 previously performed⁴⁴ by using PEI for transfecting 15 mg of the transfer plasmid and 15 mg of
825 lentiviral packaging plasmids (containing 1:1:1 pRSV, pMDL, pVSV-G) into 1.0×10⁷ HEK293T
826 cells cultured in a 15-cm dish in DMEM complete medium. 48 hours after transfection, the virus
827 was precipitated from the media supernatant using Lentivirus Precipitation Solution (Alstern,
828 VC100) and resuspended in 500 µL of PBS, and then further concentrated using the 0.5-ml Amicon
829 Ultra Centrifugal 100 kDa Column. 1.8 µL of virus plus 0.2 µL of 1% Trypan Blue was injected by
830 ICV for each neonatal mouse. Mouse brains were extracted on day 14 and sectioned coronally.
831

832 **Mouse cortical neuron primary cultures and immunocytochemistry**

833 Neonates were briefly sanitized with 70% EtOH and decapitated using sharp scissors, and the
834 brains were removed and placed into cold HBSS (Gibco, 14175095). The meninges were
835 removed under a dissecting microscope, and the cortices were transferred to a 15-ml conical tube
836 containing 10 ml of 0.25% Trypsin-EDTA (Gibco, 25200056) and incubated at 37°C for 30 min.
837 The trypsin was removed and the brains were gently rinsed twice in 5 ml of DMEM complete
838 media, followed by trituration of brains in 5 ml of DMEM complete media filtered through a 40 µm
839 nylon cell strainer (Corning, 352340), and diluted into DMEM complete media in a volume as
840 needed for plating. An equivalent of one brain was plated across each BioCoat Poly-D-Lysine 24-
841 well TC-treated plate (Corning, 356414). The following day, day in vitro 1 (DIV1), the DMEM
842 complete media was replaced with neuronal growth media composed of Neurobasal-A Medium
843 (Gibco, 10888022), 1× B-27 Supplement minus vitamin A (Gibco, 12587010), GlutaMAX
844 Supplement (Gibco, 35050079), and 1% penicillin-streptomycin (Gibco, 15140122). On DIV2, the
845 cultures were further supplemented with cytarabine (AraC) to a final concentration of 200 µM
846 (Thermo Scientific Chemicals, 449561000). The primary neuronal cultures were transduced with
847 AAV on DIV4 and imaged starting 4 days after transduction.
848

849 **RNA isolation and quantitative RT-PCR**

850 Using CRISPRi primary neurons at 11 days after transduction of AAV (sgNTC, sgHspa5, or
851 sgRabggt1), RNA was isolated with the Zymo Quick-RNA Microprep Kit (Zymo Research, R1050).
852 Samples were prepared for qPCR in technical replicate in 10 µL reaction volumes using
853 SensiFAST SYBR Lo-ROX 2× Master Mix (Bioline, BIO-94005), custom qPCR primers from

854 Integrated DNA Technologies used at a final concentration of 0.2 μ M and cDNA diluted at 1:20 by
855 volume. qPCR was performed on a Bio-Rad CFX96 Real Time System C1000 Touch
856 Thermocycler. The following cycles were run (1) 98°C for 3 min; (2) 95°C for 15 s (denaturation);
857 (3) 60°C for 20 s (annealing/extension); (4) repeat steps 2 and 3 for a total of 39 cycles; (5) 95°C
858 for 1 s; (6) ramp 1.92°C s⁻¹ from 60°C to 95°C to establish melting curve. Expression fold changes
859 were calculated using the $\Delta\Delta Ct$ method, normalizing to housekeeping gene *Gapdh*. RT-qPCR
860 primers are listed in **Supplementary Table 4**.

861

862 Digital PCR

863 Digital PCR (dPCR) was performed to quantify percent of episomal sgRNAs with an inverted
864 Handle sequence collected from mouse brains with or without hSyn1-Cre or hI65i-Cre. Episomal
865 samples from the CrAAVe-seq post-RNase treatment step were diluted 1:50 in water prior to
866 analysis. Two sets of primers were used: one for total sgRNAs (oIR020/oIR021, 132 bp amplicon)
867 and one for sgRNAs with an inverted handle (oIR022/oIR023, 137 bp amplicon) with sequences
868 in Supplemental Table 4. 15 μ l dPCR reactions were prepared using 5 μ l EvaGreen 3 \times PCR
869 master mix (Qiagen, 250111), 1 μ l of diluted episomal sample, 1.5 μ l 10X primer mix (final primer
870 concentration 0.4 μ M each), and 7.5 μ l nuclease-free water; 13 μ l of this reaction was loaded into
871 the microplate. dPCR was performed on a QIAcuity One 5-plex Digital PCR instrument (Qiagen)
872 using 8.5k-partition, 24-well nanoplates (Qiagen, 250011). The thermal cycling conditions
873 consisted of an initial 2 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 15 sec at 60°C, and
874 15 sec at 72°C, with a final 5 min cooling step at 40°C. Image capture exposure was set to 250
875 ms. Samples included M1 library + hSyn1-Cre, M1 library only, and M1 library + hI56i-Cre, with
876 four separate mice for each condition, analyzed for both total and inverted products. Analysis was
877 performed on QIAcuity Software Suite (version 2.5.0.1). A common threshold of 75 RFU was set
878 for all samples. Absolute concentration results for inverted sgRNAs were divided by total sgRNAs
879 for each sample and multiplied by 100 to determine percent inverted sgRNAs.

880

881 Mouse brain immunofluorescence staining

882 Whole brains were removed and fixed overnight at 4°C in 4% paraformaldehyde (Electron
883 Microscopy Sciences, 15710) diluted in 1 \times PBS. The following day, the fixative was replaced with
884 30% sucrose dissolved in 1 \times PBS for at least 48 hours. Fixed brains were blotting dry, cut down
885 the midline with a razorblade, and mounting into a cryomold (Epredia, 2219) using OCT
886 compound (Sakura Finetek, 4583). To snap freeze, cryomolds were partially submerged in a pool
887 of 2-propanol cooled by a bed of dry ice. Brains were sectioned in the sagittal plane at 40 μ m on
888 a cryostat (Leica, CM1950) with a 34° MX35 Premier+ blade (Epredia, 3052835). The resulting
889 brain sections were stored free-floating in 1 \times PBS + 0.05% NaN₃ at 4°C. When ready for staining,
890 representative brain sections were wasted three times in 1 \times PBS and incubated in a 24 well plate
891 at room temperature for one hour in blocking buffer: 10% goat serum (Gibco, 16210064), 1% BSA
892 (Sigma-Aldrich, A7906), and 0.3% Triton X-100 (Sigma-Aldrich, T8787) diluted in 1 \times PBS. The
893 brain sections were incubated in primary antibodies diluted in blocking buffer overnight at 4°C on
894 a gentle shaker. The sections were washed three times in 1 \times PBS, then incubated in secondary
895 antibodies for 2 hours at room temperature in the dark on a gentle shaker. Sections were washed
896 three times in 1 \times PBS and moved to charged glass microscope slides (Fisher Scientific, 12-
897 55015). After PBS was removed, Fluoromount-G with DAPI mountant (Invitrogen, 00-4959-52)
898 was added, and a No. 1.5 coverslip (Globe Scientific, 1415-15) was applied. Slides were dried at
899 room temperature in the dark overnight and sealed with nail polish. For experiments without DAPI,
900 ProLong Gold Antifade mountant (Invitrogen, P10144) was used instead. For experiments with
901 Hoechst instead of DAPI, sections were lastly incubated for 15 mins in Hoechst 33342 (BD
902 Pharmingen, 561908) diluted 2 μ g/ml in 1 \times PBS, then washed 3 times in 1 \times PBS before mounting
903 using ProLong Gold mountant.

904

905 The following primary antibodies were used: rabbit anti-CREB (1:1,000 dilution, clone: 48H2, Cell
906 Signaling Technologies, 9197), rabbit anti-SOX9 (1:2,000 dilution, polyclonal, EMD Millipore,
907 AB5535), guinea pig anti-NeuN (1:500 dilution, polyclonal, Synaptic Systems, 266004), alpaca
908 FluoTag-Q anti-TagFP nanobody (reacts to mTagBFP2 but not eGFP, 1:500 dilution, clone: 1H7,
909 Alexa647 pre-conjugated, NanoTag Biotechnologies, N0501-AF647-L. The following secondary
910 antibodies were used: goat anti-rabbit IgG Alexa Fluor 488 (1:1,000 dilution, Invitrogen A-11034),
911 goat anti-rabbit IgG Alexa Fluor 568 (1:1,000 dilution, Invitrogen, A-11011), goat anti-rabbit IgG
912 Alexa Fluor 647 (1:1,000 dilution, Invitrogen, A-21245), goat anti-guinea pig IgG Alexa Fluor 488
913 (1:1,000 dilution, Invitrogen, A-11073), goat anti-guinea pig IgG Alexa Fluor 647 (1:1,000 dilution,
914 Invitrogen, A-21450). All secondary antibodies were highly cross-absorbed.
915

916 **Microscopy, image segmentation, and analysis**

917 Slides containing brain sections were imaged using a Zeiss AxioScan.Z1 with a Zeiss Colibri 7
918 unit, $\times 20/0.8$ NA objective lens, 5-30 ms exposure, 1 \times 1 binning and 25-100% intensity using 425-
919 nm, 495-nm, 570-nm and 655-nm lasers, running ZEN version 2.6 software. The images were
920 imported into QuPath (version 0.4.2) for analysis⁴⁵. The raw CZI files are available on Dryad
921 repository (see Data Availability).

922 To identify overlap between BFP, NeuN, and SOX9, a representative region of the cortex was
923 outlined and the nuclei were segmented on the DAPI channel using the ‘Cell detection’ module
924 without expansion of the nuclei to develop virtual cell boundaries. Classifiers were created to
925 distinguish BFP⁺, NeuN⁺, and SOX9⁺ cells, and applied sequentially. Cells containing overlapping
926 NeuN and SOX9 were considered to be neurons (as there was a low, but detectable signal in the
927 SOX9 channel in all nuclei with this antibody) and only cells exclusively containing SOX9 were
928 considered astrocytes. Similar segmentation on DAPI and sequential application of classifiers
929 were used to examine overlap between nuclear BFP, mNeonGreen, and mScarlet signal in mice
930 shown in Fig. 2d,e.
931

932 To evaluate CREB1 levels, a representative region was selected as indicated by specific brain
933 regions, and the nuclei were segmented on the DAPI channel as above. The measurements for
934 the segmented nuclei were exported. The mean fluorescence intensity for the anti-Creb1 channel
935 was obtained selected by the top 200 nuclei of highest anti-mTagBFP2 fluorescence intensity. A
936 representative region of brain stained with secondary antibodies only was selected to determine
937 the background mean fluorescence intensity for that channel. The same segmentation was
938 performed in mice injected with FLEX-GFP, with the top 2% and bottom 2% of GFP⁺ or BFP⁺
939 nuclei examined for CREB1 mean fluorescence intensity.
940

941 For mouse primary neurons transduced with AAV, live imaging was performed every other day
942 using an ImageXpress Micro Confocal HT.ai High-Content Imaging System (Molecular Devices).
943 The imaging chamber was warmed to 37°C and equilibrated with 5% CO₂. The system used an
944 Andor Zyla 4.5 camera with a Plan Apo $\times 10/0.45$ NA objective lens, an 89 North LDI laser
945 illumination unit, 10-500 ms exposure time, 1 \times 1 binning, and 10% laser intensity using 405-nm,
946 475-nm, and 555-nm lasers, running MetaXpress (version 6.7.1.157). Resulting images were
947 imported into Cell Profiler (version 4.2.1)⁴⁶ and analyzed using a custom pipeline. hSyn1-Cre-
948 GFP+ nuclei were segmented using the ‘IdentifyPrimaryObjects’ module, with expected diameter
949 8-40 pixels, using an Adaptive threshold (size 50) and the Minimum Cross-Entropy method, with
950 a 1.5 smoothing scale, 1.0 correction factor, and lower- and upper-bound threshold at 0.435 and
951 1, respectively. Segmented objects were exported, and counted in each field, then summed
952 across all fields within a well to calculate the number of objects per well (n=29 fields per well, n=4
953 wells per condition), using a custom R script. This was repeated for each timepoint. Data was
954 normalized to fluorescent intensity at day 8 (as before that day, fluorescence intensity increased
955

956 linearly with time in all channels as cells manufactured fluorescent proteins) and percentage
957 change was calculated for each well from day 8, for subsequent timepoints through day 16.
958

959 A similar protocol was used to analyze *Rabggta* knockdown data with some modifications. hSyn1-
960 Cre-GFP⁺ nuclei were segmented using the 'IdentifyPrimaryObjects' module, with an expected
961 diameter of 7-40 pixels, using an Adaptive threshold (size 50) and Minimum Cross-Entropy
962 method, with a 1.3488 smoothing scale, 1.0 correction factor, and lower- and upper-bound
963 threshold at 0.101 and 1, respectively. Segmented objects were exported and counted in each
964 field, then summed across all fields within a well to calculate the number of objects per well (n =
965 29 fields per well, n = 3) using a custom R script. This was repeated for each timepoint. Data was
966 normalized to fluorescent intensity at day 10 and percentage change was calculated for each well
967 from day 10, for subsequent time points through day 26.
968

969 CRISPR Screen Analysis

970 Computational analysis of screen data was carried out using a newly developed bioinformatics
971 pipeline which is publicly available (see Code availability section). Raw sequencing results were
972 mapped to the M1 protospacer library using 'sgcount'. Briefly, 'sgcount' is a tool to match
973 protospacers against a reference protospacer library with exact pattern matching. The resulting
974 count matrices, containing guide and gene information along with count data for each sample,
975 was used as input for subsequent analyses.
976

977 'crispr_screen' was used to perform differential sgRNA abundance analysis and gene score
978 aggregation analysis. 'crispr_screen' is a reproduction of the original MAGeCK analysis but
979 performs differential sgRNA analysis using a negative binomial as originally described in the
980 study and not a truncated normal distribution as used in the current MAGeCK implementation.
981 In brief, sgRNA abundances are median normalized across samples then a weighted linear
982 regression (weighted ordinary least squares) is used to fit the log-variance to the log mean of
983 the control samples (representing sgRNA abundances in the AAV library). The fit variance and
984 mean are then used to parameterize negative binomial distributions for each sgRNA and a
985 survival function or cumulative distribution function is used to calculate a p-value for sgRNA
986 under- and over-abundance. We excluded any sgRNAs that were represented with fewer than
987 100 reads across the control AAV samples.
988

989 To calculate a gene-level aggregated metric across sgRNAs of the same gene group we
990 established a novel algorithm, geopolg. We performed the following operations on the under-
991 and over-abundance p-values in parallel. First, the differential abundance p-values for sgRNAs
992 were corrected for multiple hypothesis correction using the Benjamini-Hochberg correction
993 procedure to calculate a false discovery rate (FDR) for each sgRNA. Next, FDRs for sgRNAs
994 belonging to the same gene grouping were collected and sorted ascendingly. We then
995 calculated a weighted geometric mean FDR (q_i) for each gene ($i \in N$) across the FDRs (x_j) for
996 sgRNAs within the gene group ($j \in M$).
997

$$q_i = \exp \left(\sum_j^{M_i} \frac{w_j \ln x_j}{\sum_j^{M_j} w_j} \right)$$

998
999 We calculated a weighted geometric mean to down-weight the relative impact of the first sgRNA
1000 within the group using a "Drop-First" weighting strategy. The first sgRNA (or top performing
1001 sgRNA) is down-weighted because we generally aim to capture genes with multiple high-
1002 performing sgRNAs. The weights for each gene grouping (M_i) are defined as follows:
1003

1004 $w_j = \begin{cases} 0.5 & \text{if } x_j = \text{Min}[x_k \forall k \in M_i] \\ 1.0 & \text{if } x_j \neq \text{Min}[x_k \forall k \in M_i] \end{cases}$

1005
1006 We also performed an aggregation of the log₂-fold-changes in abundance (a gene's phenotype
1007 score) of each sgRNA (φ_j) within the gene group with an arithmetic mean:
1008

1009 $\varphi_i = \frac{1}{M_i} \left(\sum_j^{M_i} \varphi_j \right)$

1010
1011 We then created random groupings of non-targeting control sgRNAs, which we denote as the
1012 *amalgam* gene set (A), to match the gene membership distribution of the input sgRNA library.
1013 This was performed by determining the membership size (number of sgRNAs) of each gene
1014 (M_j) and sampling an equal amount of sgRNAs without replacement from the non-targeting
1015 controls. We next performed an identical calculation as above for each of the newly created
1016 *amalgam* genes.
1017
1018 We then calculated a 'gene score' for each gene and each *amalgam* gene within the dataset
1019 using the calculated weighted geometric mean of the FDR values q_i) and their phenotype score
1020 (φ_i).
1021 $\gamma_i = (\varphi_i)(-\log_{10} q_i)$

1022
1023 We next sort the gene scores (γ_i) in an ascending order or a descending order for the under-
1024 and over-abundant tests respectively.
1025
1026 Finally, we calculate an empirical false discovery rate (δ_i) by stepping through the weighted
1027 geometric mean (q) arrays and determining for each rank (i) how many *amalgam* genes ($g_i \in A$)
1028 are preceding it. Because the true empirical false discovery rate will be zero for all genes
1029 preceding the first *amalgam* gene, we provide a non-zero score by constraining the reported
1030 false discovery rate to be the maximum of the empirical false discovery rate and the weighted
1031 geometric mean of that gene

1032 $\delta_i = \max \left(\frac{c_i}{i} \mid q_i \right)$

1033 $c_i = \sum_1^i \begin{cases} 1 & \text{if } g_i \in A \\ 0 & \text{if } g_i \notin A \end{cases}$

1034
1035 This empirical false discovery is further constrained for explicit monotonicity by requiring the
1036 current score to be greater than or equal to the previous one.
1037
1038 $\delta_i = \max (\delta_i \mid \delta_{i-1}) \forall i > 1$

1039
1040 The geopagg algorithm is performed for the sgRNA under- and over-abundant p-values in
1041 parallel and the final scores for each gene are reported as the most significant of the two tests.
1042 151 genes in the M1 library and 129 genes in the M3 library are targeted at two different
1043 transcriptional start sites by different sets of sgRNAs. These sets were evaluated independently
1044 with a label of P1 and P2 (e.g. GeneA_P1 and GeneA_P2). In cases where only one set is
1045 significant and labeled on a heatmap or volcano plot, the P1 or P2 label is not shown, but this
1046 information is included in **Supplementary Tables 2 and 3**.
1047

1048

1049 Bootstrapping Analysis

1050 To estimate the required number of mice to effectively power a screen and assess the
1051 robustness of hit detection, we performed a bootstrapping analysis using a custom Python
1052 package ('rescreener'). `crispr_screen` was used to perform differential gene abundance
1053 analyses. The analysis was conducted as follows for each mouse experiment:

1054

- 1055 1. Full Dataset Analysis: The n=12 mice of the M1 library+CaMKII-Cre and n=11 mice of
1056 the M1library+hI56i-Cre were analyzed using the `crispr_screen` tool to establish
1057 baseline results at an FDR of < 0.1, providing a list of hit genes from each full cohort
1058
- 1059 2. Bootstrapped Subset Analysis: From each cohort, multiple subsets of the treatment
1060 samples (mice) were randomly selected and analyzed:
 - 1061 a. Subset sizes ranged from 1 to the total number of treatment samples (mice)
 - 1062 b. For each subset size, 50 bootstrap replicates were generated by randomly
1063 sampling without replacement from the treatment samples (mice).
 - 1064 c. Each bootstrapped subset was analyzed using `crispr_screen` with the same
1065 parameters as the full dataset analysis.
- 1066 3. Overlap Assessment: For each bootstrap replicate, the overlap between its significant
1067 hits and those from the full cohort was calculated. Hits were defined as genes with an
1068 FDR of < 0.1.
- 1069 4. Hit Recovery Analysis: For each gene identified as a hit in the full dataset analysis, we
1070 calculated the proportion of bootstrap replicates in which it was also identified as a hit.
1071 This proportion, termed the "recovery rate" was calculated as the number of times a
1072 gene was identified as a hit across all bootstraps divided by the total number of
1073 bootstrap replicates.

1074

1075 Statistics and Reproducibility

1076 No statistical methods were used to pre-determine sample sizes, but our sample sizes are
1077 similar to those reported in previous publications, as cited in the main text. Numbers of
1078 replicates are listed in each figure. No repeat measurements were made on the same samples.
1079 Data were assumed to be normally distributed except for instances within the `crispr_screen`
1080 pipeline where geopagg uses a negative binomial distribution to calculate p-value. The
1081 `crispr_screen` pipeline was used with FDR < 0.1 and controls for multiple comparisons using
1082 the Benjamini-Hochberg correction. For cell culture experiments, randomization was not
1083 performed because treatment groups of cells were derived from the same parent population of
1084 cells. Data collection and analysis were not performed blinded to the conditions of the
1085 experiments. No animals or data points were excluded from the relevant analyses. Major
1086 findings were validated using independent samples and orthogonal approaches.

1087

1088 Data availability

1089 All data are available from the corresponding author (MK), and will be made publicly available at
1090 the UCSF Dryad data repository upon publication (DOI: [10.5061/dryad.0k6djh9t](https://doi.org/10.5061/dryad.0k6djh9t)). There are no
1091 restrictions on data availability.

1092

1093 Code availability

1094 For CRISPR screen analysis, we developed a highly efficient analysis toolkit called `sgcount` for
1095 sgRNA mapping and `crispr_screen` for differential gene abundance testing. The sgRNA
1096 mapping utility ('sgcount', version 0.1.32) is also available on GitHub

1097

1099 (<https://github.com/noamteyssier/sgcount>) and Zenodo
1100 (<https://zenodo.org/doi/10.5281/zenodo.12774352>). The differential gene abundance tool
1101 ('`crispr_screen`', version 0.2.8) is also available on GitHub
1102 (https://github.com/noamteyssier/crispr_screen/) and Zenodo
1103 (<https://zenodo.org/doi/10.5281/zenodo.12774208>) All bootstrapping analyses were performed
1104 using a custom python package ('`rescreener`', version 0.1.0) available on GitHub
1105 (https://github.com/noamteyssier/bootstrap_analysis_invivo_crispr_screen).
1106

1107 The R notebooks for analysis are available at <https://kampmannlab.ucsf.edu/article/scripts-vivo-screening-manuscript>.
1108

1109
1110 The CellProfiler pipelines will be made available on request to the corresponding author (MK)
1111 and will also be submitted to the CellProfiler depository of published pipelines
1112 (https://cellprofiler.org/examples/published_pipelines.html) upon publication.
1113

1114 **Supplementary Materials**

1115
1116 **Supplementary File 1: Annotated sequence of CrAAVe-seq plasmid pAP215.** A map of
1117 pAP215 is provided in a GenBank file format.
1118

1119 **Supplementary Table 1: Summary of mice injected with AAV across different studies**
1120 Lists all mice used in the study, time points, biological sex, and virus amounts. For mice used for
1121 CRISPR screens, the number of sequencing reads obtained from each sample is included.
1122

1123 **Supplementary Table 2: The CRISPR screen results of cohorts**
1124 Provides the results file of the '`crispr_screen`' pipeline applied to the different cohorts of mice
1125 reported in this manuscript.
1126

1127 **Supplementary Table 3: CRISPR screen results for individual mice**
1128 Provides the knockdown phenotype (log2fc), pvalue, FDR, and Gene Score, obtained from the
1129 output of the '`crispr_screen`' pipeline for all individual mice relative to input the AAV library, with
1130 each tab representing a different cohort of mice.
1131

1132 **Supplementary Table 4: List of primers used for sgRNA amplification, next generation**
1133 **sequencing, and RT-qPCR**
1134 Provides the primer sequences for sgRNA amplification, the custom sequencing primers for
1135 Illumina sequencing, and the primers used for RT-qPCR of *Hspa5*, *Rabggta*, and *Gapdh*.
1136

1137 **Supplemental Table 5: Values generated from bootstrap analyses for CaMKII-Cre and**
1138 **hi56i-Cre cohorts.**
1139 Three tabs each for bootstrap analyses of the CaMKII-Cre and hi56i-Cre cohorts reported in Fig.
1140 6 are included. "Recovery by mouse number" tab shows the fraction of hits (frac_overlapping)
1141 that overlap with the hits of the full cohort when sampling different numbers of mice (subset).
1142 "All bootstraps" shows the total number (num_tests) and fraction (frac_tests) of all bootstraps
1143 that recover each hit of the full cohort. "Hits by subsample" shows the number (num_tests) and
1144 fraction (frac_tests) of bootstraps that recover each hit of the full cohort when sampling different
1145 numbers of mice (subset). Each test was run 50 times (replicate).
1146

1147 **Supplementary Video 1: Gross motor phenotypes in sgHspa5 + hSyn1-Cre injected mice**
1148 LSL-CRISPRi neonates were co-injected by ICV with AAV containing sgHspa5 (in pAP215) and
1149 AAV containing hSyn1-Cre. At 16 days post injection, mice displayed gross motor phenotypes,

1150 as recorded in the video. After video capture, mice were immediately euthanized as per IACUC
1151 protocol. Mice were littermates to those shown in Supplementary Video 2 and injected and
1152 recorded at the same timepoint.
1153

1154 **Supplementary Video 2: Lack of gross motor phenotypes in sgHspa5 only injected mice**

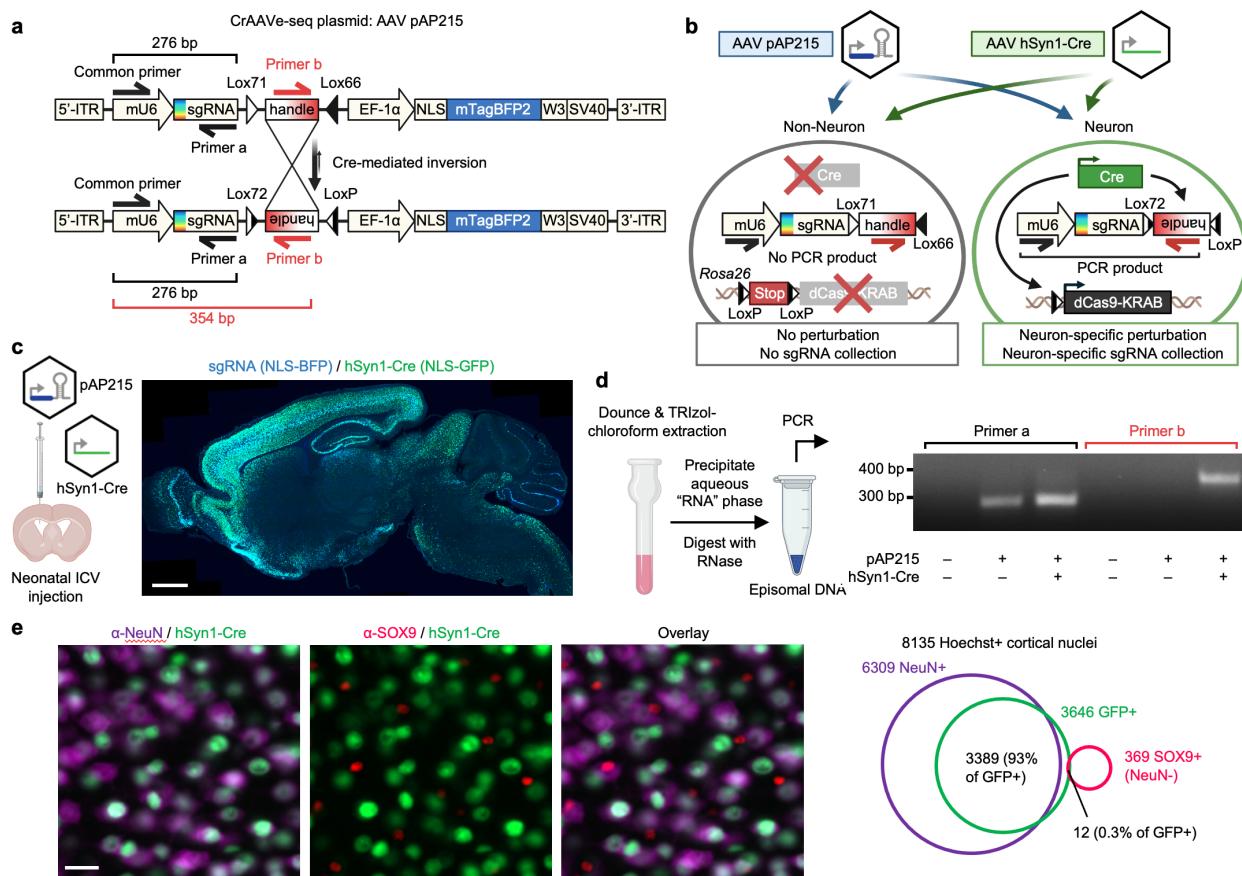
1155 LSL-CRISPRi neonates were co-injected by ICV with AAV containing sgHspa5 (in pAP215) and
1156 an equivalent volume of phosphate buffered saline. At 16 days post injection, mice displayed
1157 normal motor phenotypes, as recorded in the video. Mice were littermates to those shown in
1158 Supplementary Video 1 and injected and recorded at the same timepoint.
1159

1160 **Supplementary Material References**

- 1161 36. Duan, Y. *et al.* The Clustered, Regularly Interspaced, Short Palindromic Repeats-associated
1162 Endonuclease 9 (CRISPR/Cas9)-created MDM2 T309G Mutation Enhances Vitreous-
1163 induced Expression of MDM2 and Proliferation and Survival of Cells. *J Biol Chem* **291**,
1164 16339–16347 (2016).
- 1165 37. Kabadi, A. M., Ousterout, D. G., Hilton, I. B. & Gersbach, C. A. Multiplex CRISPR/Cas9-
1166 based genome engineering from a single lentiviral vector. *Nucleic Acids Res* **42**, e147
1167 (2014).
- 1168 38. Levy, J. M. *et al.* Cytosine and adenine base editing of the brain, liver, retina, heart and
1169 skeletal muscle of mice via adeno-associated viruses. *Nat Biomed Eng* **4**, 97–110 (2020).
- 1170 39. Huijbers, I. J. *et al.* Using the GEMM-ESC strategy to study gene function in mouse models.
1171 *Nat Protoc* **10**, 1755–1785 (2015).
- 1172 40. Biebl, M. M. *et al.* NudC guides client transfer between the Hsp40/70 and Hsp90 chaperone
1173 systems. *Mol Cell* **82**, 555-569.e7 (2022).
- 1174 41. Negrini, M., Wang, G., Heuer, A., Björklund, T. & Davidsson, M. AAV Production
1175 Everywhere: A Simple, Fast, and Reliable Protocol for In-house AAV Vector Production
1176 Based on Chloroform Extraction. *Current Protocols in Neuroscience* **93**, e103 (2020).
- 1177 42. Aurnhammer, C. *et al.* Universal real-time PCR for the detection and quantification of adeno-
1178 associated virus serotype 2-derived inverted terminal repeat sequences. *Hum Gene Ther
Methods* **23**, 18–28 (2012).
- 1179 43. Kim, J.-Y., Grunke, S. D., Levites, Y., Golde, T. E. & Jankowsky, J. L. Intracerebroventricular
1180 Viral Injection of the Neonatal Mouse Brain for Persistent and Widespread Neuronal
1181 Transduction. *JoVE (Journal of Visualized Experiments)* e51863 (2014) doi:10.3791/51863.
- 1182 44. Gilbert, L. A. *et al.* Genome-Scale CRISPR-Mediated Control of Gene Repression and
1183 Activation. *Cell* **159**, 647–661 (2014).
- 1184 45. Bankhead, P. *et al.* QuPath: Open source software for digital pathology image analysis. *Sci
Rep* **7**, 16878 (2017).
- 1185 46. Stirling, D. R. *et al.* CellProfiler 4: improvements in speed, utility and usability. *BMC
Bioinformatics* **22**, 433 (2021).
- 1186 47. Wang, G. *et al.* Mapping a functional cancer genome atlas of tumor suppressors in mouse
1187 liver using AAV-CRISPR-mediated direct *in vivo* screening. *Sci Adv* **4**, eaao5508 (2018).
1188
1189
1190
1191

1192 **Fig. 1**

1193



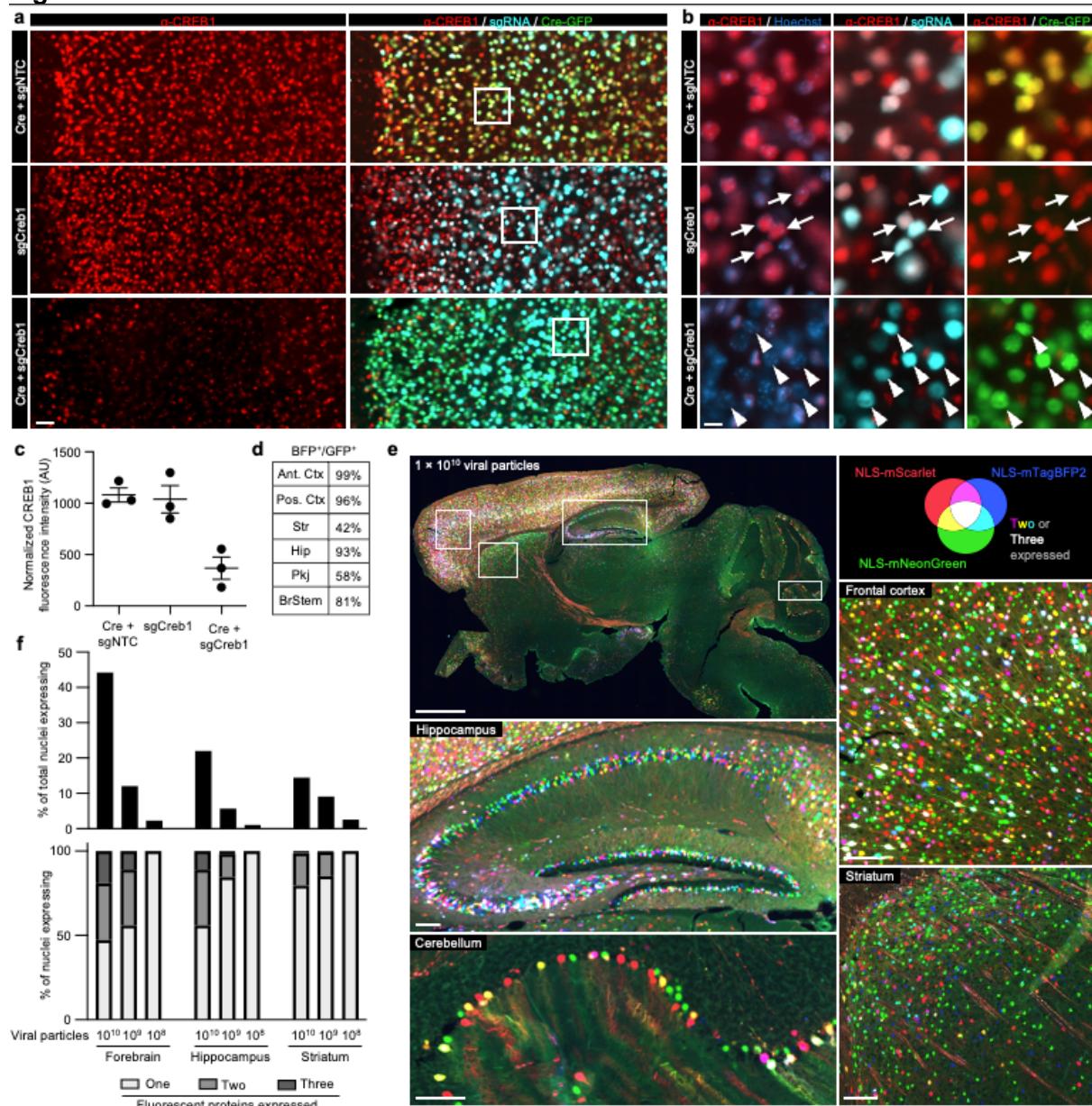
1194

Fig. 1: CrAAVe-seq strategy for cell type-specific *in vivo* CRISPR screening using Cre-sensitive sgRNAs

1195 **a**, Structure of CrAAVe-seq plasmid pAP215 (sgRNA backbone), which enables cell type-specific CRISPR screening by AAV episome sequencing (CrAAVe-seq) *in vivo*. pAP215
1196 expresses sgRNAs under an mU6 promoter and contains a Lox-flanked handle that inverts
1197 upon exposure to Cre recombinase. The plasmid also expresses a nuclear-localized BFP for
1198 visualization. **b**, Example of a strategy for CRISPRi screening in neurons using pAP215 and a
1199 Cre recombinase driven by a pan-neuronal hSyn1 promoter. Expression of Cre induces both
1200 expression of CRISPRi machinery (via recombination of LoxP sites of the Lox-Stop-Lox (LSL)-
1201 CRISPRi transgene in the endogenous Rosa26 locus) and inversion of the pAP215 "handle"
1202 element, which contains a primer site for PCR amplification on recovered episomes. **c**,
1203 Distribution and expression of AAV pAP215 (sgRNA, nuclear BFP $^{+}$) and AAV hSyn1-Cre-NLS-
1204 GFP (hSyn1-Cre, nuclear GFP $^{+}$), both packaged using PHP.eB capsid, in a mouse brain three
1205 weeks after neonatal intracerebroventricular (ICV) delivery. Scale bar, 1 mm. **d**, PCR performed
1206 on episomes recovered from mouse brains expressing pAP215 with or without hSyn1-Cre using
1207 the primers diagrammed in (a). PCR amplification with *primer a* is invariant to Cre expression,
1208 whereas amplification with *primer b* requires Cre expression. **e**, Immunofluorescent staining for
1209 neurons (NeuN $^{+}$) and astrocytes (SOX9 $^{+}$) in frontal cortex of mice injected with PHP.eB::hSyn1-
1210 Cre. Scale bar, 25 μ m. Overlap of Cre expression with NeuN and Sox9 within a cortical region
1211 demonstrates high coverage and specificity for neurons.
1212
1213
1214
1215

1216

Fig. 2



1217

1218

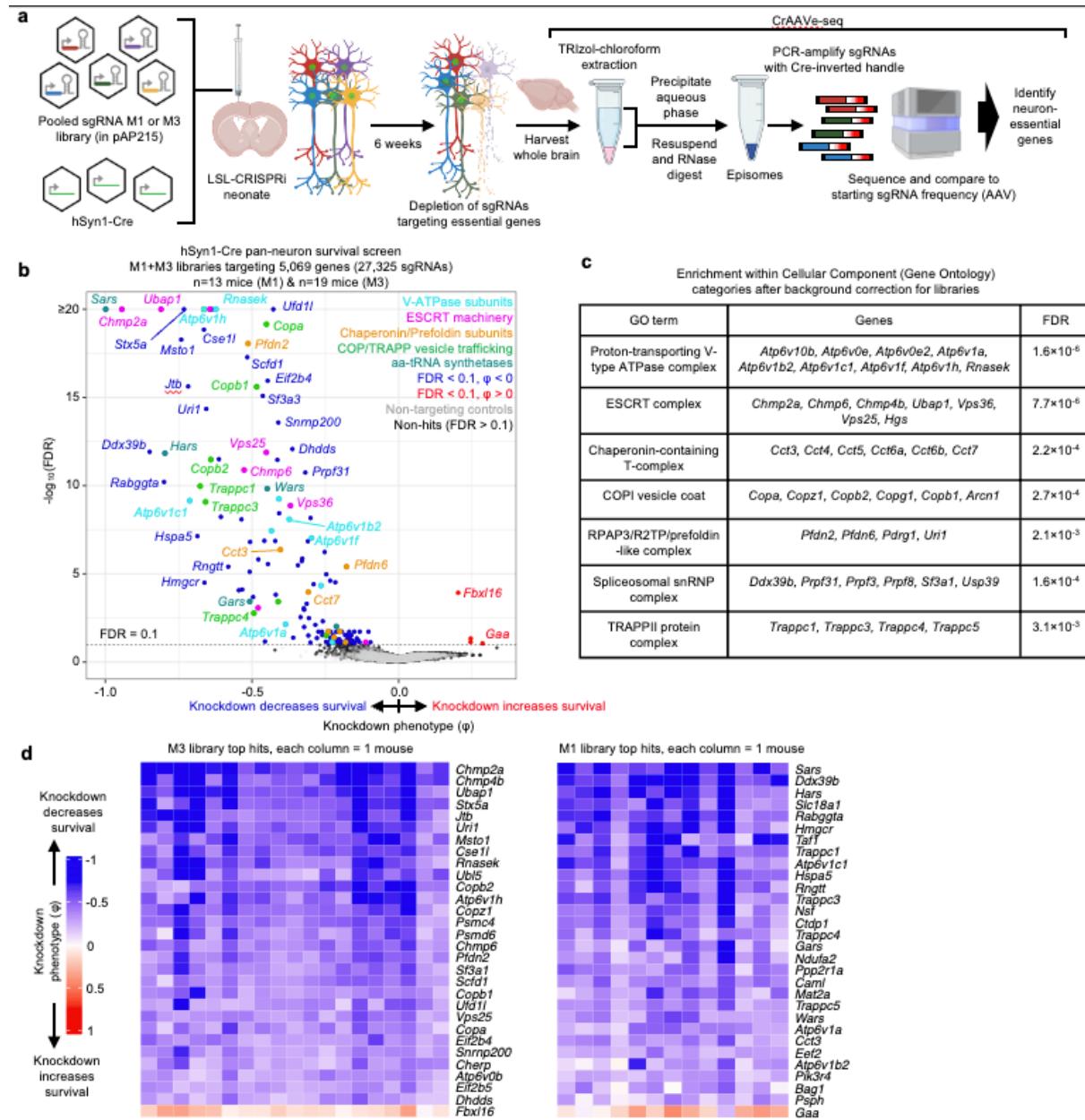
Fig. 2: Cre-dependent CRISPRi knockdown of CREB1 *in vivo* and estimating extent of AAV multiple infections

a, sgRNAs targeting the non-essential gene *Creb1* (sgCrb1) or a non-targeting control (sgNTC) were cloned into pAP215 and PHP.eB-packaged, then delivered with or without PHP.eB::hSyn1-Cre by ICV injection into neonatal LSL-CRISPRi mice. Brains were stained at 3 weeks to determine reduction in CREB1 levels; a representative area of frontal cortex is shown, with cortical layer 1 oriented to the left. Scale bar, 50 μ m. Other brain regions are shown in Extended Data Fig. 3. Images with higher brightness are used to identify cells with low GFP or BFP expression. **b**, Higher magnification of the boxed regions in panels from **a**, with arrowheads indicating examples of neurons having received both sgCrb1 and Cre, whereas arrows indicate neurons that received Cre only or sgCrb1 only. Scale bar, 10 μ m. **c**, Quantification of CREB1

1230 levels in sgRNA-containing (BFP^+) nuclei within a representative region of cortex (mean \pm s.e.m,
1231 n = 3 independent mice). Other brain regions are quantified in Extended Data Fig. 4. **d**, Table
1232 showing the percentage of neurons in a sgCreb1 + Cre mouse 2 that are positive for both BFP
1233 and GFP, and in different brain regions (Ctx: cortex; Pkj: Purkinje; Hippo: hippocampus; BrStem:
1234 brainstem; Str: striatum. **e**, To estimate the number of multiple infections, AAV (PHP.eB
1235 serotype) expressing nuclear-localized mNeonGreen, mTagBFP2, or mScarlet fluorescent
1236 proteins were co-packaged and co-injected by ICV into neonates at three different
1237 concentrations, and the highest concentration (1×10^{10} viral particles per mouse) is shown here
1238 across multiple brain regions. Lower concentrations are shown in Extended Data Fig. 5. **f**, *Top*,
1239 quantification of data from (**e**) and Extended Data Fig. 5, showing percentage of nuclei that were
1240 expressing at least one fluorescent protein in the forebrain, hippocampus, and striatum at three
1241 different concentrations of injected AAV (1×10^{10} , 10^9 , and 10^8 viral particles per mouse). *Bottom*,
1242 percent of nuclei expressing one, two, or all three fluorescent proteins for the same
1243 experiments.
1244

1245
1246

Fig. 3



1247
1248

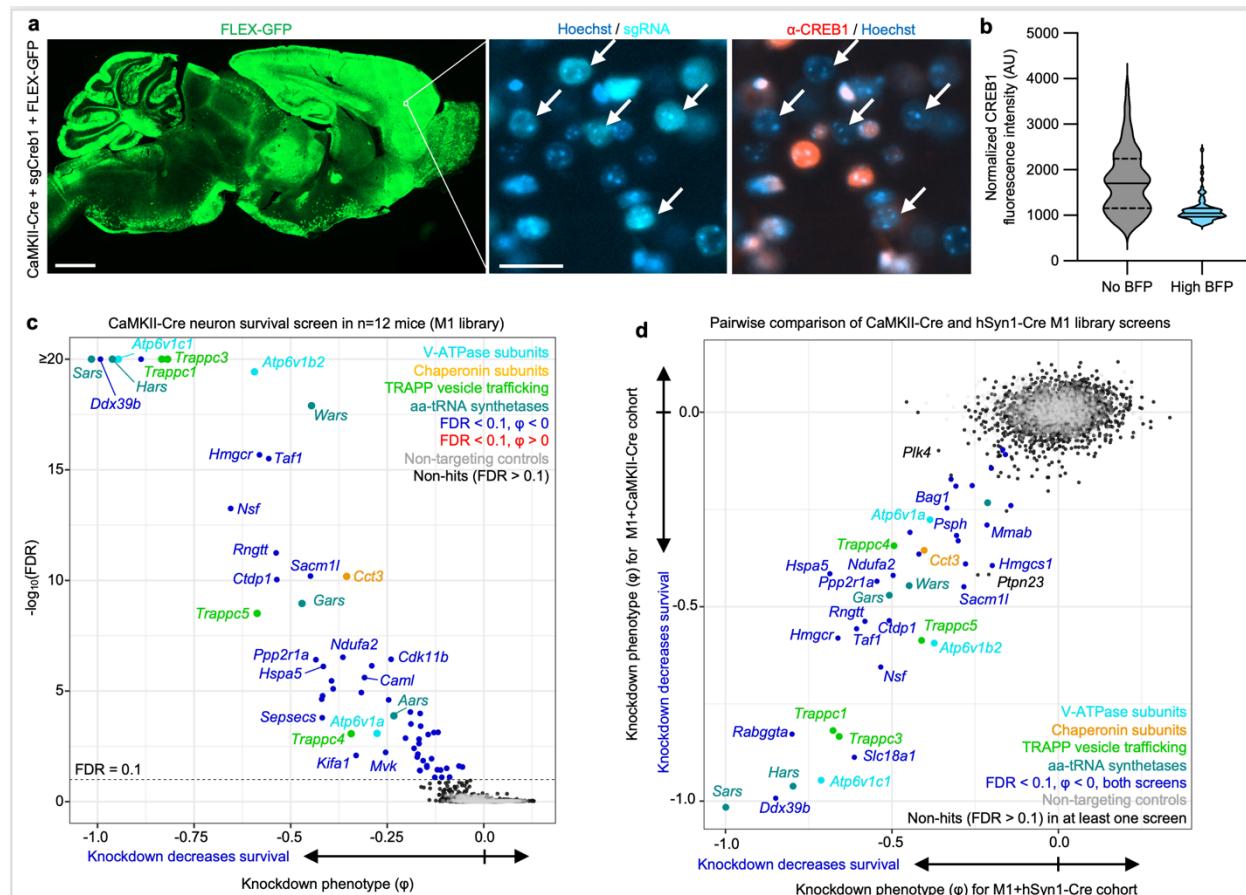
Fig. 3: In vivo CrAAVe-seq uncovers neuron-essential genes in the mouse brain.

a, Experimental design for CrAAVe-seq to identify neuron-essential genes across two different pooled sgRNA libraries (pAP215 expressing the M1 or M3 libraries). M1 targets kinases, phosphatases, and druggable targets: 2,269 genes (12,100 targeting sgRNAs and 250 non-targeting controls); M3 targets stress and proteostasis targets: 2,800 genes (14,685 targeting sgRNAs and 290 non-targeting controls)²⁶. The sgRNA libraries and hSyn1-Cre were packaged into AAV (PHP.eB capsid) and co-injected into neonatal LSL-CRISPRi mice. **b**, Results from CRISPRi screen described in (a): Knockdown phenotype (ϕ , see Methods) for 5,069 genes and non-targeting (quasi-gene) controls at 6 weeks post-injection (n=13 mice injected with pAP215-M1 library, and n=19 mice injected with pAP215-M3 library). Hit genes (FDR<0.1) within

1259 enriched pathways are color-coded. Non-targeting controls (gray) are plotted on top of non-hit
1260 genes (black). **c**, Table showing enrichment of hit genes in different Cellular Component Gene
1261 Ontology (GO) terms. **d**, Knockdown phenotype for each individual mouse (columns) for 30 top
1262 hit genes (rows, 29 with negative and 1 with positive knockdown phenotypes). Rows are ranked
1263 by average knockdown phenotype.
1264

1265
1266

Fig. 4



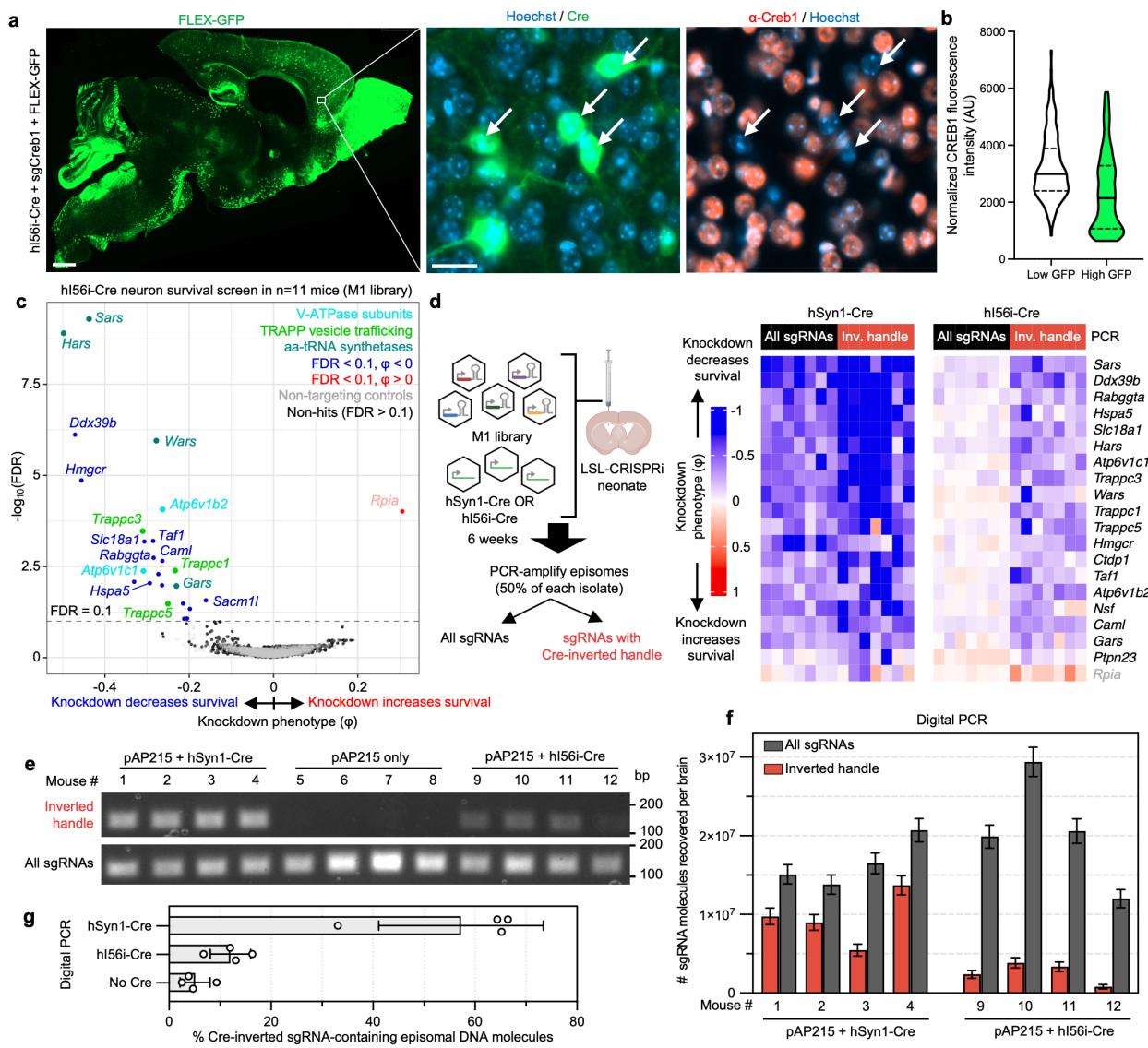
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281

Fig. 4. Genetic modifiers of survival in a CaMKII⁺ subpopulation of neurons in the mouse brain *in vivo*

a, PHP.eB::CaMKII-Cre was co-injected with PHP.eB::FLEX-GFP and PHP.eB::pAP215-sgCreb1 in LSL-CRISPRi mice. Brains were examined by immunofluorescence staining for CREB1 at 3 weeks. *Left*, low-power view showing expression of FLEX-GFP across a sagittal section of the brain. *Right*, magnified region of frontal cortex showing CREB1 knockdown in BFP⁺ (sgCreb1⁺) cells (arrows). **b**, CREB1 levels in BFP⁺ (n=79 cells) versus BFP⁻ (n=78 cells) nuclei in a representative region of cortex. Scale bars: 1 mm (left) and 20 μ m (right). **c**, Knockdown phenotypes for 2,269 genes averaged across 12 mice at 6 weeks after ICV injection of PHP.eB-packaged M1 sgRNA library and CaMKII-Cre. Experimental design is identical to Fig. 3a, except injecting CaMKII-Cre instead of hSyn1-Cre. Genes within enriched pathways are color-coded. **d**, Comparison of CRISPRi screen results between the CaMKII-Cre cohort (**c**) and the hSyn1-Cre cohort shown in Fig. 3b.

1282
1283

Fig. 5



1284

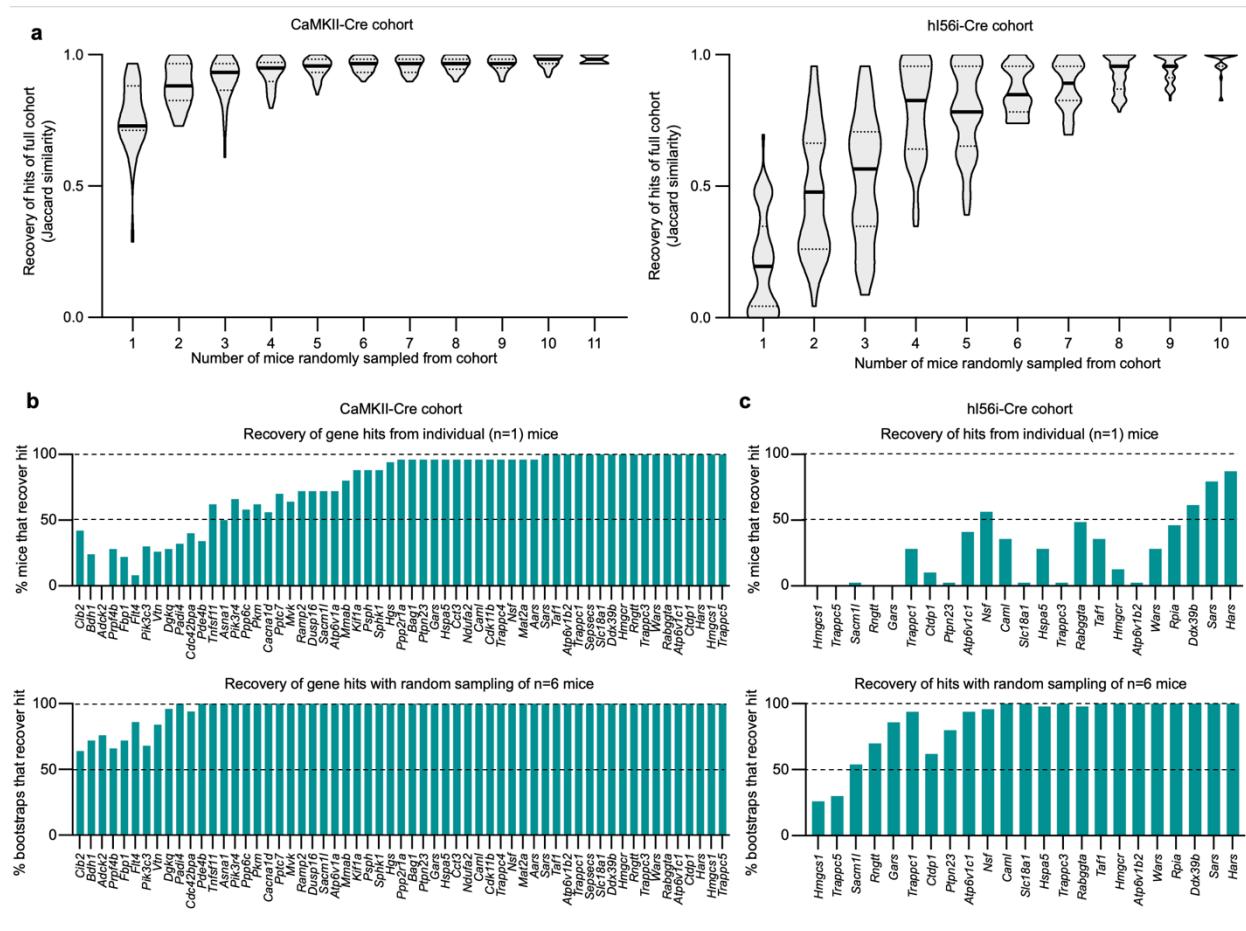
Fig. 5: Cre-dependent sgRNA recovery is critical for screening on small neuronal subpopulations

1285
1286
1287 **a**, PHP.eB::hL56i-Cre (predicted to target predominantly forebrain GABAergic interneurons)³¹
1288 was co-injected with PHP.eB::FLEX-GFP and PHP.eB::pAP215-sgCrb1 in LSL-CRISPRi mice,
1289 followed by immunofluorescence staining for CREB1 in the brain at 3 weeks. *Left*, Sagittal brain
1290 section showing FLEX-GFP expression (reporting Cre activity). *Right*, Inset showing CREB1
1291 knockdown in GFP⁺ cells (arrows). Scale bars: 800 μm (left) and 20 μm (right). **b**, CREB1 levels
1292 in GFP⁺ (n=189 cells) versus GFP⁻ (n=188 cells) cells within a representative cortical region. **c**,
1293 Averaged knockdown phenotypes for 2,269 genes across 11 mice, 6 weeks after ICV injection
1294 of PHP.eB::M1 sgRNA library and PHP.eB::hL56i-Cre. *RpiA* is labeled in light pink, indicating its
1295 phenotype was driven by only one sgRNA. **d**, *Left*, Experimental design comparing impact of
1296 PCR recovery of all sgRNAs versus only those with the Cre-inverted handle, in mice injected
1297 with M1 library and either hSyn1-Cre or hL56i-Cre. *Right*, Heatmap showing knockdown
1298 phenotypes for top hits (rows) across n=7 mice (columns) from each Cre cohort, contrasting the

1299 two PCR recovery methods. Genes listed are top hits selected from (**c**) and include 19 with
1300 negative and 1 with positive knockdown phenotypes. **e**, Ethidium bromide-stained agarose gel
1301 for PCR products from episomes recovered from brains of mice at 4 weeks post-ICV injection. **f**,
1302 Digital PCR (dPCR) performed on Cre-injected samples in (**e**) showing absolute numbers of
1303 pAP215 sgRNA-encoding episomal DNA molecules recovered from each brain using CrAAVe-
1304 seq (with 95% confidence interval from dPCR Poisson distribution), both for total episomes and
1305 episomes with cell type-specific Cre-inverted handle. **g**, Percent recovered sgRNAs from (**f**) that
1306 contains the inverted handle upon hSyn1-Cre (all neurons) or hI56i-Cre (small subset)
1307 expression, including no Cre negative control (n=4 mice per condition, mean ± s.d.).
1308

1309
1310

Fig. 6

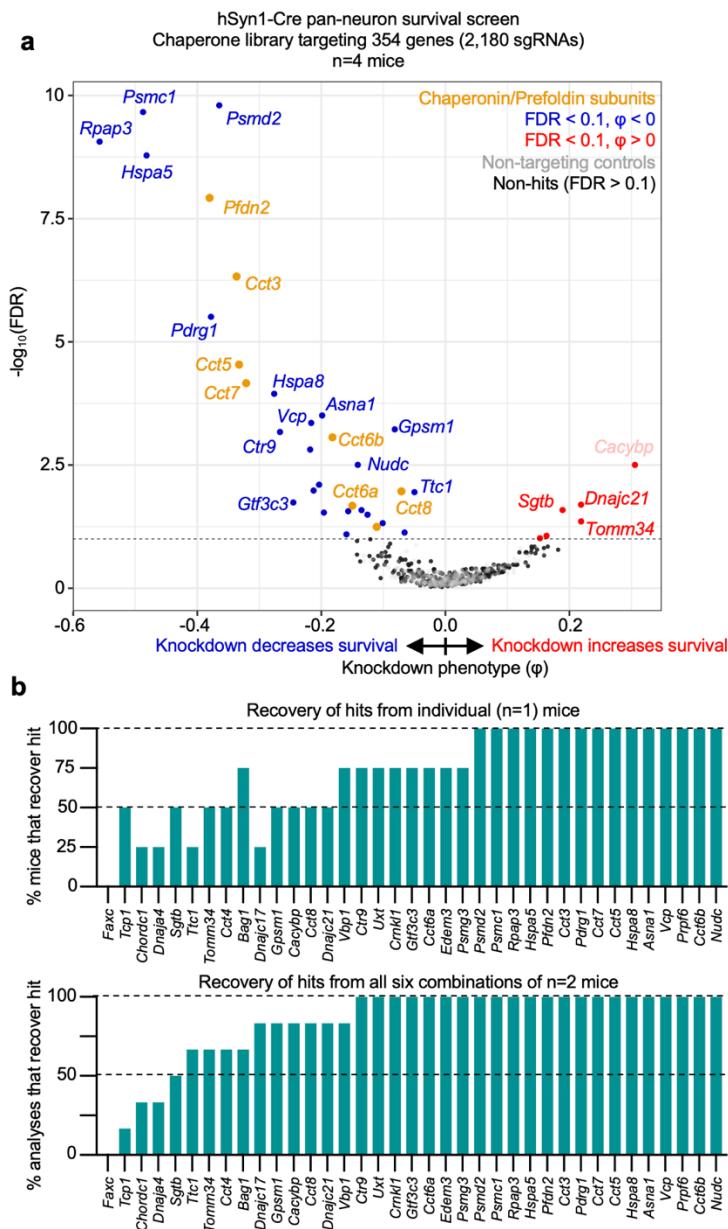


1311
1312

Fig. 6: Bootstrapping analysis estimates the number of mice required for different *in vivo* screening conditions

1315
1316
1317
1318
1319
1320
1321

1322 **Fig. 7**
 1323



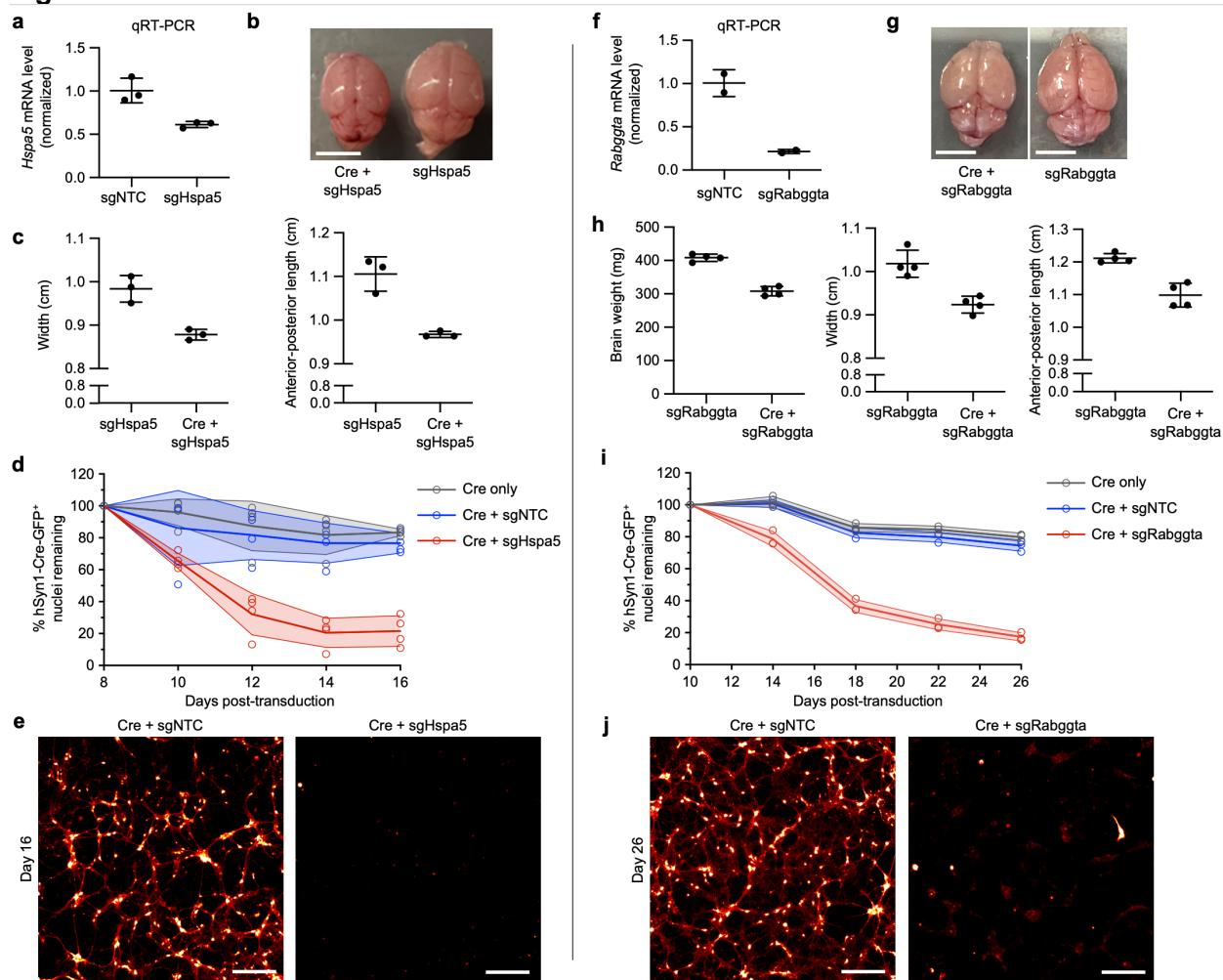
1324
 1325

1326 **Fig. 7: CRISPRi screening for neuron-essential chaperones using a smaller sgRNA library.**

1327 **a**, Knockdown phenotypes of 354 chaperone genes averaged across n=4 LSL-CRISPRi mice at
 1328 6 weeks after neonatal ICV injection of PHP.eB::hSyn1-Cre and a PHP.eB-packaged sgRNA
 1329 library targeting chaperones (354 genes, 2,180 sgRNAs, of which 350 are non-targeting
 1330 controls). Cacybp is labeled in light pink to reflect that its phenotype was driven by only one
 1331 sgRNA. **b**, Top, the percentage of individual mice that recover each of the hits defined from
 1332 analyzing the full cohort. Bottom, percentage of analyses for all six combinations of n=2 of the 4
 1333 mice that recover each of the hits of the full cohort.

1335

Fig. 8



1336

1337

1338

1339 **Fig. 8: Validation of *Hspa5* and *Rabggta* as neuron-essential genes in mouse neurons**

1340 **a**, Primary neurons cultured from constitutive CRISPRi mice were transduced with
1341 PHP.eB::pAP215 targeting *Hspa5* (sg*Hspa5*) or non-targeting control (sgNTC). *Hspa5* mRNA
1342 levels were assayed by qRT-PCR and normalized to the sgNTC control (mean \pm s.d., n=3
1343 technical replicates). **b**, Representative brains of LSL-CRISPRi mice 16 days after neonatal ICV
1344 injection of PHP.eB::pAP215-sg*Hspa5* with or without co-injection with PHP.eB::hSyn1-Cre
1345 (Cre). Scale bar: 5 mm. **c**, Quantification of brain width and length (mean \pm s.d., n = 3
1346 independent mice) from (b). Gross motor phenotypes for these mice are shown in
1347 Supplementary Videos 1 and 2. **d**, Primary neurons cultured from LSL-CRISPRi mice following
1348 transduction with PHP.eB::hSyn1-Cre and PHP.eB::pAP215-sg*Hspa5* or sgNTC (mean \pm s.d., n
1349 = 4 wells). Survival was determined by counting GFP+ nuclei over time and normalized to peak
1350 fluorescence, which occurred at day 8. **e**, Representative image of primary neuronal cultures
1351 from (d) at 16 days post transduction. Cells were co-transduced with constitutively expressed
1352 cytosolic mScarlet (PHP.eB::CAG-mScarlet), which reveals fine neuronal processes (displayed
1353 with a red to white lookup table). Scale bar: 250 μ m. **f-j**, Identical experimental designs to (a-e),
1354 except using PHP.eB::pAP215-sg*Rabggta* instead of sg*Hspa5*, with the following changes:
1355 Brains extracted from mice in (h) were weighed after extraction. Quantification of neuronal

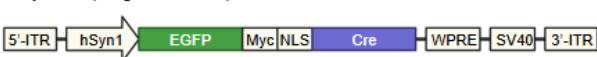
1356 survival in (i) used n=3 wells instead of n=4. Peak fluorescence in cultures quantified in (i)
1357 occurred at day 10 instead of day 8, and were normalized to that timepoint, and imaged until
1358 day 26 instead of day 16. Neurons in (j) were displayed at day 26 instead of day 16.
1359

1360 **Extended Data Fig. 1**

a

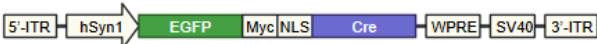
	Tissue targeted	Method of sgRNA delivery	Method of sgRNA recovery	Phenotype	Number of genes targeted	Number of sgRNA elements (incl. # NTCs)	Mechanism of cell-type specificity (beyond inherent viral tropism)	Virus serotype	CRISPR mechanism
CRISPR-based screens in brain using AAV									
This study	Brain	AAV	CrAAVe-seq	Survival	5,069	27,865 (540 NTCs)	Cre-based targeting	PHP.eB	CRISPRi
Zheng, et al. (2024)	Brain	AAV	scRNA-seq	mRNA levels	4	16 (4 NTCs)	Cell type identification by scRNA-seq	Multiple	CRISPRko
Santinha, et al. (2023)	Brain	AAV	scRNA-seq	mRNA levels	29	64 (6 NTCs)	Cell type identification by scRNA-seq	PHP.B	CRISPRko
Chow, et al. (2017)	Brain	AAV	N/A*	Tumorigenesis	56	288 (8 NTCs)	Cre-based targeting	AAV9	CRISPRko
CRISPR-based screens in brain using lentivirus									
Ruetz, et al. (2024)	Brain	Lenti	PCR from gDNA	NSC migration/aging	50	350 (100 NTCs)	None***	VSV-G	CRISPRko
Wertz, et al. (2020)	Brain	Lenti	PCR from gDNA	Survival	20,077**	80,972 (664 NTCs)**	None	VSV-G	CRISPRko
Jin, et al. (2020)	Brain	Lenti	scRNA-seq	mRNA levels	39	40 (1 NTC)	Cell type identification by scRNA-seq	VSV-G	CRISPRko
CRISPR-based screens in other tissues using AAV									
Wang, et al. (2018)	Liver	AAV	N/A*	Tumorigenesis	56	288 (8 NTCs)	Cre-based targeting	AAV9	CRISPRko

b



	Lentivirus	AAV
Biosafety level	BSL-2	BSL-1
Viral spread	Limited beyond site of injection	High spread throughout injected tissue
Packaging options	Few envelope pseudo-types (commonly VSV-G)	>20 serotypes commonly used (e.g. AAV1-9, PHP.eB)
Integration	Integrating, sgRNAs require gDNA amplification	Non-integrating, sgRNAs amplified from small episomes
PCR reaction volume to amplify sgRNAs	~15,000 µL per brain	100 µL per brain

c hSyn1-Cre (Addgene # 105540)



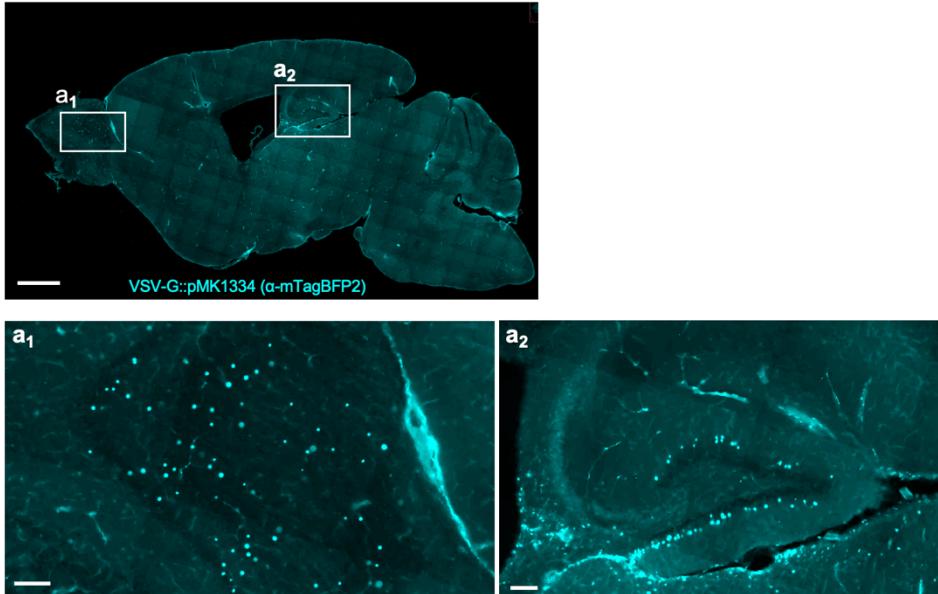
1361
1362 **Extended Data Fig. 1: CrAAVe-seq enables simultaneously highly scalable and cell type-specific in vivo screens**
1363

1364 **a**, Table summarizing published *in vivo* pooled screens of endogenous cells in the brain and
1365 AAV-based screen outside of the brain: Zheng et al.⁹, Santinha et al.¹⁰, Chow et al.⁵, Ruetz et
1366 al.⁸, Wertz et al.⁶, Jin et al.⁷, Wang et al.⁴⁷. *Did not capture sgRNAs, instead performed
1367 mutagenesis screen; **Murine genome-wide Asiago library; ***A specific brain region containing
1368 migrated neural stem cell (NSCs) provided selectivity. CRISPRko: CRISPR knockout.
1369 Highlighted cells are large-scale sgRNA libraries (green), cell-type selectivity by Cre (blue), and
1370 single-cell RNA-sequencing (scRNA-seq, red). **b**, Table comparing different aspects of AAV
1371 and lentivirus. **c**, Simplified diagram of the hSyn1-Cre plasmid construct (Addgene # 105540).
1372

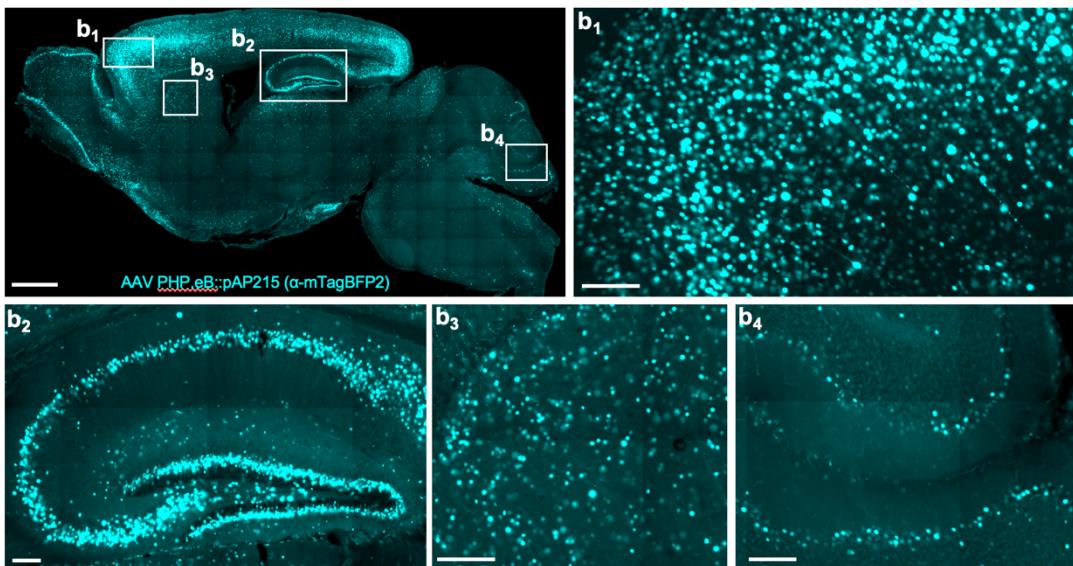
1373 **Extended Data Fig. 2**

1374

a



b



1375

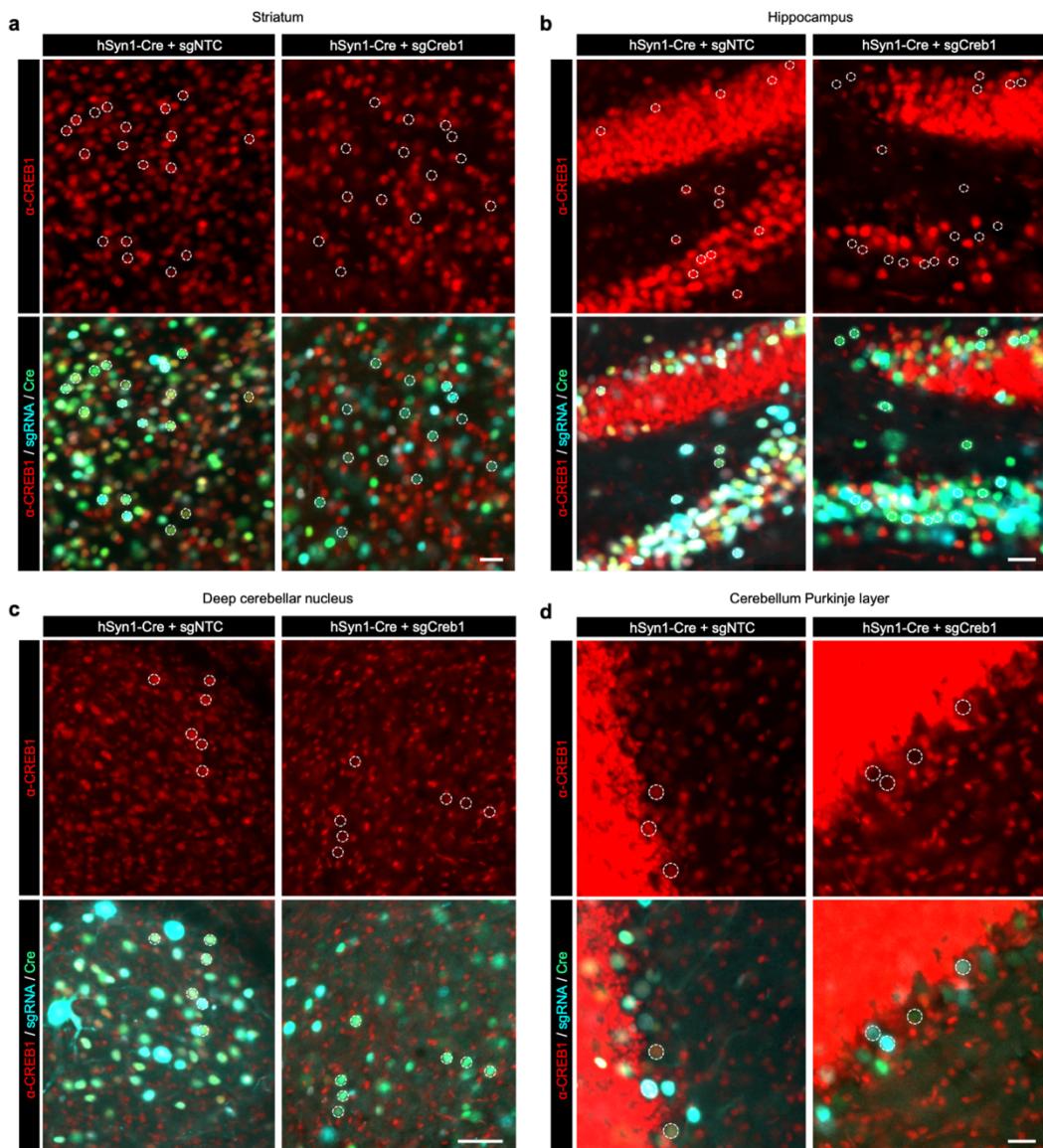
1376

1377 **a**, Sagittal section of mouse brain injected with sgRNA construct packaged in VSV-G-
1378 pseudotyped lentivirus (EF1a-NLS-mTagBFP2; pMK1334), stained with antibody recognizing
1379 mTagBFP2 at 3 weeks after ICV injection. Scalebar is 1 mm for low-magnification and 100 μ m
1380 for subpanels (a1), (a2). **b**, Sagittal section of mouse brain injected with sgRNA construct
1381 packaged in AAV PHP.eB capsid (EF1a-NLS-mTagBFP2; pAP215), stained with antibody
1382 recognizing mTagBFP2 at 3 weeks after ICV injection. This condition is sgCreb1 + hSyn1-Cre
1383 (mouse 2), used in Fig. 2 and in Extended Data Fig. 4. Scalebar is 1 mm for low magnification
1384 and 100 μ m for subpanels (b1), (b2), (b3), and (b4).

1385

1386 **Extended Data Fig. 3**

1387



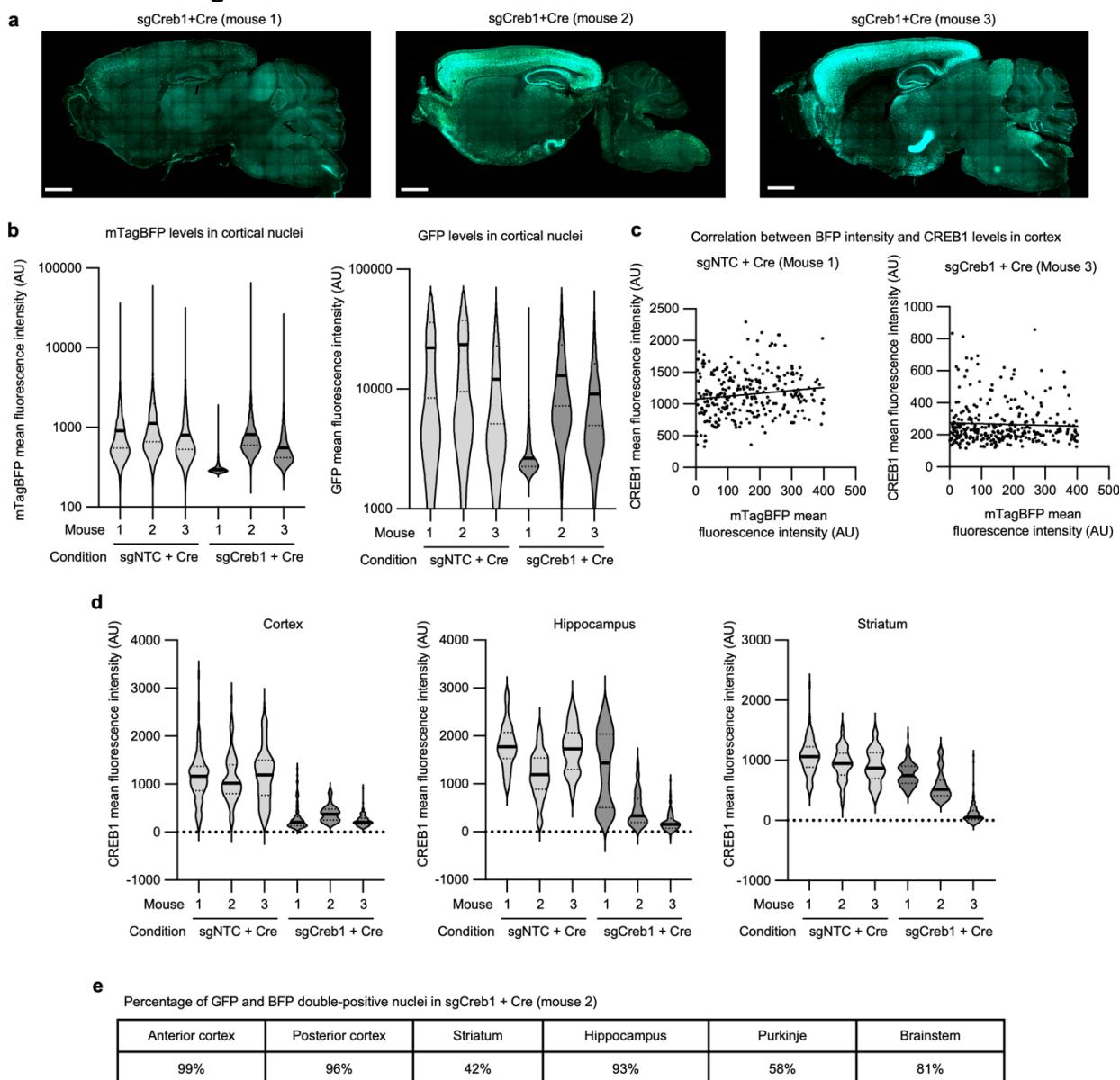
1388

1389 **Extended Data Fig. 3: Cre-dependent CRISPRi knockdown of CREB1 in different brain**

1390 **regions of a mouse.**

1391 Immunofluorescent staining for CREB1 (red) in an LSL-CRISPRi mouse 3 weeks after injection
1392 with PHP.eb::pAP215-sgNTC or -sgCrb1 (blue) along with PHP.eB::hSyn1-Cre (green), for
1393 different brain regions (**a**, Striatum, **b**, Hippocampus, **c**, Deep cerebellar nuclei, **d**, Cerebellum
1394 Purkinje layer). Representative nuclei containing both BFP and GFP are outlined with a dotted
1395 circle, with the same nuclei shown in the top and bottom panels of each brain region,
1396 highlighting markedly reduced CREB1 in double-positive nuclei with sgCrb1 as compared to
1397 sgNTC. DG: dentate gyrus; ML: molecular layer, Pkj: Purkinje cell layer (Pkj); IGL: internal
1398 granule layer. Data is quantified in Extended Data Fig. 4b.
1399

1400 **Extended Data Fig. 4**



1401

1402

1403 **Extended Data Fig. 4: Quantification of CREB1 by brain region and by BFP intensity**

1404 **a**, Sagittal sections of the three mice injected with AAV pAP215 expressing sgCrb1 and AAV

1405 hSyn1-Cre (Cre) that are used in Fig. 2. Mouse 1 has a lower expression of both the sgRNA and

1406 Cre, potentially due to injection variability. **b**, Quantification of levels of BFP and GFP in a

1407 representative region of the cortex for sgNTC + Cre and sgCrb1 + Cre mice, n=3 each. **c**,

1408 CREB1 levels plotted against BFP levels in a representative region of cortex from two different

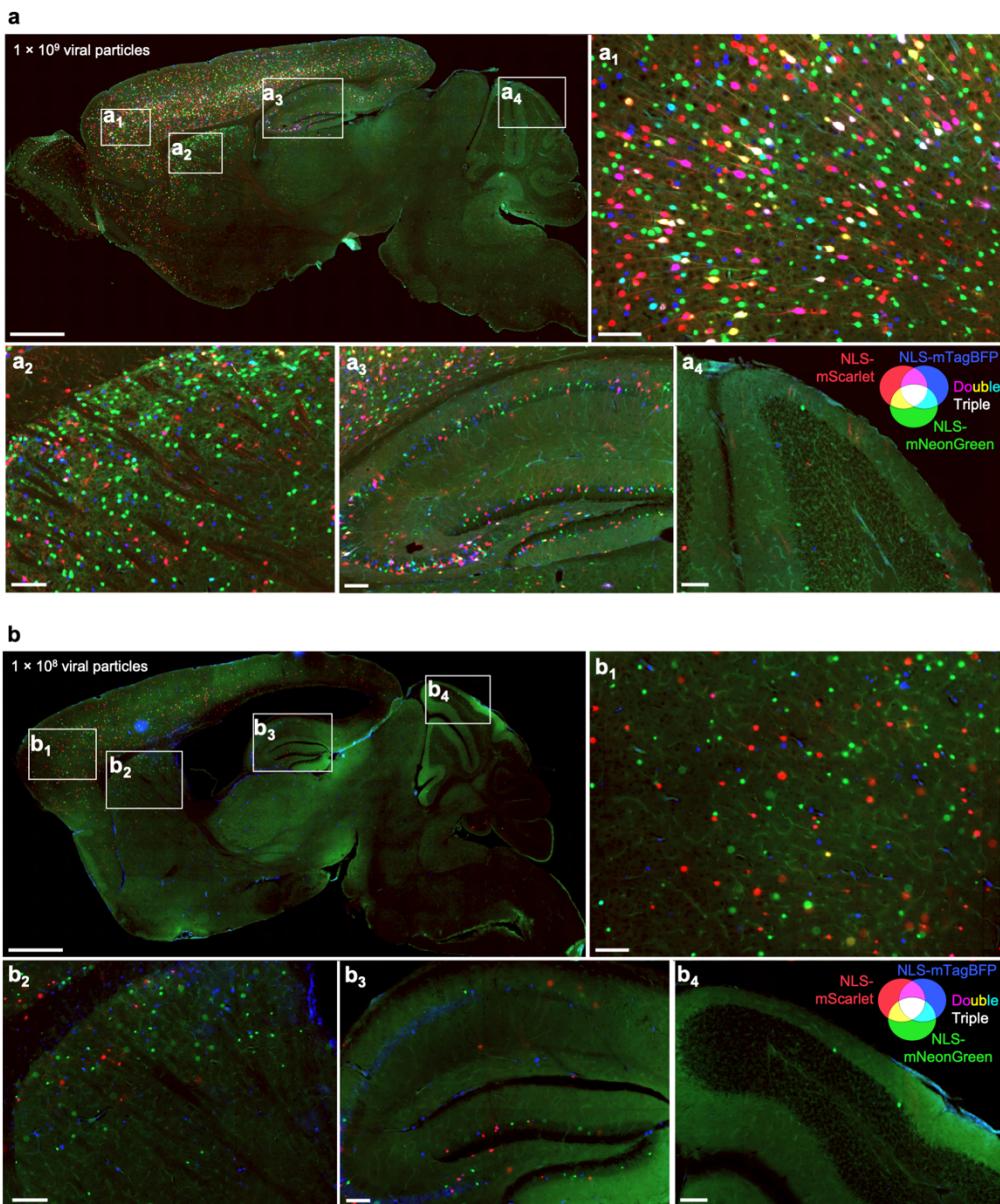
1409 mice, with trendlines generated by simple linear regression showing no correlation between

1410 BFP and CREB1 levels. **d**, Quantification of CREB1 levels in BFP⁺ nuclei of three different

1411 brain regions.

1412

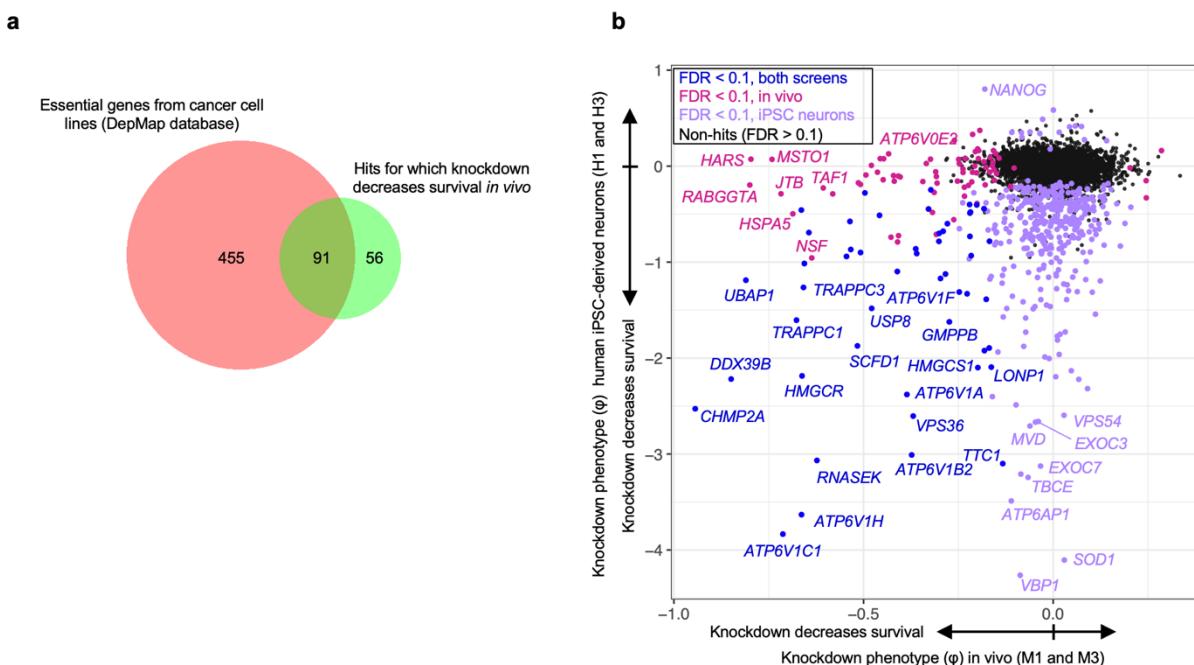
1413 **Extended Data Fig. 5**
1414



1415
1416 **Extended Data Fig. 5: Distribution and extent of multiple infections with AAV injected at**
1417 **lower concentrations**

1418 Sagittal sections of mouse brains at 3 weeks after ICV co-injection of PHP.eB-packaged AAV
1419 expressing nuclear-localized mScarlet, mNeonGreen, or mTagBFP, at the indicated viral
1420 concentrations, which are (a) 10-fold or (b) 100-fold lower than show in Fig. 2. Scale bars: 1 mm
1421 for low-magnification views and 100 µm for subpanels.
1422

1423 **Extended Data Fig. 5**



1424

1425

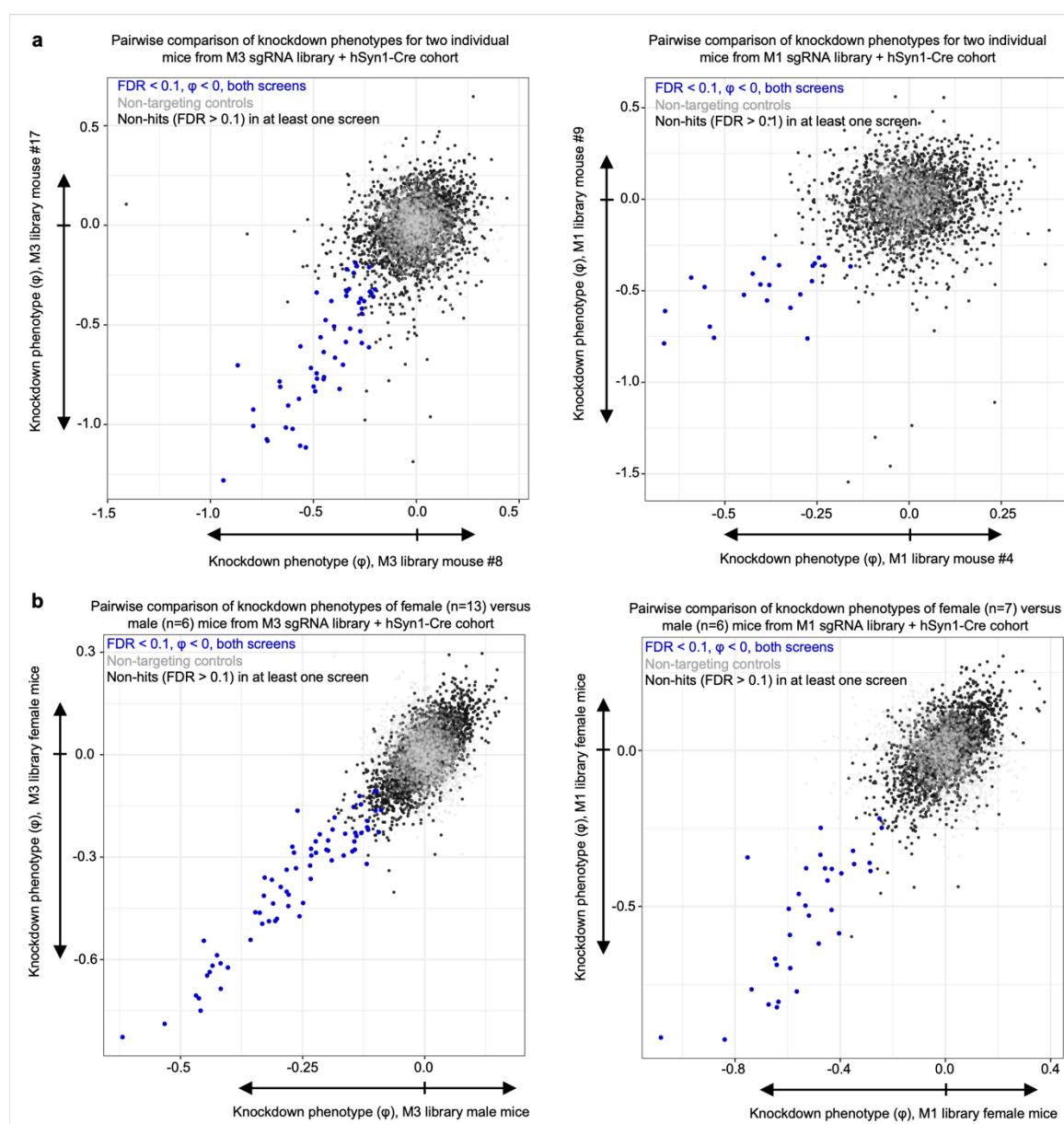
1426 **Extended Data Fig. 6: Overlap of hits of *in vivo* screens with essential genes of iPSC-derived neurons, and DepMap common essential genes**

1427 **a**, Venn diagram showing that 91 of the 147 hit genes for which knockdown decrease neuronal
1428 survival *in vivo* (Fig. 3) overlap with common essential genes of the injected libraries in cancer
1429 cell lines described in the DepMap database²⁷, P<0.0001 by Fisher's exact test, two-sided. **b**,
1430 Comparison of knockdown phenotype for genes between the *in vivo* neuronal survival screen of
1431 Fig. 3 and survival screens previously performed in iPSC-derived neurons by Tian et al.².
1432

1433

1434
1435

Extended Data Fig. 7



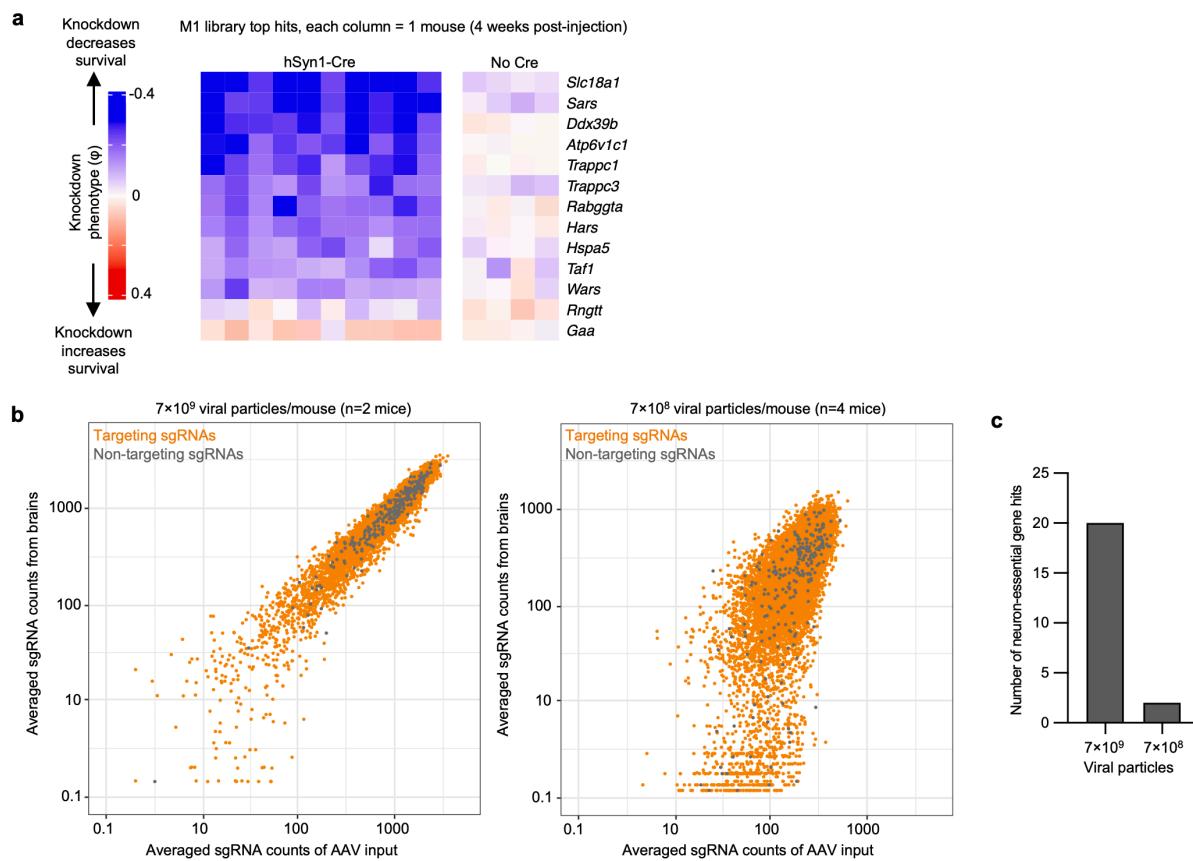
1436
1437

Extended Data Fig. 7: Pairwise comparisons of CRISPR screens by individual mice and by biological sex

a, Pairwise correlation of knockdown phenotypes between two individual mice injected with hSyn1-Cre and the M3 library (Left) and the M1 library (right). The two individual mice that called the greatest number of hits in each screen were selected for these comparisons. **b**, Pairwise correlation of combined knockdown phenotypes between male and female mice injected with hSyn1-Cre and the M3 library (left) and the M1 library (right).

1444
1445

1446 **Extended Data Fig. 8**



1447

1448

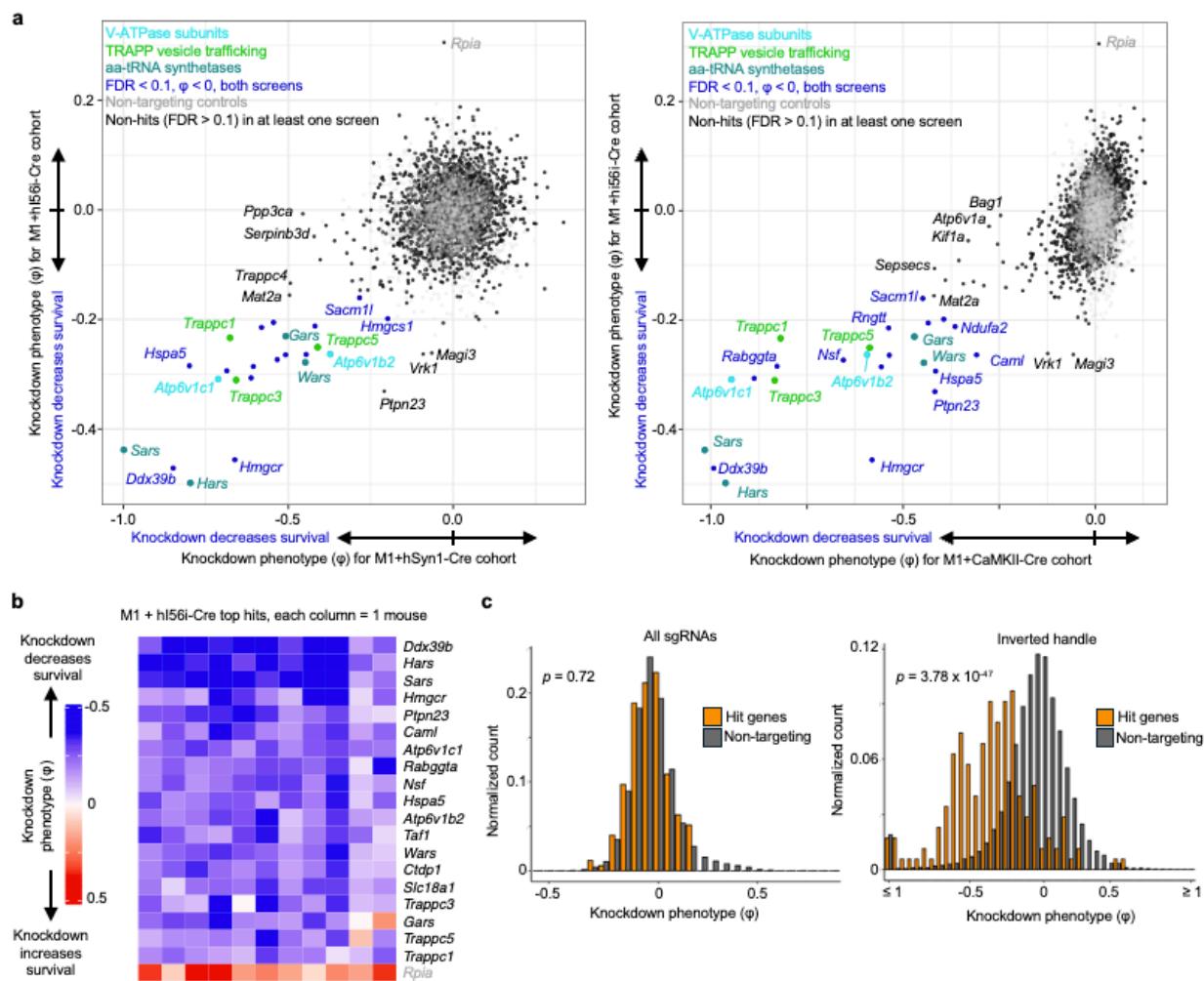
1449

1450 **Extended Data Fig. 8: Cre expression and concentration dependency for screening performance**

1451 **a**, Heatmap of a subset of top hit genes in LSL-CRISPRi mice at 4 weeks after injection of
1452 PHP.eB::pAP215-M1 library, with or without co-delivery of PHP.eB::hSyn1-Cre. **b**, A cohort of
1453 LSL-CRISPRi mice were injected with either 7×10^9 (n=2 mice) or 7×10^8 (n=4 mice) viral
1454 particles of the PHP.eB::pAP215-M1 library in littermates. Normalized and averaged sgRNA
1455 counts from the brains are plotted against the sgRNA counts from the input AAV library, showing
1456 marked dropout of both targeting and non-targeting sgRNAs when using the lower concentration
1457 of virus. **c**, number of neuron-essential genes identified based on overlap with hits identified
1458 from the M1 library cohort in Fig. 3b in the mice with different concentrations of virus.
1459

1460

1461 **Extended Data Fig. 9**



1462

1463

1464

1465 **Extended Data Fig. 9: Knockdown phenotypes of essential genes identified by hi56i-Cre**
1466 **CRISPRi screen**

1467 **a**, Comparison of the knockdown phenotypes between the hi56i-Cre cohort and the hSyn1-Cre
1468 cohort of Fig. 3 (Left) and the CaMKII-Cre of Fig. 4 (Right). *Rplia* is labeled in gray to indicate
1469 that its effect is driven by only a single (but strong) sgRNA. **b**, Knockdown phenotypes plotted
1470 on a heatmap for top hit genes (rows, 19 with negative and 1 with positive knockdown
1471 phenotype) for all of the individual mice (columns). *Rplia* is shown in grey to reflect that its
1472 knockdown phenotype is driven by only one sgRNA. **c**, Histogram showing the distribution of
1473 gene knockdown phenotypes (φ) for either hit genes (orange), from (b), or for non-targeting
1474 control-based quasi-genes (grey) from the pAP215-M1 + hi56i-Cre screening cohort of mice
1475 shown in Fig. 5d. These plots compare the distributions for either all sgRNAs (left) or sgRNAs
1476 with the inverted handle (right). Knockdown phenotypes for each gene across the cohort were
1477 binned by 0.05 increments and normalized to the total number of genes (or quasi-genes) in that
1478 respective set. *P*-value calculated by asymptotic two-sample Kolmogorov-Smirnov test.