nature biotechnology

BRIEF COMMUNICATIONS

Efficient genome editing in the mouse brain by local delivery of engineered Cas9 ribonucleoprotein complexes

Brett T Staahl[1], Madhurima Benekareddy[2], Claire Coulon-Bainier[2], Ashwin A Banfal[1], Stephen N Floor[1], Jennifer K Sabo[1], Cole Urnes[1], Gabriela Acevedo Munares[1], Anirvan Ghosh[2,3] & Jennifer A Doudna[1,4–7]

We demonstrate editing of post-mitotic neurons in the adult mouse brain following injection of Cas9 ribonucleoprotein (RNP) complexes in the hippocampus, striatum and cortex. Engineered variants of Cas9 with multiple SV40 nuclear localization sequences enabled a tenfold increase in the efficiency of neuronal editing *in vivo*. These advances indicate the potential of genome editing in the brain to correct or inactivate the underlying genetic causes of neurological diseases.

Cas9 is an RNA-guided, DNA-binding and cleaving protein that enables facile modification or perturbation of genes and non-coding genetic elements in a wide variety of organisms[1–4]. Cas9-mediated genome editing has been used to treat diseases of the eye, ear, liver and muscle in animals, but the challenge of tissue-specific delivery remains to be addressed[5–10]. *In vivo* delivery of genetically encoded Cas9 with viral vectors and plasmids is undesirable for several reasons, including potential direct integration into genomic DNA, immune responses elicited by persistent expression of the bacterial Cas9 protein in edited cells[11] and off-target editing[12]. Delivery of non-genetically encoded, preassembled and short-lived Cas9 ribonucleoprotein (RNP) complexes overcomes these concerns[5,13–16].

To test the activity of Cas9 RNPs *in vivo* we first adapted the Ai9 tdTomato mouse to report Cas9 editing (**Supplementary Fig. 1a**). This reporter mouse model provides a high-throughput and quantitative readout of site-specific genome editing that results in a red fluorescent protein gain-of-function signal in genetically modified cells[17]. We developed a single guide RNA (sgRNA), termed sgRNAtdTom, to delete the stop cassette and activate tdTomato expression in cells (**Supplementary Figs. 1** and **2a**). Upon nucleofection of neural progenitor cells (NPCs) with Cas9-2×NLS (SV40 nuclear localization sequence) sgRNAtdTom RNP complexes, we observed an RNP dose-dependent activation of tdTomato by microscopy and flow cytometry analysis (FACS; **Fig. 1a**). NexGen sequence analysis (NGS) of the locus confirmed Cas9 RNP-induced insertion/deletion (indel) mutations (**Supplementary Fig. 2b**). PCR analysis of genomic DNA from the edited tdTomato stop cassette revealed a DNA laddering pattern, and FACS sorting followed by genomic DNA PCR analysis showed *tdTomato* reporter activation required a specific double deletion (**Supplementary Fig. 2c**). Sanger sequencing of the other edited alleles identified additional edits that did not activate tdTomato (**Supplementary Fig. 2d**,**e**). Quantification of the double-deletion PCR band correlated closely with the percentage of tdTomato$^+$ cells measured by flow cytometry. Therefore, although the number of tdTomato$^+$ cells under-reports RNP total editing, the tdTomato mouse can be used to visually detect genome editing by Cas9 RNPs, and should enable visualization of edited cells *in vivo*.

Cas9 RNP has no innate cell-penetrating activity, and its direct delivery into cells requires chemical conjugation of polyarginine peptides, a strategy prone to inefficiency and heterogeneity[18], or mixing with lipid carrier molecules[5,16], which are immunogenic, inflammatory and toxic[19,20]. In previous work, arrays of Simian vacuolating virus 40 nuclear localization sequence (SV40-NLS) enhanced the innate cell-penetrating capabilities of zinc finger nucleases[21]. The Cas9 protein we used for cell-based experiments contained two SV40-NLSs on the C terminus, Cas9-2×NLS. However, we found that RNPs generated using this protein were not cell-penetrating and did not result in genome editing when added directly to the media of cultured cells (even in the absence of serum). To test potential cell-penetrating properties, we designed Cas9 proteins with increasing numbers of N-terminal SV40-NLS arrays that we call 0×, 1×, 2×, 4× and 7×NLS-Cas9-2×NLS (**Supplementary Table 1**). These were made with and without superfolder (sf)GFP[22] fused on the C terminus (**Fig. 1c**). We delivered RNPs to the media of tdTomato NPC cultures. TdTomato reporter activation increased markedly as the number of N-terminal NLS sequences on Cas9 was increased from none to four copies and decreased for 7×NLS-Cas9-2×NLS (**Fig. 1d**). TdTomato was not detected with a non-targeting sgRNA. PCR analysis of tdTomato locus confirmed the expected laddering pattern, indicating deletion edits and, as anticipated, showed higher total deletion efficiency than reported by the percentage of dTomato$^+$ cells (**Fig. 1e**).

We bypassed the requirement for protein-based cell penetration by mixing RNPs with Lipofectamine2000 to trigger RNP delivery into the cytoplasm by cell membrane fusion in HEK293 cells with a GFP reporter[5] or by using a NaCl hypertonic protein transduction protocol in tdTomato NPCs[23]. We observed gene editing in both cell types; editing activity was not significantly different between RNP variants at all RNP doses delivered (HEK293 $P = 0.9032$ and tdTomato NPCs $P = 0.8685$, **Supplementary Fig. 3a**,**b**). Similar findings were obtained in terminally differentiated neurons, where direct delivery

[1]Department of Molecular and Cell Biology, University of California, Berkeley, California, USA. [2]Roche Pharma Research and Early Development, Basel, Switzerland. [3]E-Scape Bio, San Francisco, California, USA. [4]Howard Hughes Medical Institute, University of California, Berkeley, California, USA. [5]Innovative Genomics Institute, University of California, Berkeley, California, USA. [6]MBIB Division, Lawrence Berkeley National Laboratory, Berkeley, California, USA. [7]Department of Chemistry, University of California, Berkeley, California, USA. Correspondence should be addressed to J.A.D. (doudna@berkeley.edu).

Received 22 August 2016; accepted 31 January 2017; published online 13 February 2017; doi:10.1038/nbt.3806

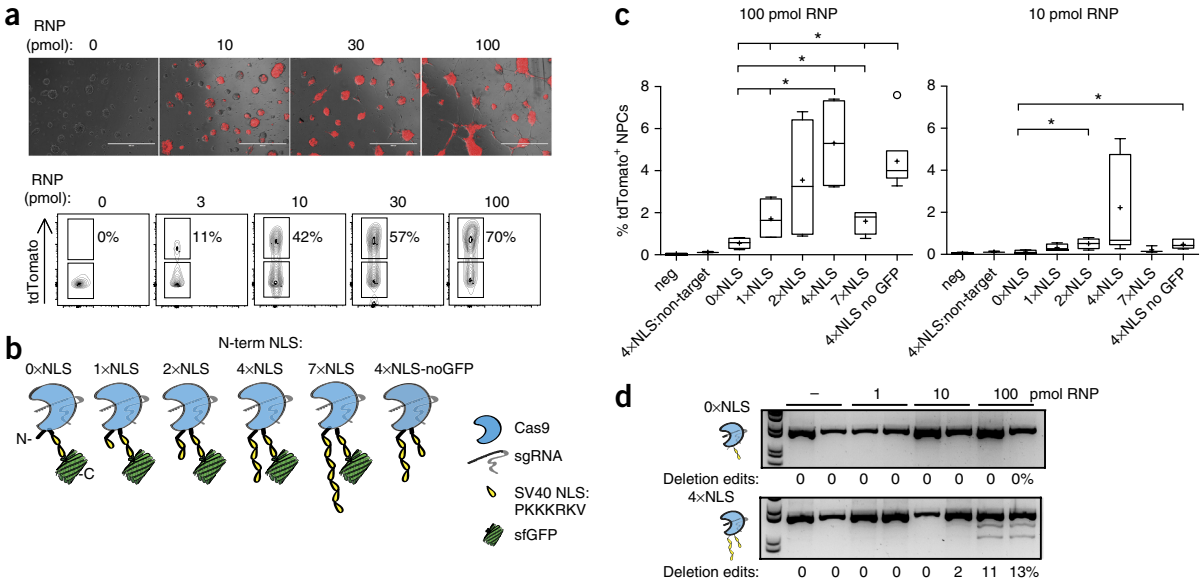


Figure 1 Generation of cell-penetrating Cas9 RNPs. (**a**) Images and flow cytometry analysis of tdTomato+ NPC neurospheres 3 d after nucleofection of Cas9 RNP dose course. Scale bars, 400 µm. Representative data from $n = 3$ independent experiments. (**b**) N-terminal 1–7×NLS-Cas9-2×NLS design. (**c**) Direct delivery of 1–7×NLS-Cas9-2×NLS with NPCs led to activation of *tdTomato* reporter in genome-edited cells. 4×NLS-Cas9-2×NLS designs are more efficient at genome-editing cells than other designs. Data are represented as Tukey box and whisker plots, box is interquartile range (IQR), whiskers are the lowest datum still within 1.5 IQR of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile, outliers are open circles, + is mean, line is median. Brackets with multiple ticks represent statistical difference ($P < 0.05$) with 4×NLS noGFP, 7×NLS and 4×NLS, respectively (100 pmol 0×NLS vs. 4×NLS-noGFP; two-tailed unpaired *t*-test with Welch's correction; $P = 0.0015$ and $F_{5,3} = 39.04$. 1×NLS vs. 4×NLS-noGFP; two-tailed unpaired *t*-test with Welch's correction; $P = 0.0099$ and $F_{5,3} = 2.434$. 100 pmol 7×NLS vs. 4×NLS-noGFP; two-tailed unpaired *t*-test with Welch's correction; $P = 0.0051$ and $F_{5,3} = 7.443$. 100 pmol 0×NLS vs. 7×NLS; two-tailed unpaired *t* test with Welch's correction; $P = 0.0283$ and $F_{3,3} = 5.246$. 100 pmol 4×NLS vs. 7×NLS; two-tailed unpaired *t*-test with Welch's correction; $P = 0.0419$ and $F_{3,3} = 15.35$. 100 pmol 0×NLS vs. 4×NLS; two-tailed unpaired *t*-test with Welch's correction; $P = 0.0236$ and $F_{3,3} = 80.50$. 100 pmol 1×NLS vs. 4×NLS; two-tailed unpaired *t*-test with Welch's correction; $P = 0.0416$ and $F_{3,3} = 5.019$. 10 pmol 0×NLS vs. 2×NLS; two-tailed unpaired *t*-test with equal s.d.; $P = 0.0096$ and $F_{2,2} = 2.597$. 10 pmol 0×NLS vs. 2×NLS; two-tailed unpaired *t*-test with equal s.d.; $P = 0.0096$ and $F_{2,2} = 2.597$. 10 pmol 0×NLS vs. 4×NLS-noGFP; two-tailed unpaired *t*-test with equal s.d.; $P = 0.0462$ and $F_{3,2} = 4.072$) $n = 4$ experimental replicates with 2 technical replicates each. (**d**) Genomic DNA PCR of *tdTomato* stop locus following RNP direct delivery validates tdTomato+ flow cytometry analysis. 4×NLS-Cas9-2×NLS RNP complexes yield deletion edits (lower two bands) while 0×NLS-Cas9-2×NLS RNP complexes do not. 100 pmol 4×NLS-Cas9-2×NLS RNP bottom DNA band (activated *tdTomato* locus) contains 5.4% ± 0.4% of the total DNA correlating with %tdTomato+ cells observed in **c**. Representative gel from $n = 4$ experimental replicates with two technical replicates each. $^*P < 0.05$.

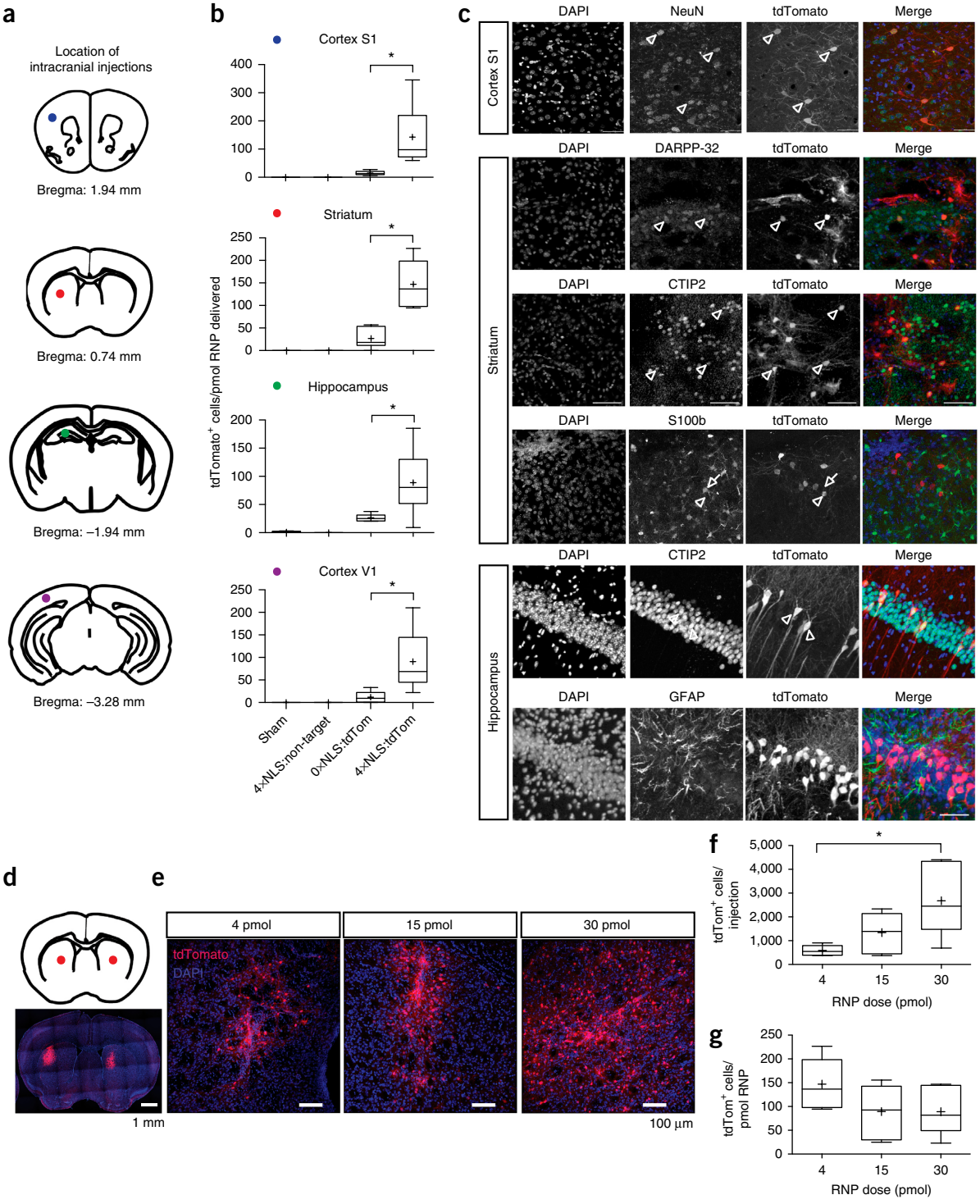
of 4×NLS-Cas9-2×NLS but not 0×NLS-Cas9-2×NLS RNPs triggered genome editing in mature neurons (**Supplementary Fig. 3c**), but when RNPs were mixed with Lipofectamine2000, both triggered genome editing (**Supplementary Fig. 3d**). Together these results support our hypothesis that 4×NLS-Cas9-2×NLS RNPs have enhanced cell-penetration capabilities.

Figure 2 Injection of Cas9 RNP into multiple brain regions in adult mice. (**a**) Color-coded dots indicate stereotaxic injection sites on coronal cartoons of mouse brain. Single Cas9 RNP injections (4 pmol/0.5 µl) with sham (injection buffer only), (Cas9 construct; sgRNA) 4×NLS-Cas9-2×NLS;non-targeting, 0×NLS-Cas9-2×NLS;tdTom, or 4×NLS-Cas9-2×NLS;tdTom into hippocampus, striatum, primary somatosensory cortex (S1), primary visual cortex V1. Male mice were 14–15 weeks old. (**b**) Quantification of tdTomato+ cells/pmol RNP delivered. Brains were analyzed 12–14 d after injection. 4×NLS-Cas9-2×NLS RNPs are significantly more efficient compared to 0×NLS-Cas9-2×NLS RNPs for *in vivo* genome-editing in all brain regions tested. Sham and 4×NLS-Cas9-2×NLS; non-targeting RNPs do not activate tdTomato indicating specificity of genome editing with Cas9 RNPs. Data are presented as Tukey box and whisker plots. Box is IQR, whiskers are the lowest datum still within 1.5 IQR of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile, + is mean, line is median. (4×NLS;tdTom vs. 0×NLS;tdTom; two-tailed unpaired *t*-test with equal s.d.; $P = 0.002$ and $F_{5,5} = 5.9$ for the striatum. Two-tailed unpaired *t*-test with Welch's correction, $P = 0.03$ and $F_{5,5} = 215$ for Cortex S1. $P = 0.03$ and $F_{5,5} = 25$ for Cortex V1. $P = 0.04$ and $F_{5,5} = 83$ for the hippocampus.) The sample size for each group is as follows: Sham and 4×NLS-Cas9-2×NLS;non-targeting: $n = 2$ animals, $n = 2$ injections per animal for each group, 0×NLS-Cas9-2×NLS;tdTom: $n = 3$ animals, $n = 2$ injections per animal, 4×NLS-Cas9-2×NLS;tdTom: $n = 3$ animals, $n = 2$ injections per animal. (**c**) Representative confocal microscopy images of 4×NLS-Cas9-2×NLS-RNP-treated brains identify tdTomato+ cells co-localizing with neuron and not astrocyte marker proteins. NeuN, neuronal-specific nuclear protein in vertebrates. CTIP2, aka BCL11a, a transcription factor present in CA1 hippocampus and striatum neurons. DARPP-32, cAMP-regulated neuronal phosphoprotein, a marker of striatum medium spiny neurons. GFAP, glial fibrillary acidic protein, an intermediate filament protein expressed in astrocytes and ependymal cells of the CNS. S100β, a highly expressed protein in striatal astrocytes. Scale bar, 50 µm. (**d**) Increasing dose of 4×NLS-Cas9-2×NLS RNP significantly increases number of tdTomato+ genome-edited cells in the striatum. Red dots indicate bilateral stereotaxic injection sites on coronal cartoon of mouse brain. Coronal section mosaic tile image of bilateral 30 pmol RNP injections with tdTomato reporting genome-editing in the striatum. Blue, DAPI staining nuclei; red, endogenous tdTomato expression. Scale bar, 1 mm. (**e**) Representative confocal images of tdTomato+ cells in single 4, 15, 30 pmol/0.5 µl injection dose course. Scale bars, 100 µm. (**f**) Quantification of total # tdTomato+ cells per injection site. Data are presented as Tukey box and whisker plots. Box is interquartile range (IQR), whiskers are the lowest datum still within 1.5 IQR of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile, + is mean, line is median. Two-tailed unpaired *t*-test with Welch's correction, $P = 0.0188$ and $F_{5,5} = 46.03$ for 4 pmol vs. 30 pmol. $P = 0.0536$ and $F_{5,5} = 13.43$ for 4 pmol vs. 15 pmol. Two-tailed unpaired *t*-test with equal s.d.; $P = 0.0845$ and $F_{5,5} = 3.428$ for 15 pmol vs. 30 pmol. $n = 3$ animals, $n = 2$ injections per animal for each group. (**g**) Quantification of tdTomato+ cells per pmol RNP delivered (1 tdTomato+ cell per 10 fmol RNP). Data are presented as Tukey box and whisker plots. Box is IQR, whiskers are the lowest datum still within 1.5 IQR of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile; + is mean, line is median ($P = 0.213$; Kruskal–Wallis test. $n = 3$ animals, $n = 2$ injections per animal for each group). $^*P < 0.05$.

We next tested the activity of the 4×NLS-Cas9-2×NLS and 0×NLS-Cas9-2×NLS RNPs in adult tdTomato mouse brains using an intracranial RNP injection system. To test RNP activity in diverse neuronal subtypes, we performed stereotaxic injections (4 pmol per 0.5 µL) in four brain regions: hippocampus, dorsal striatum, primary somatosensory cortex (S1) and primary visual cortex (V1) (**Fig. 2a**). We analyzed brains 12–14 d after injection to allow time for tdTomato protein to be expressed and to accumulate in genome-edited cells.

In vivo genome editing was observed for both the 4×NLS-Cas9-2×NLS and 0×NLS-Cas9-2×NLS RNPs that targeted tdTomato, but not for RNPs loaded with non-targeting sgRNA, showing RNP specificity. Similar to *in vitro* results, the 4×NLS-Cas9-2×NLS RNP was tenfold more efficient at editing cells in the brain compared to the 0×NLS-Cas9-2×NLS RNP in all four brain regions tested (**Fig. 2b**).

We next performed dual-color immunofluorescence to identify the cell types that 4×NLS-Cas9-2×NLS RNP edits *in vivo*. In the cortex, tdTomato$^+$ cells co-localized with NeuN (post-mitotic neuronal marker). In the hippocampus, tdTomato$^+$ cells co-localized with CTIP2 (also known as BCL11a), a transcription factor present in CA1 hippocampus and striatal post-mitotic neurons. Interestingly, in the



hippocampus tdTomato$^+$ cells did not co-localize with GFAP, an astrocyte marker protein. In the striatum, tdTomato$^+$ cells co-localized with CTIP2 and DARPP-32 (cAMP-regulated neuronal phosphoprotein), a well-documented marker of GABAergic striatal medium spiny neurons. As in the hippocampus, genome-editing was not detected in astrocytes as there was no tdTomato$^+$ co-localization with GFAP or S100β, (a protein highly expressed in striatal astrocytes) (**Fig. 2c**). Therefore, our cellular analysis showed 4×NLS-Cas9-2×NLS-RNP-mediated editing of diverse neuronal subtypes and not astrocytes.

To investigate whether a higher dose of 4×NLS-Cas9-2×NLS RNPs would increase the number or volume of genome-edited cells, we performed a 4-, 15- and 30-pmol RNP per 0.5 µl intrastriatal injection dose course. We observed significantly more tdTomato$^+$ cells with increasing RNP dose, 4 pmol (588 ± 90 cells), 15 pmol (1,339 ± 331 cells), 30 pmol (2,675 ± 613 cells), 4 pmol vs. 30 pmol ($P = 0.0188$) (**Fig. 2e**,**f**). We noted the highest RNP dose (30 pmol) had a threefold larger volume of tdTomato$^+$ tissue compared to the lowest RNP dose (4 pmol), ~1.5 mm^3 vs. ~1 mm^3 (**Supplementary Fig. 4**). Since the same injection volume was used for each RNP dose, this result hints at a volume-independent mechanism of RNP spreading through the striatum interstitial space. We also observed increased density of tdTomato$^+$-edited cells in the 30-pmol Cas9 RNP (**Fig. 2e** and **Supplementary Fig. 4c**). Notably, the efficiency of 4×NLS-Cas9-2×NLS-RNP-mediated genome editing was ~100 tdTomato$^+$ cells per pmol of RNP delivered and was not significantly different across the three RNP doses ($P = 0.213$) (**Fig. 2g**). This suggests that calculating the amount of RNP needed to edit a given number of neurons could be possible (**Supplementary Fig. 5**).

To assess whether there is an innate immune response to Cas9 RNP injected into the dorsal striatum, we analyzed activation of microglia, CNS resident immune cells, by IBA-1 immunostaining and relative transcript expression of a panel of immune marker genes by qPCR (**Supplementary Table 2**). We did not observe significant IBA-1 intensity differences or morphological differences among uninjected, sham-injected and RNP-injected brains, and qPCR of immune-related transcripts suggests an undetectable to mild microglia-mediated innate immune response following Cas9 RNP injection (**Supplementary Fig. 6**). The increased TNF-alpha transcript levels at 12 d after injection could reflect an adaptive response to RNP delivery that reduces synaptic strength of the corticostriatal pathway for neuroprotection[24] and not an innate immune response. Future studies will explore this possibility.

Overall, our findings demonstrate efficient Cas9-RNP-mediated genome editing of neural progenitor cells *in vitro* and neurons in distinct brain regions *in vivo*. Although the mechanism of 4×NLS-Cas9-2×NLS RNP *in vivo* neurotropism is not yet understood, it can be exploited for neuron-specific targeting. Therefore, this technology has the potential to be applied to treat genetic neurological diseases that are manifested in distinct populations of neurons. The evidence that functional Cas9 RNPs can be effectively, precisely and safely delivered to neurons in the brains of adult animals in a non-genetically encoded manner has implications for future therapeutic use of Cas9-RNP complexes to treat neurological disease and for tissue-specific editing in general.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank L. Harrington, T. Gaj, S. Lin, R. Rouet, C. Fellmann and D. Schaffer for productive discussions and comments on the manuscript as well as L. Bai for technical support. This work was supported by a F. Hoffmann-La Roche Postdoctoral Fellowship, RPF311, award to B.T.S. and by a Roche Pharmaceutical's Roche Alliance with Distinguished Scientists (ROADS) Fund award to J.A.D. J.A.D. is an HHMI Investigator and a Paul Allen Frontiers in Science investigator. M.B. and C.C.-B. are employed by F. Hoffmann-La Roche. A.G. was employed by F. Hoffmann-La Roche during the time of this study.

AUTHOR CONTRIBUTIONS

B.T.S., A.G. and J.A.D. conceived the study and analyzed the data. B.T.S., J.K.S., C.U., S.N.F. and G.A.M. conducted *in vitro* studies. B.T.S. did Cas9 protein and sgRNA design and RNP assembly. B.T.S. and A.A.B. conducted protein production. M.B., C.C.-B. B.T.S. and J.K.S. conducted *in vivo* studies. B.T.S., M.B., A.G. and J.A.D. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

1. Cong, L. *et al. Science* **339**, 819–823 (2013).
2. Mali, P. *et al. Nat. Biotechnol.* **31**, 833–838 (2013).
3. Jinek, M. *et al. Science* **337**, 816–821 (2012).
4. Jinek, M. *et al. eLife* **2**, e00471 (2013).
5. Zuris, J.A. *et al. Nat. Biotechnol.* **33**, 73–80 (2015).
6. Yin, H. *et al. Nat. Biotechnol.* **32**, 551–553 (2014).
7. Nelson, C.E. *et al. Science* **351**, 403–407 (2016).
8. Long, C. *et al. Science* **351**, 400–403 (2016).
9. Tabebordbar, M. *et al. Science* **351**, 407–411 (2016).
10. Wu, W.-H. *et al. Mol. Ther.* **24**, 1388–1394 (2016).
11. Chew, W.L. *et al. Nat. Methods* **13**, 868–874 (2016).
12. Ran, F.A. *et al. Nat. Protoc.* **8**, 2281–2308 (2013).
13. Kim, S., Kim, D., Cho, S.W., Kim, J. & Kim, J.-S. *Genome Res.* **24**, 1012–1019 (2014).
14. Lin, S., Staahl, B.T., Alla, R.K. & Doudna, J.A. *eLife* **3**, e04766 (2014).
15. Woo, J.W. *et al. Nat. Biotechnol.* **33**, 1162–1164 (2015).
16. Wang, M. *et al. Proc. Natl. Acad. Sci. USA* **113**, 2868–2873 (2016).
17. Madisen, L. *et al. Nat. Neurosci.* **13**, 133–140 (2010).
18. Ramakrishna, S. *et al. Genome Res.* **24**, 1020–1027 (2014).
19. Dokka, S., Toledo, D., Shi, X., Castranova, V. & Rojanasakul, Y. *Pharm. Res.* **17**, 521–525 (2000).
20. Armeanu, S. *et al. Mol. Ther.* **1**, 366–375 (2000).
21. Liu, J., Gaj, T., Wallen, M.C. & Barbas, C.F. III. *Mol. Ther. Nucleic Acids* **4**, e232 (2015).
22. Pédelacq, J.-D., Cabantous, S., Tran, T., Terwilliger, T.C. & Waldo, G.S. *Nat. Biotechnol.* **24**, 79–88 (2006).
23. D'Astolfo, D.S. *et al. Cell* **161**, 674–690 (2015).
24. Lewitus, G.M., Pribiag, H., Duseja, R., St-Hilaire, M. & Stellwagen, D. *J. Neurosci.* **34**, 6146–6155 (2014).

ONLINE METHODS

Neural progenitor cell (NPCs) line creation. NPCs were isolated from cortices from embryonic day 13.5 Ai9-tdTomato homozygous mouse embryos[17]. Cells were cultured as neurospheres in NPC medium: DMEM/F12 with glutamine, Na-Pyruvate, 10 mM HEPES, non-essential amino acid, penicillin and streptomycin (100×), 2-mercaptoethanol (1,000×), B-27 without vitamin A, N2 supplement, bFGF and EGF, both 20 ng/ml as final concentration. NPCs were passaged using MACS Neural Dissociation Kit (Papain) cat# 130-092-628 following manufacturer's protocol. bFGF and EGF were refreshed every other day and passaged every 6 d. The NPC line was authenticated by immunocytochemistry marker protein staining. They were tested for mycoplasma using Hoechst stain with visual analysis and were negative.

Cas9 RNP direct delivery. Direct delivery RNP in NPC experiments was added to media and incubated with cells for 24 h, then cells were washed 2× with 200 U/ml heparin in DMEM media and allowed to grow for 24 additional hours.

HEK293T-EGFP-PEST cell line creation. The d2EGFP reporter construct was created in a modified lentivirus backbone with EF1-a promoter driving the gene of interest and a second PGK promoter driving production of a gene that confers resistance to hygromycin. The EGFP is destabilized by fusion to residues 422–461 of mouse ornithine decarboxylase, giving an *in vivo* half-life of ~2 h. Transduced 293T cells were selected with hygromycin (250 µg/ml). d2EGFP clones were isolated by sorting single cells into 96-well plates and characterized by intensity of d2EGFP. Lentivirus was produced by PEI (Polysciences Inc., 24765) transfection of 293T cells with gene delivery vector co-transfected with packaging vectors pspax2 and pMD2.G essentially as described[25]. The parental HEK293T cell line was obtained from UC Berkeley Scientific Facilities and authenticated using short tandem repeat analysis. They were tested for mycoplasma using Hoechst stain with visual analysis and were negative.

HEK 293T EGFP disruption assay. GFP disruption assays were based on those previously described[26]. HEK-293T-d2EGFP cells were used in these experiments because they are efficiently transfected with Cas9 RNP mixed with lipofectamine2000 and, therefore, useful for this experiment which analyzes the activity of our 0×NLS- and 4×NLS-Cas9-2×NLS RNPs after cell penetration. Briefly, HEK293T-d2EGFP cells were cultured in 10-cm dishes using Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5g/L glucose L-glutamine and sodium pyruvate (Corning Cellgro) plus 10% FBS, 1× MEM Non-Essential Amino Acids Solution (Gibco), and penicillin and streptomycin (Gibco). One day before transfection, ~3×10^4 cells were plated into each well of a 96-well plate with the DMEM medium plus hygromycin and allowed to settle. The next day Cas9 RNP was complexed with Lipofectamine 2000 (Life Technologies) at 0.005–50 pmol RNP+ 1 µl Lipofectamine in 20 µl OMEM media and added to the cells. Cells were analyzed for EGFP expression at 48 h after transfection using a BD LSR Fortessa high-throughput sequencer.

NaCl hypertonic protein transduction. RNPs were added to NaCl hypertonic protein transduction solution, CRISPR/Cas9 Transduction Media: Opti-MEM media (Life Technologies) supplemented with 542 mM NaCl, 333 mM GABA, 1.673 N_2, 1.673 B27 minus vitamin A, 1.673 non-essential amino acids, 3.3 mM glutamine, 167 ng/ml bFGF2, and 84 ng/ml EGF[23]. This mixture was added to NPCs that were plated 1 d before at 9×10^4 cells per well of 96-well plate. The CRISPR/Cas9 Transduction Media + Cas9 RNPs were removed after 1 h and replaced with growth media. Editing efficiency was assessed by FACS and genomic DNA PCR analysis.

Primary neuron culture. E18.5 cortical neurons were dissociated with MACS Neural Dissociation Kit (Papain) cat# 130-092-628 following manufacturer's protocol. Cells were seeded in NBActive media (Brainbits) at 1,500 cells/mm^2 on poly-ornithine-, fibronectin-, laminin-coated, imaging-quality, 96-well plates (Greiner Bio One). On day *in vitro* (DIV) 4, 1/3 media was changed, on DIV 8, 1/3 media was changed, and RNP or RNP + Lipofectamine2000 added to cells. Per well, 0.5 µl Lipofectamine 2000 + 5 µl OMEM was incubated for 10 min at room temp. To this, 10 pmol RNP + 5 µl OMEM was added and incubated for 10 min at room temp, then added to cells. The RNP or RNP + LNP was incubated for 16 h, at which point media was removed and pre-equilibrated, 50% conditioned media added to cells.

Cas9 purification. The recombinant *Streptococcus pyogenes* Cas9 used in this study carries two C-terminal SV40 nuclear localization sequences. The protein was expressed with an N-terminal hexahistidine tag and maltose binding protein in *Escherichia coli* Rosetta 2 cells (EMD Millipore, Billerica, MA) from plasmids based on pMJ915 (Addgene plasmid # 69090)[14]. N-terminal nuclear localization sequence peptide arrays and sfGFP modifications were cloned into the plasmid using Gibson DNA assembly technique (**Supplementary Table 1**). The His tag and maltose binding protein were cleaved by TEV protease, and Cas9 was purified by the protocols described previously[3]. Cas9 was stored in "Buffer 5": 20cmM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) at pH 7.5, 150 mM KCl, 10% glycerol, 1 mM tris(2-chloroethyl) phosphate (TCEP) and stored at −80 °C. For *in vivo* experiments, Cas9 was buffer exchanged into "Buffer#1" (25 mM Na phosphate pH 7.25, 300 mM NaCl, 200 mM trehalose) before size exclusion column chromatography and stored at −80 °C. Cas9 protein endotoxin levels were measured using Pierce LAL Chromogenic Endotoxin Quantification Kit Cat. # 88282.

sgRNA target site prediction. sgRNA target sequences were selected using the website http://crispr.mit.edu[27].

***In vitro* T7 transcription of sgRNA.** The DNA template encoding: T7 promoter, a 20-nt target sequence and an optimized sgRNA scaffold[28] was assembled from synthetic oligonucleotides (Integrated DNA Technologies, San Diego, CA) by overlapping PCR. Target sequences are: sgRNA-tdTom aka sgRNA298 (targets STOP cassette in tdTomato locus), 5′-AAGTAAAACCTCTACAAATG-3′, sgRNA-non-targeting aka sgRNA339 (targets Gal4 sequence that is not present in mouse genome), 5′-AACGACTAGTTAGGCGTGTA-3′, sgRNA-NT3 (targets EGFP gene) 5′-GGTGGTGCAGATGAACTTCA-3′. Briefly, for the sgRNA-tdTom template, the PCR reaction contains 20 nM premix of BS298 (5′-TAA TAC GAC TCA CTA TAG AAG TAA AAC CTC TAC AAA TGG TTT AAG AGC TAT GCT GGA AAC AGC ATA GCA AGT TTA AAT AAG G-3′) and BS6 (5′-AAA AAA AGC ACC GAC TCG GTG CCA CTT TTT CAA GTT GAT AAC GGA CTA GCC TTA TTT AAA CTT GCT ATG CTG TTT CCA GC-3′), 1 µM premix of T25_long (5′-GAA ATT AAT ACG ACT CAC TAT AG-3′) and BS7 (5′-AAA AAA AGC ACC GAC TCG GTG C-3′), 200 µM dNTP and Phusion Polymerase (NEB, Ipswich, MA) according to the manufacturer's protocol. The thermocycler setting consisted of 40 cycles of 95 °C for 10 s, 59 °C for 10 s and 72 °C for 10 s. The PCR product was extracted once with phenol:chloroform:isoamyl alcohol and then once with chloroform, before isopropanol precipitation overnight at −20 °C. The DNA pellet was washed three times with 70% ethanol, air-dried and resuspended in elution buffer.

A 400-µl T7 *in vitro* transcription reaction consisted of 50 mM Tris–HCl (pH 8), 30 mM $MgCl_2$, 0.01% Triton X-100, 2 mM spermidine, 20 mM fresh dithiothreitol, 5 mM of each ribonucleotide triphosphate, 100 µg/ml T7 Polymerase and 1 µM DNA template. The reaction was incubated at 37 °C for 4 h-to-overnight, and 5 units of RNase-free DNaseI (Promega, Madison, WI) was added to digest the DNA template 37 °C for 1 h. The reaction was quenched with 2×STOP solution (95% deionized formamide, 0.05% bromophenol blue and 20 mM EDTA) at 60 °C for 5 min. The RNA was purified by electrophoresis in 10% polyacrylamide gels containing 6 M urea. The RNA band was excised from the gel, crushed in a 15-ml tube, and eluted with five volumes of 300 mM sodium acetate (pH 5) overnight at 4 °C. The supernatant was filtered through a 0.2-µm filter to remove acrylamide fragments. 2.5 equivalents of ethanol was added and the RNA precipitated overnight at −20 °C. The RNA pellet was collected by centrifugation, washed three times with 70% ethanol, and briefly air-dried or vacuum-dried. To refold the sgRNA, the RNA pellet was re-dissolved in dPBS-Ca,-Mg. The sgRNA was heated to 70 °C for 5 min and cooled to room temperature for 5 min. $MgCl_2$ was added to a final concentration of 1 mM. The sgRNA was again heated to 50 °C for 5 min, cooled to room temperature for 5 min and kept on ice. The sgRNA concentration was determined by OD_{260nm} using Nanodrop (Thermo Fisher Scientific, Waltham, MA). The sgRNA was stored at −80 °C.

Cas9 RNP assembly. Cas9 RNP either was prepared immediately before experiments or prepared and snap-frozen in liquid nitrogen and stored at −80 °C for later use. We did not measure any loss in activity upon freeze-thawing Cas9 RNP complexes. To prepare the Cas9 RNP complexes, we incubated Cas9 protein with sgRNA at 1:1.2 molar ratio. Briefly, we added sgRNA to Buffer#1 (25 mM NaPi, 150 mM NaCl, 200 mM trehalose, 1 mM $MgCl_2$), then added the Cas9 to the sgRNA, slowly with swirling, and incubated at 37 °C for 10 min to form RNP complexes. RNP complexes were filtered before use through a 0.22-µm Costar 8160 Filter pre-wet with 200 µl Buffer#1. If needed, the RNP sample was concentrated with a 0.5 ml Millipore Ultra 100-Kd cutoff filter, part # UFC510096, until the desired volume was obtained.

Cas9 nucleofection. Neural progenitor cell neurospheres were dissociated by the MACS Neural Dissociation Kit (Papain) cat# 130-092-628, spun down by centrifugation at 80*g* for 3 min, and washed once with dPBS-Ca,-Mg. Nucleofection of NPCs with Cas9 RNP was performed using Lonza (Allendale, NJ) P3 cell kits and program EH-100 in an Amaxa 96-well Shuttle system. Each nucleofection reaction consisted of approximately 2.5×10^5 cells in 20 µl of nucleofection reagent and mixed with 10 µl of RNP. After nucleofection, 70 µl of growth media was added to the well to transfer the cells to tissue culture plates. For plasmid nucleofection, we used a modified pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (Addgene plasmid # 42230 (ref. 1)) that contained the puromycin *N*-acetyltransferase (PuroR) gene and optimized sgRNA scaffold[28]. Nucleofection was performed using 700 ng plasmid with 4×10^5 NPCs and Lonza P3 cell kit and program DS-113 in an Amaxa 96-well Shuttle system. The cells were incubated at 37 °C for 1–5 d depending on the assay. For genomic DNA analysis the media was removed by aspiration, and 100 µl of Quick Extraction solution (Epicentre, Madison, WI) was added to lyse the cells (65 °C for 20 min and then 95 °C for 20 min) and extract the genomic DNA. The cell lysate was stored at −20 °C. The concentration of genomic DNA was determined by NanoDrop. tdTomato activation in NPCs was analyzed by flow cytometry. UC Berkeley FACS Core facilities were used.

Animals. Mice were maintained on a 12-h light-dark cycle with *ad libitum* access to food and water. All animals were group-housed and experiments were conducted in strict adherence to the Swiss federal ordinance on animal protection and welfare as well as according to the rules of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and with the explicit approval of the local veterinary authorities. Animals at University of California, Berkeley were maintained on a 12-h light-dark cycle with *ad libitum* access to food and water. All animals were group-housed and experiments were conducted in strict adherence to the University of California, Berkeley's Animal Care and Use Committee (ACUC) ethical regulations. No randomization was used to allocate animals to experimental groups.

Stereotaxic infusion of Cas9 RNPs. 0×NLS-Cas9-2×NLS and 4×NLS-Cas9-2×NLS RNPs were prepared and shipped by Brett Staahl, UC Berkeley to Roche Pharmaceuticals Basel, Switzerland. 15- to 20-week-old male Ai14 tdTomato mice (which lack the NeoR cassette present in Ai9 tdTomato mice but are otherwise identical)[17] were anesthetized using injectable anesthesia (fentanyl 0.05 mg/kg + medetomidine 0.5 mg/kg + midazolam 5 mg/kg; s.c.). The anesthetized mouse was then aligned on an Angle two stereotactic frame (Leica, Germany) and craniotomies were performed with minimal damage to brain tissue. All stereotaxic coordinates are relative to bregma. Mouse stereotaxic surgery targets: striatum (+0.74 mm anterioposterior, ± 1.74 mm mediolateral, −3.37 mm dorsoventral), hippocampus (−1.94 mm anterioposterior, ± 1.12 mm mediolateral, −1.75 mm dorsoventral), cortex V1 (−3.28 mm anterioposterior, ± 2.4 mm mediolateral, −1.25 mm dorsoventral), cortex S1 (+1.94 mm anterioposterior, ± 2.37 mm mediolateral, −2.00 mm dorsoventral). Cas9 RNPs were infused (0.5 µl/side) using a Neuros 75 5 µL syringe (Hamilton). After infusion, the injector was left at the injection site for 5 min and then slowly withdrawn. After the injections, the operation field was cleaned with sterile 0.9% NaCl and closed with suture (Faden Monocryl Plus 5-0, Aichele Medico) and surgical glue (3M Vetbond Tissue Adhesive). The mouse was kept warm at 37 °C during the surgical procedure and also post-surgery. To avoid drying of the eyes during surgery, an ointment was applied outside of the eyes of the mouse. The mice were left undisturbed for 12 d before cellular analysis. Sample size was chosen based on expected effect size. No randomization was applied while allocating animals to groups.

Immunofluorescence analysis. For immunofluorescence analysis, mice were perfused with 4% paraformaldehyde and post-fixed overnight. Brains were sectioned (coronal plane sections) on a vibratome and 50-µm thick sections were used for antibody labeling. Sections were first treated with blocking solution (0.3% Triton X-100, 10% goat serum in 1× PBS) and incubated with the primary antibody (in blocking solution) overnight at 4 °C. Sections were washed with 1× PBS and incubated in the secondary antibody at room temperature for 3 h. Finally, sections were washed three times in 1× PBS, stained with the DNA binding fluorescence probe DAPI (1 µg/ml, Roche Life Science, Switzerland) and mounted on glass slides in a Prolong gold anti-fade medium (Thermo Fischer, USA).

***In vivo* tdTomato reporter.** For tdTomato reporter analysis, 12- to 14-d post-injection mice were perfused with 4% paraformaldehyde and brains were post-fixed overnight. Brains were sectioned (coronal plane sections) on a vibratome and 50-µm thick sections were DAPI counterstained and endogenous $tdTomato^+$ cells counted.

Primary antibodies (**Supplementary Table 3**) used were polyclonal rabbit anti-IBA1 (1:100, Wako, #019-19741), polyclonal rabbit anti-S100β (1:1,000; Abcam,# ab41548), monoclonal rabbit anti-DARPP32 (1:100; Cell Signaling Technologies,# 2306), monoclonal mouse anti-NeuN (1:500; Millipore, #MAB377), polyclonal chicken anti-GFAP (1:500; Abcam,# ab4674) and monoclonal rat anti-CTIP2 (1:100; clone 25B6, Abcam,# ab18465). Secondary antibodies used were donkey anti-rabbit Alexa Fluor 488 (1:500, Jackson Lab, USA, #711-545-152), and donkey anti-rat Alexa Fluor 488 (1:500, Thermo Fischer, USA, #A-21208), donkey anti-chicken Alexa Fluor 488 (1:500; Jackson Labs, Bar Harbor, ME, #703-545-155), donkey anti-mouse Alexa Fluor 488 (1:500; Jackson Labs, Bar Harbor, ME, #715-545-150).

Confocal Imaging. Confocal fluorescent images were acquired using a Leica TCS SP5 (Leica Microsystems) inverted microscope. Image analysis and maximum intensity projections of images acquired along the *z* axis were done using LAS-AF software.

Cell counting. Images for cell counting were acquired using a Leica TCS SP5 (Leica Microsystems) inverted confocal microscope with 20× dry objective. Image analysis and maximum intensity projections of images acquired along the *z* axis was done using LAS-AF software. Every sixth section from the dorsal striatum was stained with DAPI and used for cell counting. Quantification of Td-tomato and DAPI double-positive cells was done using ImageJ. The total number of edited cells per brain was quantified by multiplying the number of cells counted with the section periodicity (here it is 6). The experimenter was blinded to treatment condition while performing cell counting.

Quantification of fluorescence intensity. Images for quantification were acquired using a Leica TCS SP5 (Leica Microsystems) inverted microscope with 20× dry objective. All parameters were kept constant to allow comparative measurements between images. To quantify the fluorescence intensity corresponding to microglia (IBA-1 staining) at the injection site, we performed reconstruction of the injection site of the slice by recording at least 140 single optical layers (step size system optimized of 0.18 mm) at a 512-512 pixel resolution. The brightest sample was used to define optimal confocal settings with such settings used for the acquisition of all subsequent *z* stacks. LAS AF Lite software was used to reconstruct 3D projection of the section. Quantification of the intensity of IBA-1 staining in the striatum was done with ImageJ Software. Data are represented as mean ± s.e.m. (One way ANOVA; $P = 0.4496$ and $F_{2,7} = 0.898$). $n = 3$ animals per condition with 2–8 sections analyzed per animal. For each section, fluorescence intensity of the IBA-1-labeled channel for the entire 20× field was measured with image J. The experimenter was blinded to treatment condition while performing quantitation of fluorescence intensity.

RNA extraction from brain tissue slices and quantitative RT-PCR. Bilateral intrastriatal stereotaxic infusion of sham (buffer only) or 50 pmol

doi:10.1038/nbt.3806

4×NLS-Cas9-2×NLS RNPs with sgRNA-tdTomato in 0.5 µl were performed as described earlier. Mice were perfused with ice-cold PBS at 3 d and 12 d after injection. Brains were harvested and cut into 1-mm sections using a brain slicer matrix around the injection site. The slices were transferred to ice-cold PBS and then onto frozen glass slides. The dorsal injected striatum (1 mm thick × 1–1.25 mm wide × 2 mm long) was cut out and frozen on dry ice. On day3, tdTomato$^+$ signal was not yet present so we therefore identified the needle track to excise the RNP-treated tissue in the dorsal striatum. On day 12, tdTomato$^+$ signal accumulated in edited cells, and we used the needle track in addition to the tdTomato signal to identify RNP-treated tissue in the dorsal striatum. One milliliter of TRIzol reagent (Invitrogen) was added to 50 or 150 mg tissue for 3-day and 12-day post-treatment samples, respectively, and triturated to dissociate tissue. One microgram of total RNA was treated with DNase I to remove potential contamination of genomic DNA. One microgram of DNase I-treated RNA was reverse-transcribed using a First Strand Synthesis kit (Invitrogen). qRT-PCR analysis was performed using SYBR green master mix on a Applied Biosystems Step One Plus. The relative expression from RNA samples was determined using the $2^{-\Delta\Delta CT}$ method. Values were normalized to the expression of the *PPIA* housekeeping gene. Error bars represent s.e.m. $n = 3$ animals, $n = 2$ injections per animal for each group. To test the statistical significance of observed gene expression differences, we performed two-tailed unpaired *t*-tests with equal s.d. Primers are listed in **Supplementary Table 2**. Sample size was chosen based on expected effect size. No randomization was applied while allocating animals to groups. The experimenter was blinded to treatment condition while collecting tissue.

NexGen sequence analysis. The genomic region flanking the CRISPR target site was amplified by two-step PCR method using primers listed in **Supplementary Figure 2a**. First, the genomic DNA from the edited and control samples was isolated and PCR amplified 15 cycles using Kapa Hot start high-fidelity polymerase (Kapa Biosystems, Wilmington, MA) according to the manufacturer's protocol. The resulting amplicons were purified by AMPure beads to remove primers and subjected to five cycles of PCR to attach Illumina P5 adapters as well as unique sample-specific barcodes followed by bead purification. Berkeley Sequencing facility performed the AMPure bead cleanup. Barcoded and purified DNA samples were quantified by Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), size analyzed by BioAnalyzer, quantified by qPCR and pooled in an equimolar ratio. Sequencing libraries were sequenced with the Illumina MiSeq Personal Sequencer (Life Technologies, Carlsbad, CA). Amplicon sequencing data were analyzed with CRISPR-GA[29]. This work used the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley.

25. Tiscornia, G., Singer, O. & Verma, I.M. *Nat. Protoc.* **1**, 241–245 (2006).
26. Gilbert, L.A. *et al. Cell* **154**, 442–451 (2013).
27. Hsu, P.D. *et al. Nat. Biotechnol.* **31**, 827–832 (2013).
28. Chen, B. *et al. Cell* **155**, 1479–1491 (2013).
29. Güell, M., Yang, L. & Church, G.M. *Bioinformatics* **30**, 2968–2970 (2014).