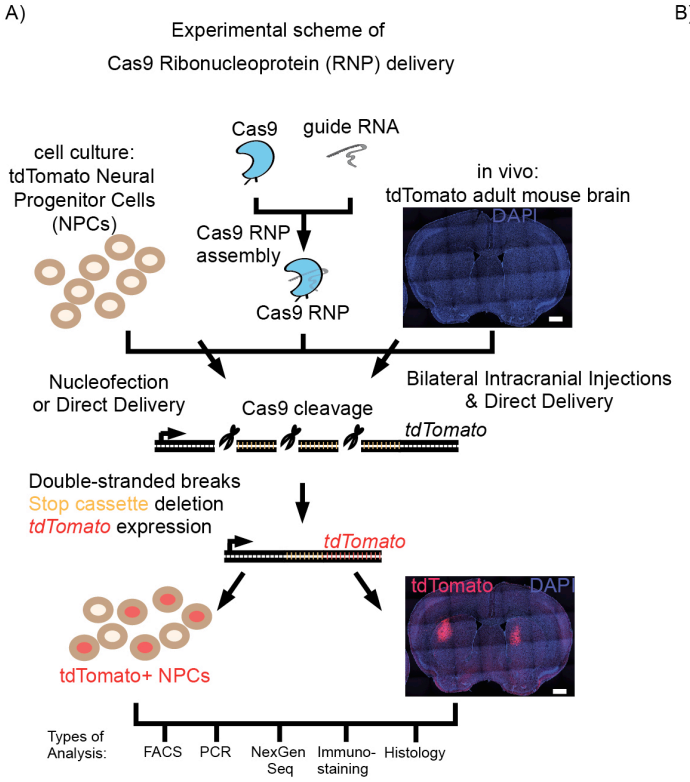
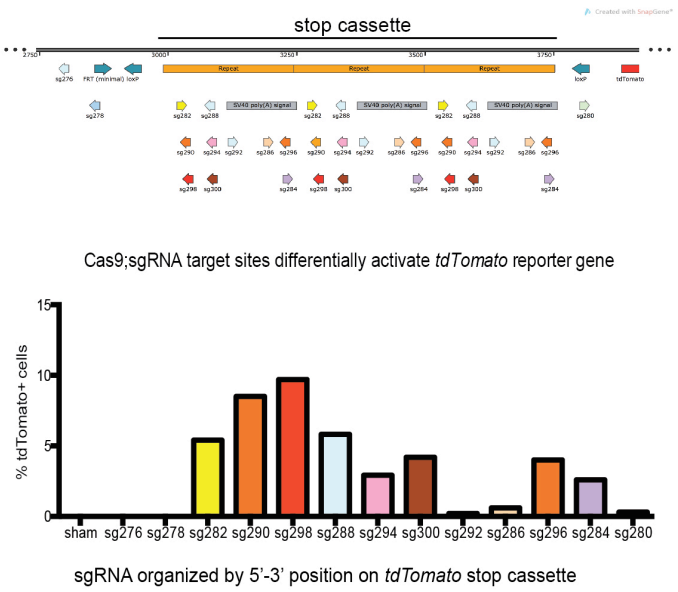
A)



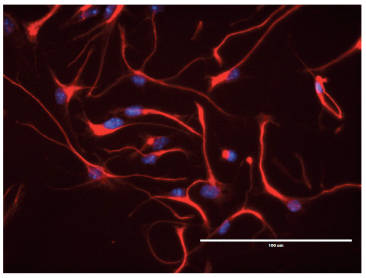
B)



C)

sgRNA#	score	guide	PAM	# target sites	%tdTomato+ cells
sham	-	-	-	-	0
sg276	85	CAATAACCAGCACGTTGCCC	AGG	1x	0
sg278	84	TAGGAACTTCTTAGGGCCCG	CGG	1x	0
sg282	73	CAGCCATACCACATTTGTAG	AGG	3x	5.4
sg290	63	AAACCTCTACAAATGTGGTA	TGG	3x	8.5
sg298	54	AAGTAAAACCTCTACAAATG	TGG	3x	9.7
sg288	65	TATGTTTCAGGTTCAGGGGG	AGG	3x	5.8
sg294	57	TTTTATGTTTCAGGTTCAGG	GGG	3x	2.9
sg300	50	ATTTTATGTTTCAGGTTCAG	GGG	3x	4.2
sg292	59	TTGTTTATTGCAGCTTATAA	TGG	3x	0.2
sg286	66	TTTTCACTGCATTCTAGTTG	TGG	3x	0.6
sg296	55	ATAAGATACATTGATGAGTT	TGG	3x	4
sg284	68	TCAATGTATCTTATCATGTC	TGG	3x	2.6
sg280	79	GTATGCTATACGAAGTTATT	AGG	1x	0.3

D)

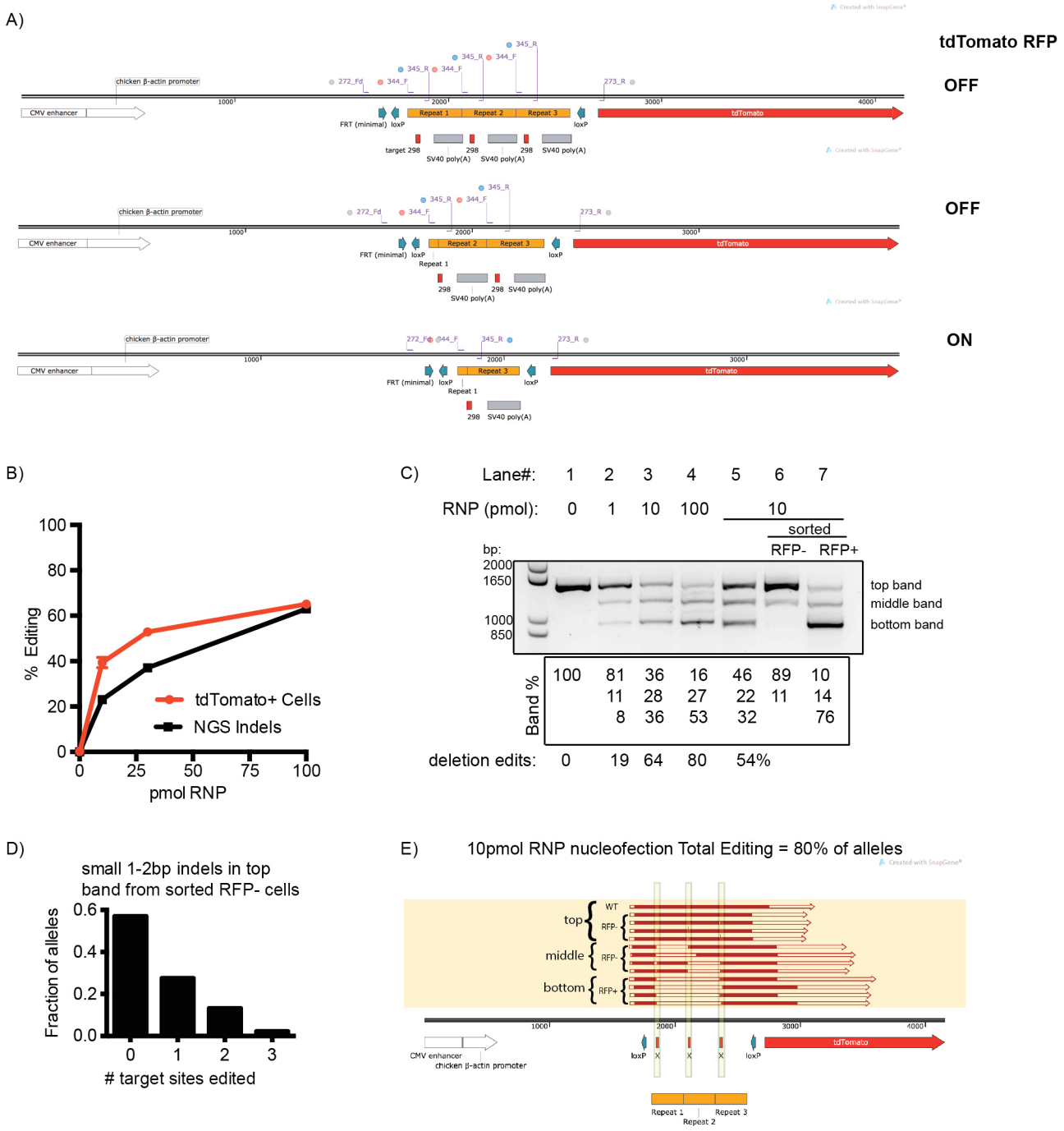


Supplementary Figure 1

Adaptation of Ai9/Ai14 *tdTomato* mouse model into a Cas9-genome editing reporter system in neural cells.

These mice harbor a modification at the *Rosa26* locus with a ubiquitously expressed CAGGS promoter and a *loxP*-flanked stop cassette (three repeats of the SV40 polyA sequence) that prevents expression of the tdTomato fluorescent protein. This system was designed for Cre-mediated recombination at the *loxP* sites leading to excision of the stop cassette and *tdTomato* expression. This mouse model provides a robust, high-throughput, quantitative

readout of site-specific genome modification at the *loxP*-flanked stop cassette locus with a gain-of-function fluorescent signal in modified cells[17]. However, one of the two *loxP* sites lacks a Protospacer Adjacent Motif (PAM) site necessary for *S. pyogenes* Cas9-mediated DNA cleavage, and therefore, two unique single-guide RNAs (sgRNAs) would be needed for Cas9 to cut as Cre does at the *loxP* sites. Therefore we set out to find a unique targetable sequence within the stop cassette that is capable of activating *tdTomato* expression. To test this we isolated E13.5 Neural Progenitor/Stem cells (NPCs) from homozygous tdTomato mice. We nucleofected these cultures with plasmids encoding *S. pyogenes* Cas9 and various sgRNA's that targeted the stop cassette. From this experiment we determined a stop cassette-targeting sgRNA, sgRNA298 hereafter referred to as sgRNAtdTom, which most efficiently activated *tdTomato* expression. A) Experimental scheme of Cas9 RNP delivery *in vitro* or *in vivo* for genome editing of *tdTomato* locus and genetic or phenotypic characterization. Coronal brain sections with native tdTomato signal in edited cells. Scale bar 1mm. B) Location of sgRNA's on *tdTomato* stop cassette. C) Cas9;sgRNA target sites differentially activate *tdTomato*. sgRNAs organized on histogram by 5'-3' position on *tdTomato* stop cassette. D) Table of sgRNA sequences with Off target hit scores[26]. E) Neural Progenitor Cell cultures are Nestin^{+}, a neural progenitor/stem cell marker protein. Scale bar = 100um.

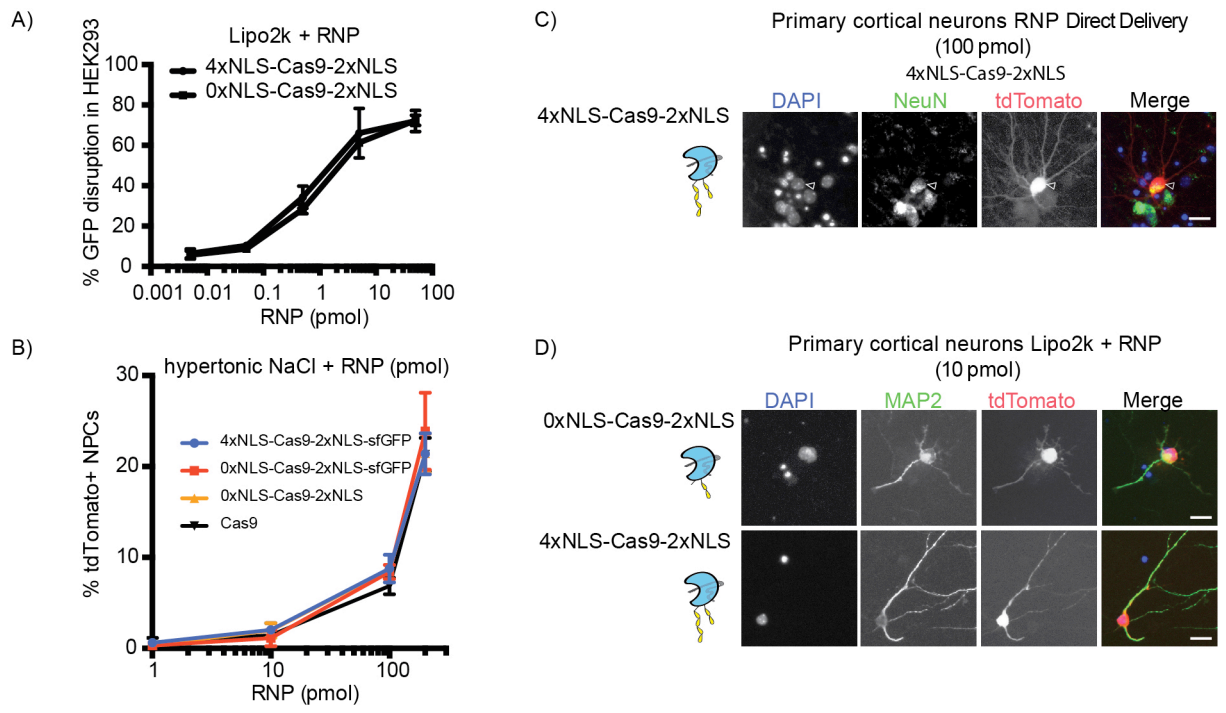


Supplementary Figure 2

Cas9 RNP-mediated editing of Ai9 mouse *tdTomato* stop cassette.

A) Cartoon illustrating *tdTomato* stop cassette locus before and after Cas9 editing. sgRNA-tdTom (aka target298) has three target sites in the stop

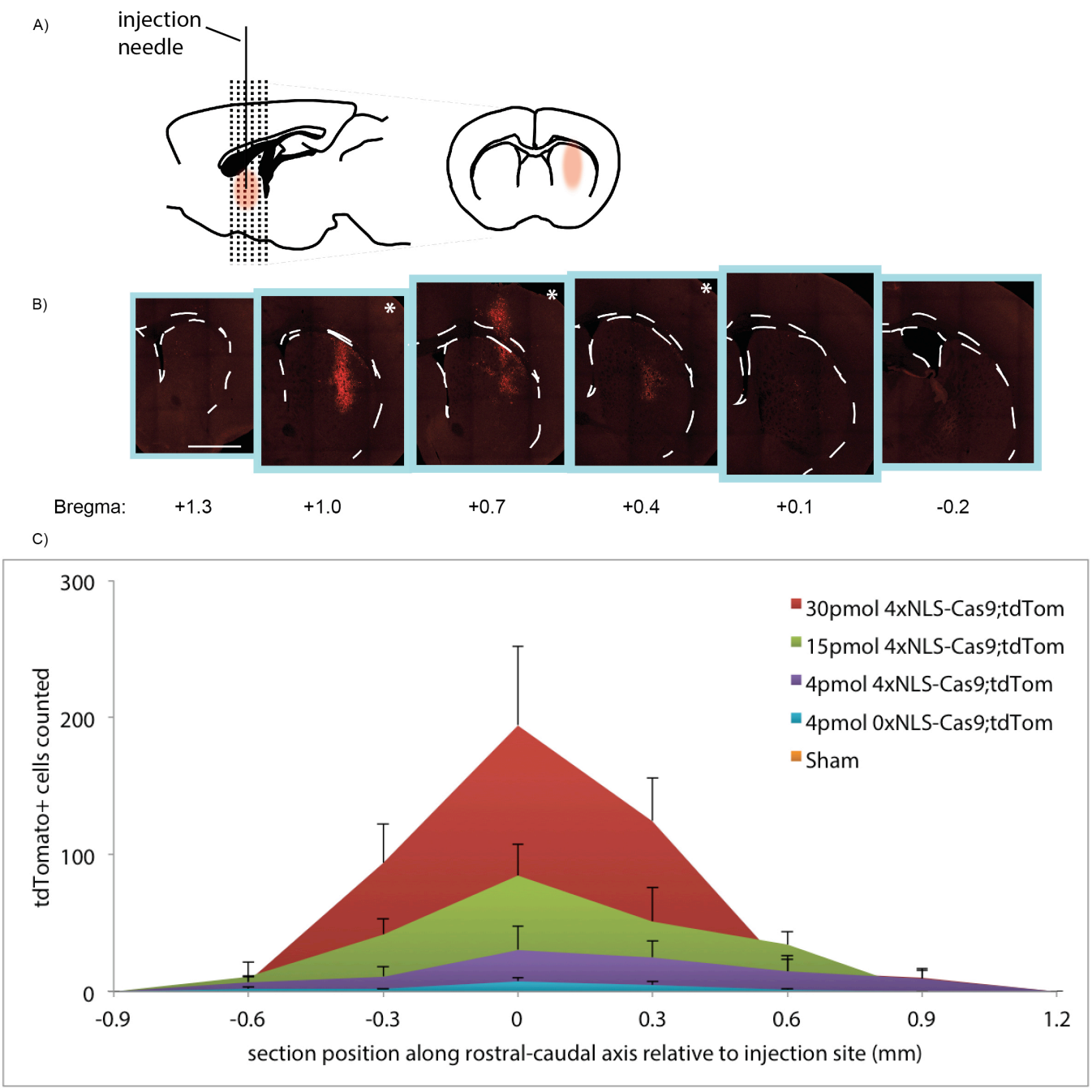
cassette (red boxes) and therefore can generate six types of edits, including three different small 1-2bp indels that do not generate a deletion, two types of single-repeat deletions and one double-repeat deletion. The tdTomato NPCs are homozygous with two *tdTomato* reporter genes per cell. The double repeat deletion activated *tdTomato* expression. PCR primers used for genomicDNA PCR, 272F/273R. B) Quantification of activation of native tdTomato protein by flow cytometry and editing efficiency by NexGen Sequencing[28] in NPCs, primers 344F/345R. n=3 independent experiment replicates for flow cytometry analysis. C) PCR analysis of edited stop cassette, 3 days post RNP nucleofection reveals the expected triple DNA laddering pattern in edited cells; Top band = unedited or small 1-2bp indels, middle band = single repeat deletion, bottom band = double repeat deletion pattern. These three observed bands correspond to the 6 expected products due to the shared sizes of some of the products. To determine which type of edits activate *tdTomato* expression we used fluorescence-activated cell sorting (FACS) to separate fluorescent tdTomato$^+$ (RFP$^+$) and non-fluorescent (RFP$^-$) cells within samples of 10pmol RNP nucleofected NPCs in which 39% +/-2% of the cells are tdTomato$^+$ (Supplementary Fig. 2B). PCR analysis of FACS sorted RFP$^-$ and RFP$^+$ cells, indicates that only double repeat deletion/bottom band in stop cassette activates *tdTomato* RFP, whereas the single repeat deletion occurred in both RFP$^+$ and RFP$^-$ cell populations. Therefore, activation of *tdTomato* required double deletion that removed two of three SV40 polyA repeat sequences, in at least one of two reporter alleles, while single repeat deletions did not activate *tdTomato*. D) Sanger sequencing of 100 clones of the top DNA band from RFP$^-$ FACS sorted cells revealed that 45% of these alleles contained small 1-2 bp indels at 1, 2 or 3 of the sgRNA298 target sites that did not activate *tdTomato* expression. E) Representative WT and edited alleles amplified from genomicDNA with primers 272F/273R. DNA bands were gel purified, cloned, Sanger sequenced and aligned to tdTomato STOP cassette. Based on these data, we estimate that 80% of alleles ([0.45 x 36%] + 28% + 36% = 80.2%) in the 10pmol RNP nucleofected samples were edited, with ~34+/-2% of the alleles acquiring the multiple edits necessary to activate *tdTomato* expression. This correlates well with 39% +/-2% tdTomato$^+$ cells in 10pmol RNP nucleofected samples (Supplementary Fig. 2B). The correlation of *tdTomato* RFP reporter activation and genomicDNA quantitative PCR-based analysis of the bottom DNA band, validates the quantitative PCR analysis.



Supplementary Figure 3

Cas9 RNP variants are equally active in mitotic and post-mitotic cells when assisted across the cell membrane but only 4xNLS-Cas9-2xNLS is active in post-mitotic cortical neurons under direct delivery conditions.

A) Cas9 RNP complexes targeting d2EGFP are mixed with Lipofectamine2000 and delivered to HEK293T-d2EGFP cells. Percent EGFP gene disruption is measured by FACS analysis. RNPs mixed with Lipofectamine2000 resulted in robust GFP disruption and 0xNLS-Cas9-2xNLS was not significantly different from 4xNLS-Cas9-2xNLS at all RNP doses. Data are represented as mean ± SD (4xNLS-Cas9;sgRNA-NT3 vs. 0xNLS-Cas9;sgRNA-NT3; Two-tailed Unpaired t test with equal SD; $p = 0.9032$ and $F_{5,5} = 1.034$). n=3 experimental replicates with 3 technical replicates each. B) NaCl hypertonic protein transduction protocol to deliver RNPs into tdTomato NPCs [23]. Editing activity for 4xNLS-Cas9-2xNLS-sfGFP, 0xNLS-Cas9-2xNLS-sfGFP, 0xNLS-Cas9-2xNLS and Cas9 with no NLS was not significantly different at all RNP doses. (2way ANOVA with Tukey's multiple comparisons test; $p=0.8685$ and $F_{9,19}=0.4825$). n=3 experimental replicates with 2 technical replicates each. C) We assessed the ability of 4xNLS-Cas9-2xNLS and 0xNLS-Cas9-2xNLS to edit primary cortical neurons (which are post-mitotic and no longer breakdown the nuclear envelope during cell division) in cell culture by direct delivery of RNP complexes or by LNP carrier assisted delivery, RNP+Lipofectamine2000. The efficiency of neuron editing was low in all cases, <0.05% of all seeded cells were tdTomato$^+$ / MAP2$^+$ or NeuN$^+$ (marker proteins of post-mitotic mature neurons). Direct delivery of 4xNLS-Cas9-2xNLS results in edited post-mitotic neurons (NeuN$^+$/tdTomato$^+$) while 0x-NLS-Cas9-2xNLS does not. n=3 experimental replicates with 2 technical replicates each. D) In contrast, when RNPs were mixed with Lipofectamine2000 both 4xNLS-Cas9-2xNLS and 0xNLS-Cas9-2xNLS RNPs triggered genome editing in mature neurons (MAP2$^+$, tdTomato$^+$). Therefore, both 4xNLS-Cas9-2xNLS and 0xNLS-Cas9-2xNLS RNPs are capable of editing post-mitotic neurons if assisted across the cell membrane by Lipofectamine2000, but only 4xNLS-Cas9-2xNLS is capable of penetrating and triggering genome editing in neurons when directly added to the media.
n=3 experimental replicates with 2 technical replicates each. Scale bar 10μm.

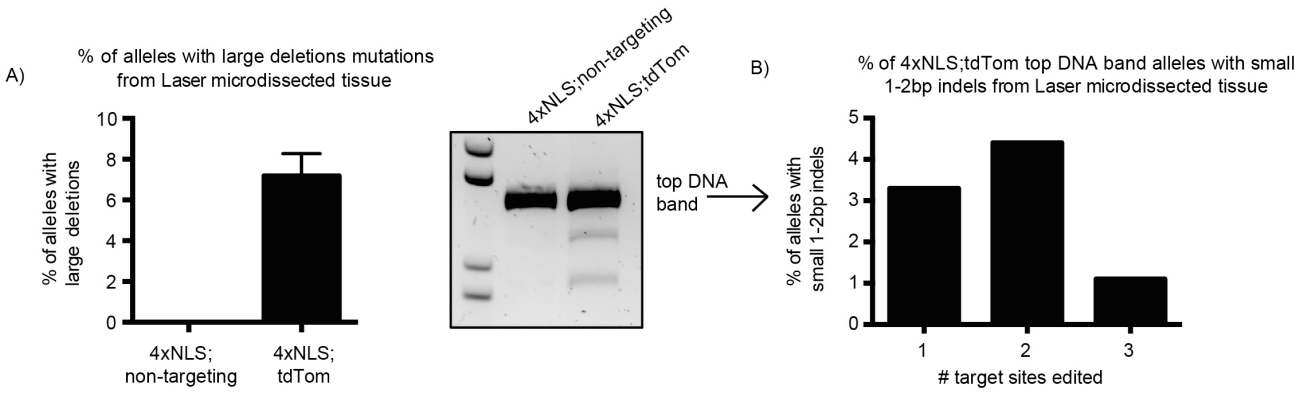


Supplementary Figure 4

Bilateral intrastriatal injection measurements of tdTomato$^+$ cell volume and density indicates RNP dose dependent increase in edited tissue volume.

A) Red oval indicates region of tdTomato$^+$ cells on sagittal and coronal cartoons of the mouse brain. Injection needle with 200µm outer diameter is drawn for reference. Dashed lines on sagittal section represent approximate positions of 50µm coronal sections along the rostral-caudal axis. B)

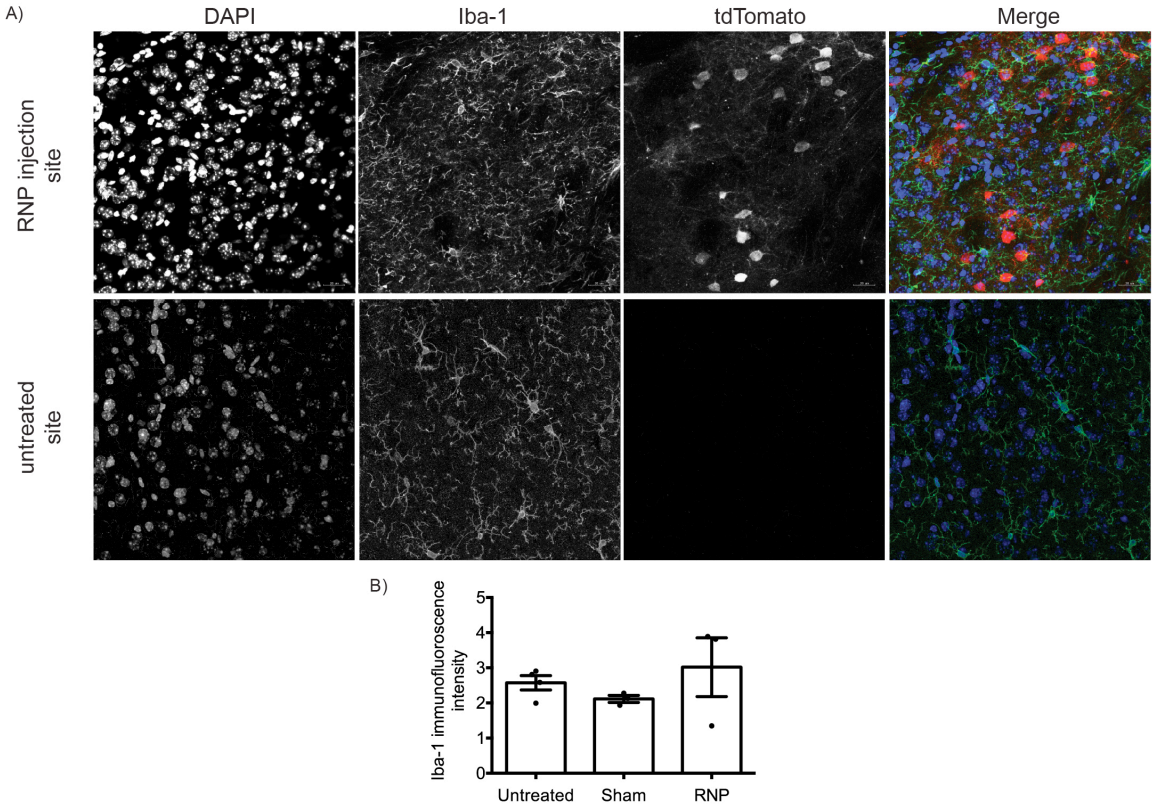
Representative images of serial coronal sections from a 30pmol 4xNLS-Cas9-2xNLS injected striatum are presented here with approximate Bregma coordinates. With serial sectioning at the periodicity of 1 in 6, each coronal section represented here samples 300μm of tissue making the volume of edited cells 1.5mm^3. Asterisks (*) indicate position of tissue sections used for Laser microdissection of $tdTomato^+$ dorsal striatal tissue. Scale bar = 1.4mm. C) Area representation with # of $tdTomato^+$ cells on Y-axis and rostral-caudal position of coronal sections analyzed on X-axis. X-axis units are millimeters (mm). 4pmol 0xNLS-Cas9-2xNLS RNP edits fewer cells and extends for ~1mm along rostral-caudal axis. Density and volume of $tdTomato^+$ cells along rostral-caudal axis increases with increasing dose of 4xNLS-Cas9-2xNLS. n=3 animals, n=2 bilateral injections per animal for each group. Data are represented as mean ± SEM.



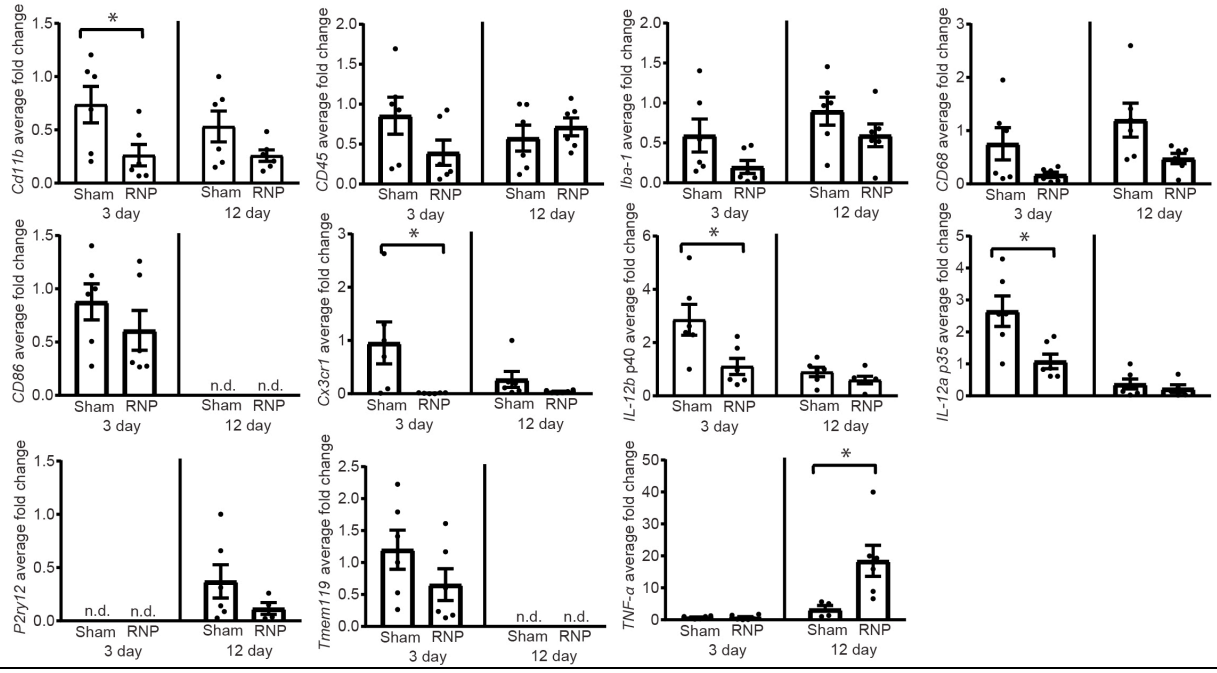
Supplementary Figure 5

Quantification of *in vivo* editing efficiency by genomicDNA PCR analysis of *tdTomato* stop locus.

A) Laser microdissection of tissue from 30pmol RNP treated dorsal striatum was used for this analysis. Rectangles of tissue containing tdTomato$^+$ cells, ~1mm x ~1.5mm, were microdissected from three 50µm thick sections spanning ~1mm along rostral-caudal axis (marked with asterisk in Supplementary Fig. 4B) and combined. PCR analysis of genomic DNA isolated from this tissue confirmed the expected deletions in 7.18% +/-2.18% of the alleles of which 3.7%+/-0.18% of alleles have the double deletion edit that activates *tdTomato* expression. n=3 animals, n=2 bilateral injections per animal for each group. B) Sanger sequencing of the top band PCR product (corresponding in size to wild-type and/or small 1-2bp indel editing events) revealed an additional 8.8% of alleles with small 1-2bp indels at 1,2, or 3 target sites, as was observed for tdTomato mouse NPCs edited *in vitro*. Based on this analysis we estimate tdTomato$^+$ cells is reporting ~23% of the total editing in the dorsal striatum. (*tdTomato*$^+$ "double deletion" alleles / total edited alleles i.e. 3.7% / (7.18% (deletion edits) + 8.8% (small indels)=16%) = 23%. Therefore we can extrapolate that 30pmol 4xNLS-Cas9-2xNLS RNP edits ~4x as many alleles as tdTomato$^+$ cells report, i.e. 2675 +/-613 tdTomato$^+$ cells / 0.23 = 11,630 cells in ~1.5mm^3 volume of tissue.



C) 3 and 12 day post injection qPCR transcript analysis



Supplementary Figure 6

Analysis of innate immune response in sham treated, RNP treated and untreated brains.

A) Morphological appearance of microglia in the mouse striatum in Cas9 RNP complex RNP treated and untreated mice visualized in green by immunostaining with anti-Iba-1 antibody. Iba-1 (ionized calcium-binding adapter molecule 1, also known as Allograft inflammatory factor 1 (AIF-1)) is useful as an indicator of activated microglia because 1) its levels increase and 2) the cytoplasmic staining pattern can be used to assess microglia morphological changes that occur upon activation, i.e. microglia enlarge their cell bodies and thicken their processes, which closely enwrap neuronal cell bodies [29]. In Cas9 RNP treated mice, microglia have small cell bodies and long and slender processes indicating they are not activated. B) Quantification of Iba-1 protein intensity in untreated, sham (buffer only) and 4x-NLS-Cas9-2xNLS treated mice. Data are represented as mean ± SEM with scatter plots of mean intensity of 2-8 sections per animal (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 20X field was measured with image J. The experimenter was blinded to treatment condition while performing quantitation of fluorescence intensity.
C) A panel of microglia markers of activation were analyzed at two timepoints, 3 and 12 days post injection by qPCR transcript analysis. The panel contains: CD11b, CD45, CD68, CD86, Cx3cr1, IBA-1, IL-12b p40, IL-12a p35, Tmem119 and TNF-α. Dorsal striatum of sham injected (buffer only) and Cas9 RNP (50pmol/0.5ul) injected mice were collected at 3 days post injection (n=3 animals, n=2 bilateral injections per animal) and 12 days post injection (n=3 animals, n=2 bilateral injections per animal). Transcripts that are significantly different in sham vs. RNP injected at 3 days post injection are Cd11b, Cx3cr1, IL-12b p40 and IL-12a p35. All are depleted in RNP-treated samples relative to sham-treated. At 12-day post injection, TNF-α is the only transcript significantly different and increased 5.65-fold in RNP-treated relative to sham-treated samples. Data are presented as fold change relative to sham for respective timepoint. Data are represented as mean ± SEM with scatter plots of individual samples. Data for 3 days post injection. Two-tailed unpaired t test with equal SD was used to assess significance for the following genes: *Cd11b* $p=0.0378$ and $F_{5,5}$ 2.903, *CD45* $p=0.1298$ and $F_{5,5}$ 2.133, *CD68* $p=0.0718$ and $F_{5,5}$ 32.36, *CD86* $p=0.3150$ and $F_{5,5}$ 1.22, *Cx3cr1* $p=0.0371$ and $F_{5,5}$ 12239, *IBA-1* $p=0.1100$ and $F_{5,5}$ 6.714, *IL-12b p40* $p=0.0322$ and $F_{5,5}$ 3.71, *IL-12a p35* $p=0.0138$ and $F_{5,5}$ 4.44, *Tmem119* $p=0.1946$ and $F_{5,5}$ 1.518, *TNF-α* $p=0.9248$ and $F_{5,5}$ 2.776. Data for 12 days post injection. Two-tailed unpaired t test with equal SD was used to assess significance for the following genes: Cd11b $p=0.1072$ and $F_{5,5}$ 7.168, CD45 $p=0.4906$ and $F_{5,5}$ 2.177, CD68 $p=0.0562$ and $F_{5,5}$ 10.72, Cx3cr1 $p=0.1548$ and $F_{5,5}$ 217, IBA-1 $p=0.3129$ and $F_{5,5}$ 3.355, IL-12b p40 $p=0.0625$ and $F_{5,5}$ 4.272, IL-12a p35 $p=0.0657$ and $F_{5,5}$ 14.54, P2ry12 $p=0.0760$ and $F_{5,5}$ 67.41, *TNF-α* $p=0.0384$ and $F_{5,5}$ 23.81.

Supplementary Table 1 **Staahl et al. 2016**

TEVscarSV40NLSGGSlinkerATG of Cas9

N-terminal NLS DNA sequences

1xNLS:
TCCAATGCCACCCCAAAGAAGAAACGGAAAGTAGGCATCCACGGAGTCCCAGCAGCTACCATG

2xNLS:
TCCAATGCCACCCCAAAGAAGAAACGGAAAGTAGGCGGCTCCCCCAAGAAGAAGCGGAAGGTAGGTATCCACGGAGT
CCCAGCAGCTACCATG

4xNLS:
TCCAATGCCACCCCCAAGAAGAAGCGAAAGGTGGGTGGGTCCCCAAAGAAGAAACGGAAAGTAGGCGGCTCCCCCAA
AAAGAAGCGAAAAGTAGGGGGTAGCCCCAAGAAGAAGCGGAAGGTAGGTATCCACGGAGTCCCAGCAGCTACCATG

7xNLS:
TCCAATGCCACCCCCAAGAAGAAGCGAAAGGTGGGTGGGTCCCCAAAGAAGAAACGGAAAGTAGGCGGCTCCCCCAA
AAAGAAGCGAAAAGTAGGGGGTAGCCCCAAGAAGAAGCGGAAGGTAGGTGGGTCCCCAAAGAAGAAACGGAAAGTA
GGCGGCTCCCCCAAAAAGAAGCGAAAAGTAGGGGGTAGCCCCAAGAAGAAGCGGAAGGTAGGTATCCACGGAGTCCC
AGCAGCTACCATG

Supplemental Table 1 Staahl et al. 2016

qPCR primers used in analysis of microglia activation and gene description.

A)

Primer Name	Sequence	Reference
Cd11b Fd	AAGTGGAGCCATATGAAGTTCACA	Jonas *et al*., J Clin Invest 2014
Cd11b Rv	GCACCAGGCCCCCAAT	Jonas *et al*., J Clin Invest 2014
TNF-α Fd	CACAAGATGCTGGGACAGTGA	Jonas *et al*., J Clin Invest 2014
TNF-α Rv	TCCTTGATGGTGGTGCATGA	Jonas *et al*., J Clin Invest 2014
CD68 Fd	GGACTACATGGCGGTGGAATA	Binder *et al*., J Neurosci 2008
CD68 Rv	GATGAATTCTGCGCCATGAA	Binder *et al*., J Neurosci 2008
CD45 Fd	TCATGGTCACACGATGTGAAGA	Barnum *et al*., J Neuroinflammation 2012
CD45 Rv	AGCCCGAGTGCCTTCCT	Barnum *et al*., J Neuroinflammation 2012
CD86 Fd	TCTCCACGGAAACAGCATCT	Ye *et al*., J Endocrinology 2011
CD86 Rv	CTTACGGAAGCACCCATGAT	Ye *et al*., J Endocrinology 2011
IL-12a p35 Fd	AGACGGCCAGAGAAAAACTGAA	Im *et al*., Cell Metabolism 2011
IL-12a p35 Rv	GTTTGGTCCCGTGTGATGTCTT	Im *et al*., Cell Metabolism 2011
IL-12b p40 Fd	TGTGGGAGAAGCAGACCCTTA	Im *et al*., Cell Metabolism 2011
IL-12b p40 Rv	GGGTGCTGAAGGCGTGAA	Im *et al*., Cell Metabolism 2011
P2ry12 Fd	CAAGGGGTGGCATCTACCTG	Bennett *et al*., PNAS 2016
P2ry12 Rv	AGCCTTGAGTGTTTCTGTAGGG	Bennett *et al*., PNAS 2016
Tmem119 Fd	GTGTCTAACAGGCCCCAGAA	Bennett *et al*., PNAS 2016
Tmem119 Rv	AGCCACGTGGTATCAAGGAG	Bennett *et al*., PNAS 2016
Cx3cr1 Fd	CAGCATCGACCGGTACCTT	Bennett *et al*., PNAS 2016
Cx3cr1 Rv	GCTGCACTGTCCGGTTGTT	Bennett *et al*., PNAS 2016
Aif1 (IBA-1) Fd	TGAGGAGCCATGAGCCAAAG	Sant'Anna *et al*., Sci Reports 2016
Aif1 (IBA-1) Rv	GCTTCAAGTTTGGACGGCAG	Sant'Anna *et al*., Sci Reports 2016
PPIA Fd	AGCTCTGAGCACTGGAGAGA	Staahl *et al*., J Neurosci 2013
PPIA Rv	GCCAGGACCTGTATGCTTTA	Staahl *et al*., J Neurosci 2013

B)

Gene Name	Function
Cd11b	Complement receptor 3
TNF-α	Proinflammatory cytokine
CD68	lysosomal protein highly expressed in activated phagocytes
CD45	common leukocyte antigen
CD86	membrane protein expressed by antigen-presenting cells, ligand for two proteins at the cell surface of T cells, CD28 antigen and cytotoxic T-lymphocyte-associated protein 4
IL-12a p35	proinflammatory cytokine
IL-12b p40	proinflammatory cytokine
P2ry12	purinergic receptor P2Y, G-protein coupled 12, microglia marker
Tmem119	transmembrane protein 119, specific microglia marker protein
Cx3cr1	chemokine (C-X3-C) receptor 1, microglia marker protein
IBA-1 (Aif1)	IBA-1 (ionized calcium-binding adapter molecule 1, Allograft inflammatory factor 1 (AIF-1), microglia marker protein
PPIA	Peptidylprolyl Isomerase A, housekeeping gene used for cDNA normalization