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Title: CRISPR/Cas9-mediated *in vivo* gene editing reveals that neuronal 5-HT_{1A} receptors in the dorsal raphe nucleus contribute to body temperature regulation in mice

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Authors:

Naoya Nishitani¹, Yu Ohmura^{1*}, Kazuki Nagayasu², Norihiro Shibui², Shuji Kaneko², Akiko Ohashi³, Takayuki Yoshida¹, Akihiro Yamanaka⁴, Mitsuhiro Yoshioka¹

Author Addresses: ¹ Department of Neuropharmacology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, N15 W7 Kita-ku, Sapporo 060-8638, Japan

² Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan

³ Department of Anatomy, Nihon University School of Dentistry, Tokyo 101-8310, Japan

⁴ Department of Neuroscience II, Research Institute of Environmental Medicine (RIEM), Nagoya University, Nagoya 464-8601, Japan

***Corresponding author:**

Yu Ohmura.

E-mail: gwd0701@yahoo.co.jp; yohmura@med.hokudai.ac.jp

TEL: +81-11-706-5059; FAX: +81-11-706-7872.

ABSTRACT (250/250 words)

Serotonin (5-HT) in the central nervous system regulates a variety of biological functions, from the basic homeostatic control to higher brain functions, by acting on fourteen known receptor subtypes. However, it is still usually unclear which receptor subtype is responsible for a specific function due to the lack of highly selective ligands for most of these receptors. Although 5-HT receptor knockout mice are useful, the brain-wide distribution of various receptors makes it difficult to dissect receptor functions in specific brain regions and cell types. Recent advances in CRISPR/Cas9-mediated *in vivo* genome editing technology may overcome this problem. In this study, we constructed a viral vector expressing a single guide (sg)RNA targeting *Htr1a* (sgHtr1a) and Cre recombinase under the control of a neuron-specific promoter. Injection of the viral vector into the dorsal raphe nucleus (DRN) of Cre-dependent Cas9 knock-in mice induced Cre-dependent Cas9 expression mainly in DRN serotonin and GABA neurons. Mismatch cleavage assay and Sanger sequencing showed insertion or deletion formation at the target site. 5-HT_{1A} receptor agonist-induced hypothermia was attenuated and antidepressant effect of a selective serotonin reuptake inhibitor (SSRI) was enhanced by microinjection of the viral vector expressing sgHtr1a into the DRN of Cre-dependent Cas9 knock-in mice. These results suggest that this *in vivo* CRISPR/Cas9-mediated 5-HT receptor gene knockout strategy provides a reliable and low-cost method for elucidating 5-HT receptor functions in specific cell types and brain regions. Further, we demonstrate that the neuronal 5-HT_{1A} receptor in the DRN regulates body temperature and antidepressant effect of SSRI.

KEYWORDS: genome editing; serotonergic; rectal temperature

1. Introduction

The functions of central serotonergic systems remain elusive partly because of the diversity of serotonin receptors. Indeed, 14 subtypes of the serotonin (5-HT) receptor have been identified so far (Hannon and Hoyer, 2008; Hoyer et al., 1994), and in most cases, pharmacological methods have failed to manipulate each receptor exclusively partly because of amino acid sequence similarity among 5-HT receptor subtypes (Hannon and Hoyer, 2008; Hoyer et al., 1994). Gene knockout mice can overcome shortcomings of pharmacological techniques, but it is impractical to obtain and breed knockout mice for all subtypes in most laboratories. Moreover, it is still difficult to specify the brain regions and types of cells involved in the observed functional changes. Thus, we need an easier and more cost-effective strategy to knockout 5-HT receptor genes in specific brain regions and cell types.

Recent developments in CRISPR/Cas9-mediated gene editing techniques (Jinek et al., 2012) may provide a solution. A recent study demonstrated that a specific gene in a selected brain region can be knocked out by injecting viral vector expressing single guide (sg)RNA and Cre recombinase to that target specific brain regions of Cre-dependent Cas9 knock-in mice (Platt et al., 2014). Therefore, we speculated if it is feasible to knockout 5-HT receptor genes by injecting a viral vector expressing a targeted sgRNA into Cre-dependent Cas9 knock-in mice. This approach would provide easier and lower-cost knockout of 5-HT receptor genes in specific cell types and brain regions because only one type of transgenic mice (Cre-dependent Cas9 knock-in) is required to knockout any one of the 14 5-HT receptor subtypes.

In the present study, we chose the gene encoding the 5-HT_{1A} receptor and targeted the dorsal raphe nucleus (DRN), an origin of serotonergic projections to the forebrain, because 5-HT_{1A} receptors are densely expressed in the DRN and their function, body temperature regulation and

1 inhibition of serotonin neuron firing, are well established (Craven et al., 1994; Gartside et al., 1995;
2 Heisler et al., 1998; Hillegaart, 1991; Richardson-Jones et al., 2010). Moreover, the 5-HT_{1A}
3 receptor is the most thoroughly studied of 5-HT receptors, so it is a particularly suitable target to
4 test the utility of this gene knockout strategy.

5 First, we tested whether the 5-HT_{1A} receptor gene could be edited by designed guide RNAs *in*
6 *vitro*. Second, we injected viral vectors expressing sgRNA and Cre recombinase into
7 Cre-dependent Cas9 knock-in mice, and examined whether Cas9 was expressed in virus-infected
8 cells by immunohistochemistry, and whether the 5-HT_{1A} receptor gene was edited by PCR
9 amplification of genomic DNA. A resolvase detecting the mismatched heteroduplex DNA and a
10 conventional sequencer were used to confirm the gene editing. Third, we determined the types of
11 neurons infected by our viral vectors because some studies have demonstrated that some
12 selectively infect specific cell types (Nathanson et al., 2009). Moreover, 5-HT_{1A} receptors are
13 expressed not only in serotonin neurons but also in GABA neurons of the DRN (Bonnavion et al.,
14 2010). Finally, we tested whether 5-HT_{1A} receptor agonist-induced hypothermia was reduced and
15 antidepressant effect of selective serotonin reuptake inhibitor (SSRI) was enhanced in
16 Cre-dependent Cas9 knock-in mice microinjected with sgRNA expression viral vectors into the
17 DRN. In addition, we examined whether 5-HT_{1A} receptor agonist or 5-HT_{1A} receptor knockout in
18 the DRN could affect 5-HT metabolism.

20 **2. RESULTS**

21 *2.1. In vitro screening of gRNAs for editing efficacy at the Htr1a gene locus*

22 First we assessed the efficacy of designed gRNAs for gene editing of the mouse Htr1a (mHtr1a)
23 gene locus *in vitro* using an EGxxFP assay (Mashiko et al., 2013). In this assay, the efficacy of
24 gRNAs is assessed by the strength of the reconstituted enhanced green fluorescent protein (EGFP)

fluorescence without Cel-I nuclease digestion of the PCR-amplified target region (Mashiko et al., 2013). The Htr1a gene locus was cloned into the pCAG-EGxxFP vector, and the resultant construct (pCAG-mHtr1a-EGxxFP) was co-transfected with pX330 expressing SpCas9 and AAV:ITR-U6-sgRNA-hSyn-Cre expressing gRNAs into HEK293T cells. Forty-eight hours after co-transfection, EGFP-positive cells were abundantly observed (Fig. 1A-C), whereas few EGFP-positive cells were observed when cells were transfected with gRNA only or pCAG-mHtr1a-EGxxFP only (Fig. 1D-G). Although this *in vitro* screening showed that all three gRNAs had comparable efficacy, we selected gRNA#1 because it was most likely to influence protein expression and function because it targets the most upstream region of the mHtr1a gene locus.

2.2. *In vivo* expression of Cas9 and gene editing of the 5-HT_{1A} receptor

The viral vectors used in the present study conferred expression of green fluorescent protein (GFP) and Cre recombinase in the infected cells, and transgenic mice used in the present study expressed FLAG-tagged Cas9 in a Cre-dependent manner. Thus, we first verified that all GFP-positive cells expressed FLAG and *vice versa* in the virus-injected region (DRN) of Cre-dependent Cas9 knock-in mice (Fig. 2A). In addition, the viral infection was localized predominantly in the DRN (Supplementary Fig. 1).

A resolvase detecting the mismatched heteroduplex DNA generated cleavage products only in the virus-injected region (DRN) of Cre-dependent Cas9 knock-in mice (Fig. 2B). The sizes of the cleavage products were close to those expected from the targeted cleavage site (370 bp and 270 bp). The frequency of insertion and deletion mutation (Indel) formation was estimated to be 8.65% ± 1.67%. No cleavage products were observed when we used the amplicons of the top 4 candidate sequences for off-target cleavage (Fig. 2C).

Furthermore, the results of DNA sequencing showed multiple chromatogram peaks in

virus-injected Cre-dependent Cas9 knock-in mice than in virus-injected wild type (WT) littermates. The multiple chromatogram peaks were observed only around the targeted cleavage site (Fig 2D; Supplementary Fig. 2). Recently, the Tracking of Indels by Decomposition (TIDE) assay was reported to detect indel formation at a frequency similar to next generation sequencing (Sentmanat et al., 2018). TIDE analysis revealed that overall efficiency was $17.94\% \pm 2.58\%$ and that the introduced indels led mainly to a frameshift mutation (Fig 2E).

2.3. Characteristics of neurons infected by viral vector

Histological analysis revealed that $10.6\% \pm 1.4\%$ of GFP-positive cells were immunoreactive for tryptophan hydroxylase 2 (TPH2), a specific marker for serotonin neurons, and $83.5\% \pm 5.5\%$ of GFP-positive cells were immunoreactive for GAD67, a marker for GABA neurons, in virus-injected Cre-dependent Cas9 knock-in mice (Fig. 3). Similarly, in virus-injected WT littermates, $14.0\% \pm 6.0\%$ and $80.6\% \pm 0.5\%$ of GFP-positive cells were immunoreactive for TPH2 and GAD67, respectively (Fig.3). In addition, $15.97\% \pm 2.08\%$ of TPH2-positive cells were GFP-positive in Cre-dependent Cas9 knock-in mice and $20.63\% \pm 7.29\%$ of TPH2-positive cells were GFP-positive in WT littermates. There were no significant differences in these immunopositivity ratios between Cre-dependent Cas9 knock-in and WT mice (Table 1; TPH2-positive cells in GFP-positive cells: $\chi^2 = 2.03$, $p = 0.153$, GAD67-positive cells in GFP-positive cells: $\chi^2 = 3.74$, $p = 0.053$, GFP-positive cells in TPH2-positive cells: $\chi^2 = 2.65$, $p = 0.103$).

2.4. Cas9-mediated 5-HT_{1A} receptor knockout reversed 5-HT_{1A} receptor agonist-induced hypothermia

Two-way ANOVA showed that i.p. injection of 8-OH-DPAT (0.5 mg/kg), a 5-HT_{1A/7} receptor agonist, induced hypothermia (Fig. 4A; a main effect of time, $F_{2,38, 26.22} = 46.97$, $p < 0.001$, with Greenhouse–Geisser correction). However, the reduction in body temperature was significantly

smaller in Cre-dependent Cas9 knock-in mice that received microinjection of viral vectors expressing sgRNA into the DRN than in WT littermates receiving the same microinjections (Fig. 4; time $\times$ genotype interaction, $F_{2,38, 26,22} = 5.56$, $p = 0.007$, with Greenhouse–Geisser correction). One-way ANOVA for each time point revealed that body temperature differed between Cas9 transgenic and WT controls at 10, 20, and 30 min after 8-OH-DPAT injection (Fig. 4A; $F_{1, 11} = 5.67$, 13.04, and 9.77, $p = 0.036$, 0.004, and 0.01, respectively), while there were no differences between genotypes at other time points (Fig. 4A; $F_{1, 11} < 4.37$, NS).

Moreover, two-way ANOVA revealed that NLX-112, a selective 5-HT_{1A} receptor agonist, also induced hypothermia (Fig. 4B; a main effect of time, $F_{1,49, 22,32} = 47.99$, $p < 0.001$, with Greenhouse–Geisser correction). This hypothermic effect was significantly attenuated in Cas9 transgenic compared to WT controls at 10-60 min after NLX-112 injection (Fig. 4B; two-way ANOVA, time $\times$ genotype interaction, $F_{1,48, 22,32} = 9.58$, $p = 0.002$, with Greenhouse–Geisser correction; one-way ANOVA for each time point, $F_{1, 15} = 10.56$, 5.942, 6.119, 8.146, 9.392, and 8.923, $p = 0.005$, 0.028, 0.026, 0.012, 0.008, and 0.009, respectively).

2.5. Cas9-mediated 5-HT_{1A} receptor knockout enhanced selective serotonin reuptake inhibitor-induced antidepressant effect in the forced swim test.

Immobility time in the forced swim test was significantly shorter in Cas9 transgenic than in WT controls after i.p. injection of fluoxetine (10 mg/kg), a SSRI (Fig. 4C; $t_{16} = 3.929$, $p = 0.0012$).

2.6. Cas9-mediated 5-HT_{1A} receptor knockout did not alter 5-HT metabolism.

NLX-112 administration significantly reduced 5-HIAA/5-HT ratio in the DRN (Fig. 5A, $t_6 = 2.82$, $p = 0.03$) while it did not alter 5-HT and 5-HIAA levels in the DRN ($ts_6 < 0.52$, $ps > 0.22$). Although NLX-112 administration tended to increase 5-HT levels in the ventral hippocampus (VH, $t_6 = 2.35$, $p = 0.057$), it did not alter 5-HIAA or 5-HIAA/5-HT ratio ($ts_6 < 1.90$, $ps > 0.10$). In the other brain regions, NLX-112 administration did not affect 5-HT, 5-HIAA levels, or 5-HIAA/5-HT

ratio ($ts_6 < 1.55$, $ps > 0.17$). NLX-112 administration tended to reduce 5-HIAA/5-HT ratio in urine (Fig. 5B, $t_6 = 1.97$, $p = 0.096$), but it did not affect 5-HT or 5-HIAA levels in urine ($ts_6 < 1.47$, $ps > 0.19$). However, 5-HT, 5-HIAA levels, and 5-HIAA/5-HT ratio in the brain tissues and urine were comparable between Cas9 transgenic and WT controls after NLX-112 administration (Figs. 5C and 5D, $ts_{15} < 1.36$, $ps > 0.19$).

3. DISCUSSION

We observed efficient *in vivo* editing of *Htr1a* in Cre-dependent Cas9 knock-in mice injected with a targeted sgRNA expression vector as predicted by *in vitro* screening (Figs. 1 and 2), indicating that this *in vitro* screening test could be useful for choosing the optimal guide RNA sequence (Mashiko et al., 2013). Cas9 was expressed only in virus-infected cells (Fig. 2A), minimizing the risk of constitutive Cas9 expression-induced damage to cells during development. Although a previous study reported that constitutive Cas9 expression did not impair the physiological functions of CA1 pyramidal neurons in the hippocampus (Platt et al., 2014), another study found that constitutive overexpression of an exogenous protein such as GCaMP6 caused aberrant cortical activity (Steinmetz et al., 2017). Using this strategy, we demonstrated that 5-HT_{1A} receptors in the DRN contribute to the regulation of body temperature and antidepressant effect of SSRI (Fig. 4), though it might not be 5-HT_{1A} receptors expressed by serotonin neurons (Fig. 3).

The efficiency of editing was approximately 8% to 18% in the present study (Figs. 2B and 2E). Considering that we used a neuron-specific promotor, this efficiency is relatively high because the isolated DRN slices also contain glia and stromal cells as well as neurons. Given that the glia/neuron ratio in rodents ranges from around 3 to 6 (Herculano-Houzel, 2014), it is possible that the gene encoding the 5-HT_{1A} receptor was edited in most infected neurons.

Unfortunately, we could not find any 5-HT_{1A} receptor selective antibodies. However, our

1 CRISPR/Cas9-mediated 5-HT_{1A} receptor knockout strategy will advance the development of
2 selective antibodies in the future and overcome this kind of problems (Baker, 2015). Although it is
3 best to check the selectivity of antibodies by using gene knockout mice, knockout mice are not
4 always available for most researchers. Easier and cost-effective CRISPR/Cas9-mediated knockout
5 strategy will resolve it because this strategy will be available for most researchers and because we
6 can check the selectivity of antibodies by using a brain slice of Cas9-mediated knockout mice. That
7 is, if an antibody labels Cas9-negative cells but not Cas9-expressing cell, the antibody will be
8 selective for the targeted protein.

9 A previous pharmacological study reported that microinjection of 8-OH-DPAT into the DRN,
10 but not the median raphe nucleus, decreased body temperature (Hillegaart, 1991). However, it is
11 known that 8-OH-DPAT is a 5-HT₇ receptor agonist as well as a 5-HT_{1A} receptor agonist (Hedlund
12 et al., 2004; Wood et al., 2000), and 5-HT₇ receptors are also involved in regulating body
13 temperature (Hedlund et al., 2003; Hedlund et al., 2004). Although genetic studies using 5-HT_{1A}
14 receptor knockout/knockdown mice have shown that the 5-HT_{1A} receptor regulates body
15 temperature (Heisler et al., 1998; Richardson-Jones et al., 2010), the responsible brain regions have
16 not yet been determined. Therefore, our results filled the gap between pharmacological and genetic
17 findings by showing a specific contribution of DRN 5-HT_{1A} receptors. More, we demonstrate a
18 direct effect of 5-HT_{1A} receptor-expression neurons. The 5-HT_{1A} receptor is expressed in glial
19 cells as well as neurons (Hirst et al., 1998; Miyazaki et al., 2013; Whitaker-Azmitia et al., 1990),
20 but previous pharmacological and genetic studies did not discriminate between neurons and glia. In
21 the present study, we expressed Cas9 only in neurons by utilizing the hSyn promotor, and
22 demonstrate that neuronal 5-HT_{1A} receptors in the DRN are responsible for regulating body
23 temperature.

24 It has been reported that the stimulation of 5-HT_{1A} receptor by 5-HT inhibits serotonin neuron

firing and the inhibitory effect of SSRIs via 5HT_{1A} receptor offsets increase of 5-HT release in the frontal cortex (Craven et al., 1994; Gartside et al., 1995). Due to the offsetting effect, SSRIs have often failed to exert sufficient antidepressant effects especially when fluoxetine and C57BL/6 mice were used (Lucki et al., 2001). Indeed, we also failed to find antidepressant effects of fluoxetine in WT mice (C57BL/6N mice, Supplementary Fig. 3B). Moreover, it has been suggested that the blockade of 5-HT_{1A} receptor might accelerate antidepressant effects of SSRIs (Artigas et al., 2001) while a previous study showed that knockdown of 5-HT_{1A} autoreceptor itself only slightly affected immobility in the forced swim test (Richardson-Jones et al., 2010). Thus, the enhancement of antidepressant effect in Cas9-mediated 5-HT_{1A} receptor knockout mice (Fig. 4C) likely reflect the absence of this inhibitory effect.

In addition, 5-HT_{1A} receptor agonist reduced 5-HT turnover in the DRN (Fig. 5A), consistent with the previous study showing that 5-HT_{1A/7} receptor agonist reduced 5-HT turnover in the brainstem (Veenema et al., 2005). However, 5-HT, 5-HIAA levels, and 5-HIAA/5-HT ratio were comparable in urine and several brain tissues between Cas9-mediated 5-HT_{1A} receptor knockout mice and WT mice (Fig. 5C, D). Because only a part of serotonin neurons (approx. 16%) were GFP-positive in Cre-dependent Cas9 knock-in mice, the 5-HT_{1A} receptor agonist-induced reduction of 5-HT turnover might not be fully reversed by 5-HT_{1A} receptor knockout in the present study.

Most neurons infected by viral vector were GABA neurons while only a minority were serotonin neurons (Fig. 3). Given that 5-HT_{1A} receptors are expressed in both serotonin and GABA neurons of the DRN (Bonnavion et al., 2010), GABA neurons in the DRN may play a dominant role in body temperature regulation. However, stimulation of 5-HT_{1A} autoreceptors may also induce hypothermia by decreasing serotonin release in the hypothalamus (Lin et al., 1998). This uncertainty over the precise mechanisms could be addressed by taking advantage of Cre-dependent

Cas9 expression; for example, SERT-Cre or GAD-Cre mice might be useful (Taniguchi et al., 2011; Zhuang et al., 2005).

We cannot completely exclude the possibility that expression of GFP impaired cell functions, thereby counteracting the effects of the 5-HT_{1A} receptor agonist in Cre-dependent Cas9 knock-in mice. However, our preliminary results using a viral vector lacking the guide RNA sequence showed that the expression of GFP alone did not mitigate the effects of the 5-HT_{1A} receptor agonist (Supplementary Fig. 3). Thus, it is unlikely that GFP expression interfered with the hypothermic effects of the 5-HT_{1A} receptor agonist.

We found GFP-positive cells in the adjacent brain areas to the DRN such as the periaqueductal gray (Supplementary Fig. 1). However, the previous study showed that 5-HT_{1A} receptor is localized in the raphe nucleus in the midbrain (Chalmers and Watson, 1991). We confirmed that any GFP-positive cells were not observed in other serotonergic raphe nuclei such as the median raphe nucleus. Therefore, the physiological changes observed are most likely due to 5-HT_{1A} receptor knockout in the DRN.

Although this strategy is promising, there is uncertainty about the off-target effects of CRISPR/Cas9 (Zhang et al., 2015). We reduced the risk of off-target effects by utilizing recently suggested algorithms (Naito et al., 2015) and checked some potential off-target sites (Fig. 2), but we cannot completely exclude the possibility of off-target effects. However, the risk of off-target effects is not as problematic compared to other methods such as pharmacological agents or RNAi knockdown for preclinical studies (Boettcher and McManus, 2015). For clinical applications, more precise tools are being developed (Chen et al., 2017; Shen et al., 2018). It might be prudent for the time being to interpret the results obtained by CRISPR/Cas9 together with pharmacological results or to combine CRISPR/Cas9 with pharmacological agents as in the present study.

Taken together, we successfully achieved *in vivo* genetic manipulation of the 5-HT_{1A} receptor in

a specific brain area and cell type, and revealed that neuronal 5-HT_{1A} receptors in the DRN can induce hypothermia. If this approach becomes possible for all 5-HT receptor subtypes, double/triple knockout and/or conditional knockout in specific brain regions and cell types will be feasible at reasonable cost. Such an approach may be necessary for clinical studies. For instance, it is unlikely that a single gene is responsible for any major psychiatric disorders (Arnedo et al., 2015). Further, the physiological roles of specific 5-HT receptor subtypes depend on the expressing cells as shown for other neurotransmitters (de Almeida and Mengod, 2008; Rey et al., 2012). This realization will surely accelerate progress on elucidation of the distinct roles of 5-HT receptor subtypes in psychiatric disorders. While only the first step, the present study could facilitate such research by providing a tool for *in vivo* knockout of the 5-HT_{1A} receptor gene.

4. Experimental procedures

4.1. Design of guide RNAs and prediction of off-target sites.

Three guide RNA candidates for the 5-HT_{1A} receptor gene (*Htr1a*) were selected using CRISPRdirect (Naito et al., 2015). We checked “show highly specific target only” on the website (<https://crispr.dbcls.jp/>), and chose three sequences from the 18 candidates expected to produce cleavage in the middle of the coding region:

Guide Candidate 1, GGGACAGGCGGCAACGATAC (TGG)

Guide Candidate 2, TGTGCGCCATCGCGCTAGAC (AGG)

Guide Candidate 3, (CCA)TCGCGCTAGACAGGTACTGG

Protospacer adjacent motif sites are in parentheses.

These three candidates were cloned into AAV:ITR-U6-sgRNA(backbone)-hSyn-Cre-2A-EGFP-KASH-WPRE-shortPA-ITR (AAV:ITR-U6-sgRNA-hSyn-Cre) via the SapI restriction enzyme site. The

AAV:ITR-U6-sgRNA(backbone)-hSyn-Cre-2A-EGFP-KASH-WPRE-shortPA-ITR was a gift from Feng Zhang (Addgene plasmid # 60231; <http://n2t.net/addgene:60231>; RRID:Addgene_60231).(Platt et al., 2014)

Candidates for off-target sites were selected using CROP-IT (Singh et al., 2015). We submitted the sequence used in the present *in vivo* study (Guide Candidate 1) to the web (<http://cheetah.bioch.virginia.edu/AdliLab/CROP-IT/homepage.html>). The top 4 off-target candidates were tested by a resolvase assay as described in Section 4.5. The candidate sites were as follows:

Off-Target Candidate 1. GaGAgAaGaGGCAACGATAgaGG

Off-Target Candidate 2. aGaACAGGgtGCAACGATAgaGG

Off-Target Candidate 3. cGGggtGGCtGCAACGATAtgGG

Off-Target Candidate 4. caGgaAGGgGGCAACGATAggGG

Capital letters in these off-target candidates indicates matches with the target sequence and lower-case letters indicate mismatches with the target sequence. No off-target candidate sites with fewer than six mismatches were found by this tool.

4.2. *In vitro* screening of gRNAs by EGxxFP assay

The Htr1A gene locus was PCR-amplified from mouse genomic DNA by Q5 DNA polymerase (New England Biolabs, Ipswich, MA, USA). Primer sequences were as follows: mHtr1a target Fw: 5'-GAGGCTAGCTGCAGGCAGGCATGGATATGTTTCAG-3', mHtr1a target Rv: 5'-CTTGTCGACGCGGGATGGAGATGAGAAAGCCAAT-3'. The PCR-amplified DNA fragment was digested by NheI and SalI and then cloned into pCAG-EGxxFP (Mashiko et al., 2013) (Addgene #50716) also digested by NheI and SalI using a DNA Ligation kit according to manufacturer's instructions (Takara Bio, Kusatsu, Japan). Vector pX330 (Cong et al., 2013) was obtained from Addgene (Watertown, MA, USA; Addgene #42230). HEK293T cells were cultured

1 in Dulbecco's modified Eagles medium with 10% fetal bovine serum and passaged using
2 trypsin-EDTA. On the day of transfection, cells were trypsinized and seeded onto 24-well tissue
3 culture plates (Greiner Bio-One, Kremsmünster, Austria) at 1×10^5 cells/well. Then, 267 ng of
4 AAV:ITR-U6-sgRNA-hSyn-Cre expressing gRNAs, 267 ng of pCAG-mHtr1A-EGxxFP, and 267
5 ng of pX330 were co-transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham,
6 MA, USA). As negative controls, 267 ng of AAV:ITR-U6-sgRNA-hSyn-Cre expressing gRNAs
7 and 534 ng of empty vector, or 267 ng of pCAG-mHtr1A-EGxxFP and 534 ng of empty vector
8 were co-transfected using Lipofectamine 2000. Forty-eight hours after transfection, EGFP
9 fluorescence was recorded under an inverted fluorescence microscope (TE200, Nikon, Tokyo,
10 Japan) equipped with a digital camera (DS-L2, Nikon).

11 *4.3. Animals*

12 Adult male and female mice (C57BL/6N WTs or transgenic mice on the C57BL/6N
13 background, aged > 56 days at the start of the virus injection procedure) were used following the
14 National Institutes of Health (NIH) policy (Clayton and Collins, 2014). Approximately 50% of the
15 animals were female, and we combined the data of male and female mice because there were no
16 significant sex differences in the present study (data not shown). Cre-dependent Cas9 knock-in
17 mice (Stock No: 024857, the Jackson Laboratory) were backcrossed to the C57BL/6N strain for
18 more than four generations. The mice were housed in groups under an alternating light-dark cycle
19 (light from 7 p.m. to 7 a.m.) at approximately 25 °C. All testing was performed during the dark
20 period. The treatment of animals complied with the Guidelines for the Care and Use of Laboratory
21 Animals of the Animal Research Committee of Hokkaido University. Two independent cohorts of
22 mice were used, where cohort 1 underwent the body temperature measurement using 8-OH-DPAT,
23 mismatch cleavage assay, Sanger sequencing and immunohistochemistry. Cohort 2 underwent the
24 body temperature measurement using NLX-112, the forced swim test and the measurement of

1 5-HT/5-HIAA tissue contents.

2 *4.4 Adeno-associated virus (AAV) production and purification*

3 All AAV vectors were produced using the AAV Helper-Free System (Agilent Technologies,
4 Inc., Santa Clara, CA, USA) and purified as described previously (Inutsuka et al., 2016). Briefly,
5 HEK293 cells were transfected with an AAV vector plasmid that included a gene of interest,
6 pHelper, and pAAV-RC (serotype DJ; Cell Biolabs Inc, San Diego, CA, USA) using a standard
7 calcium phosphate method. Three days later, transfected cells were collected and suspended in
8 artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 3 mM KCl, 26 mM NaHCO₃, 2 mM CaCl₂, 1
9 mM MgSO₄, 1.25 mM KH₂PO₄, and 10 mM D-Glucose). Cells were disrupted by four freeze-thaw
10 cycles, and the lysate was treated with benzonase nuclease (Merck, Darmstadt, Germany) at 45 °C
11 for 15 min, followed by centrifugation two times at 16,000 g for 10 min. The supernatant was used
12 as the virus-containing solution. To measure the titer of purified virus, the supernatant was
13 dissolved in artificial CSF and quantitative PCR was performed using the following primer pairs:
14 woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), WPRE-Forward: 5'
15 -ACTGTGTTTGCTGACGCAAC-3', WPRE-Reverse: 5' -CAACACCACGGAATTGTCAG-3';
16 human growth hormone polyA-Forward: 5' -TGGGAAGACAACCTGTAGGG-3', human growth
17 hormone polyA-Reverse: 5' -GTGAAACCCCGTCTCTACCA-3'. The AAV vector was stored at
18 -80 °C in small aliquots until the day of experiment. The titer of AAV was estimated to be about 1
19 $\times 10^{13}$ copies/ml.

21 *4.5. Surgical and virus injection procedure*

22 Mice were anesthetized with isoflurane and fixed in a stereotaxic frame (Narishige, Tokyo,
23 Japan). An injection cannula (35 gage; Nanofil, NF35BL-2; World Precision Instruments, Sarasota,
24 FL, USA) was inserted into the DRN at coordinates 4.5 mm posterior to bregma, 1.5 mm lateral to

the midline, 2.8 mm ventral to the dura, and at a 30° angle from the vertical plane to avoid the sagittal sinus. The virus solution (0.3 µl) was infused over a period of 3 min at constant flow by a microinjection pump (Ultra Micro Pump II and Micro4 controller, World Precision Instruments), and the injector was left in place for 5 min after injection to allow diffusion. After surgery, the mice were singly housed. Experiments (Sections 4.6–4.11) were conducted more than 5 weeks after virus injection.

4.6. Immunohistochemistry

Immunohistochemistry and confocal microscopy were performed as described previously (Shikanai et al., 2012). In brief, after intracardial perfusion with 4% paraformaldehyde in PBS at pH 7.2, brains were post-fixed overnight, placed in 0.1 M PB containing 30% sucrose, and sectioned at 50 µm. The primary antibodies used were mouse anti-GAD67 (1:5,000; MAB5406, Chemicon, Temecula, CA, USA), rabbit anti-tryptophan hydroxylase 2 (1:10,000) (Nakamura et al., 2008), and mouse anti-FLAG (1:1,000; F1804, Sigma Aldrich, St. Louis, MO, USA). The secondary antibodies used were Alexa 647 (Invitrogen, Carlsbad, CA, USA) and indodicarbocyanine (Cy5; Jackson ImmunoResearch, West Grove, PA, USA). Images were captured with a confocal laser-scanning microscope (FV10i, Olympus, Tokyo, Japan). Quantitative analyses were conducted on representative images from three mice per genotype.

4.7. Detection of CRISPR-Cas9-induced mutation using a resolvase

We prepared brain slices as described in Section 4.6., and further isolated the visual cortex and DRN regions using surgical knives under a microscope. The isolated slices were placed in a tube containing 100 µL tissue lysate solution (20 mM Tris HCl [pH 8.0], 5 mM EDTA, 400 mM NaCl, 0.3% SDS, and 200 µg/mL proteinase K). These sample tubes were placed in a thermocycler (Verti Thermal Cycler, Applied Biosystems, Waltham, MA, USA) and subjected to the following

thermocycling parameters: 55 °C for 30 min, 95 °C for 10 min, and cooling at 4 °C. Then, PCR was conducted, followed by mutation detection using the Guide-it Mutation Detection Kit (Takara, Kyoto, Japan) according to manufacturer's protocol. In brief, PCR reactions were run using the following thermocycle: an initial denaturation step at 98 °C for 2 min, 40 cycles of 98 °C for 10 s, 70 °C for 15 s, and 76°C for 1 min, and then cooling at 4 °C. The primers are listed below:

Target (Htr1a) primer (forward): CGCCGGTGAGTGCTCCTCTC

Target (Htr1a) primer (reverse): CCGGGGCGTCCTCTTGTTCA

Off-Target Candidate 1 primer (forward): TGCCTGTCAATACGTGCTGT

Off-Target Candidate 1 primer (reverse): GAATGGGCCAACACACCAAC

Off-Target Candidate 2 primer (forward): AACTCAGGTCTCCCAGTGGA

Off-Target Candidate 2 primer (reverse): GAAAGGCCAGAGGACACAGG

Off-Target Candidate 3 primer (forward): GCAGAGAGTTCATGCCTGGA

Off-Target Candidate 3 primer (reverse): ACCAAACTGTAGACAAGCCGA

Off-Target Candidate 4 primer (forward): CCACGGAGCTATGTGTCCAG

Off-Target Candidate 4 primer (reverse): CTTCTTGTGGGTCCCGAACA

The PCR products were re-annealed to facilitate heteroduplex formation and then subjected to the following thermocycling parameters: 95 °C for 5 min, 95 °C to 85 °C ramping at 2 °C/s, 85 °C to 25 °C at 0.1 °C/s, and cooling at 4 °C. Re-annealing products were treated with Guide-it Resolvase for 30 min at 37 °C and electrophoresed on 2% agarose gels with a marker (100 bp DNA Ladder, Douourika, Sapporo, Japan). Intensity of each band was measured using ImageJ (<http://imagej.nih.gov/ij/>) and the percentage of insertion and deletion formation (%Indel) was calculated using the formula $\%Indel = (1 - [1 - f_{cut}]^{0.5}) \times 100$ (Guschin et al., 2010), where f_{cut} is the total density of cleaved bands divided by the sum of the density of uncleaved and cleaved bands.

4.8. DNA sequencing and TIDE analysis

The PCR products were purified and sequenced by FASMAC Co., Ltd. (Kanagawa, Japan). Sanger chromatograms were analyzed using the TIDE web tool (<http://tide.nki.nl>) (Brinkman et al., 2014).

4.9. Drug Treatment

(2R)-(+)-8-Hydroxy-2-(di-n-propylamino)tetralin hydrobromide (8-OH-DPAT) was purchased from Tocris Bioscience (Bristol, UK). NLX-112 was purchased from Sigma-Aldrich (St. Louis, USA). Fluoxetine was purchased from Tokyo Chemical Industry (Tokyo, Japan). 8-OH-DPAT and fluoxetine were dissolved in 0.9% saline. NLX-112 was dissolved in 0.9% saline with 1% DMSO. All drugs were dissolved to a concentration allowing injection at 10 ml/kg.

4.10. Body temperature measurement

A microcomputer thermometer (MODEL 7001H; Physitemp, Clifton, NJ, USA) was used to assess body temperature. Mice were subjected to a sham handling procedure once a day for two consecutive days before the test. The procedure consisted of gently touching and restraining the animal for approximately 3 min. On the test day, the mice were gently hand-restrained and body temperature was measured intrarectally using a probe smeared with Vaseline (WAKO, Osaka, Japan). The probe was inserted to approximately 2 cm and kept in position for at least 5 s before recording. Body temperature was monitored every 10 min. We started the drug injection procedure only after body temperature was stabilized (± 0.2 °C in three consecutive values) to avoid stress-induced changes. Three baseline values were recorded, and transgenic or WT littermate control mice received intraperitoneal (i.p.) injection of 8-OH-DPAT (0.5 mg/kg) or NLX-112 (0.5 mg/kg). After injection, body temperature was monitored over 60 min.

4.11. Forced swim test

The forced swim test was performed as described previously (Tsutsui-Kimura et al., 2017). In brief, 30 min after intraperitoneal (i.p.) injection of fluoxetine (10 mg/kg), mice were placed in a

cylinder glass (diameter, 23 cm; height, 30 cm; AGC techno glass, Shizuoka, Japan) containing water at a temperature of $23\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and a depth of 12 cm. The test lasted 6 min and behaviors were video-recorded and scored later by an experimenter who was blind to the treatments. Only the last 4 min of behaviors were analyzed, and they were categorized as follows: floating, twitching/kicking, swimming, and climbing. The immobility was calculated as the sum of the durations of floating and twitching/kicking behaviors

4.12 5-HT and 5-HIAA measurement

Thirty minutes after the intraperitoneal injection of NLX-112 (0.5 mg/kg), mice were sacrificed by decapitation. The brain tissue including dorsal raphe (DRN), median raphe (MRN), dorsal striatum (DS) and ventral hippocampus (VH) were dissected on ice. Each sample was weighed, placed in a tube, immediately frozen in liquid nitrogen and kept frozen at -80° until analysis. For extraction of 5-HT and 5-HIAA, samples were added 200 μL each of ice cold homogenization solution (0.9 M perchloric acid containing 1 mM DTT and 0.05 μM N-methylserotonin as an internal standard). The tissue block was dispersed by a 5 sec sonication and followed by another 5 sec for further homogenization. The homogenates were stored at -80° for determination of 5-HT, 5-HIAA (HPLC). Urine was taken at the time of decapitation and stock frozen at -80° until determination of 5-HT, 5-HIAA (HPLC) and creatinine (LabAssayTM Creatinine, FUJIFILM Wako 290-65901). Prior to HPLC analysis, the frozen homogenates were unfrozen on ice and spun to remove any precipitates. HPLC for determination of 5-HT and 5-HIAA was performed as described previously (Inoue et al., 1985) with slight modification. In brief, HPLC system was equipped with a fluorescence monitor (JASCO model, FP920) set at 290 nm and 350 nm for excitation and emission, respectively. The solid phase was ODS (4.6 $\times$ 250 mm, JASCO, Finepak SIL-C18T5), and the mobile phase was a 100 : 5 : 7 mixture of 40 mM sodium acetate (adjusted to pH 3.5 with formic acid), acetonitrile and methanol and the flow rate was 1 mL/min).

4.13. Statistical analysis

Body temperature was analyzed using two-factor ANOVA with time as the within-subject factor and genotype as the between-subject factor. When there was a significant time $\times$ genotype interaction, one-factor ANOVA was conducted for each time point. If Mauchly's sphericity test was significant, Greenhouse–Geisser correction was used. In immunohistochemical analysis, we used a chi-square test to compare the proportion of labeled cells. Immobility time and 5-HT/5-HIAA tissue contents were analyzed using unpaired *t*-test. The alpha level was set at 0.05 (two-tailed) for all comparisons. All statistical procedures were conducted using SPSS (version 23.0).

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ADDITIONAL INFORMATION

Supplemental information accompanies this paper.

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2 **Competing interests:** The authors declare no competing interests.

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1 Table 1. Immunohistochemical analysis of GFP-positive cells
4

TPH2	Number of labeled cells (mean $\pm$ S.E.M.)		
	GFP ⁺	TPH2 ⁺	GFP ⁺ /TPH2 ⁺
WT	265.0 $\pm$ 24.7	172.0 $\pm$ 3.5	35.3 $\pm$ 11.8
Cas9	309.3 $\pm$ 23.5	205.3 $\pm$ 15.2	33.3 $\pm$ 6.2

GAD67	Number of labeled cells (mean $\pm$ S.E.M.)		
	GFP ⁺	GAD67 ⁺	GFP ⁺ /GAD67 ⁺
WT	253.7 $\pm$ 17.4	-	204.7 $\pm$ 15.3
Cas9	261 $\pm$ 22.3	-	220.3 $\pm$ 32.9

	Percentages of co-labeled cells (mean $\pm$ S.E.M.)		
	TPH2 ⁺ in GFP ⁺	GAD67 ⁺ in GFP ⁺	GFP ⁺ in TPH2 ⁺
WT	14.4 $\pm$ 5.61	80.6 $\pm$ 0.53	20.59 $\pm$ 6.80
Cas9	10.61 $\pm$ 1.4	83.5 $\pm$ 5.54	15.97 $\pm$ 2.08

Figure Legends

Fig. 1. In vitro assessment of gRNAs by EGxxFP assay.

HEK293T cells were transfected with vector pAAV-U6-sgRNA-hSyn-Cre encoding gRNAs targeting the *Htr1A* locus plus vectors pX330 and pCAG-mHtr1A-EGxxFP (A-C), AAV:ITR-U6-sgRNA-hSyn-Cre alone (D-F), or pCAG-mHtr1a-EGxxFP alone (G). Forty-eight hours after transfection, EGFP fluorescence was recorded under fluorescence microscopy. Representative images obtained from two independent experiments are shown.

Fig. 2. Cre-dependent Cas9 expression and in vivo genome editing of Htr1a in the dorsal raphe nucleus by viral vector.

(A) Cre-dependent Cas9 knock-in mice (Cas9 mice) were microinjected with the viral vector into the dorsal raphe nucleus (DRN). Five weeks after injection, coronal sections containing the DRN were prepared and stained with anti-FLAG antibody. After staining, GFP (Green) and FLAG (Red) signals were observed by confocal microscopy. Bar = 100 μ m. (B) The mismatch detection assay demonstrated insertion and deletion mutation (indel) formation in the DRN, but not in the cortex of viral vector-injected Cas9 mice. Frequency of indel formation (%Indel) is expressed in mean $\pm$ SEM. n=7. The uncleaved band was 640 bp. The cleaved bands were 370 bp and 270 bp. (C) The mismatch detection assay of the top four candidates for off-target site cleavage showed no detectable indel formation. (D) Sanger sequencing chromatograms of the PCR products containing the target site obtained from the DRN of viral vector-injected Cas9 mice and wild type (WT) littermates showed indel formation only in the Cas9 mice (Cas9). Arrow indicates the predicted cut site. (E) TIDE analysis showed mostly frameshift mutations in *Htr1a* of viral vector-injected Cas9 mouse DRN. n=5. The bars represent the mean, and the lines represent the SEM.

Fig. 3. Histological characterization of Cas9 expressing cells in the dorsal raphe nucleus.

Cre-dependent Cas9 knock-in mice (Cas9; lower panels) and wild type littermates (WT; upper panels) were microinjected with viral vector into the dorsal raphe nucleus (DRN). Five weeks after microinjection, coronal sections containing the DRN were prepared and stained with anti-TPH2 (left panels) or anti-GAD67 (right panels) antibody. After staining, GFP and TPH2/GAD67 signals were observed by confocal microscopy. Bar = 100 μ m.

Fig. 4. Effect of in vivo CRISPR/Cas9-mediated Htr1a gene knockout in DRN neurons on 5-HT_{1A} agonist-induced hypothermia.

(A) Five weeks after microinjection of viral vector into the DRN, hypothermia induced by intraperitoneal (i.p.) administration of 8-OH-DPAT, a 5-HT_{1A} agonist, was attenuated in Cre-dependent Cas9 knock-in mice (Cas9; n=7) compared to wild type littermates (WT; n=6). (B) Hypothermia induced by i.p. administration of NLX-112, a selective 5-HT_{1A} agonist, was also attenuated in Cas9 (n=10) compared to WT (n=8). (C) Immobility time in the forced swim test was reduced in Cas9 (n=10) compared to WT (n=8) after i.p. injection of fluoxetine, a selective serotonin reuptake inhibitor. **P* < 0.05, ***P* < 0.01.

Fig. 5. The effects of 5-HT_{1A} receptor agonist on 5-HT metabolism in CRISPR/Cas9-mediated 5-HT_{1A} receptor knockout mice and wildtype mice.

(A-D) Thirty minutes after administration of NLX-112, the brain tissue (DRN: dorsal raphe nucleus, MRN: median raphe nucleus, DS: dorsal striatum, VH: ventral hippocampus) and urine contents of 5-HT and 5-HIAA were measured. The 5-HIAA/5-HT ratio was calculated as a measure of 5-HT turnover. (A, B) In the brain (A) or urine (B), 5-HT, 5-HIAA and 5-HIAA/5-HT were not changed by the administration of NLX-112 in normal C57BL/6N mice (saline: n=4, NLX-112: n=4), except for 5-HIAA/5-HT in the DRN. (C, D) In the brain (C) or urine (D) 5-HT, 5-HIAA or 5-HIAA/5-HT did not differ between Cas9 transgenic (n=9) and WT controls (n=8). * $P < 0.05$

Supplementary figure legends

Supplementary Fig. S1. A representative image of virally infected area.

Cre-dependent Cas9 knock-in mice were microinjected with the viral vector containing sgRNA for *Htr1a* (sgHtr1a) into the dorsal raphe nucleus. Five weeks after microinjection, GFP fluorescence was observed by fluorescence microscopy. (A) The representative image of the maximal spread of the GFP-positive cells. (B) The representative image of the minimum spread of the GFP-positive cells. (C) The diagram of the spread of the GFP-positive cells. Black dotted line and gray dotted line indicate the boundary of the maximum and the minimum spread of GFP fluorescence, respectively. aq= cerebral aqueduct.

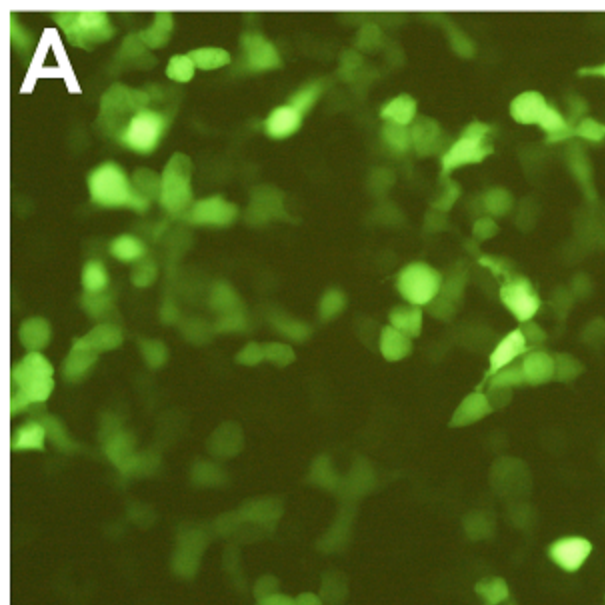
Supplementary Fig. S2. Sanger sequencing chromatograms of the PCR products containing target site.

The viral vector containing sgRNA for *Htr1a* (sgHtr1a) or lacking sgRNA (sgRNA(backbone)) was microinjected into the dorsal raphe nucleus (DRN) of Cre-dependent Cas9 knock-in mice (Cas9) and wild type littermates (WT). Five weeks after injection, PCR products were obtained from the dorsal raphe nucleus and sequenced. Arrow indicates the predicted cut site.

Supplementary Fig. S3. 5-HT_{1A} agonist-induced hypothermia in Cre-dependent Cas9 knock-in mice injected with viral vector lacking sgRNA and the effect of fluoxetine on wildtype naïve mice in the forced swim test.

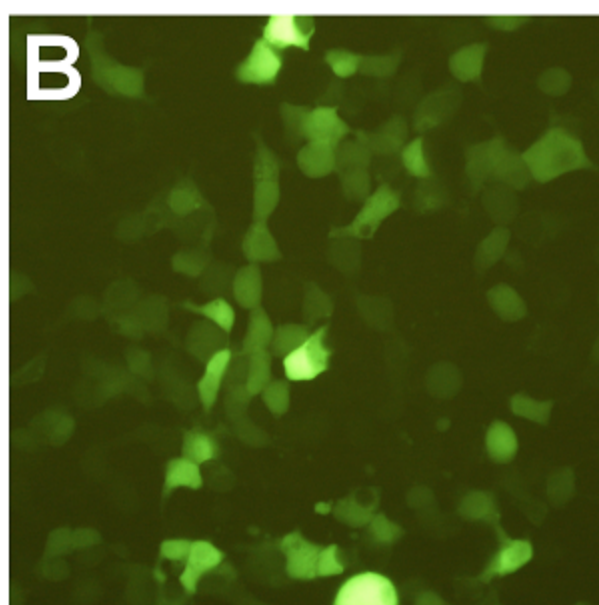
(A) Cre-dependent Cas9 knock-in mice were microinjected with a viral vector lacking sgRNA (Cas9: n=3). Wild type littermates were microinjected with the viral vector containing sgRNA for *Htr1a* (WT: n=6). 8-OH-DPAT-induced hypothermia level was comparable between groups. (B) Wild type mice were injected with saline (n=6) or fluoxetine (10 mg/kg; n=5). Thirty minutes after injection, the forced swim test was performed. Immobility time was comparable between groups.

**gRNA #1, pX330
mHtr1A-EGxxFP**



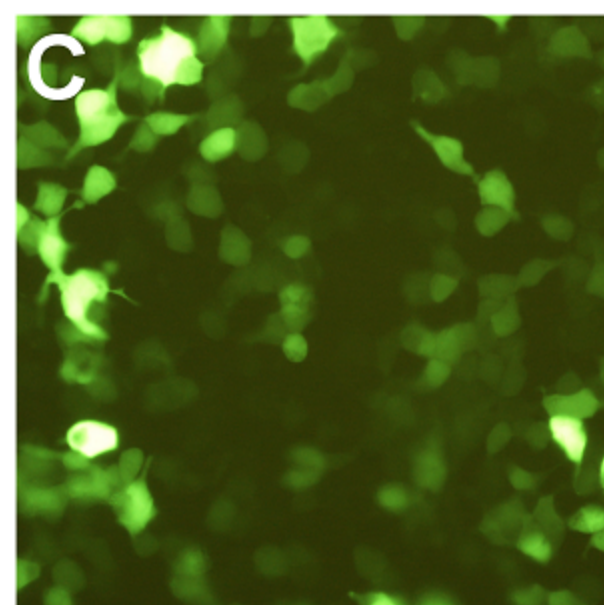
**gRNA #1
only**

**gRNA #2, pX330
mHtr1A-EGxxFP**

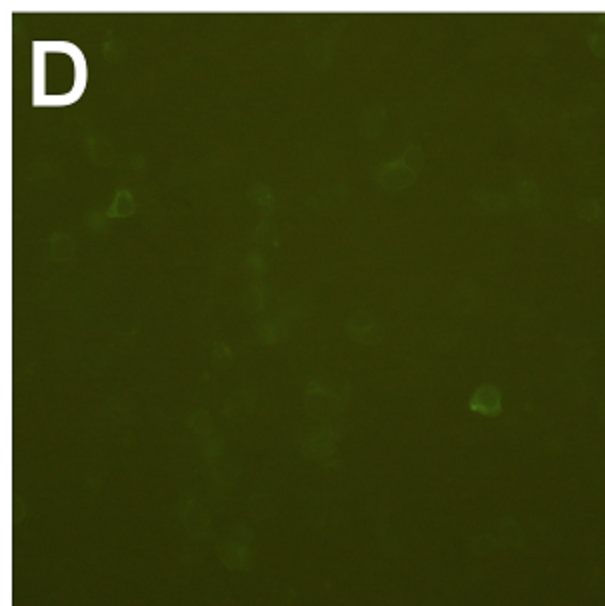


**gRNA #2
only**

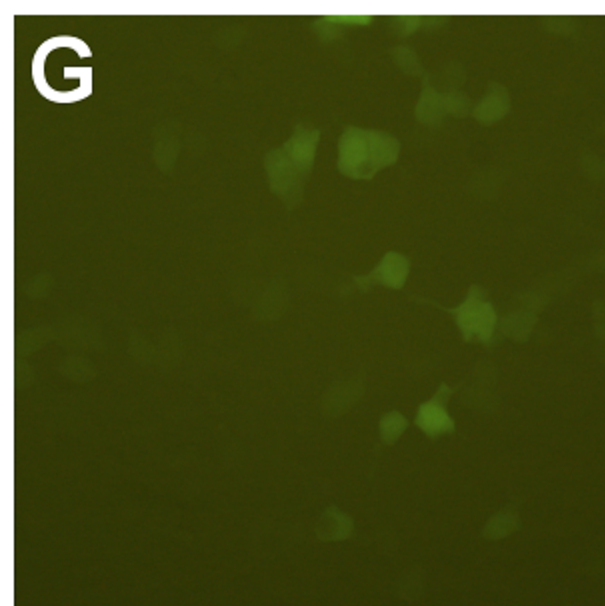
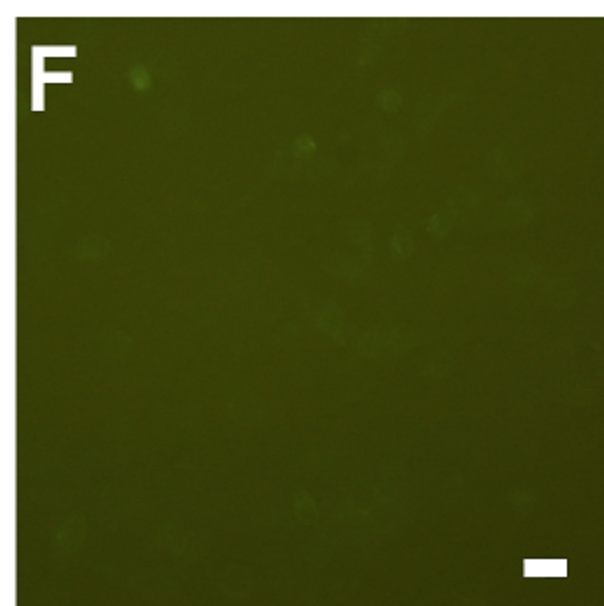
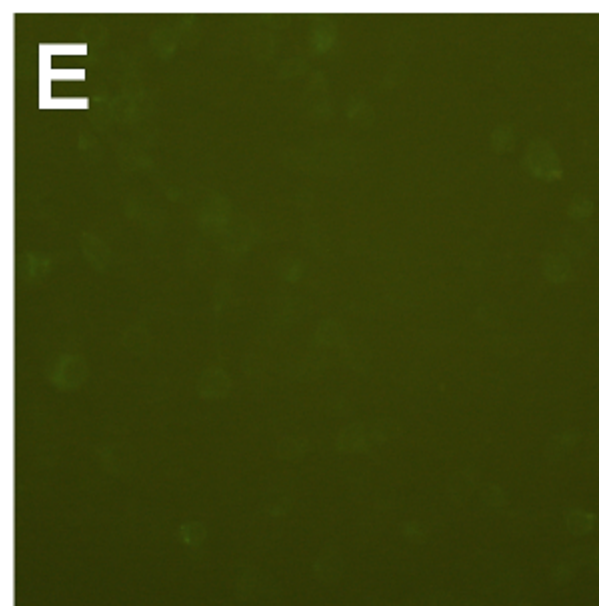
**gRNA #3, pX330
mHtr1A-EGxxFP**

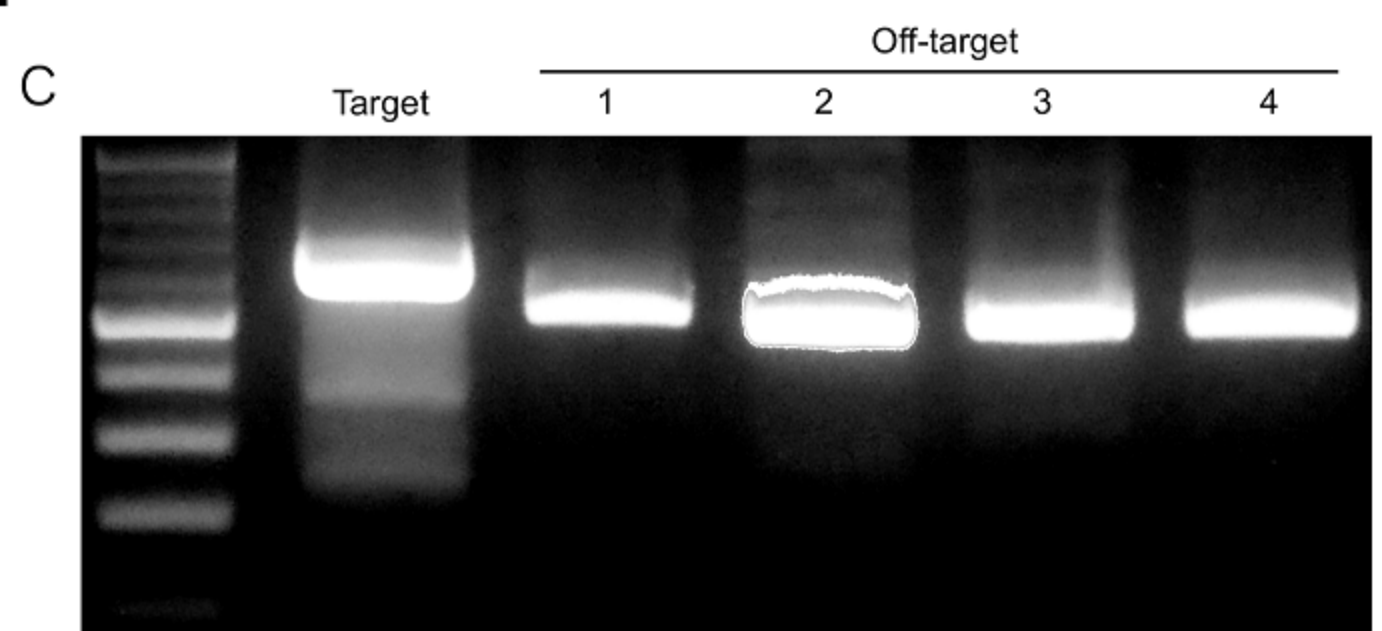
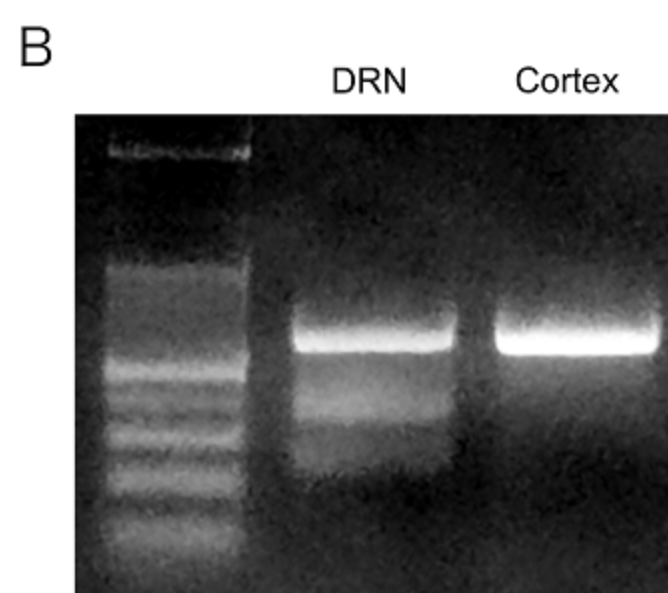
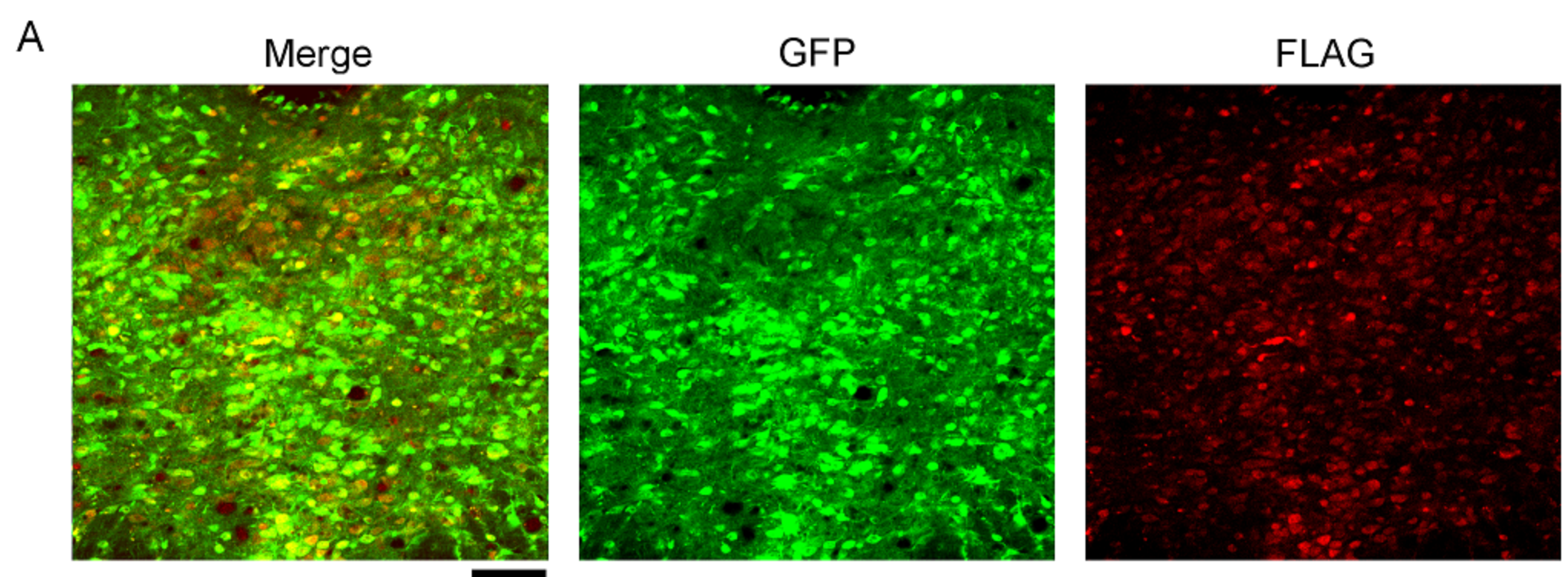


**gRNA #3
only**

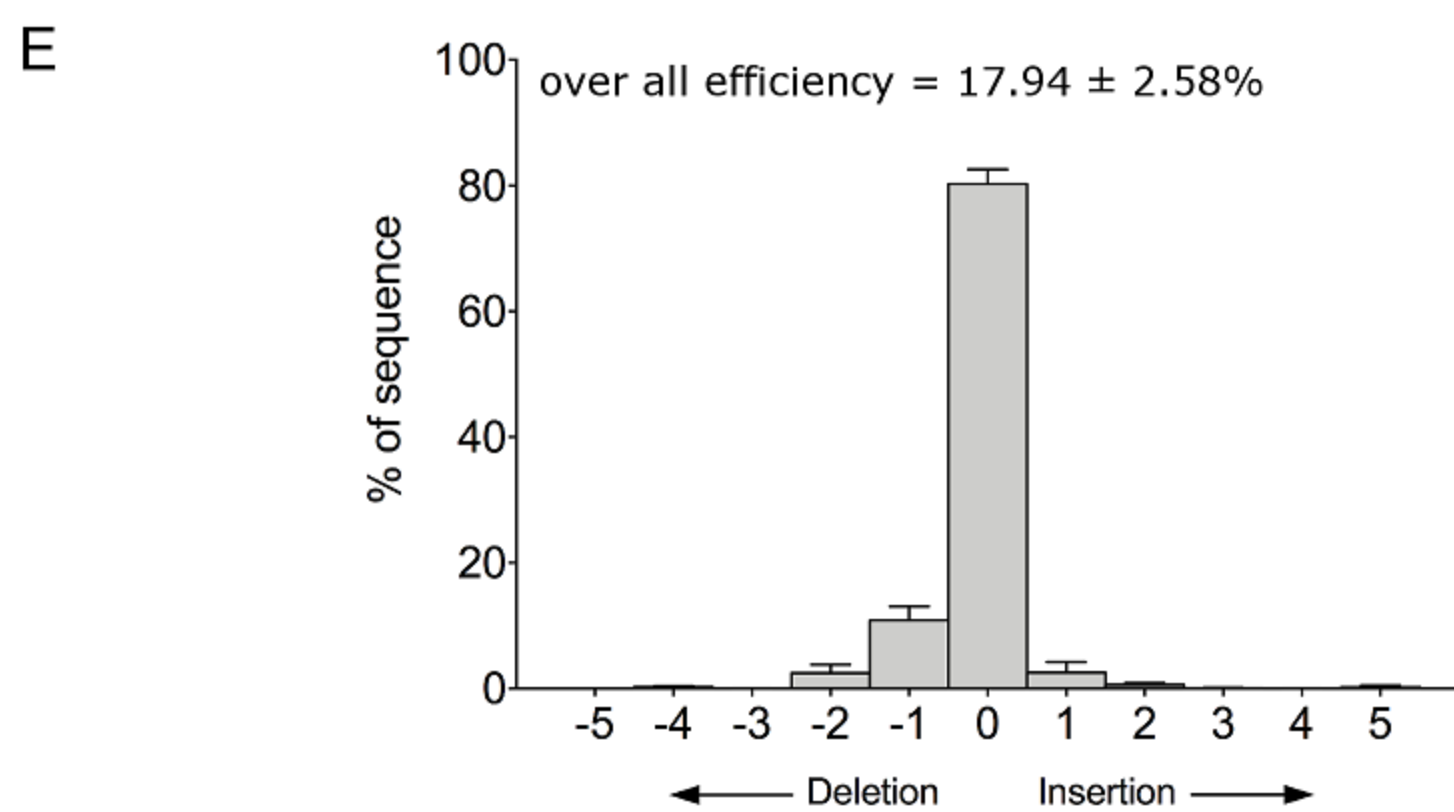
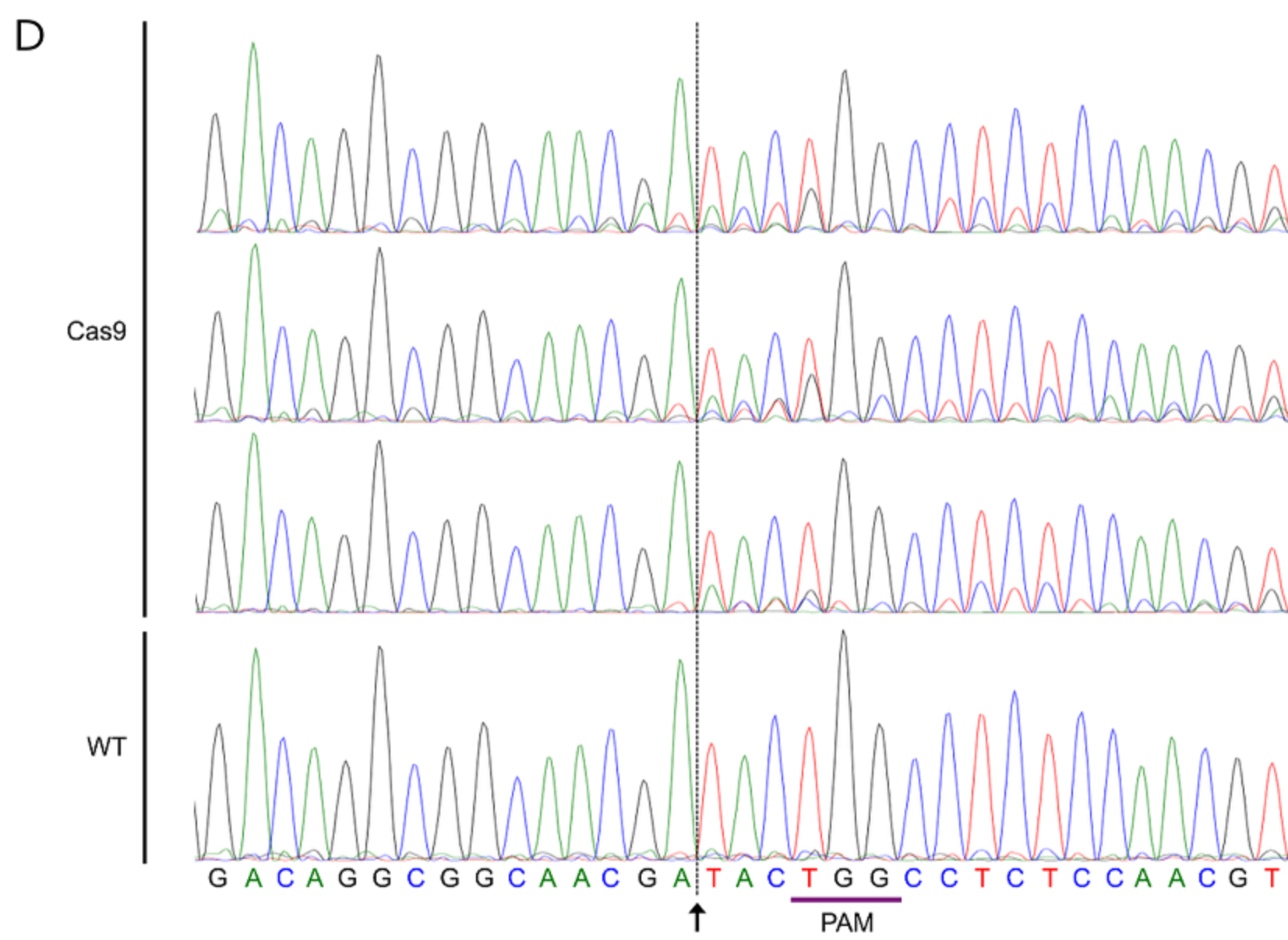


**mHtr1A-EGxxFP
only**





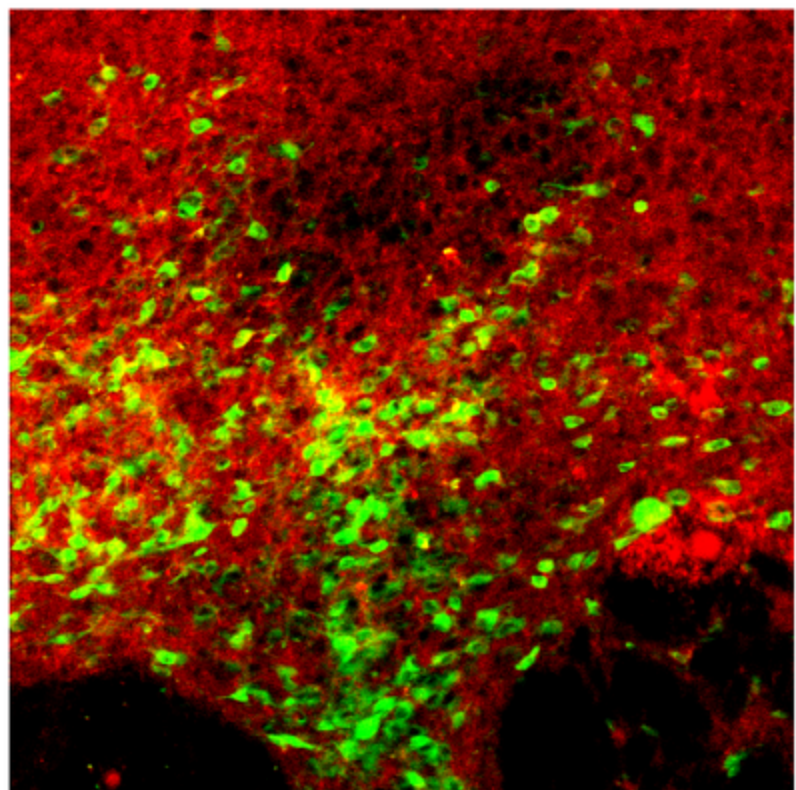
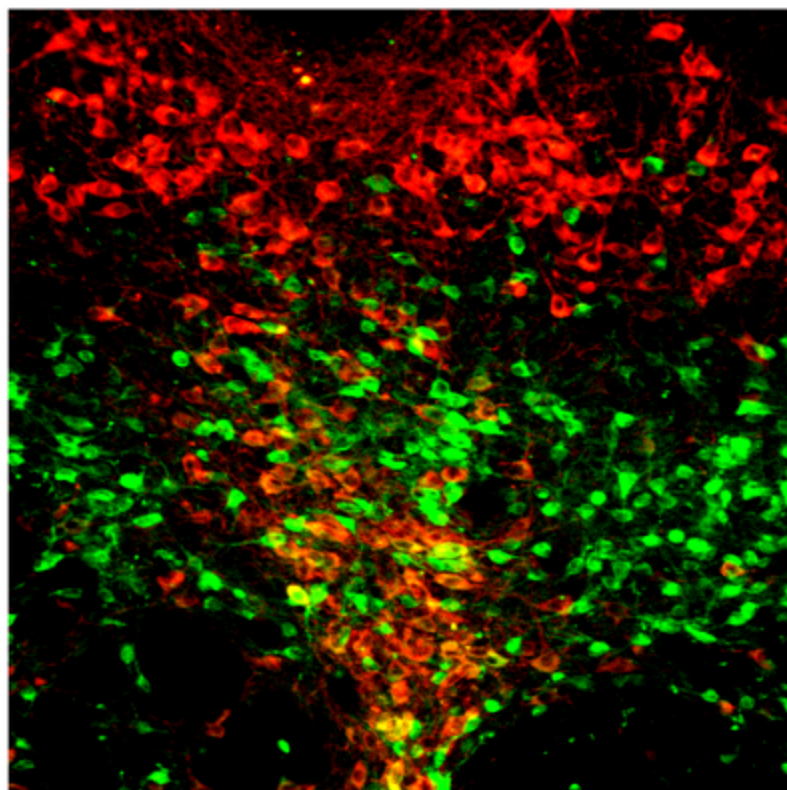
%Indel = 8.65 ± 1.67



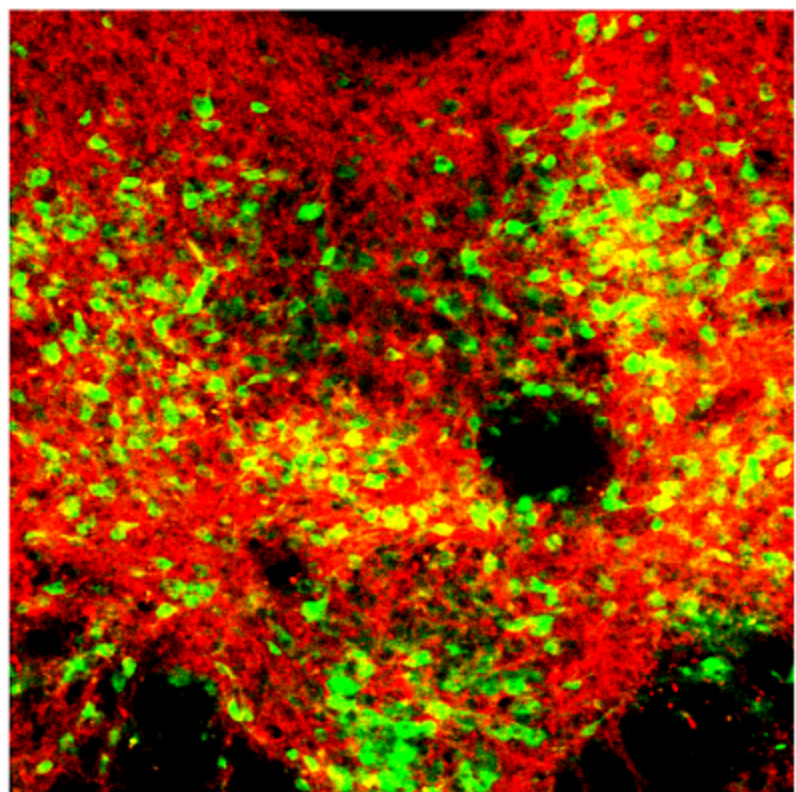
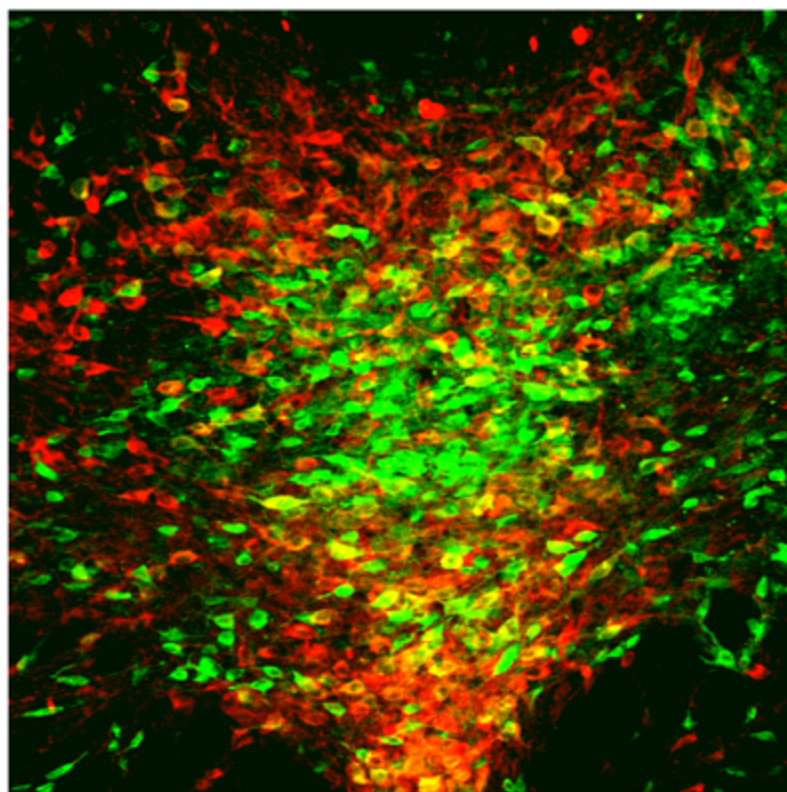
GFP / TPH2

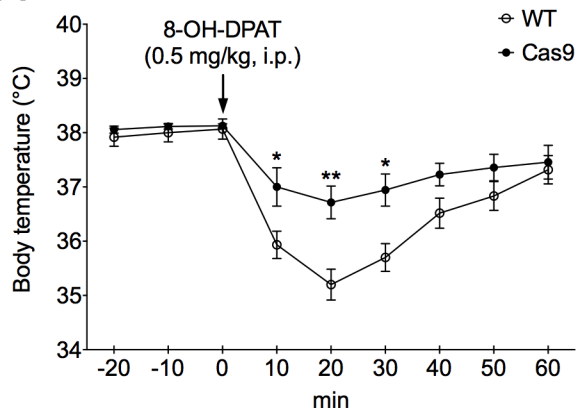
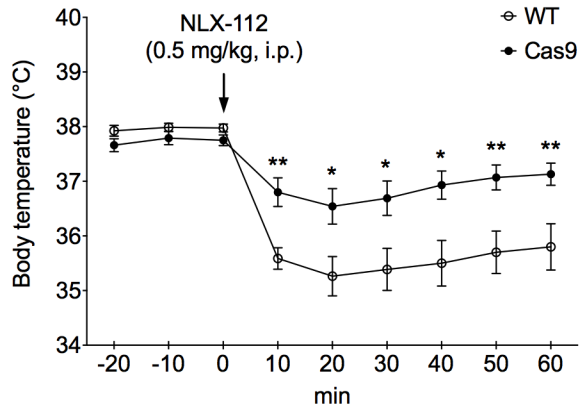
GFP / GAD67

WT



Cas9



A**B****C**