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Citation

Published Version
doi:10.1371/journal.pone.0105584

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CRISPR/Cas9-Mediated Gene Knock-Down in Post-Mitotic Neurons

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Abstract

The prokaryotic adaptive immune system CRISPR/Cas9 has recently been adapted for genome editing in eukaryotic cells. This technique allows for sequence-specific induction of double-strand breaks in genomic DNA of individual cells, effectively resulting in knock-out of targeted genes. It thus promises to be an ideal candidate for application in neuroscience where constitutive genetic modifications are frequently either lethal or ineffective due to adaptive changes of the brain. Here we use CRISPR/Cas9 to knock-out Grin1, the gene encoding the obligatory NMDA receptor subunit protein GluN1, in a sparse population of mouse pyramidal neurons. Within this genetically mosaic tissue, manipulated cells lack synaptic current mediated by NMDA-type glutamate receptors consistent with complete knock-out of the targeted gene. Our results show the first proof-of-principle demonstration of CRISPR/Cas9-mediated knock-down in neurons in vivo, where it can be a useful tool to study the function of specific proteins in neuronal circuits.


Editor: Ya-Ping Tang, Louisiana State University Health Sciences Center, United States of America

Received May 19, 2014; Accepted July 22, 2014; Published August 20, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are contained within the paper.

Funding: Funding provided by National Institute of Neurological Disorders and Stroke NS077907; Howard Hughes Medical Institute; DAAD Postdoctoral fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The typical approach to investigate the function of a given protein is to delete the genomic sequence encoding it and study the physiological effect of this ‘knock-out’ [1]. In neuroscience this approach has been used widely, but results are often limited by several technical difficulties. First, a significant subset of genes is essential for survival, and genomic deletion is lethal [2]. Second, the plasticity of the brain can lead to developmental compensation for deleted genes, thereby obscuring the effect [3]. These problems have been circumvented by generating conditional knock-out mice, in which genomic deletion of a gene depends on the presence of a recombinase whose expression can be spatially and/or temporally restricted [4]. However, this approach still requires the generation of germline genetically modified mice, a process that takes at least several months.

The difficulties of conventional transgenic mouse models could be overcome by efficient genome editing methods that would allow controlled perturbation in post-mitotic neurons. The type II prokaryotic adaptive immune system (clustered regularly interspaced short palindromic repeats (CRISPR)) [5] with the endonuclease CRISPR-associated (Cas) 9 has recently been engineered for this use in mammalian cells [6,7]. In addition to the endonuclease Cas9, CRISPR/Cas9-mediated editing requires two short RNAs, a target-recognizing CRISPR-RNA, and a Cas9-recruiting tracer-RNA; both RNAs can be linked together as single guide-RNA (gRNA) [8]. When co-expressed with an appropriate gRNA, Cas9 is recruited to the genomic DNA in a sequence-specific manner, and cuts both strands at a precise location. The genomic DNA is then repaired by non-homologous end joining (NHEJ), introducing mutations that effectively interrupt the open reading frame, and thereby results in a functional knock-out of the encoded protein [8].

Since its first application in mammalian cells, CRISPR/Cas9 has been used in many different organisms and applications [9–15]; however, it is still unknown if this system can be used in mammalian neurons to generate genetically mosaic brain tissue. Here we demonstrate that CRISPR/Cas9-mediated knock-down can be used to effectively delete proteins in individual post-mitotic neurons of an otherwise unperturbed brain.

Materials and Methods

Animal research and ethics statement

All experiments that included animals were carried out in accordance with protocols approved by the Harvard Standing Committee on Animal Care following guidelines described in the US National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all efforts were made to minimize suffering. For in-utero electroporation, mice were anesthetized using 2% isoflurane and injected with 0.1 mg/kg of buprenorphine as anesthetic. For euthanasia, mice were anesthetized with isoflurane. This study, and all procedures in it, was approved by the Harvard Medical School Institutional Animal Care and Use Committee (IACUC), protocol no. 03551.
Cloning and constructs

The genomic sequence surrounding the sequence encoding for the second transmembrane region of mouse GluN1 (±200 bp) was analyzed for potential CRISPR/Cas9 targets in silico [16]. The two sequences with the highest predicted ‘on-target score’, ‘CRISPR/Cas9 against Grin1.1/2’ (‘CC_Grin1.1’ and ‘CC_Grin1.2’; Figure 1B) were synthesized and subcloned into a vector (pX330, addgene plasmid #42230) containing the flanking gRNA sequences and a codon-optimized Cas9 [8].

In-utero electroporations

Neuronal transfections were performed by in-utero electroporation of E15 wild-type C57BL/6J mice as described previously [17]. Briefly, an E15 pregnant mother was anesthetized using 2% isoflurane and injected with 0.1 mg/kg of buprenorphine as anesthetic. Embryonic pups within the intact uterus were temporarily removed from the abdomen and injected into the left hemisphere with 1 μl of DNA mixture, containing the appropriate CRISPR-construct and soluble GFP (10:1), using a ~50 μm-diameter pipette sharply beveled at 15°–20° (Narishige, Japan), visually confirming the proper site of correct injection by mixing 0.005% fast green with the DNA. To target transfection to the hippocampus, the head of the embryonic pup was placed between paddles of tweezer electrodes (CUY21 electroporator, NEPA GENE, Japan), with the positive terminal covering the lateral surface of the right hemisphere and the negative terminal covering the left hemisphere. Each injected embryo was then subjected to 5×50 ms/35 V electric pulses. Following electroporation, the intact uterus containing the pups was returned to the abdomen, and the mother’s abdomen sutured shut. Recordings were made from transfected pups 14–20 days following birth.

Biolistic transfection of organotypic slice cultures

Biolistic transfection of post-mitotic neurons was achieved using organotypic hippocampal slice cultures. Slice cultures were prepared from P6–8 wild-type Sprague-Dawley rats as described previously [18]. Slice cultures were maintained at 34°C with 5% CO2 on 30 mm Millicell Cell Culture inserts with 0.4 um pores.
(Millipore) in slice culture media containing: MEM (8.32 g/L), 20% heat-inactivated horse serum, 1 mM L-glutamine, 1 mM CaCl₂, 2 mM MgCl₂, insulin (1 mg/l), 13 mM D-Glucose, 5.2 mM NaHCO₃, 30 mM HEPES, and 0.00125% ascorbic acid. Media was changed every 2–3 days. For biolistic transfection, a DNA mixture containing 50 μg of the CRISPR-construct and 30 μg of a GFP-expressing plasmid were precipitated onto 1 μm diameter gold particles, washed with ethanol, and coated onto the inside of Tefzel tubing (Bio-rad). After 2–3 days in vitro, slice cultures were shot with the DNA-coated gold cartridges using 100 PSI ultra-pure helium with a Helios GeneGun (BioRad).

Transfected neurons were identified by the presence of both GFP epifluorescence and a gold particle in the neuronal cell body. Neurons that were GFP positive, but lacking a gold particle or visibly fused with neighboring cells were excluded from analysis.

**Electrophysiology**

Whole-cell voltage clamp recordings were obtained from transfected hippocampal CA1 and CA3 pyramidal neurons as identified by GFP epifluorescence, as well as neighboring untransfected control cells. Initial target sequence validation was performed in neurons transfected via *in utero* electroporation. Transfected CA1 and CA3 neurons were recorded from 300 μm acute, horizontal brain slices of P14–20 mice, cut using a vibratome (Leica Biosystems) in a chilled sucrose cut solution containing (in mM): 2.5 KCl, 7 MgSO₄, 1.25 NaH₂PO₄, 25 NaHCO₃, 7 glucose, 210 sucrose, 1.3 ascorbic acid, and 3 sodium pyruvate. Following cutting, the slices recovered for 30 minutes in 34°C artificial cerebral spinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26.2 NaHCO₃, 11 glucose, 2.5 CaCl₂, and 1.3 MgCl₂. Both the cut solution and ACSF were bubbled with 95% O₂/5% CO₂ gas throughout dissection, slicing and recording. During recording, slices were perfused at a rate of 250 ml/hr with ACSF. 10 μM SR 95531 (Tocris) was added to the ACSF to block inhibition. For recordings from organotypic slice cultures, CaCl₂ and MgCl₂ concentration were both increased to 4 mM and 10 μM 2-chloroadenosine (Tocris) added to prevent runaway excitation following stimulation. Synaptic responses were evoked using electrical stimulation of stratum radiatum from a tungsten bipolar electrode (FHC). To minimize polysynaptic responses, a cut was made at the border of CA3 and CA1 using a microscalpel (Electron Microscopy Sciences). Whole-cell recordings were made using 3–5 MΩ glass pipettes filled with internal solution containing (in mM): 135 CsMeSO₄, 8 NaCl, 10 HEPES, 0.3 EGTA, 5 QX-314, 4 Mg-ATP, 0.3 Na-GTP, and 0.1 spermine, at 294 mOsm and pH 7.34. Whole-cell voltage clamp was performed using a Multiclamp 700B amplifier (Axon instruments) with a 3 kHz Bessel filter, digitized at 10 kHz using a National Instruments data acquisition board, and recorded using custom-written MatLab (MathWorks) software [19].

**Data analysis and statistics**

Data were analyzed offline in Igor Pro (Wavemetrics). For each cell the average peak response at −70 mV holding potential was quantified from 10–20 sweeps as AMPAR mediated current, whereas an equal number of recordings at +40 mV where quantified at 100 ms post-stimulus as NMDAR current. These two values were used to calculate the NMDAR/AMPA ratio, and for each cell the holding potential was switched several times back and forth during the recording, to account for potential changes in the evoked response over time. Different conditions were compared using non-parametric statistical tests, Mann-Whitney test for two groups and Kruskal–Wallis analysis of variance (ANOVA) followed by Dunn’s Multiple Comparison Test for multiple group comparisons.

![Figure 2. CRISPR/Cas9-mediated knock-down in postmitotic neurons.](image-url)

(A) Example traces of AMPAR currents (recorded at −70 mV, inward) and NMDAR currents (recorded at +40 mV, outward) from organotypic slice cultures. Shown are examples from untransfected cells and CC_Grin1.1 transfected cells following 10 days expression (left) and 18 days expression (right). Scale bars are 20 pA (left) or 50 pA (right) and 100 ms. (B) NMDAR/AMPA current ratio for all cells as described before. Untransfected and CC_Grin1.1 transfected cells were compared individually for different time points, using the Mann-Whitney test.

*doi:10.1371/journal.pone.0105584.g002*
Results and Discussion

To test CRISPR/Cas9-mediated gene knockdown in post-mitotic mouse neurons, we targeted 
Grin1, the gene encoding the GluN1 subunit of the N-methyl-D-aspartate-type glutamate receptor 
(NMDAR). This subunit is essential for NMDAR function [20], and the degree of GluN1 loss can be 
easily assayed by the amplitude of synaptic NMDAR currents [21]. Additionally, 
constitutive genomic deletion of 
Grin1 is embryonic lethal [22], whereas a hypomorphic allele shows decreased NMDAR currents 
and severe neural dysfunction early in development [18]. A 
conditional GluN1 knockout mouse has been generated [23], and sparse transfection of Cre has been used to examine the effect of 
NMDAR deletion in individual hippocampal neurons [24,25]. 
Thus, targeting GluN1 provides a well-characterized and robust 
experimental system to assay for the deletion of an essential 
protein in individual neurons of an otherwise unperturbed brain.

We identified two genomic CRISPR/Cas9 target sequences (see 
methods) within or near the region encoding for the second, 
per-forming transmembrane domain of GluN1 (Figure 1A, B). ‘CC_Grin1.1’ is located on the sense strand of exon 14, spanning the 
sequence that encodes the beginning of the second 
transmembrane domain; ‘CC_Grin1.2’ is on the intron anti-sense 
strand between exon 13 and 14, about 130 bp upstream of 
CC_Grin1.1. The appropriate sequences were subcloned into a vector containing all other elements required for CRISPR/Cas9 
mediated knock-down [8]. Identification of target sequences and the 
required cloning required less than three days, emphasizing the 
simplicity and effectiveness of this approach.

To functionally assay for NMDAR-deletion, CC_Grin1.1 and 
CC_Grin1.2 were transfected by 
in-utero electroporation into the 
hippocampus of wildtype mice, and a plasmid encoding GFP was 
co-transfected at a 1:10 ratio. We measured the ratio of NMDAR-
amplified AMPAR-mediated excitatory postsynaptic currents (EPSCs) in 
untransfected pyramidal neurons was 0.61±0.04 (n = 36), and 
transfection of a CRISPR/Cas9 construct lacking the targeting 
sequence (gX330) had no effect on this ratio (0.53±0.03, n = 8). In contrast, cells transfected with CC_Grin1.1 showed complete 
loss of NMDAR currents (0.04±0.01, n = 12). Importantly, the functional loss of NMDAR by CC_Grin1.1 was observed in every 
cell tested, demonstrating effective CRISPR/Cas9 mediated 
knockdown with full penetration and suggesting disruption of both 
genomic alleles. Using the same approach with CC_Grin1.2 did 
not show any effect (0.67±0.11, n = 10), and every cell tested 
showed robust NMDAR currents (Figure 1C). Together, the 
results demonstrate that CRISPR/Cas9-mediated knockdown can be 
achieved in acutely 
transfected post-mitotic neurons.

Acute knockdown in the brain requires genetic modification in 

Acknowledgments

The authors thank all members of the Sabatini lab for helpful discussions.

Author Contributions

Conceived and designed the experiments: CS AG BS. Performed the 
experiments: CS AG JS. Analyzed the data: CS AG. Contributed 
reagents/materials/analysis tools: CS AG JS. Contributed to the writing 
of the manuscript: AG CS BS JS.

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database of essential genes that includes both protein-coding genes and 
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