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CRISPR/Cas9 system-mediated impairment of synaptobrevin/VAMP function in postmitotic hippocampal neurons

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Abstract

Background—The use of the CRISPR/Cas9 system is becoming widespread, however current studies have predominantly focused on dividing cells. It is currently unknown if CRISPR/Cas9 can be used in a postmitotic setting to examine non-cell autonomous/presynaptic phenotypes in the resulting genetically heterogeneous cell population.

New method—A single CRISPR/Cas9 lentivirus was used to transfect a high percentage of primary cultured neurons and target synaptobrevin 2 (Syb2, also called VAMP2).

Results—Primary hippocampal cultures infected with the Syb2 targeting virus displayed dramatic reductions in Syb2 protein and immunocytochemical staining. In many boutons Syb2 was completely undetected. These cultures recapitulated the known functional phenotypes of Syb2 knockout neurons, which are non-cell autonomous and presynaptic in origin, indicating that Syb2 was knocked out in a large fraction of neurons.

Comparison with existing method(s)—Previous methods used multiple viruses or sparse transfection methods and only examined cell autonomous or postsynaptic phenotypes. The current method demonstrates that the CRISPR/Cas9 system can be used to alter network dynamics by removing or lowering the target gene from a majority of cells in the culture.

Conclusions—A combination of CRISPR/Cas9 system and single high efficiency lentivirus infection can be used to examine non-cell autonomous and presynaptic phenotypes in postmitotic neurons.

Graphical Abstract

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Conflicts of interest

The authors declare no conflicts of interest with regard to this work.

Keywords

neurons; gene knockout; synaptobrevin; synaptic transmission

1. Introduction

The CRISPR/Cas9 system is a powerful molecular tool used to edit the genome via creation of a double stranded break at a specified genomic locus (Jansen et al., 2002; Jinek et al., 2012). Since its introduction as a biological tool, the CRISPR/Cas9 system has been harnessed to create model organism genetic knockouts (KO), label chromatin, and even modulate gene activity (Dahlman et al., 2015; Konermann et al., 2015; Platt et al., 2014; Sung et al., 2014; Wang et al., 2016). Advantages of the CRISPR/Cas9 system include its ease of use and versatility, making it the tool of choice for genetic manipulation.

At its most basic level, Cas9 is directed to a specific genomic site by a guide RNA molecule whereon it cleaves both strands of DNA. This double stranded break is then repaired by the cell using either homology directed repair (HDR) or non-homology-end-joining (NHEJ). HDR accurately repairs the break by using the homologous chromosome or provided homologous DNA, whereas NHEJ causes the insertion or deletion (indel) of base pairs at the site of cleavage. Consequently, a plethora of indels are possible any time NHEJ is used. Moreover, although both pathways are used by dividing cells, HDR is most prevalent in the S and G2 phases of the cell cycle (Pardo et al., 2009; van Gent and van der Burg, 2007), while NHEJ is assumed to be the major repair pathway used in postmitotic cells. Because multiple different mutations may be formed at the double stranded break, the vast majority of work utilizing the CRISPR/Cas9 system has been done in dividing cells. This allows the user to select a cell with the mutation they want and then clone or create a cell line from it so as to obtain a genetically homogenous population. However, the use of CRISPR/Cas9 in non-dividing cells, such as postmitotic neurons, has not been as extensively explored.

Previous studies have used CRISPR/Cas9 in postmitotic neurons to examine cancer generation (Platt et al., 2014), hair cell manipulation (Zuris et al., 2015), and even create region specific inducible knock outs (de Solis et al., 2016). To date, four groups have successfully used CRISPR/Cas9 to KO proteins and examine neuronal function. Incontro *et al.* demonstrated that CRISPR could successfully delete the GluN1 subunit of the NMDA receptor and the GluA2 subunit of the AMPA receptor in hippocampal slice culture

(Incontro et al., 2014). The authors used a gene gun to sparsely transfect neurons and record from transfected and neighbor cells and found that transfected cells displayed deficits in NMDA or AMPA current, depending on which receptor subunit had been targeted. Straub *et al.*, also used a gene gun to sparsely transfect neurons in hippocampal slice culture to target GluN1 and saw a similar deficit in NMDA current (Straub et al., 2014). Swiech *et al.* used an adeno-associated virus (AAV) version of CRISPR/Cas9 to delete the methyl-CpG binding protein 2 (MeCP2) *in vivo* from the dentate gyrus and visual cortex (Swiech et al., 2015). This group injected the target region with two separate viruses, one carrying the Cas9 gene, and one carrying the guide RNA and recorded firing rate, a postsynaptic phenotype, from transfected cells and neighboring control cells. More recently, Wei *et al.* used calcium phosphate to sparsely transfect cultured hippocampal neurons and KO α/β -Hydrolase domain-containing 6 (ABHD6) (Wei et al., 2016). Again, they recorded from transfected cells or un-transfected controls and measured postsynaptic cell autonomous phenotypes.

Although these studies used CRISPR/Cas9 to create functional KOs of different genes in postmitotic neurons, they all examined postsynaptic and cell autonomous phenotypes and had low transfection rates. Therefore, it remains an open question whether the CRISPR/Cas9 system can be used to generate high levels of functional gene KO required to evaluate presynaptic and non-cell autonomous phenotypes in postmitotic neurons. To address this question we used a lentiviral CRISPR/Cas9 construct to target synaptobrevin2 (Syb2, also called VAMP2) in primary hippocampal cultures. Syb2 is a presynaptic soluble NSF attachment protein receptor (SNARE) protein required for synaptic vesicle release (Baumert et al., 1989; Schiavo et al., 1992; Sudhof et al., 1989; Trimble et al., 1988) with a well-characterized loss-of-function phenotype (Deak et al., 2004; Schoch et al., 2001). Specifically, Syb2 KO hippocampal cultures display decreased miniature excitatory postsynaptic currents (mEPSCs) frequency and reduced evoked EPSC amplitudes (Schoch et al., 2001). Heterozygous mice display no overt phenotypes consistent with the fact that synaptic vesicles contain a large number (70 – 100) of Syb2s (Schoch et al., 2001; Takamori et al., 2006), and only a small number of Syb2s are sufficient to execute fusion (Mohrmann et al., 2010; Sinha et al., 2011). This extensive redundancy necessitates a complete loss-of-function strategy to examine Syb2's function. Therefore we specifically tested the ability of a lentiviral CRISPR/Cas9 construct to fully KO Syb2 in postmitotic neurons.

2. Materials and Methods

2.1 Animals

C57BL/6 pups post-natal day 1–3 were used for all experiments. Animal procedures conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center.

2.2 Plasmid Preparation

Five test single guide RNAs (sgRNA) were designed targeting exon 2 of the synaptobrevin 2 (Syb2) gene using the online tool CRISPR.MIT.edu. sgRNAs were cloned into the LentiCRISPRv2 (Sanjana et al., 2014; Shalem et al., 2014) (Abcam) plasmid using the BsmBI cloning sites. Following lentivirus production, these 5 guides were tested by

infecting primary mouse hippocampal cultures with each guide and measuring Syb2 protein levels by western blot after 16 days *in vitro* (DIV16) unless otherwise noted. The most effective guide had the following sequence: CCGTCCCGCCTGCCGCCCCG, and was used in all experiments described in this manuscript. A control sequence (AACGCCTTTCCTTTCATCTT) was also cloned into LentiCRISPRv2 plasmid and used in all experiments.

2.3 Lentivirus production

HEK 293-T cells were transfected with FuGENE6, necessary viral coat and packaging protein constructs (pVSVG, pRsv-Rev, and pPRE), and LentiCRISPRv2 (Sanjana et al., 2014; Shalem et al., 2014) (Abcam) with one of five test sgRNAs, a control sequence, or with no guide. Before transfection, HEK 293-T cell media was replaced with 0 μ M cytosine arabinoside (ARAC; Sigma) media. On DIV3 virus was harvested from the HEK 293-T cells and added directly to neuronal media at DIV4.

2.4 Cell Culture

Dissociated hippocampal cultures were prepared as previously described (Kavalali et al., 1999). Whole hippocampi were dissected from PN1–3 mice, trypsinized (~4 mg/mL, Sigma), mechanically dissociated, and plated on matrigel (BD Biosciences) coated glass coverslips. Neurons were plated in MEM containing 27.8 mM of Glucose, 2.4 mM of NaHCO₃, 1.3 μ M of Transferrin (Calbiochem), 2 mM of L-Glutamine, 4.4 μ M of insulin, and 10% FBS. On DIV1, FBS concentration was reduced to 5%, L-Glutamine concentration was reduced to 500 μ M, and 1 $\times$ B-27 supplement (Gibco) and 4 μ M of cytosine arabinoside (ARAC; Sigma) were added. On DIV4 the concentration of ARAC was reduced to 2 μ M. Also on DIV4, cells were infected with 100 μ L of lentivirus carrying Cas9 and the specified guide strand targeting the Syb2 gene locus. Cells were maintained at 37°C in 5% CO₂ atmosphere.

2.5 Western Blotting

On DIV6, 12, 16, or 20, cells were collected in 125 μ L PBS and 125 μ L Laemmli sample buffer (BioRad). 15 μ L of cell lysate was run on an SDS/PAGE gel and then transferred to a nitrocellulose membrane using the Trans-Blot Turbo transfer system (BioRad). Primary antibodies were used at the following concentrations: GAPDH (14C10; Cell Signaling) 1:50,000; synaptobrevin2 (104 211; Synaptic Systems) 1:5,000; synaptotagmin1 (105 011; Synaptic Systems) 1:5,000; synapsin1 (106 103; Synaptic Systems) 1:2,000; Vti1a (611220; BD Biosciences) 1:500. After incubation in primary antibodies at 4°C overnight, membranes were incubated in secondary antibodies at the following concentrations: GAPDH 1:10,000 (anti-rabbit); synaptobrevin2 1:5,000 (anti-mouse); synaptotagmin1 1:5,000 (anti-mouse); synapsin1 1:5,000 (anti-rabbit); Vti1a 1:5,000 (anti-mouse). Protein bands were detected using ECL and exposure to film. Quantification was performed using ImageJ to detect protein band intensities. Values were normalized to GAPDH before comparison.

2.6 Immunocytochemistry and imaging

Cells were fixed on glass coverslips by incubation in 4% paraformaldehyde at room temperature for 20 minutes on DIV20. Coverslips were then incubated in blocking solution (1% albumin, 125 μ M of saponin, and 2% FBS in PBS) at room temperature for 2 hours. Following block, coverslips were incubated in primary antibodies at 4°C overnight. Primary antibodies were used at the following concentrations: synaptobrevin 2 (104 211; Synaptic Systems) 1:1,000; synapsin 1 (106 103; Synaptic Systems) 1:1,000. The next day, coverslips were incubated in fluorescent secondary antibody at room temperature for 90 minutes. Secondary antibodies were used at the following concentrations: Alexa Fluoro 488 goat anti-rabbit IgG (A-11008, ThermoFischer) 1:500; Alexa Fluoro 594 goat anti-mouse IgG (A-11005, ThermoFischer) 1:500. Cells were then stained with DAPI nuclear stain diluted 1:10,000 in PBS at room temperature for 10 minutes. Coverslips were mounted on glass slides using Aqua Poly/Mount (18606; Polysciences Inc.).

Following mounting, cells were imaged on a Nikon ECLIPSE 80i Upright microscope using a 20 $\times$ objective and NIS-element software. Representative images in figure 2 are cropped from full images in order to better highlight the punctate staining. Quantification was completed on full images using ImageJ software. Intensities were compared by multiplying the area by the mean intensity of an image to obtain total intensity. Total Syb2 intensities were normalized to synapsin1 intensities before comparison. For particle analysis, synapsin1 images were thresholded using the percentile method and then the particle analyzer was used to find particles $1\mu\text{m}^2$, which corresponded to synaptic boutons. These regions of interest (ROIs) were then transferred to the corresponding Syb2 image and the intensity of each particle was calculated. In all image analysis, background intensity (determined by averaging intensity of coverslips which were not stained with primary antibody) was subtracted.

2.7 Electrophysiology

Electrophysiology was performed as previously described (Nelson et al., 2006). On DIV16–21 cells were recorded using the whole cell voltage clamp configuration of the patch clamp technique. Extracellular Tyrode solution, adjusted to pH 7.4 and 319 mOsm, contained: 150 mM of NaCl, 4 mM of KCl, 2 mM of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mM of glucose, 10 mM of HEPES, and 2 mM of Ca^{2+} . Intracellular pipette solution, adjusted to pH 7.3 and 304 mOsm, contained: 115 mM of Cs-methanesulphonate, 10 mM of CsCl, 5 mM of NaCl, 10 mM of HEPES, 20 mM of TEA.Cl hydrate, 4 mM of MgATP, 0.3 mM of GTP, and 0.6 mM of EGTA. Miniature excitatory postsynaptic currents (mEPSCs) were measured in extracellular Tyrode solution supplemented with 1 μ M of tetrodotoxin and 50 μ M of picrotoxin. mEPSCs were analyzed using MiniAnalysis software. For evoked EPSC recordings the peak amplitude of responses to a train of stimulations of a single cell was measured in extracellular Tyrode solution. Cells were stimulated by bath stimulation through parallel platinum electrodes for 100 pulses at 1Hz. Evoked EPSCs were analyzed using pCLAMP10 software.

2.8 Statistical Analyses

All statistical analyses were performed using GraphPad Prism 7 software. One-way ANOVA's were performed to compare not infected, control guide infected, and Syb2 guide infected groups in all experiments. Comparisons between groups were made using Tukey's *post hoc* multiple comparisons test. Paired t-tests were used to compare each time point in figure 2D. The Kolmogorov-Smirnov (K-S) test was used to compare frequency distributions in figure 3C–D. A *p* value of $< .05$ was considered statistically significant in all experiments. For immunostaining particle analysis histograms were also created using GraphPad Prism software.

3. Results

3.1 Lentiviral CRISPR system reduces overall protein level in neuronal cultures

To test the ability of the lentiviral CRISPR/Cas9 system to alter gene expression in neuronal cultures, we infected dissociated hippocampal cultures on DIV4 with a construct expressing Cas9 and a sgRNA. We collected total protein on DIV16, or 12 days post infection (dpi). Levels of Syb2 were reduced in the Syb2 guide infected compared to not infected ($p < .0001$) and control guide infected cultures ($p = .0054$) (Fig. 1A–B; one-way ANOVA $F_{(2, 44)} = 14.39$, $p < .0001$; Syb2 guide: $9.5 \pm 3.1\%$ of not infected, $n = 16$; control guide: $70.0 \pm 18.0\%$ of not infected, $n = 15$; not infected: $100 \pm 11.8\%$ of not infected, $n = 16$). There were no significant differences in Syb2 expression between not infected and control guide infected cultures. In order to ensure that Syb2 levels were not decreasing due to neuronal death or a decrease in synaptic boutons, we also measured the protein levels of three other presynaptic proteins: synaptotagmin1 (Syt1), synapsin1, and Vti1a. There was a small decrease in Syt1 levels in control guide ($p = .0429$) and Syb2 guide ($p = .0131$) conditions compared to uninfected cultures (Fig 1C; one-way ANOVA $F_{(2, 44)} = 5.113$, $p = .0101$; Syb2 guide: $60.1 \pm 12.2\%$ of not infected, $n = 16$; control guide: $73.8 \pm 16.9\%$ of not infected, $n = 15$; not infected: $100 \pm 13.8\%$ of not infected, $n = 16$). Although there were no significant differences in the level of synapsin1 or Vti1a, we did notice slight decreases in these proteins in the control guide and Syb2 guide infected cultures compared to not infected cultures as well (Fig. 1D–E).

The guide strands used in these experiments were selected because of their low likelihood of alternative genomic targets. However, given that we observed a small decrease in protein levels following infection with the target and control guide, we examined whether the decrease could be due to expression of Cas9 itself. Indeed we found that expressing our lentiviral CRISPR/Cas9 construct without any guide produced a similar decrease in protein levels as the guide infected cultures alone. These data demonstrate the decrease in protein levels in the guide infected cells was not due to a nonspecific effect but rather that expression of Cas9 in postmitotic cells can impact protein expression (Fig 2B–C).

In initial experiments we had determined that 12 dpi produced a robust decrease in Syb2 protein expression. Given that our data revealed Cas9 expression by itself could impact protein expression, we sought to more fully examine whether the decrease in protein expression was stable. We infected neurons with the guide or Syb2 construct and determined

Syb2 protein expression at 4 dpi, 8 dpi, 12 dpi, and 16 dpi. We found that 4 dpi and 8 dpi were not sufficient to produce a robust KO of Syb2, however Syb2 was maximally deleted by 12 dpi and this level of reduction was indistinguishable at 16 dpi (Fig 2D; 12 dpi: paired t-test $t_{(5)} = 4.294$, $p = .0078$; 16 dpi: paired t-test $t_{(3)} = 3.732$, $p = .0335$).

3.2 Syb2 is completely removed from presynaptic terminals

Immunocytochemistry was used to visualize the distribution of Syb2 in neurons. An analysis of the overall intensity of Syb2 showed that there was no difference in Syb2 intensity between the not infected and control guide infected cultures. In contrast, Syb2 guide infected cultures had significantly reduced levels of Syb2 compared to not infected ($p < .0001$) and control guide infected cultures ($p < .0001$) (Fig. 3A; one-way ANOVA $F_{(2,33)} = 48.04$, $p < .0001$; Syb2 guide: 34.8 ± 2.5 % of not infected, $n = 12$; control guide: 106.7 ± 7.7 % of not infected, $n = 14$; not infected: 100 ± 4.3 % of not infected, $n = 10$). A decrease in Syb2 protein levels and overall staining intensity could be due to either the amount of Syb2 reduced in all neurons or Syb2 completely knocked out from some neurons and present in others.

In order to investigate these two alternatives, we analyzed individual synaptic boutons in our images. Boutons were identified by synapsin1 staining in region of interest (ROIs) less than $1\mu\text{m}^2$ in area, and then the intensity of Syb2 was measured at each of these ROIs. There were no differences in the bouton densities between groups (Fig 3B). Additionally, the number of nuclei identified by DAPI staining did not vary between groups. The average synapsin1 intensities per bouton did not vary between groups (Fig 3C inset), and distributions showing synapsin1 intensity at each bouton clustered at approximately the same value. There were no differences in the average Syb2 intensities between not infected and control guide infected cultures. However, the average Syb2 intensity per bouton was significantly lower in the Syb2 guide infected cultures compared to not infected ($p < .0001$) and control guide infected cultures ($p < .0001$) (Fig 3D inset; one way ANOVA $F_{(2,33)} = 55.42$, $p < .0001$; Syb2 guide: 7.97 ± 1.37 ; control guide: 29.4 ± 2.35 ; not infected: 34.8 ± 1.25). Indeed, the distribution of Syb2 intensity across boutons in cultures infected with the Syb2 guide virus was drastically shifted to the left (Fig. 3D). Importantly, when we tested for significance between the distributions using the Kolmogorov-Smirnov test, given the sensitivity of this test, we detected a significant difference among all distributions ($p < .0001$). However the test statistic Dn values — a measure of the least upper bound normalized distance between the distributions — comparing Syb2 guide to not infected (0.8858) and control guide (0.7671) were much higher than the comparison between not infected and control guide infected cultures (0.1816). Additionally, the Dn value comparing not infected and control guide infected syb2 intensity distributions was very similar to those found when comparing synapsin1 intensity distributions (Fig. 3B; not infected vs. control guide K-S test = 0.2273; not infected vs. Syb2 guide K-S test = 0.1698; control guide vs. Syb2 guide K-S test = 0.0602). Interestingly, the Syb2 guide distribution clustered strongly around 0 suggesting that in many of the boutons measured, Syb2 was completely absent and had been fully knocked out. Additionally, a large majority of boutons in the Syb2 guide infected cultures had Syb2 intensities below half of the mean intensity of the not infected

and control guide infected cultures. This likely indicates that in most Syb2 guide infected cells at least one Syb2 allele was targeted and disrupted by CRISPR/Cas9.

3.3 Syb2 Guide Infected cultures recapitulate functional phenotypes of Syb2 KO

To further assess the effectiveness of the lentiviral CRISPR/Cas9 system to KO genes in postmitotic neurons we assessed the functional phenotype of the Syb2 guide infected neurons. Specifically, Syb2 is a presynaptic protein involved in vesicle fusion (Schiavo et al., 1992; Schoch et al., 2001). Syb2 KO neurons show decreases in miniature excitatory postsynaptic current (mEPSC) frequency, as well as severely decreased evoked release (Schoch et al., 2001). Because these phenotypes are presynaptic in origin, but measured on a postsynaptic cell, it is necessary for a majority of cells to be missing Syb2 in order to detect them. Importantly, we did not select for transfected cells when recording synaptic phenotypes.

In our first set of experiments, we measured mEPSCs. As is seen in Syb2 KO cells, the Syb2 guide infected neurons had decreased mEPSC frequency compared to not infected ($p = .0106$) and control guide infected neurons ($p = .0071$) (Fig. 4B; one-way ANOVA $F_{(2, 30)} = 6.71$, $p = .0039$; Syb2 guide: 0.62 ± 0.17 Hz, $n = 10$; control guide: 2.58 ± 0.50 Hz, $n = 12$; not infected: 2.52 ± 0.45 Hz, $n = 11$). There was no difference in mEPSC frequency between not infected and control guide infected neurons. A decrease in mEPSC frequency could be due to either decreased spontaneous presynaptic vesicle fusion or decreased synapse number. Since we did not detect any difference in synaptic bouton density between conditions (Fig. 3B), the decrease in mEPSC frequency indicates a decrease in spontaneous presynaptic vesicle fusion. We did not detect any difference in mEPSC amplitude between any of the groups (Fig. 4C).

Next we measured evoked EPSCs. Unlike Syb2 KO neurons, Syb2 guide infected neurons still responded to stimulation with no failures seen. However, the average peak amplitude of the evoked EPSCs was reduced by more than half in Syb2 guide infected neurons compared to not infected ($p = .0168$) and control guide infected neurons ($p = .0030$) (Fig. 5B; one-way ANOVA $F_{(2, 40)} = 7.04$, $p = .0024$; Syb2 guide: 881 ± 150 pA, $n = 14$; control guide: 2137 ± 274 pA, $n = 15$; not infected: 1925 ± 304 pA, $n = 14$). There was no difference in evoked EPSC peak amplitude between not infected and control guide infected neurons. The decrease in peak EPSCs in Syb2 guide infected neurons does not appear to be the result of decreased synapse number, as no difference in bouton density was observed (Fig. 3B). It is also not a result of decreased postsynaptic receptors as there is no difference in mEPSC amplitude between conditions (Fig. 4C). The decrease in evoked EPSC peak without complete failure of response is likely due to the fact that, as shown with immuno-staining, there are neurons in the culture that continue to express Syb2. These cells should supply the normal presynaptic vesicle fusion events in response to the stimulus while the neurons completely lacking Syb2 would fail to respond, and thus would cause an average lowered response seen in the postsynaptic cell.

4. Discussion

This study demonstrates that lentivirus expressing the CRISPR/Cas9 system can be used to infect primary neuronal cultures to delete a gene of interest at high efficiency. We successfully showed that the lentiviral CRISPR/Cas9 system could knockout Syb2 in primary hippocampal neuronal culture and replicate the presynaptic non-cell autonomous phenotype of Syb2 loss-of-function seen in genetic knockout mice. Specifically, Syb2 guide infected cells had reduced mEPSC frequency and evoked EPSC peak amplitude without any reduction in synaptic terminal number or alteration in mEPSC amplitude. To our knowledge this is the first time the CRISPR/Cas9 system has been used to investigate presynaptic or non-cell autonomous phenotypes in postmitotic neurons.

To examine whether a specific gene has cell autonomous effects, or impacts presynaptic function, it is critical that a large majority of cells carry the desired manipulation. We have previously observed that this lentivirus system can transfect primary neurons at high levels of infectivity (Leitz and Kavalali, 2011; Nelson et al., 2006). This is supported in the current study by the dramatic reduction in the Syb2 level in Syb2 guide infected cultures (Fig 1–3). Since the infected cells in our cultures were genetically heterogeneous at the Syb2 location, it was unclear whether all of the individual indels would be able to disrupt gene function enough for the entire culture to act as a KO. Our data suggests that this is indeed possible using the lentiviral CRISPR/Cas9 system which opens the door for the use of this system as an alternative to knock down approaches.

There are key differences between the CRISPR/Cas9 system and knock down approaches such as RNAi, in that the CRISPR/Cas9 system targets the genome. Therefore, with CRISPR/Cas9 any reduction in gene expression is the result of gene knock out rather than knock down and will most likely be complete and permanent as observed in our system via immunocytochemistry (Fig 3). This conclusion is further supported by the analysis of individual synaptic boutons in which cultures infected with the Syb2 guide revealed numerous boutons lacking Syb2 expression. We were also able to detect a reduction in mEPSC frequency (Fig 4) and evoked EPSC peak amplitude (Fig 5) demonstrating the KO was sufficient to impact synaptic transmission. Rather intriguingly these phenotypes are not seen in Syb2 knock down or Syb2^{-/+} cultures suggesting that only a small amount of Syb2 is needed to carry out its essential functions.

In the current CRISPR/Cas9 study, we used a virus with a control guide rather than neighboring un-transfected cells as a control. This approach has the advantage of assessing potential off target effects of Cas9. We did observe a slight decrease in protein level in all of our infected groups. In fact, over expressing Cas9 without any guide also replicates this small protein decrease (Fig 2). However, this decrease is not on the order of the targeted protein, Syb2. Additionally, the Syb2 guide infected cells recapitulated the known Syb2 KO phenotypes, decreased mEPSC frequency and evoked EPSC peak amplitude, without alterations in mEPSC amplitude or synapse number suggesting specificity of the system. The rapid development of new CRISPR/Cas9 constructs, which are specialized to reduce off target effects (Kleinstiver et al., 2016; Slaymaker et al., 2016), should further ensure proper fidelity and decrease toxicity.

5. Conclusions

In the current study, we demonstrated that lentivirus expressing the CRISPR/Cas9 system can be used to efficiently delete an endogenous gene in postmitotic neurons. Lentivirus has several advantages in that it is relatively safe (Biosafety Level 2), can infect a wide variety of cell types depending on the pseudotype used, and can hold larger inserts than adeno-associated virus. Our current data suggests lentivirus is an efficient tool for delivery of CRISPR/Cas9 to neurons for high levels of KO to examine non-cell autonomous phenotypes.

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Highlights

- Use of lentivirus to express CRISPR/Cas9-mediated gene editing in postmitotic neurons.
- -Ability to detect cell autonomous and non-cell autonomous effects.
- -Examination of presynaptic as well as postsynaptic phenotypes.

Figure 1.

Syb2 protein, but not other pre-synaptic proteins, is depleted in syb2 guide infected cultures.

(A) Representative blots of protein levels in not infected, control guide infected, and Syb2 guide infected lanes. Samples are lysates from dissociated hippocampal cultures DIV 16.

(B–E) Quantification of western blot images. Bar graphs are average $\pm$ SEM, ** indicates $p < .01$, **** indicates $p < .0001$, n.s. indicates values are not significantly different from each other.

Figure 2.

Cas9 only infection has similar effect as control guide infection. (A) Representative blots of protein levels in not infected, Cas9 only infected, control guide infected, and Syb2 guide infected lanes. (B) Quantification Syb2 and (C) Syt1 levels from western blot images. Samples are lysates from dissociated hippocampal cultures DIV 16. (D) Time course of Syb2 and Syt1 protein levels 4, 8, 12, and 16 days post infection (dpi). Syb2 levels are maximally deleted by 12 dpi (DIV 16). All graphs are average $\pm$ SEM, * indicates $p < .05$, ** indicates $p < .01$, n.s. indicates values are not significantly different from each other.

Figure 3.

Syb2 is fully deleted from presynaptic terminals. (A) Representative images of synapsin1, syb2, and DAPI staining of not infected, control guide, and syb2 guide infected cultures. Scale bar is 100 μm. (B) No difference was detected in the number of puncta identified in each image. (C) Histograms showing synapsin1 and (D) Syb2 intensities in individual synaptic boutons identified from synapsin1 staining. Insets are averaged intensities of all puncta in each image analyzed. Bar graphs are average $\pm$ SEM, n.s. indicates values are not significantly different from each other.

Figure 4.

Syb2 guide infected cultures have lowered mEPSC frequency. (A) Representative traces of whole cell patched voltage clamp mEPSC recordings. (B) Quantification of mEPSC frequency and (C) amplitude. mEPSC frequency, but not amplitude, is decreased in syb2 guide infected cultures. Bar graphs are average $\pm$ SEM, * indicates $p < .05$, ** indicates $p < .01$, n.s. indicates values are not significantly different from each other.

Figure 5.

Syb2 guide infected cultures have lowered average evoked EPSC peak amplitudes (A) Representative averages of a cell's response to a train of 100 stimulations at 1Hz. (B) Quantification of whole cell patched voltage clamp evoked EPSC recordings. Average peak amplitude is decrease in syb2 guide infected cultures. Bar graphs are average $\pm$ SEM, * indicates $p < .05$, ** indicates $p < .01$.