CRISPR/dCas9-Mediated Upregulation of Reelin and Neuronal Excitability

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Abstract

Reelin is an extracellular matrix protein involved in brain development, synaptic plasticity, and long-term potentiation. Overexpression of *Reln* mRNA, which codes for Reelin protein, enhances long-term potentiation and memory formation, and *Reln* dysfunction plays a role in many neuropsychiatric disorders including Alzheimer's disease, autism spectrum disorders, and schizophrenia. However, the specific mechanisms by which Reelin regulates these processes is currently unclear. Previous strategies to upregulate *Reln* and other genes relied on genetic manipulations such as global overexpression or knockout. Here, we employ a CRISPR-dCas9 strategy to alter gene expression through recruitment of transcription activators to understand how manipulating the endogenous *Reln* locus will impact gene expression and neuronal function. Using a specific guide RNA (gRNA) targeting the promoter of *Reln* in cultured hippocampal neurons, we found that recruitment of the transcriptional activator VPR to the *Reln* promoter induced robust and selective upregulation of *Reln* mRNA. To explore whether *Reln* upregulation altered neuronal physiology, hippocampal neurons expressing CRISPR components were plated on multi-electrode arrays to record neuronal activity, bursting, and network synchrony. Hippocampal neurons overexpressing *Reln* appear to exhibit increased spontaneous activity, suggesting that *Reln* manipulation may globally alter neuronal excitability. Our results demonstrate that CRISPR-dCas9 tools are an effective way to control the *Reln* gene expression and impact neuronal physiology, and also suggest that these tools could be useful in further understanding *Reln* expression and signaling.

Introduction

Many chemical interactions are involved in cultivating the developing brain, but one of the most critical molecules in this process is the extracellular matrix protein, Reelin. Reelin plays a modulatory role in brain functions such as synaptic plasticity, dendrite development, and long-term potentiation (1). Reelin is multifunctional; it is involved in the growth, maturation, and activity of neurons in the adult brain. The *Reln* gene is very large, taking up about 450 kb of genomic DNA (19). It is also fairly complex, and the mechanisms that regulate *Reln*, as well as the molecular function of Reelin, are still poorly understood. It is, however, understood that Reelin is needed to promote axon projection growth and a lack of *Reln* is linked to abnormalities in dendrite development, specifically a delay in dendrite development (4). Both increases and decreases in the expression of Reelin have been shown to affect neural function (16-19). A lack of Reelin has been associated with abnormalities in dendrite growth and with disruption during brain formation (6). During the development of the central nervous system in the embryonic stage, Reelin is secreted by Cajal Retzius cells and functions as a marker for positioning during brain development (3). Disruption in the production of Reelin also disrupts many signaling pathways, resulting in a morphologically abnormal brain (7-8).

The capacity of the brain to modulate synapses at a molecular level allows for plasticity within the brain and higher cognitive functions. In the adult brain, chronic increases in Reelin lead to increases in dendritic spine density (16). Reelin application has been correlated with spine density as well as Dab1 and CREB activation, both of which are

genes important to memory formation. *Reln* not only has the ability to promote growth and supplement LTP, but also aids in pruning dendritic spines in cortical neurons. Reelin has been found to accumulate around synapses, and a large pool of work now exists to support the idea that one primary function of Reelin may be in synaptic function (18). There are several electrophysiological studies using neuronal cultures, which have shown that Reelin can increase synaptic activity by affecting NMDA receptor activity. Long term Reelin treatment has been found to alter the composition of NMDA receptors, causing it to mature and reduce inactive synapses (19). These and other studies are only a few examples of the importance of Reelin in LTP.

Another key component of memory formation and maintenance involves epigenetic modifications that alter translation and transcription of genetic material (1). Prior research has also shown that modulating gene expression through epigenetics can potentially promote cellular behaviors indicative of brain plasticity. Epigenetic mechanisms function as important regulatory processes that affect both genomic architecture and gene expression, and are often implicated in neuropsychiatric disease states. Strikingly, the expression of Reelin is heavily regulated by promoter DNA methylation, a critical epigenetic modification. This observation has led to subsequent interest in modulation of the *Reln* gene since reduced expression of *Reln* is associated with disorders such as Schizophrenia, bipolar disorder, and Autism (9-11, 20). Research has suggested that downregulation of *Reln* is related to vulnerability to psychosis (9). Postmortem brain studies have indicated that *Reln* mRNA is reduced in brains from both bipolar and schizophrenia patients, which has contributed to the idea that *Reln* dysfunction is related to the pathogenesis of psychiatric disorders (20). Bipolar disorder and Schizophrenia have been found to share similar neural mechanisms; both of these disorders have been characterized as having a relationship to abnormal expression of *Reln* (20). Additionally, reduction in Reelin has been reported in studies on autism spectrum disorders. Single nucleotide polymorphisms that result in decreased Reelin in the brain have been identified as risk factors for both autism spectrum disorders, as well as Schizophrenia (20). This potential relevance to neuropsychiatric disease highlights the importance of understanding how *Reln* functions both during brain development and also in the adult central nervous system.

Previous methods to modulate the *Reln* gene have relied on methods such as virally mediated knockdown, overexpression, or protein infusion. While these are all effective ways to study *Reln* and the role it assumes, these approaches neglect the endogenous gene and lack the specificity to define what changes must occur to produce a change in the genes expression. We know that *Reln* is heavily regulated by DNA methylation of the promoter region (19); modulation of the endogenous gene may alter the epigenetic landscape in a way that promotes learning. The objective of this study was to target changes to the *Reln* gene itself and measure changes in cellular function. A catalytically deactivated CRISPR system is used to allow tethered proteins to provide gene specific epigenetic regulation.

CRISPR originates from an immune mechanism in bacteria (12). To carry out an immune response, these small organisms can take up invading DNA fragments and first incorporate them into the CRISPR locus. A host CRISPR locus incorporates the invading fragments as spacers between crRNA. When Cas proteins are expressed the sequence is transcribed into pre-crRNA. The pre-crRNA is cleaved and processed, at this point it functions as a guide to target the invading DNA, the spacer sequences allow it to be

recognized by the Cas proteins. The Cas protiens are able to locate the target with the guide sequence and cleave the invading genetic material, protecting the host (13). CRISPR has been adapted into a tool that is becoming widely popular due to the ability to precisely edit and manipulate the genome (15). To edit the regulation of genomic information CRISPR uses two parts, a DNA binding sequence and an effector that regulates transcription near the location of the binding site (15). Cas9 is a commonly used DNA binding protein; it can be made to target a different site by changing the attached gRNA sequence (14). For sequence specific regulation, Cas9 can be tethered to effectors such as transcriptional activators, repressors, or epigenetic modifiers. dCas9 is utilized in this project, it is typically used to study proteins that interact with specific loci (14). It is different from Cas9 because it is a mutated version of the molecule that sits on the gene as opposed to cleaving elements out. However, it was still tethered to an effector, in this case VPR, a strong transcriptional activator. Various versions of Cas-9 mediated gene editing systems have been used to study the roles of specific genes, making models of diseases, and may be later implemented into gene therapy techniques. In this study, CRISPR/dCAS9 was utilized to target the Reln gene and upregulate its activity in order to investigate how the gene is relevant to cellular function in a hippocampal culture, and eventually gather insight into the relationship of the gene to LTP and brain development.

Method

C6 Cell Experiments: C6 cells are part of a cell line that is cultured for these experiments. At about 80% confluence, cells were nucleofected in order to introduce plasmids containing CRISPR constructs. C6 media was aspirated and cells were washed with 5mL of PBS prior to detachment with 0.25% trypsin. Once detached, 5mL of warm media was added to stop the reaction. The cells were transferred to a tube and spun in a centrifuge at 900rpm for 5 minutes. The media was aspirated and then fresh media was added. Some media and cells were transferred to a new T75 flask and placed in the incubator. The remaining cells were spun in the microcentrifuge at 200g. The media was then removed and 100uL of nucleofection buffer was added along with the DNA. Contents were transferred to a cuvette, which was placed in the 2b nucleofector device, a cuvette based instrument that allows for the transfection of cell lines. Once the gene transfer system has been run, the cells can be transferred to a fresh microcentrifuge tube until plated.

Next, RNA could be extracted from the C6 cells that have taken up the CRISPR construct during nucleofection.

RNA quantification with RT-qPCR: Total RNA was extracted using the RNeasy Mini kit following the included instructions. RNA was reverse transcribed using iScript RT-PCR kit. PCR amplification was performed using a CFX96 real-time PCR system at 95 degrees C for 3 minutes, followed by 40 cycles of 95 degrees C for 10 seconds and 58 degrees C for 30 seconds. *Gapdh* was used as an internal control.

Cultured Neuron Experiments: Primary rat hippocampal neuron cultures were created from embryonic day 18 rat hippocampal tissue. Cell culture wells were coated with poly-L-lysine and rinsed three times with diH2O. Collected hippocampal tissue was incubated with

papain for 20 minutes at 37 degrees C. A suspension of the tissue was suspended in Neurobasal media by titration. Cells were grown in Neurobasal media with a supplement for 4-5 days *in vitro* in a 37 degree C incubator.

After 5 days, cells were transduced with a lentivirus containing the CRISPR-dCas9 constructs. Cells were incubated for 11 days *in vitro* before RNA was extracted or 7 days *in vitro* before MEA recordings were collected.

Construction of lentivirus for neuronal transduction: HEK293T Cells were transfected with the FuGene protocol. Transfer plasmids were slowly thawed on ice while warm UltraCulture media was put into the T225 flasks. All 4.5 mL of the OptiMem transfection mix was added. At 40-48 hours later, virus concentration was performed. The L 100k ultracentrifuge, was set to 4 degrees, and 1 hour and 45 minutes at 25,000 rpm. Media was collected from each flask and placed into a 50mL tube. The tube was then spun at 2300g for 5 minutes. 36 mL supernatant was pipetted into a 30mL syringe and filtered back into the tube. The tubes were run in the L100k again. A couple of Kimwipes were placed in the tube, avoiding the pellet at the bottom. Once all the liquid evaporated, 150 uL PBS was added to the tubes without disturbing the pellet. The tubes were covered with parafilm and left in the fridge overnight. The following day the PBS was transferred to cold microtubes. The tubes were spun at 2300g for 5 minutes at 4 degrees. The supernatant was transferred to a new tube and a mix of 1mg polybrene in milliQ water was diluted into it 250x. Neurons were then transduced after 5 days *in vitro*.

Multi Electrode Recordings: Neurons were plated on 6-well Multi-Electrode Arrays (MEAs; Figure 1) enabling neuronal activity to be recorded at 7 days *in vitro*. MEA plates were removed from the incubator and placed on the recording stage (Multi-Channel Systems MEA 2100). If neurons were present and noise was eliminated, recording would begin by pressing the play button. Channels with active neurons were identified, and thresholds for neuronal action potential detection were set. Thresholds were set to include neuronal activity and exclude as much electronic noise as possible. Next, the Offline Sorter program was used to identify waveforms indicative of neuronal spikes and exclude residual electronic noise. After sorting was completed, the final data was accessed through the Neuroexplorer software. Neuroexplorer data was exported to a Microsoft Excel file for further processing and graphical analysis.

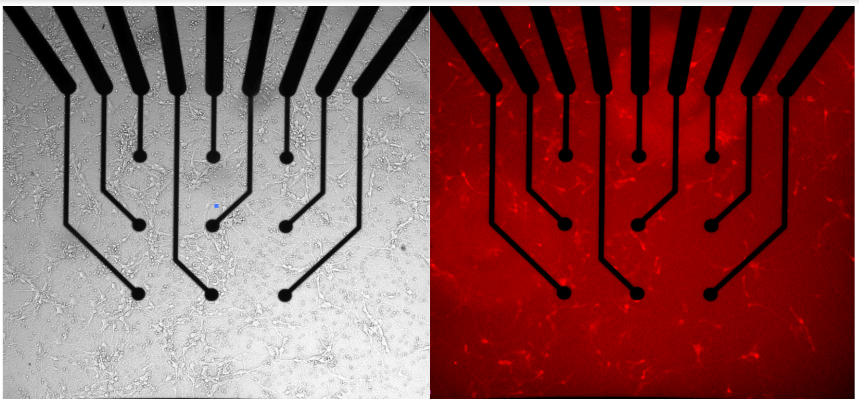

Figure 1: *Microscopic images of electrodes in MEA plates and hippocampal culture.* Electrodes in MEA plates recorded electrical activity from the cell culture. Cells appearing red indicate they contain the CRISPR construct since the construct contains a fluorophore called mCherry.

Results

CRISPR-dCas9 upregulation of Reln. To modulate *Reln*, CRISPR-dCas9 with VPR (Figure 2) was used to target specific areas of the *Reln* gene promoter to induce transcription of *Reln* mRNA. As described earlier, dCas9 is a variation of Cas9, this variation does not cleave DNA, it functions as a vehicle to carry VPR. VPR is a three-part activator that has been engineered for strong transcriptional activation. First, C6 rat cells were nucleofected with the CRISPR-dCas9 construct containing 3 different *Reln* gRNAs (Figure 3), *Lac Z* gRNA, or *Fos* gRNA. Validation of the guides through C6 cells offers a faster method to test their effectiveness before moving on to hippocampal culture experiments. The *Lac Z* group functions as a non-targeting, negative control, while *Fos* functions as a positive control. *Lac Z* is a non-targeting gRNA, since this is a bacterial gene, it does not exist in the rat genome and therefore, the construct should not be effective. The inclusion of a *Fos* gRNA group indicates guide specificity. *Fos* is an immediate early gene, this is a gene which reacts rapidly in response to cellular stimuli. The upregulation of *Fos* indicates effectiveness as a positive control since we have a gRNA that is known to be highly effective in upregulating this gene. Additionally, since *Reln* is not upregulated with *Fos*, and likewise *Fos* is not upregulated with *Reln*, it serves to show these two guides are specific to their target. RNA extraction was used to validate increases in gene activity. Upregulation of a gene can be identified using rt-qPCR to analyze RNA extracted from cell cultures. Total RNA was extracted from C6 cultures. The initial groups consisted of a multiplex group containing all three *Reln* gRNAs, a *Lac z* group, and a *Fos* group. Since it was found that *Reln* was significantly upregulated (Figure 4), next the guides needed to be tested separately to identify which one was effective. The three *Reln* guides were separated into different groups. Cultures were nucleofected again, and RNA was extracted from the C6 cultures and analyzed using rt-qPCR 16 hours after nucleofection. The *Reln* 1 guide was effective at upregulating *Reln* at a 3-fold change (Figure 4). Next a primary hippocampal culture was created from E18 embryonic pups and transduced with a lentivirus containing the CRISPR dCas9 construct at 4 days *in vitro*. RNA was extracted from the neuronal cultures prepared from embryonic rat hippocampus at 11 days *in vitro* to allow for neuronal maturation and virus expression. Comparison between data from *Reln* gRNA groups and *Lac Z* gRNA groups indicated a significant 4-fold increase in activity in the *Reln* 1 guide group (Figure 5).

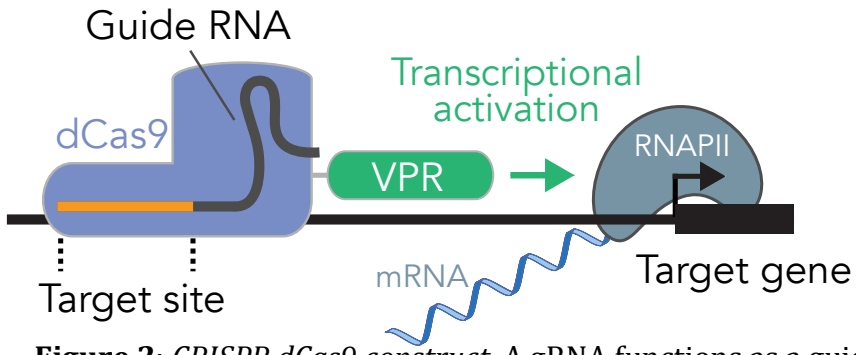

Figure 2: *CRISPR dCas9 construct*. A gRNA functions as a guide to direct the construct to a specific location on the gene. dCas9 is mutated so that instead of cutting the gene, it only sits and carries VPR, a strong transcriptional activator.

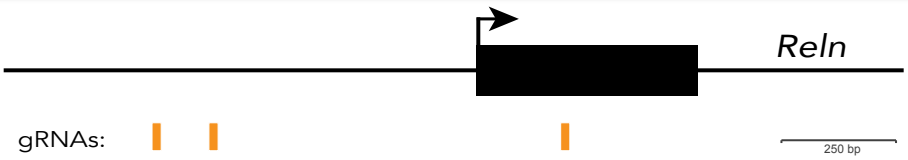

Figure 3: *Representative Reln gene.* Three gRNA guide sequences were designed to target different locations on the Reln gene.

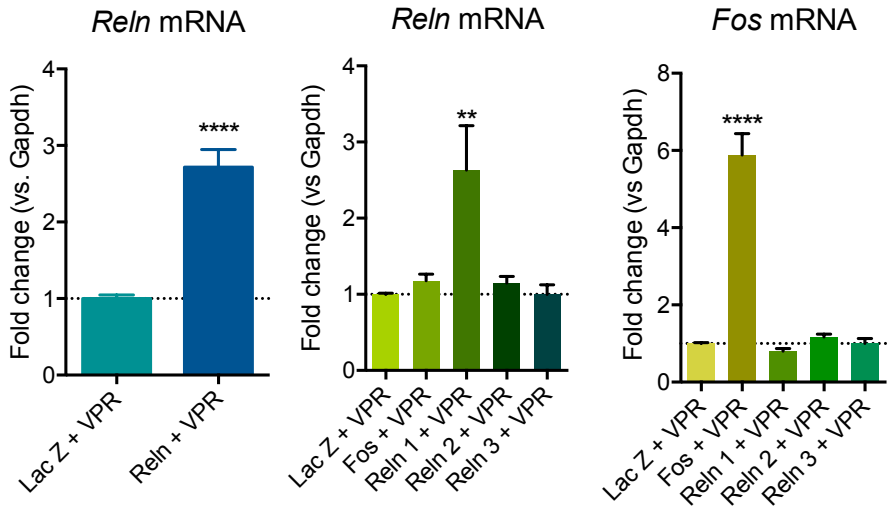

Figure 4: *Total RNA extraction from C6 cells*. As compared to a *Lac Z* negative control, the *Reln* multiplex group was significantly upregulated. When the *Reln* guides were separated, it was found that the *Reln* 1 guide was effective at upregulating at a 3-fold change. The inclusion of *Fos* indicates that each guide is specific and provides a positive control.

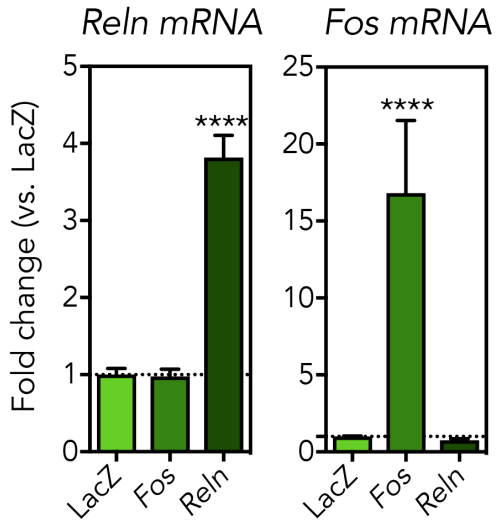

Figure 5: *Total RNA extraction from hippocampal culture*. RNA was extracted from primary hippocampal culture created from E18 embryonic pups and transduced with a lentivirus containing the CRISPR dCas9 construct at 11 days *in vitro*. When compared to the *Lac Z* group, it was found that the *Reln* 1 guide upregulated the gene at a 4-fold change. *Fos* results are included to indicate guide specificity.

Recording neuronal behavior with multi electrode arrays. Neuronal cultures from embryonic rat hippocampus were prepared in a 6-well multi electrode array. At 5 days *in vitro*, cells were transduced with a lentivirus. Wells on each MEA alternated between groups that had been transduced with a CRISPR dCas9 construct containing *Lac Z* gRNA or *Reln* gRNA. At 7 *days in vitro*, MEA recordings began. Plates were recorded from for 20-minute intervals during the same time of day from 7 days *in vitro* to 11 days *in vitro*. Recording for multiple days allows for the visualization of the effect time has on the experiment as well as identification of the best day(s) after plating to record from this type of culture.
After recording, data collected was sorted in the spike sorter program. Sorted MEA recordings analyzed in Matlab (Figures 6-8) indicated that across all days *in vitro* there were significantly more total active neurons in the *Reln* group when compared to the *Lac Z* control (Figure 7, Figure 8). Burst and spike rates were also analyzed, although there were no significant findings in these areas. A spike refers to a single action potential that crosses the threshold so it is distinct from electronic noise. A burst refers to a cluster of many action potentials occurring nearly simultaneously (Figure 6). If cells were to become more active in the *Reln* group, they may be more developed than the groups that did not receive this treatment.

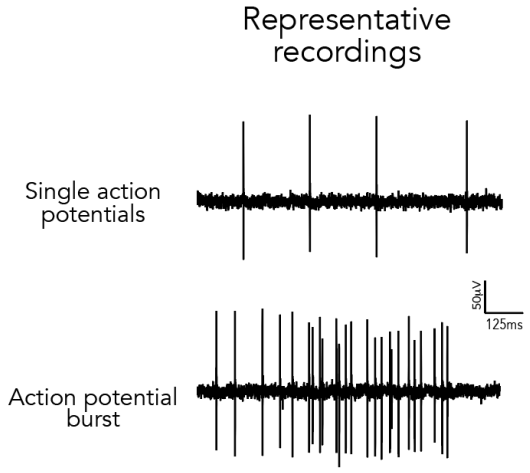

Figure 6: *Representative recordings from a hippocampal culture.* A single action potential is referred to a spike. A spike is an electrical communication that is very distinct from the electronic noise. Similarly, a burst is a cluster of many action potentials.

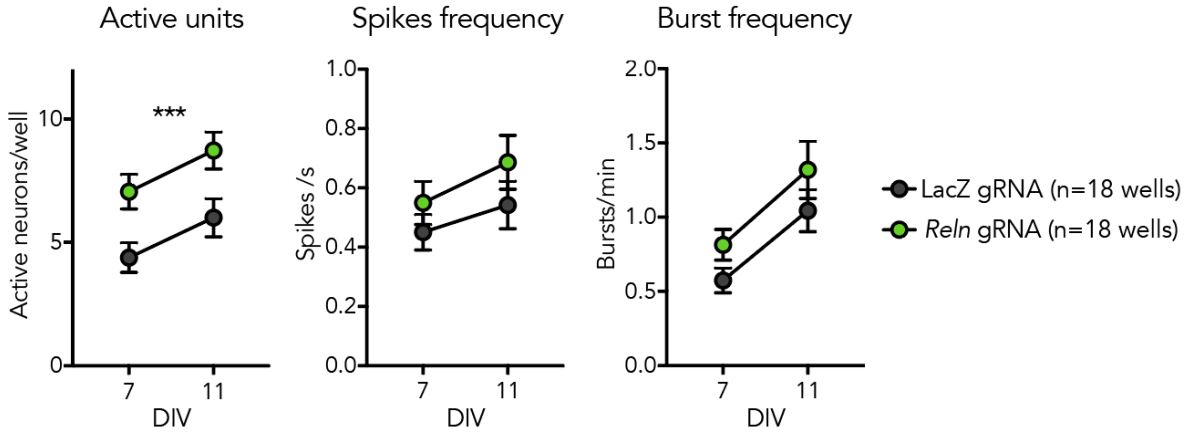

Figure 7: *Multi Electrode Array Recordings*. Analysis of MEA recordings indicated that although there was not a significant increase in individual cell activity through spikes or bursts, there were consistently more neurons becoming active in the *reln* group across all time-points.

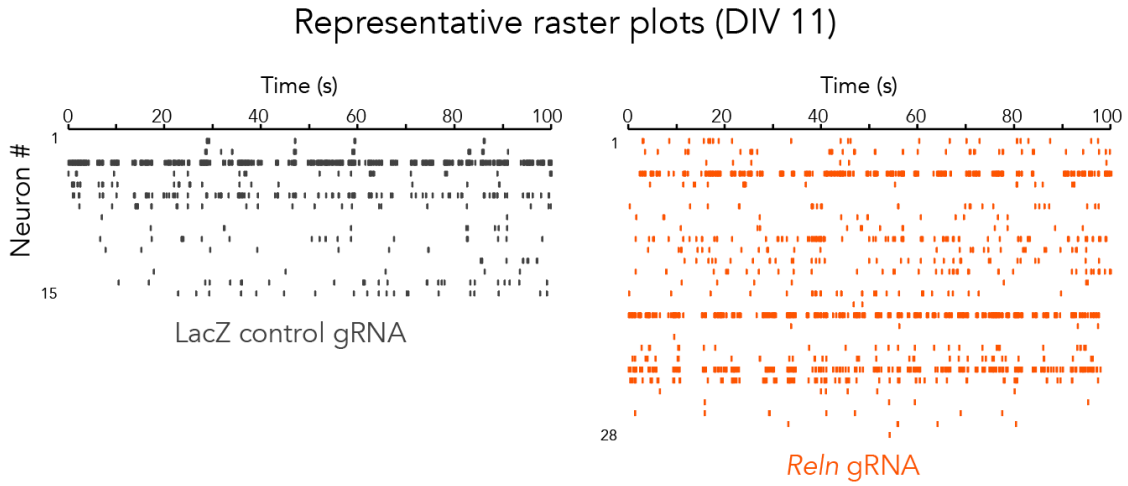

Figure 8: *Multi electrode array raw data.* Each line in the raster plot represents an action potential; the darker lines represent burst activity. Each row of lines represents an active cell that was recorded from. Across all days recorded, the *Reln* group had consistently many more active cells than the *Lac Z* group.

Discussion

Our results indicate CRISPR dCas9 with VPR is an effective method to upregulate the *Reln* gene in a hippocampal culture in a robust and specific manner. Additionally, the significant increase in active neurons in the *Reln* groups indicates a change in cellular behavior as a result of transcriptional manipulation. Previous research has traditionally been invested in the study of *Reln* in the context of embryonic CNS development (1). However, recent studies have found Reelin to be relevant in synaptic function even in the adult brain, studies indicated high levels of *Reln* restricted to particular areas, notably in the hippocampus. The highly restricted and regulated expression of *Reln* in specific memory related areas of the brain suggest that it is of importance to cognitive functioning (1). Our results are in line with these ideas. In hippocampal culture, neurons with the *Reln* gene upregulated became active at an earlier timepoint when compared to the *Lac Z* control group. The increased expression of *Reln* of the gene led to changes in cellular function, specifically, an increase in more mature, actively firing cells. Although the cause of the increase is unknown, it can be speculated that cells with upregulated *Reln* may be becoming more developed at an earlier date, and subsequently, causing an increase in cellular signaling and more output in firing.

In the adult hippocampus, *Reln*-expressing cells are associated with the induction of long-term potentiation (2). LTP in particular areas of the hippocampus require NMDA receptor activation, it is known that Reelin interacts with NMDA receptors to cause downstream signaling cascades associated with memory related functions of the brain. To verify an increase in signaling cascades related to functions such as dendrite development, it would be important to investigate dendritic spine development after the upregulation of the *Reln* gene via the CRISPR construct. Our results demonstrate that changes to this gene produce a significant change in output in terms of cell activity. Changes to morphology of dendrites or the structure of a synapse can affect the activity of neurons (16). However, to

further investigate the cause of these activity changes, additional experiments at a molecular level should be performed. To address the lack of findings in terms of spike and burst frequency, it is possible a homeostatic mechanism is at work preventing individual cells from displaying the behavioral increases seen at a population level. Some previous research with MEA recordings has suggested difficulty in producing increases in spikes and bursts as a result of cellular resistance to changes; this can potentially be attributed to an internal system of regulation that prevents excessive increases in action potentials, homeostatic regulation (5). Our data indicates a number of interesting routes to follow to further investigate the function of this gene and how it relates to memory.

Endnote: Citations

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