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# A Cre-dependent CRISPR/dCas9 activation system for gene expression regulation in neurons

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Site-specific genetic and epigenetic targeting of distinct cell populations is a central goal in molecular neuroscience and is crucial to understand the gene regulatory mechanisms that underlie complex phenotypes and behaviors. While recent technological advances have enabled unprecedented control over gene expression, many of these approaches are focused on selected model organisms and/or require labor-intensive customizations for different applications. The simplicity and modularity of CRISPR-based systems have transformed this aspect of genome editing, providing a variety of possible applications and targets. However, there are currently few available tools for cell-selective CRISPR regulation in neurons. Here, we optimized a CRISPR activation (CRISPRa) system for Cre recombinase-dependent, cell type-specific targeting. Unexpectedly, CRISPR systems based on a traditional double-floxed inverted open reading frame (DIO) strategy exhibited leaky target gene induction in the absence of Cre. Therefore, we developed an intron-containing Cre-dependent CRISPRa system (SVI-DIO-dCas9-VPR) that alleviated leaky gene induction and outperformed the traditional DIO system at endogenous genes in both HEK293T cells and rat primary neuron cultures. Using gene-specific CRISPR sgRNAs, we demonstrate that SVI-DIO-dCas9-VPR can activate highly inducible genes (*GRM2* and *Tent5b*) as well as moderately inducible genes (*Sstr2* and *Gadd45b*) in a Cre-specific manner. These results provide a robust framework for Cre-dependent CRISPR-dCas9 approaches across different model systems, and will enable cell-specific targeting when combined with common Cre driver lines or Cre delivery via viral vectors.

## INTRODUCTION

Genetic and epigenetic targeting are fundamental strategies to study gene regulation and function, and also provide novel therapeutic avenues for genetic diseases. In recent years, clustered regularly interspaced short palindromic repeats (CRISPR) systems have revolutionized the field as tools for site-specific DNA and RNA editing (Cong et al., 2013; Jinek et al., 2012; Pickar-Oliver and Gersbach, 2019; Savell and Day, 2017). In these systems, a Cas nuclease is directed to target DNA by an engineered CRISPR single guide RNA (sgRNA), resulting in nuclease-mediated cleavage of the targeted nucleic acid sequence. In addition to classical CRISPR-Cas9 systems, a number of new approaches have been developed based on a mutated, catalytically dead Cas9 (dCas9). dCas9 strategies provide a powerful system to target specific genomic loci without causing double-strand breaks. Instead, dCas9 constructs can be fused to an array of different effector proteins (Savell and Day, 2017). dCas9 systems are commonly used to induce transcriptional activation (CRISPRa) or interference (CRISPRi), epigenetic modifications, chromatin looping, tagging, or can be used as an anchor system to deliver non-coding RNAs (CRISPR-Display) (Carullo et al., 2020, Chen et al., 2019; Hilton et al., 2015; Konermann et al., 2015; Savell et al., 2019; Shechner et al., 2015).

DNA recombinases are commonly used to enable inversion, deletion, or integration of transgenes in a cell-specific manner. The Cre/Lox system is likely the best understood and most commonly used recombination approach, in which the Cre recombinase recognizes specific 34bp palindromic Lox sites within a DNA sequence (Anton and Graham, 1995; Gibb et al., 2010). Cre-mediated inversion or deletion occurs when flanking Lox sites are oriented in opposing or parallel orientations, respectively. Importantly, Cre recombinase expression driven under cell type-specific promoters enables targeted gene manipulation of these populations (Daigle et al., 2018; Madisen et al., 2012). Due in part to the ease and versatility of this popular system, hundreds of different Cre-driver transgenic lines have been generated for cell-targeted approaches.

Cre-dependent genetic FLEX switches (flip-excision; also known as double-inverted open reading frame, or DIO switches) invert DNA sequences to enable gene activation or silencing (Schnütgen et al., 2003). All FLEX/DIO systems capitalize on Lox sequence variants that provide additional specificity to Cre recognition. Since antiparallel LoxP sites cause continuous inversion, DIO systems use two heterotypic sets of Lox sequences. Recombination at one Lox pair causes transgene inversion, allowing deletion of the genetic material (including a Lox site) between the other Lox pairs. This strategy stops the inversion cycle through the excision of one antiparallel Lox site. A common FLEX/DIO strategy uses LoxP and Lox2272 sequence variants due to their efficient and specific recombination charac-

teristics (Lee and Saito, 1998). This approach has widely been used in neuroscience, including for cell type-specific optogenetic and chemogenetic activation, and specific transgene overexpression or knockdown (Atasoy et al., 2008; Erwin et al., 2020; Gong et al., 2007; Kuhn et al., 1995; Madisen et al., 2012; Tsien et al., 1996).

While CRISPR/dCas9 approaches have enabled targeted induction of transcriptional or epigenetic states at selected genes, inducible cell type-specific CRISPR tools based on these platforms remain limited (Bäck et al., 2019; Kumar et al., 2018). Here, we developed a novel Cre-dependent CRISPRa system that incorporates a synthetic intron into the dCas9 transgene. Creating an intron-containing dCas9 construct offers two advantages: (1) Introns can increase transgene expression compared to intron lacking counterparts (Shaul, 2017), and (2) Introns provide a non-coding locus for insertion sites of Lox sequences without disturbing the integrity of the coding protein. By separating the dCas9 gene into two segments with a short SV40 intron (SVI), we created a CRISPRa system in which the first segment of dCas9 is double-floxed and inverted. This approach prevents leaky expression of a functional CRISPRa construct by requiring Cre-induced inversion to orient both dCas9 segments into an open reading frame. Furthermore, the construct is designed for easy rearrangement and replacement of promoters and effector fusions and can be applied for various CRISPR dCas9 systems.

## RESULTS

### *Creation and validation of a traditional FLEX-CRISPRa system*

AAV-driven FLEX systems are among the most common Cre/Lox approaches used in neurons, enabling inducible expression by combination with Cre-driver animal models. While this strategy has proven to be a versatile approach for cell-specific targeting, few tools have been described for Cre-dependent CRISPR manipulation. Given the flexibility of CRISPRa and CRISPRi systems for transcriptional manipulation at endogenous loci, development of Cre-dependent platforms for these systems are of interest. To create a Cre-inducible system for transcriptional activation, we adapted a CRISPRa tool recently validated in neurons (Savell et al., 2019) using a common DIO strategy. In this DIO system, an inverted dCas9-VPR cassette was flanked by LoxP and Lox2272 sites (**Figure 1a**), enabling Cre-dependent inversion of dCas9-VPR into the correct orientation followed by the excision of one antiparallel Lox site to prevent further inversion events. To validate this system in dividing cells, HEK293T cells were co-transfected with either a constitutively active dCas9-VPR construct or the DIO-dCas9-VPR construct, in tandem with sgRNA plasmids targeting either the human *GRM2* promoter, or a non-targeting control (sgRNA for the bacterial *lacZ* gene). The DIO-dCas9-VPR construct was tested with or without transfection of a Cre-2A-mCherry plasmid

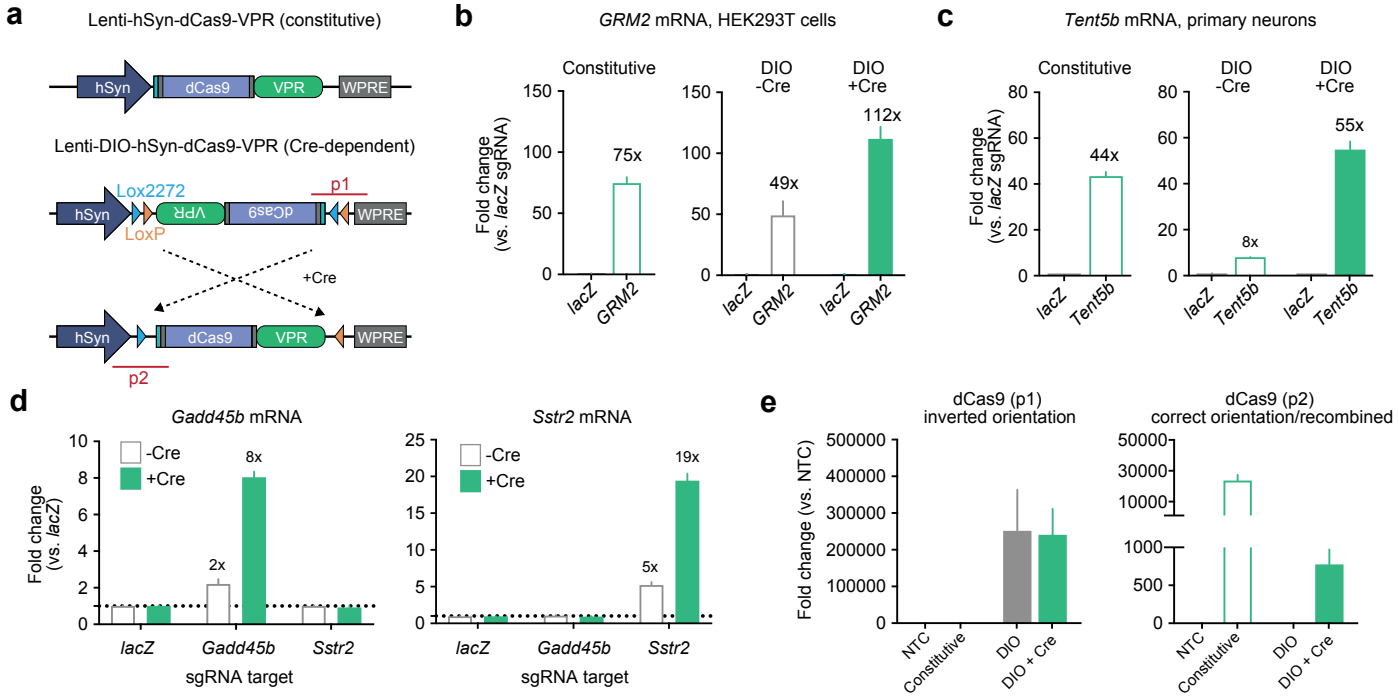


**Figure 1.** Design and validation of a DIO-dCas9-VPR CRISPRa plasmid. **a**, Illustration of CRISPRa construct designs for a traditional constitutive dCas9-VPR (top) and a Cre-dependent double-floxed inverted open reading frame (DIO) dCas9-VPR (bottom). PCR products for primer sets used in panel E are illustrated as p1 and p2 (red). **b**, In transfected HEK293T cells, the constitutive dCas9-VPR construct induced the endogenous target gene *GRM2* by 75 fold compared to the non-targeting *lacZ* sgRNA control (Welch's *t*-test $t(2) = 14.43$, $p = 0.0048$). DIO-dCas9-VPR significantly induced transcription of *GRM2* with and without Cre ($n = 3$ per group, two-way ANOVA $F(1,8) = 32.89$, $p = 0.0004$). **c**, Lentiviral expression of constitutive dCas9-VPR construct as well as the DIO-dCas9-VPR construct in rat striatal neurons revealed increased mRNA for the target gene *Tent5b* (Welch's *t*-test for constitutive $t(2) = 22.81$, $p = 0.0019$, two-way ANOVA for DIO with $n = 3$ per group, $F(1,8) = 487.9$, $p < 0.0001$). **d**, Additional CRISPRa target genes tested in neurons demonstrated leaky induction in the absence of Cre recombinase (two-way ANOVA with $n = 3$ per group for *Gadd45b* $F(1,12) = 91.03$, $p < 0.0001$, and for *Sstr2* $F(1,12) = 142.1$, $p < 0.0001$). **e**, qPCR on transfected HEK293T cell DNA showed high levels of the inverted dCas9-VPR casette with and without Cre (p1, left, $n = 4$ per group, Kruskal-Wallis test $F(3,12) = 13.06$ $p < 0.0001$). Inversion into the correct orientation only occurred in the presence of Cre (p2, right, n = 4 per group, Kruskal-Wallis test $F(3,12) = 12.11$ $p = 0.0002$). Constitutive VPR and DIO groups (with and without Cre) are compared to a non-transduced control (NTC). Data expressed as mean ± s.e.m.

that was driven under the human synapsin (hSYN) promoter (**Figure 1b**). Constitutive activation of the endogenous *GRM2* gene yielded a 75-fold increase of *GRM2* mRNA compared to a non-targeting *lacZ* control sgRNA. While the Cre-dependent DIO-dCas9-VPR system was able to induce *GRM2* mRNA transcription by 112-fold, we also observed a 49-fold increase in the absence of Cre. Next, to examine gene induction in neurons, we transduced rat primary striatal neuron cultures with lentiviruses expressing sgRNAs and CRISPR machinery (**Figure 1c**). CRISPR sgRNAs targeting the *Tent5b* (also known as *Fam46b*) promoter resulted in strong upregulation of *Tent5b* mRNA with both the constitutive and Cre-dependent constructs. However, in neurons the DIO system also exhibited leaky expression, indicated by an 8-fold increase of *Tent5b* mRNA in the absence of Cre. *Tent5b* is a highly inducible gene which could be more susceptible to baseline/leaky induction compared to other genes. Therefore, we tested the DIO-dCas9-VPR system at two other genes including a gene encoding the neuropeptide receptor *Sstr2* and DNA-damage inducible gene *Gadd45b* (**Figure 1d**). Although these genes are less inducible than *Tent5b* (with induction rates of 8-19-fold compared to *lacZ* controls), we still observed non-specific gene induction in the absence of Cre (2-5-fold induction without Cre). This baseline induction of target genes is likely due to leaky expression of the DIO-dCas9-VPR, since no transgene inversion could be detected in PCR verification using genomic DNA isolated from cells lacking Cre (**Figure 1e**). These results are consistent with recent evidence that DIO transgenes can be expressed in the inverted orientation at low levels in the absence of Cre recombinase (Fischer et al., 2019).

### *Intron insertion into dCas9 increases expression and enables creation of a split-dCas9 FLEX system*

Given that an inverted dCas9-VPR cassette still resulted in target gene induction in the absence of Cre, we next aimed to design a system that would prohibit expression of the full length dCas9-VPR fusion protein without Cre-mediated recombination. To achieve this goal, we designed an intron-containing FLEX system. First, we inserted a small SV40 intron (SVI) into the dCas9 sequence to create a constitutively active, intron-containing construct (SVI-dCas9-VPR) in which the dCas9 cassette is divided into two segments by the SV40 intron (**Figure 2a**). We created two versions (1.0 and 2.0) of this SVI-dCas9-VPR construct to determine optimal intron positioning and maximal splicing effi-

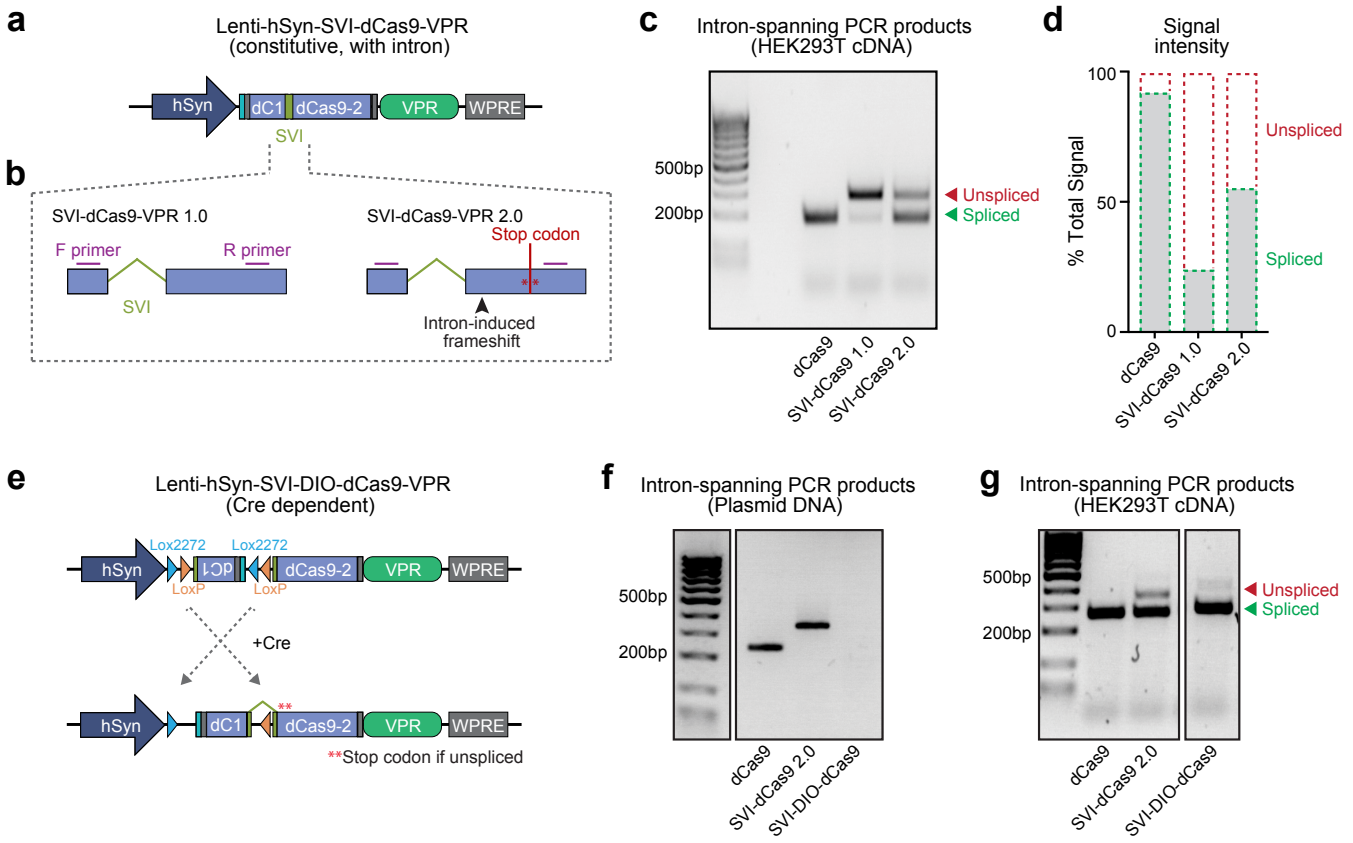


**Figure 2.** Design of an intron-containing, Cre-dependent CRISPRa system. **a**, Illustration of CRISPR construct design for an intron-containing constitutive dCas9-VPR cassette. **b**, Design of the two different intron positions. **c**, SVI-dCas9-VPR 1.0 and 2.0 intron positions were tested in HEK293T cells. PCR amplification of cDNA with primers spanning the intron within dCas9 showed more efficient splicing of the SVI-dCas9-VPR 2.0 construct. **d**, Quantification of PCR product intensities shown in panel C. **e**, Illustration of a Cre-dependent SVI-DIO-dCas9-VPR construct in which the first dCas9 segment is double-floxed and inverted. **f**, PCR amplification of plasmid DNA with intron-spanning primers verify that no product results from the SVI-DIO-dCas9-VPR plasmid prior to recombination. **g**, PCR amplification of cDNA following HEK293T cell transfection with dCas9-VPR, SVI-dCas9-VPR, and SVI-DIO-dCas9-VPR with Cre showed that SVI-DIO-dCas9-VPR transfected cells express spliced dCas9-VPR transcripts in the presence of Cre recombinase.

ciency. While the intron was only slightly shifted between versions 1.0 and 2.0, SVI-dCas9-VPR 2.0 created a frameshift immediately after the intron, resulting in a premature stop codon within less than 100 bases if the product was not spliced (**Figure 2b**). PCR amplification using cDNA with primers spanning this intron sequence following HEK293T plasmid transfection revealed that both constructs produced detectable dCas9 expression. However, splicing efficiency of the SVI-dCas9-VPR 2.0 version was higher as compared to SVI-dCas9-VPR 1.0 (**Figure 2c, d**).

Using the more efficient SVI-dCas9-VPR 2.0, we next generated a split-dCas9 DIO cassette by inverting and flanking the first segment of dCas9 with LoxP and Lox2272 sites to prevent leaky transgene expression (**Figure 2e**). With only part of the dCas9 cassette inverted, Cre-dependent inversion and recombination is required to yield a full-length functional dCas9-VPR fusion protein. Using the same intron-spanning primer set as in previous validations, we compared PCR products amplified from the original constitutive dCas9-VPR, the intron-containing constitutive VPR (SVI-dCas9-VPR 2.0), and the new Cre-dependent SVI-dCas9-VPR (SVI-DIO-dCas9-VPR) plasmids. As predicted, the SVI-dCas9-VPR 2.0 plasmid yielded a longer product compared to the original dCas9-VPR with no intron (**Figure 2f**), due to the 97bp intron in the SVI-dCas9-VPR 2.0 plasmid. However, because the SVI-DIO-dCas9-VPR contains an inverted dCas9 segment, the validation primers target the same strand and yield no PCR product. These results further validate the absence of non-specific recombination in plasmids amplified in STBL3 cells.

We next transfected HEK293T cells with each of these three constructs to compare recombination, expression, and splicing efficiency of the SVI-DIO-dCas9-VPR construct. The SVI-DIO-dCas9-VPR groups also received a Cre-EGFP plasmid that was driven under the hSYN promoter to initiate recombination. PCR amplification of cDNA generated from the transfected HEK293T experiments with intron-spanning primers revealed strong signals for the short, spliced PCR product for all three groups (**Figure 2g**). This indicates that all three constructs were expressed in HEK293T cells and that both SVI-dCas9-VPR 2.0 and SVI-DIO-dCas9-VPR transgenes generated mRNA transcripts with efficient splicing and intron exclusion. Together, these experiments demonstrate that this newly developed intron-containing FLEX system is capable of efficient splicing and transcription and that Cre-dependent recombination is required for inversion and expression of the dCas9-VPR transgene.

### *Intron-containing FLEX system drives Cre-dependent CRISPR targeting with no leaky gene induction.*

Next, we sought to test this newly developed

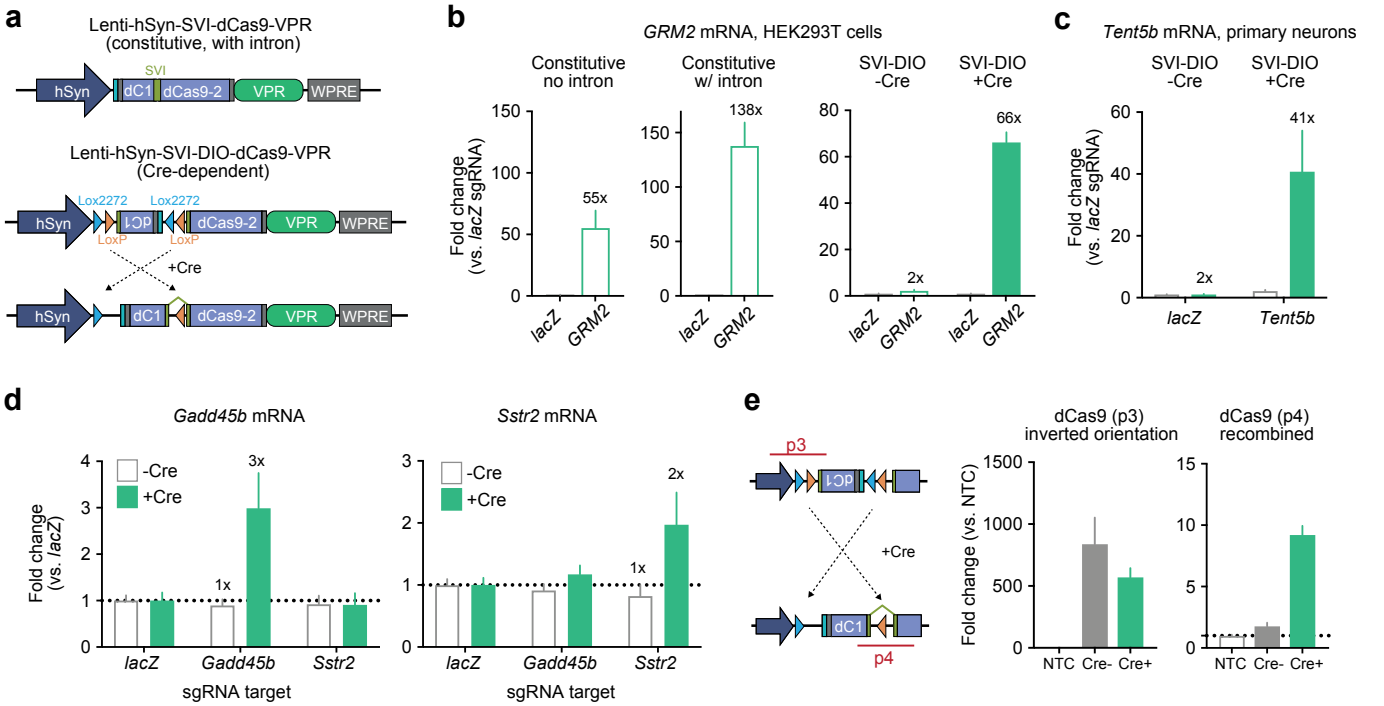


**Figure 3.** Validation of the SVI-DIO-dCas9-VPR CRISPRa system. **a**, Illustration of CRISPR construct designs for the intron-containing SVI-dCas9-VPR (top) and a Cre-dependent double-floxed inverted open reading frame (DIO) SVI-dCas9-VPR (bottom). **b**, In transfected HEK293T cells the constitutive VPR construct with and without the SV40 intron induced the endogenous target gene *GRM2* by 55 fold and 138 fold compared to the non-targeting *lacZ* control (Welch's *t*-test for constitutive $t(2) = 3.846$, $p = 0.0614$, and constitutive with intron $t(2) = 6.322$, $p = 0.0241$). SVI-DIO-dCas9-VPR significantly induced transcription of *GRM2* with but not without Cre ($n = 3$ per group, two-way ANOVA $F(1,8) = 179.5$, $p < 0.0001$). **c**, Lentiviral expression of SVI-DIO-dCas9-VPR in striatal neurons increased mRNA for the target gene *Tent5b* ($n = 10$ per group, two-way ANOVA $F(1,36) = 34.97$, $p < 0.0001$). **d**, Additional target genes tested in neurons demonstrated specific induction only in presence of Cre recombinase (two-way ANOVA with $n = 3$ per group for *Gadd45b* $F(1, 48) = 4.578$, $p = 0.0375$, and for *Sstr2* $F(1,48) = 6.615$, $p = 0.0133$). **e**, Illustration of primer and PCR product position for recombination validation experiments (left). qPCR on transduced neuronal DNA revealed high levels of the inverted dCas9-VPR cassette (p3, middle, $n = 2$ per group, Kruskal-Wallis test $F(2, 4) = 3.714$ $p = 0.2$) with and without Cre. Inversion into the correct orientation only occurred in the presence of Cre (p4, right, n = 2 per group, Kruskal-Wallis test $F(2, 4) = 4.57$ $p = 0.0667$). SVI-DIO-dCas9-VPR groups with and without Cre are compared to a non-transduced control (NTC). Data expressed as mean ± s.e.m.

intron-containing FLEX CRISPR system in vitro to validate Cre-specific expression and gene induction (**Figure 3a**). Using a similar experimental design as in the DIO-dCas9-VPR experiments (**Figure 1**), we transfected HEK293T cells with plasmids expressing constitutive dCas9-VPR, SVI-dCas9-VPR, and SVI-DIO-dCas9-VPR constructs, along with sgRNA targeting the *GRM2* gene promoter (or *lacZ* control). Notably, the intron-containing SVI-dCas9-VPR caused stronger gene induction than the original constitutive dCas9-VPR when compared to their respective *lacZ* controls (**Figure 3b**). This is in line with previous reports suggesting that introns can promote and increase expression rates of transgenes (Shaul, 2017). However, unlike the classic DIO-dCas9-VPR construct, the SVI-DIO-dCas9-VPR plasmid exhibited very little induction without Cre (2-fold) and strong induction with Cre (66-fold).

Similarly, targeting the highly inducible *Tent5b* gene in cultured striatal neurons using lentiviral transgene delivery resulted in strong upregulation of *Tent5b* mRNA with the Cre-dependent constructs (41-fold), but not in the absence of Cre (2-fold; **Figure 3c**). These patterns of Cre-dependent induction without leaky effects were also observed for the both moderately inducible target genes tested in **Figure 1** (*Sstr2* and *Gadd45b*; **Figure 3d**). PCR amplification of genomic DNA that was extracted from transduced primary neurons further validated that recombination required expression of Cre (**Figure 3e**). Together, these data demonstrate that this novel SVI-DIO-dCas9-VPR construct mitigates the leaky gene induction seen in conventional DIO systems for CRISPRa approaches while maintaining the capacity for robust and specific gene induction.

## DISCUSSION

The mammalian brain consists of heterogeneous cell populations with distinct characteristics and functions. Technologies to study gene function in specific tissues, brain regions, or cell populations are therefore necessary to understand their role in behavior and disease. For example, cell type-selective promoters have been used for specific targeting of excitatory neurons (*Camk2a*) (Mima et al., 2001), glia (*Gfap*) (Morelli et al., 1999), and cell populations that express specific enzymes or receptors such as tyrosine hydroxylase *(Th)* (Savitt et al., 2005), dopamine or serotonin transporters (*Slc6a3*, *Slc6a4*) (Bäck et al., 2019; Zhuang et al., 2005), or dopamine receptors (*Drd1*, *Drd2*) (Lobo et al., 2006; Zhang et al., 2006). In this study we developed and optimized a Cre-dependent CRISPRa system that enables gene and cell type-specific transcriptional activation. A traditional Cre/Lox approach in which the entire dCas9-VPR cassette was double-floxed and inverted was prone to leaky target gene induction in the absence of Cre. This leaky expression could be enhanced in constructs carrying strong transcriptional activators such as VPR that potentially facilitates its own transcription in inverted orientation. To avoid this leaky expression, we generated a novel SVI-DIO-dCas9 system in which dCas9 was broken up into two segments by an SV40 intron. This intron provided a location for insertion of Lox sites to invert and double-flox the first dCas9 fragment while leaving the second fragment undisturbed. Without Cre-mediated recombination, the dCas9 segments remain in opposing orientations thus cannot yield functional dCas9 protein. Additionally, introduction of the compact SV40 intron resulted in higher expression efficiency compared to a CRISPRa construct without an intron in HEK293T cells. In cultured dividing and non-diving cell lines, we demonstrate that the SVI-DIO-dCas9 based CRISPRa system alleviates the leaky transcriptional induction detected in DIO CRISPRa systems (**Figure1, Figure 3**). This work provides evidence that the SVI-DIO-dCas9-VPR construct can activate highly inducible endogenous genes in HEK293T cells (*GRM2* gene) and striatal neurons (*Tent5b*), without undesired gene induction in the absence of Cre recombinase (**Figure 3**). This system also performed well when targeted to moderately inducible genes such as *Sstr2* and *Gadd45b* in neurons (**Figure 3**), demonstrating a wide range of effect size and application possibilities.

One of the first cell type-specific CRISPR systems used in neurons was based on Cre-dependent expression of CRISPR sgRNAs. Bäck et al. developed a Lox-stop-Lox based sgRNA construct and used it for CRISPR-Cas9 mediated gene knock out (Bäck et al., 2019). This study tested this approach in transgenic rats that expressed Cre recombinase under the rat dopamine transporter (*Slc6a3*) promoter to specifically target and knockout the tyrosine hydroxylase *(Th)* gene in dopaminergic neurons. While this approach allowed for cell-type specific expression of sgRNA constructs, a concern with similar systems is the untargeted overexpression of Cas9 or dCas9 fusion proteins, which could potentially cause unintended and non-specific effects on gene expression.

Our system extends this previous work in two ways. First, the SVI-DIO-dCas9 approach alleviates some of these concerns, as the dCas9 construct itself is Cre-dependent and therefore functional fusion proteins are not expressed without Cre recombinase. Secondly, our SVI-DIO-dCas9 system is compatible with traditional validated sgRNA constructs, which can be multiplexed for effect size titration at a single gene (Savell et al., 2019). Additionally, this approach is also compatible with more complex sgRNA arrays that target entire gene programs (Savell et al., 2020). Thus, this study adds another approach for gene regulation to the CRISPR toolbox, while also outlining a novel intron/DIO strategy to avoid leaky Cre-independent transgene expression. Compared to traditional Cre-dependent overexpression vectors, Cre-dependent CRISPRa provides several advantages as it enables targeting of one or multiple endogenous genomic loci and titration of effect size for more physiological expression levels.

Prior work has incorporated temporal specificity into Cre/Lox systems, either via use of optically or

chemically inducible proteins. For example, selective estrogen receptor modulator (SERM) inducible systems have been generated by fusion of the ligand binding domain of the estrogen receptor to Cre (Cre-ER). In this approach, a mutated version of the mouse ER that binds tamoxifen but not estrogen was used to create a Tamoxifen-inducible Cre system (Danielian et al., 1993; Metzger and Chambon, 2001). Cre activity can therefore be activated or inactivated upon Tamoxifen injections, increasing temporal control of gene editing events.

In addition to cell-type specificity, selective promoters can be used to drive expression in response to stimulation or experience. Bacterial artificial chromosome (BAC) transgenic animals were generated to express GFP for tracking or channelrhodopsin (ChR) for activation under an immediate early gene promoter such as *Fos* or *Arc* (Guenthner et al., 2013). Using activity-induced approaches in combination with our SVI-DIO-dCas9 system would allow for experience-dependent genetic and epigenetic manipulations.

Cre/Lox systems are also among the most common approaches for intersectional circuit-specific manipulations in neuroscience. For example, in recent work, circuit-specific CRISPR genome editing was achieved by injection of a Cre-dependent Cas9 viral vector in the nucleus accumbens (NAc) and injection of a monosynaptic rabies virus expressing a sgRNA targeting the *Fosb* gene in the ventral hippocampus (vHPC) (Eagle et al., 2020). While the sgRNA virus successfully spread through all hippocampal projections, significant knockout of *Fosb* occurred only in vHPC neurons that projected to the NAc. In future studies, our SVI-DIO-dCas9 system could similarly be used to gain a better understanding of projections between specific cell types in the brain. The intron-containing effector construct could be delivered to one brain region and a Cre-expressing construct to the second brain region through a monosynaptic rabies virus or retrograde AAV serotype. Genetic or epigenetic editing would then only occur in cells with projections between the two targeted brain regions without leaky expression or side effects related to effector protein overexpression in non-targeted cells.

While the use of our SVI-DIO-dCas9 system in Cre driver animal models is an expected application, our approach does not require transgenic organisms. In addition to sgRNA and SVI-DIO-dCas9-VPR delivery, a separate construct expressing Cre can be delivered as necessary to target brain tissues. Additionally, this intron-containing dCas9 provides a basic framework that can be customized and combined with a number of effector proteins to introduce a variety of genetic or epigenetic modifications. The system can easily be customized for expression in various cell types and brain regions and adjusted for inducible efforts and enhanced temporal specificity.

## MATERIALS AND METHODS

***Cultured neuron experiments.*** Primary rat neuronal cultures were generated from embryonic day 18 rat striatal tissue as described previously (Savell et al., 2019). Briefly, cell culture wells were coated overnight at 37° C with poly-L-lysine (0.05 mg/ml for culture wells supplemented with up to 0.05 mg/ml Laminin) and rinsed with diH2O. Dissected tissues were incubated with papain for 25 min at 37°C. After rinsing in Hank's Balanced Salt Solution (HBSS), a single cell suspension of the tissue was re-suspended in Neurobasal media (Invitrogen) by trituration through a series of large to small fire-polished Pasteur pipets. Primary neuronal cells were passed through a 100 μM cell strainer, spun and re-suspended in fresh media. Cells were then counted and plated to a density of 125,000 cells per well on 24-well culture plate with or without glass coverslips (60,000 cells/cm). Cells were grown in Neurobasal media plus B-27 and L-glutamine supplement (complete Neurobasal media) for 11 DIV in a humidified CO2 (5%) incubator at 37° C.

For viral transduction, cells were transduced with lentiviruses on DIV 4 or 5. All viruses had a minimum titer of $1x10^9$ GC/ml, with a target multiplicity of infection (MOIs) of at least 1000. After an 8-16 hr incubation period, virus-containing media was replaced with conditioned media to minimize toxicity. A regular half-media change followed on DIV 8. On DIV 11, transduced cells were imaged and virus expression was verified prior to RNA extraction. EGFP and mCherry expression was also used to visualize successful transduction using a Nikon TiS inverted epifluorescence microscope.

***RNA extraction and RT-qPCR***. Total RNA was extracted (RNAeasy kit, Qiagen) with DNase treatment (RNase free DNAse, Qiagen), and reverse-transcribed (iScript cDNA Synthesis Kit, Bio- Rad). cDNA was subject to qPCR for genes of interest, as described previously (Savell et al., 2016). A list of PCR primer sequences is provided in **Supplementary Data Table 1**.

***CRISPR-dCas9 construct design.*** To achieve transcriptional activation, lentivirus-compatible plasmids were engineered to express dCas9 fused to VPR (Addgene plasmid # 114196 (Savell et al., 2019). A Cre-dependent DIO version of this dCas9-VPR construct was generated by insertion of LoxP and Lox2272 sequences flanking the dCas9-VPR cassette. dCas9-VPR was PCR-amplified to insert restriction sites and inserted in reversed orientation to create an intermediate construct via sequential digest and ligation (AgeI, KpnI, EcoRI, BmtI, and BspDI). Lox sites were amplified from a Lox-stop-Lox construct (Addgene plasmid # 113685 (Don et al., 2017))) to create restriction sites and inserted into the intermediate construct via sequential restriction digest and ligation (AgeI, KpnI, and BmtI). Intron-containing dCas9-VPR was constructed by the insertion of a gBlock containing the SV40 intron into

the original dCas9-VPR plasmid via Gibson assembly (Gibson Assembly kit, New England BioLabs). The SVI-FLEX construct was built via Gibson assembly of the original dCas9-VPR backbone and two gBlocks encoding the SV40 intron sequence, LoxP and Lox2272 sites (sequences based on DIO construct described above) and dCas9-part1 in inverted orientation. A Cre-encoding construct (Addgene plasmid # 49056 (Kaspar et al., 2002)) was used to amplify and insert a Cre transgene into lentivirus compatible backbones that expressed mCherry or GFP for visualization via Gibson assembly. dCas9-VPR-expressing constructs were co-transduced with sgRNA-containing constructs. Gene-specific sgRNAs were designed using an online sgRNA tool, provided by the Zhang Lab at MIT (crispr.mit.edu) and inserted in a previously described lentivirus compatible sgRNA scaffold construct (Addgene plasmid # 114199 (Savell et al., 2019)). To ensure specificity, all CRISPR crRNA sequences were analyzed with the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) and Cas-OFFinder (http://www.rgenome.net/cas-offinder/). sgRNAs were designed to target *GRM2*, *Tent5b*, *Sstr2*, and *Gadd45b* respectively. A list of the target sequences is provided in **Supplementary Data Table 1**. crRNA sequences were annealed and ligated into the sgRNA scaffold using the BbsI or BsmBI cut site. Plasmids were sequence-verified with Sanger sequencing; final crRNA insertion was verified using PCR.

***Lentivirus production.*** Viruses were produced in a sterile environment subject to BSL-2 safety by transfecting HEK293T cells with specified CRISPR-dCas9 plasmids, the psPAX2 packaging plasmid, and the pCMV-VSV-G envelope plasmid (Addgene 12260 & 8454) with FuGene HD (Promega) for 40-48 hrs as previously described (Savell et al., 2019). Viruses were purified using filter (0.45 µm) and ultracentrifugation (25,000 rpm, 1 hr 45 min) steps. Viral titer was determined using a qPCR Lentivirus Titration Kit (Lenti-X, qRT-PCR Titration Kit, Takara). For smaller scale virus preparation, each sgRNA plasmid was transfected in a 12-well culture plate as described above. After 40-48 hr, lentiviruses were concentrated with Lenti-X concentrator (Takara), resuspended in sterile PBS, and used immediately. Viruses were stored in sterile PBS at -80°C in single-use aliquots.

***HEK293T cell culturing and transfection.*** HEK293T cells were obtained from American Type Culture Collection (ATCC catalog# CRL-3216, RRID:CVCL_0063) and cultured in DMEM (DMEM High glucose, pyruvate; Gibco 11995081) supplemented with 10% bovine serum (Qualified US Origin; BioFluid 200-500-Q) and 1U Penicillin-Streptomycin (Gibco 15140122). Cells were maintained in T75 or T225 tissue flasks. At each passage, cells were trypsinized for 1-3 min (0.25% trypsin and 1 mM EDTA in PBS pH 7.4) at room temperature. For transfection experiment cells were plated in 24 well plates and transfected with FuGene HD (Promega).

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**AUTHOR CONTRIBUTIONS**
N.V.N.C., J.S.R. and J.J.D. conceived of and performed experiments. N.V.N.C. and J.J.D. wrote the manuscript. J.S.R. designed and built the traditional DIO-VPR construct. N.V.N.C. designed and built SVI-VPR and SVI-DIO-VPR constructs with assistance from J.J.T. and A.J.B.. HEK cell transfections, RNA processing were conducted by J.S.R. and N.V.N.C.. DNA and cDNA PCR-verifications were conducted by N.V.N.C. Lentivirus production, and transductions were performed by J.S.R. and N.V.N.C. with help and assistance from J.J.T.. Primary neuronal cell cultures were generated by A.J.B., J.J.T. and N.V.N.C.. All data was analyzed by N.V.N.C.. J.J.D. supervised all work.

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

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**Supplemental Data Table 1.**
**Sequences of primers and sgRNAs**

**cDNA primers (RT-qPCR)**

| Gene | Species | Forward sequence | Reverse sequence | Template length | Notes |
|---|---|---|---|---|---|
| *Gapdh* mRNA | *H. Sapiens* | TGTCAAGCTCATTTCCTGGTAT | CTCTCTTCCTCTTGTGCTCTTG | 133 bp | intron-spanning |
| *GRM2* mRNA | *H. Sapiens* | CATTCCTGCCCATCTTCTATGT | GTGGCTAACCACGTTCTTCT | 161 bp | intron-spanning |
| *Gapdh* mRNA | *R. norvegicus* | ACCTTTGATGCTGGGGCTGGC | GGGCTGAGTTGGGATGGGGACT | 217 bp | intron-spanning |
| *Tent5b* mRNA | *R. norvegicus* | CCAGCAGATTGTACAGGTAGT | CTCACTCTGCAGGTCCATCT | 163 bp | intron-spanning |
| *Gadd45b* mRNA | *R. norvegicus* | GACAACGCGGTTCAGAAGAT | TCCTCCTCTTCTTCGTCTATGG | 173 bp | intron-spanning |
| *Sstr2* mRNA | *R. norvegicus* | CGTGGAAAAGCAAGATGTCAC | TATGGCTCTGTCTGGTTGGA | 213 bp | intron-spanning |

**gDNA primers**

| Gene | Location | Forward sequence | Reverse sequence | Template length |
|---|---|---|---|---|
| human *GAPDH* | 642 bp upstram of *GAPDH* TSS | GAGCCTCGAGGAGAAGTTCC | GGACCCTTACACGCTTGGAT | 167 bp |
| rat *Gapdh* | 174 bp upstream of *Gapdh* TSS | GGTCGGAGCCCACACGCTTG | TCCCGCTCGGCTCATCCAGT | 161 bp |
| "P1" inverted DIO-dCas9 | forward primer in beginning of dCas9 with reverse primer in WRPE | GGCCGATGCTGTACTTCTTG | TGTAATCCAGAGGTTGATTGTC | 324 bp |
| "P2" correct orientation/ecombined DIO-dCas9 | forward primer in hSYN with reverse primer in beginnig of dCas9 | GAGTCGTGTCGTGCC | GGCCGATGCTGTACTTCTTG | 180bp (consitutive), 242 bp DIO |
| "P3" inverted SVI-DIO | forward primer in hSYN with reverse primer in inverted first segment of dCas9 | GAGTCGTGTCGTGCC | ATTCTGCGGCGGCAGGAAGA | 359 bp |
| "P4" recombined SVI-DIO | intron-spanning in recombined SVI-DIO | ACCTTCCGCATCCCCTACTA | TCGAAGTTGGTCATCCGCTCG | 262bp (spliced)/421 bp (unspliced) |

**CRISPR guide RNA (sgRNA)**

| Genomic locus | Species | Target Sequence | Genomic coordinates |
|---|---|---|---|
| *lacZ* | *E. coli* | TGCGAATACGCCCACGCGAT | *E. coli* strain 99-3165:3,886,546-3,886,565 |
| *Tent5b* | *R. norvegicus* | GAATTCGGTCCGAAGAGAAG | Rn6: Chr5: 155354031 |
| *Gadd45b* | *R. norvegicus* | GGCGGTGGAGCAGCTGCTGG | Rn6: Chr7: 11,647,911-11,647,930 |
| *Sstr2* | *R. norvegicus* | TGCGCCGCTAGCATTGGCCG | Rn6: Chr10: 10,2136,282-102,136,263 |