Cell type–specific manipulation with GFP-dependent Cre recombinase

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There are many transgenic GFP reporter lines that allow the visualization of specific populations of cells. Using such lines for functional studies requires a method that transforms GFP into a molecule that enables genetic manipulation. We developed a method that exploits GFP for gene manipulation, Cre recombinase dependent on GFP (CRE-DOG), a split component system that uses GFP and its derivatives to directly induce Cre/loxP recombination. Using plasmid electroporation and AAV viral vectors, we delivered CRE-DOG to multiple GFP mouse lines, which led to effective recombination selectively in GFP-labeled cells. Furthermore, CRE-DOG enabled optogenetic control of these neurons. Beyond providing a new set of tools for manipulation of gene expression selectively in GFP+ cells, we found that GFP can be used to reconstitute the activity of a protein not known to have a modular structure, suggesting that this strategy might be applicable to a wide range of proteins.

A challenge to the understanding of brain function is the ability to monitor and/or manipulate the activity of the many different cell types comprising the nervous system. To label specific cell types, many transgenic reporter lines using GFP1,2 as a marker of gene expression3 have been generated for various model organisms. Notably, over 1,000 transgenic GFP mouse lines have been characterized for labeling specific cell populations in the CNS (http://gensat.org/index.html)4–6. Until recently, GFP lines have only been used for labeling purposes. However, studies probing cellular function often require genetic manipulations, such as using mouse lines expressing site-specific DNA recombinases in specific cell types to drive the expression or deletion of particular genes. Compared with GFP reporter lines, the availability of mouse lines expressing the widely used Cre recombinase in specific cell types is more limited, and Cre expression patterns have not been as extensively characterized (http://gensat.org/index.html and http://brain-map.org/). Manipulation of GFP-labeled cell types previously required the generation of new mouse lines, for example, using the same cis-regulatory sequences that drove GFP in specific cell types to drive Cre expression in the same cell types. For more complex model organisms, such as the mouse, such an approach is costly and time-consuming. Furthermore, the expression pattern of Cre in these lines could differ from that of the equivalent GFP reporter line, potentially as a result of a combination of high sensitivity of Cre and/or position effects exerted by neighboring genomic sequences.

Recently, we developed GFP-dependent transcription factors (T-DDOGs) that enable retrofitting of transgenic GFP lines for functional manipulation studies7. In this system, GFP acts as a scaffold, bringing together modular transcription domains to assemble a hybrid transcription factor for activation of a target gene of interest. GFP recognition is mediated by pairs of GFP-binding proteins (GBPs), which were derived from the antigen recognition portion of camelid antibodies8,9. These ‘nanobodies’ are easily expressed in living cells and can be used as fusion proteins7–10. T-DDOGs opened the door for quick genetic manipulation of GFP-labeled cells via DNA electroporation, bypassing the need to generate transgenic Cre lines for cell-specific gene manipulations in certain cases7.

Several reasons motivate the continued development of new GFP-dependent systems. First, unlike transcription factors11,12, many proteins such as enzymes are not composed of simple modular structures. It is unclear whether GFP can be used to directly control the activity of such non-modular proteins. Second, many genetic tools depend on the action of Cre recombinase. Although T-DDOG can induce Cre expression, this effect requires the action of three genetically encodable components. Although electroporation can efficiently deliver multiple plasmids encoding T-DDOG to single cells, this approach is restricted to certain tissues and developmental time points. In contrast, the popular recombinant adeno-associated viral (rAAV) vectors can efficiently deliver multiple transgenes to single cells, across diverse tissues and over different developmental and/or mature stages. rAAVs encoding the recombinase-dependent flip-excision (FLEX) switch further enable convenient and selective expression of transgenes in specific cell types when injected into transgenic Cre and/or Flp lines13–15. The integration of GFP-inducible systems with FLEX responder genes in a rAAV delivery scheme would
provide a simple protocol for rapid manipulation of many types of genes selectively in GFP-labeled cell types. Furthermore, such a method would increase the number of specific cell types that could be manipulated with intersectional approaches, as transgenic animals with a combination of GFP and Flp in specific cell types could activate transgenes that are Cre and Flp dependent\(^{14,16}\).

We used GFP to control the activity of Cre, in the form of split Cre-GFP fusion proteins that together make up CRE-DOG. The GBP-Split Cre fusion proteins assembled as a complex on the GFP scaffold, initiating Cre recombination (Fig. 1a). This system requires only two components for direct activation of Cre recombination (Supplementary Fig. 1). We generated rAAV reagents that enabled delivery of CRE-DOG to diverse tissues and brain regions that cannot be electroporated, for rapid and tight GFP-regulated Cre-dependent transgene expression across GFP reporter lines and cell types. To demonstrate the utility of this approach, we showed that CRE-DOG could be used for optogenetic manipulation of GFP-expressing cells in the nervous system.

**RESULTS**

CRE-DOG refers to the combined action of two chimeric proteins bearing complementary split Cre fragments\(^2\), each fused to a different GBP. We tested the GBP1+GBP6 and GBP2+GBP7 combinations, as they have been used to assemble a hybrid transcription factor on the GFP scaffold in living cells\(^7\). Using a CAG-loxP-Neo-loxP-luc2 (CALNL-luc2) construct (or floxed luciferase reporter), we screened pairs of the GBP-split Cre fusion proteins for GFP-dependent activity. Almost all of the GBP-split Cre pairs had either low GFP-induced recombination activity or high background activity (data not shown). We considered whether it would be possible to increase efficiency by generating a full-length Cre recombinase via protein splicing\(^{18}\). To this end, we inserted the artificially split \(S.\ cerevisiae\) vacuolar ATPase (VMA1) intein elements 19,20 into the GBP-split Cre (Fig. 2a). This truncated fusion protein, hereafter named N-Cre\(\text{int}G\), was combined with C-Cre\(\text{int}G\) to give the optimized CRE-DOG (CRE-DOG\(\text{OPT}\)) (Fig. 1b). CRE-DOG\(\text{OPT}\) activity depended on all of the components of the system and was specific for GFP and its derivatives (Fig. 1c,d). Furthermore, CRE-DOG\(\text{OPT}\) activity was dependent on GFP dosage in a manner similar to that observed for T-DDOGs\(^7\) (Fig. 1e).

We next tested whether CRE-DOG could retrofit existing transgenic GFP reporter lines for cell type-specific manipulations. The Tg(CRX-GFP) line expresses GFP strongly in photoreceptors and weakly in inner nuclear layer (INL) cells\(^3\). Tg(CRX-GFP) retinas electroporated with CRE-DOG\(\text{OPT}\) and CALNL-DsRed plasmids showed strong DsRed labeling of photoreceptors and occasional labeling of INL cells, whereas electroporated retinas that were GFP-negative showed little to no DsRed expression (Fig. 2a,b and Supplementary Fig. 6a–c). In addition, 100 ± 0% (hereafter, data are presented as mean ± s.d., unless indicated otherwise) of DsRed cells labeled in the outer nuclear layer (ONL) were GFP\(^+\) (Supplementary Fig. 6g). CRE-DOG\(\text{OPT}\) induced DsRed expression in 76 ± 4% of electroporated

**Figure 1** An optimized Cre recombinase dependent on GFP (CRE-DOG\(\text{OPT}\)). (a) Schematic of CRE-DOG\(\text{OPT}\) action. (b) Schematic of chimeric constructs comprising CRE-DOG\(\text{OPT}\). (c–e) Luciferase reporter assays in transected 293T cells assessing CRE-DOG\(\text{OPT}\) specificity for all components of the system (c), specificity for different fluorescent proteins (d) and dependency on GFP level (e). \(n = 18\) replicate samples per condition for c and d; \(n = 6\) or 9 for e. Results are representative of at least three independent transfections. Boxplots indicate minimum to maximum range. Box boundaries range from 25th to 75th percentile. The line in box indicates median.
cells in the ONL (Supplementary Fig. 6a). This is an estimate of CRE-DOG\textsubscript{OPT} efficiency in vivo, as all electroporated cells in the ONL were GFP\textsuperscript{+}. The value is likely an underestimate of efficiency, as it has not been corrected for the percentage of cells that were co-electroporated with all constructs.

Given that a considerable retinal diversity lies in the bipolar cell class, we tested whether CRE-DOG\textsubscript{OPT} would be effective in this cell class. We used a bipolar-selective promoter (ChAG) to drive strong expression of CRE-DOG\textsubscript{OPT} components in various bipolar cell types of the Tg(PROX1-GFP) line, and assayed Cre activity with a ChAG-loxP-TagBFP-loxP-mCherry reporter (ChAG-LtBFPL-mCherry). In contrast with Tg(CRX-GFP), electroporated Tg(PROX1-GFP) retinas showed strong mCherry labeling of bipolar cells, as well as sparse labeling of photoreceptors, and rare labeling of Müller glia and amacrine cells (Fig. 2c). Although only 83 ± 10% of mCherry\textsuperscript{+} cells were GFP\textsuperscript{+} (Supplementary Fig. 6g), analysis of electroporated GFP-negative retinas revealed that the system was absolutely GFP dependent (Fig. 2c and Supplementary Fig. 6d,e). Thus, developmental expression of Tg(PROX1-GFP) in progenitor cells giving rise to multiple cell classes or weak/transient GFP expression may contribute to the labeling pattern. These results establish the utility of CRE-DOG for genetic manipulation of specific cell types in transgenic GFP animals.

rAAVs are widely used vectors for delivering transgenes to the mammalian nervous system given their high infectivity of postmitotic neurons and low toxicity\textsuperscript{24}. The ability to deliver rAAV-encoded CRE-DOG\textsubscript{OPT} along with rAAV carrying the tight Cre-responsive FLEX switch\textsuperscript{13} in a single injection mixture would provide a fast and versatile approach for manipulating genes in GFP-labeled cell types across wide areas of the nervous system and across a broad age window. Indeed, rAAV-encoding CRE-DOG\textsubscript{OPT} could be used to turn on rAAV-FLEX-tdTomato (tdT) in a GFP-dependent manner in vivo (Supplementary Fig. 7a). To test this approach in transgenic GFP lines, we co-injected rAAV-CRE-DOG\textsubscript{OPT} and rAAV-FLEX-tdT into retinas of Tg(TRHR-GFP)\textsuperscript{25} and Tg(CDH3-GFP)\textsuperscript{26} lines, both of which express GFP in specific retinal ganglion cell (RGC) subtypes. Functional studies of RGCs have been hampered by the scarcity of Cre driver lines that are selectively active in the same RGC subtypes seen in multiple GFP reporter lines. Single injection of the aforementioned rAAV mixture into GFP-negative retinas resulted in no detectable tdT expression in Cre-immunopositive cells of the ganglion cell layer (GCL) (0 tdT\textsuperscript{+} cells in 140 anti-Cre\textsuperscript{+} cells, sampled across three retinas; Supplementary Fig. 7b). In contrast, we found highly selective tdT expression in GFP\textsuperscript{+} RGCs, with 89 ± 7% of tdT\textsuperscript{+} cells being TRHR-GFP\textsuperscript{+} (207 of 243 tdT\textsuperscript{+} cells, 5 retinas) and 85% of tdT\textsuperscript{+} cells being CDH3-GFP\textsuperscript{+} (11 of 13 tdT\textsuperscript{+} cells, 2 retinas); Figure 3a,b.
Figure 4  rAAV delivery of CRE-DOG\textsuperscript{OPT} to the lumbar spinal cord of a GFP line labeling dorsal horn interneurons. (a) Schematic of viral injection into the spinal cord at lumbar level L3–5. Viruses were injected bilaterally into the spinal cord. (b,c) Sagittal lumbar sections of Tg(CBLN2-GFP) (b) and GFP-negative littermate (c) mice. (b) GFP antibody–boosted fluorescence (green, left), tdT (red, middle) and merged image (right). IB4 stain (blue) is in all panels and delineates lamina Ilo. Arrowheads show no tdT induction in Cre\textsuperscript{+} cells of c show obvious examples of GFP and tdT overlap. (c) Cre immunostaining (magenta, left) and tdT (right). DAPI (not shown) was used to delineate lamina II/III. Arrowheads show no tdT induction in Cre\textsuperscript{+} cells of GFP-negative animals. Scale bars represent 100 \(\mu\text{m}\). Images are representative of two spinal cords per condition.

Figure 5  rAAV delivery of CRE-DOG\textsuperscript{OPT} to the motor cortex of GFP line. (a) Left, schematic of viral injection into mouse motor cortex. rAAV-2/1 viruses were injected into the cortex at 4 weeks of age and brains were harvested 3 weeks post-injection. Right, coronal section of the mouse brain. (b) Quantifications including specificity and efficiency of CRE-DOG\textsuperscript{OPT} labeling in cortical interneurons. Plot shows percentage of cells positive for a marker, given the presence of additional marker(s) (first marker/second, third marker). For GFP\textsuperscript{+}/tdT\textsuperscript{+} and tdT\textsuperscript{+}/GFP\textsuperscript{+} parameters, 735 GFP\textsuperscript{+} and 475 tdT\textsuperscript{+} cells were counted from ten sections, three Tg(GAD67-GFP) animals. For quantifications involving Cre immunostaining, 371 GFP\textsuperscript{+}, 203 tdT\textsuperscript{+} and 1,238 Cre\textsuperscript{+} cells were counted from nine sections, three Tg(GAD67-GFP) animals. 2,560 Cre\textsuperscript{+} and 10 tdT\textsuperscript{+} cells were counted from 2 GFP-negative animals. Box extends from the 25th to 75th percentiles, and the center line represents the median. (c) Coronal cortical section of Tg(GAD67-GFP) mouse infected with rAAV showing GFP (green) and tdT (red) expression. Arrowheads indicate injection site. Cortical layers are indicated in GFP\textsuperscript{+} section. Scale bar represents 1 mm. (d) CRE-DOG\textsuperscript{OPT} induced tdT expression in Tg(GAD67-GFP) (top), but not in GFP-negative brains (bottom). Panels show GFP (green), Cre immunostaining (blue), tdT (red) and merged images of all three channels (right). Scale bar represents 100 \(\mu\text{m}\). Images in c and d are representative of three Tg(GAD67-GFP) and two GFP-negative brains.

and data not shown for Tg(CD3-H3-GFP)). The sparser number, as well as non-uniform spatial distribution of CDH3-GFP\textsuperscript{+} RGCs, may contribute to the low number of cells labeled by tdT.\textsuperscript{26,27} Notably, strong tdT expression enabled tracing of labeled RGC projections into various brain targets; the resultant patterns were consistent with the identity of GFP\textsuperscript{+} RGCs previously characterized for Tg(TRHR-GFP) (Fig. 3c,d).\textsuperscript{15} Taking together the rAAV and electroporation results, we found that one can use CRE-DOG\textsuperscript{OPT} to selectively target GFP cells residing in all three major cellular layers of a neural tissue, the retina.

To evaluate whether CRE-DOG is useful outside of the retina, we co-injected rAAV-CRE-DOG\textsuperscript{OPT} and rAAV-FLEX-tdT into additional neural tissues. We targeted GFP cells of the spinal cord in the Tg(CBLN2-GFP) line, which expresses GFP in a subset of interneurons in the dorsal horn (unpublished data, V.E.A., and Fig. 4a). Although rAAV injection of wild-type spinal cords did not result in detectable tdT\textsuperscript{+} expression (0 tdT\textsuperscript{+} of 108 anti-Cre\textsuperscript{+} cells, sampled from two spinal cords), infection of Tg(CBLN2-GFP) spinal cords led to the appearance of tdT\textsuperscript{+} cells, some of which expressed tdT\textsuperscript{+} at levels strong enough for visualization of fine processes (Fig. 4b,c). We found that 75% of tdT\textsuperscript{+} cells were GFP\textsuperscript{+} (n = 105 tdT\textsuperscript{+} cells, sampled from two spinal cords). Thus, the labeling pattern may include cells that had transient and/or low levels of GFP, below the detection threshold. To estimate the efficiency of rAAV-CRE-DOG\textsuperscript{OPT} gene manipulation protocol in the spinal cord, we quantified the number of tdT\textsuperscript{+} cells among the GFP\textsuperscript{+} cells that also expressed split-Cre fragments, as detected by a polyclonal Cre antibody. Using this approach, we estimated the efficiency to be 51% (n = 37 Cre\textsuperscript{+}, GFP\textsuperscript{+} cells, sampled from two spinal cords).
We also tested for CRE-DOG<sup>OPT</sup> activity in the brain. The Tg(GAD67-GFP) line expresses GFP in GABAergic cell types in multiple brain regions<sup>28</sup>. We injected the rAAV mixture encoding CRE-DOG<sup>OPT</sup> and FLEX-tdT into the motor cortex as well as into the cerebellum of Tg(GAD67-GFP) animals (Figs. 5 and 6, and Supplementary Table 2). Cortical infection resulted in GFP-dependent induction of tdT expression throughout all cortical layers of Tg(GAD67-GFP) brains (Fig. 5<sup>b</sup>–d). A high percentage of tdT<sup>+</sup> cells was GFP<sup>+</sup>, at 98 ± 2% (Fig. 5b). In addition, 78 ± 10% of GFP<sup>+</sup> and anti-Cre<sup>+</sup> cells were also tdT<sup>+</sup>, providing an estimate of efficiency of the rAAV-CRE-DOG<sup>OPT</sup> injection protocol in the brain (Fig. 5b).

Notably, despite efficient infection of cortical pyramidal neurons, as evidenced by Cre immunostaining, no pyramidal neurons, and rarely any other cell type, were tdT<sup>+</sup> in GFP-negative mice (8 tdT<sup>+</sup> cells in 2,560 anti-Cre<sup>+</sup> cells, ten sections, two GFP-negative brains; Fig. 5c,d and Supplementary Table 2). Injection of the rAAV-CRE-DOG<sup>OPT</sup> and FLEX-tdT mixture into Tg(GAD67-GFP) cerebella resulted in strong GFP-dependent labeling of multiple GABAergic cerebellar cell types, including Purkinje cells (PCs), molecular layer interneurons (MLIs) and granule layer interneurons (GLIs), as confirmed by morphology and immunostaining for cell type markers (Fig. 6, Supplementary Figs. 8 and 9, and Supplementary Table 2). Anti-Cre staining revealed that there was little to no CRE-DOG<sup>OPT</sup> activity in GFP-negative cerebellum infected with the rAAV mixture (Fig. 6c,d, Supplementary Figs. 8 and 9e–g, and Supplementary Table 2). In infected Tg(GAD-GFP) cerebella, we found that 96 ± 2% of tdT<sup>+</sup> cells were GFP<sup>+</sup> and, in areas of dense infection, 42 ± 10% of all GFP<sup>+</sup> cells were tdT<sup>+</sup> (Fig. 6e). To more accurately evaluate the efficiency of the CRE-DOG<sup>OPT</sup> system, we quantified the percentage of tdT<sup>+</sup> cells that were both anti-Cre<sup>+</sup> and GFP<sup>+</sup>, which was 55 ± 26% and ranged between 44–67% depending on cell type (Fig. 6d and Supplementary Table 2).

To evaluate the utility of CRE-DOG<sup>OPT</sup> for functional studies, we co-infected Tg(GAD67-GFP) with rAAV-CRE-DOG<sup>OPT</sup> and rAAV-FLEX-ChR2-tdT to manipulate GFP<sup>+</sup> cells optogenetically (Fig. 7a–c). We were able to directly trigger light-evoked action potentials and photocurrents in ChR2-tdT–labeled PCs (n = 9) and MLIs (n = 6) (Fig. 7d,e). We also were able to optogenetically inhibit spontaneous activity in ChR2-tdT–negative PCs that received GABAergic synaptic input from ChR2-tdT<sup>+</sup> MLIs (n = 4; Fig. 7f). GFP<sup>+</sup> PCs in uninfected areas, as well as PCs in infected areas of GFP-negative littermates, did not show any light-induced photocurrents or light-triggered synaptic currents (n = 9 PCs for GFP-negative animal, n = 8 PCs in uninfected area; Fig. 7g). Finally, infected PCs and MLIs did not differ from uninfected cells in intrinsic electrical properties, as assessed by membrane resistance and spontaneous action potential frequency (P = 0.92 and 0.73, respectively, one-way ANOVA for PCs, and P = 0.87 and P = 0.81, respectively, t test, for MLIs; Fig. 7h,i).

Together, these results demonstrate the utility of CRE-DOG for cell-specific manipulation of neural circuit activity in the brain.

**DISCUSSION**

We developed a high signal-to-noise system, CRE-DOG<sup>OPT</sup>, that provides a reliable and rapid means of genetically manipulating GFP-expressing cells in the many GFP lines available for life science research. We demonstrated the repurposing of GFP reporter lines for cell-specific gene manipulation via electroporation and/or viral infection. Given the widespread use of rAAVs among neuroscientists, rAAV-encoded CRE-DOG<sup>OPT</sup> should immediately be useful for many neuroscience applications. rAAV allows delivery over a broader time window and into more tissues than electroporation, the method originally used to deliver T-DOG. It should be straightforward to apply rAAV-encoded CRE-DOG<sup>OPT</sup> in a single injection mixture with rAAV-encoded Cre-dependent transgenes or into transgenic GFP animals with Cre-responsive alleles. Further refinement in cell targeting should be possible by intersecting GFP-dependent CRE-DOG<sup>OPT</sup> activity with other recombinases<sup>14,16</sup>.

The CRE-DOG<sup>OPT</sup>-encoding plasmids that we developed also will be immediately useful for targeting GFP cells for gene manipulation...
Figure 7 CRE-DOG OPT allows optogenetic manipulation of GFP-labeled neurons. (a) Schematic of experiment. (b, c) Expression of ChR2-tdT in lobule V/VI of a parasagittal cerebellar slice (b) and at the cellular level (c) in PCs and MLIs (marked by asterisks). Scale bars represent 1 mm (b) and 50 µm (c). Note that ChR2-tdT labeled membranes. (d–g) Optogenetic control of GFP-labeled cells in the cerebellar circuit. Cell-attached and whole-cell recordings are aligned with blue light stimulation (blue vertical bars at top). (d) Light-evoked spikes (marked in red) in addition to spontaneous action potentials (black) in cell-attached recording, and underlying photocurrent in a whole-cell recording of the same cell (bottom trace) in ChR2-tdT+ PC (representative of n = 9). Cells were voltage-clamped at the indicated potentials. (e) ChR2-tdT+ MLI showed light-evoked spiking (top trace, red), photocurrent (middle trace) and inhibitory current (bottom trace) from stimulation of synaptic responses connecting MLIs. The GABA_A receptor antagonist SR95531 (5 µM) blocked the inhibitory current (representative of n = 5). (f) Example of GFP+, tdT– PC (green) receiving inhibitory input from ChR2-tdT+ MLI. Activation of the ChR2-tdT+ MLI briefly inhibited PC spiking in cell-attached recording (top trace). Bottom, light-evoked inhibitory current blocked by SR95531 (representative of n = 4). (g) GFP+, tdT– cells (gray) did not respond to light stimulation (n = 9). (h, i) Membrane resistance and spontaneous action potential (AP) frequency of PCs (GFP+, tdT–: 77.2 ± 18.6 MΩ, n = 8; GFP+, tdT+: 86.8 ± 14.6 MΩ, n = 9; GFP–, tdT–: 81.0 ± 17.6 MΩ, n = 9; P = 0.92; GFP–, tdT+: 43.4 ± 7.9 Hz, n = 6; GF, tdT–: 43.2 ± 6.6 Hz, n = 8; GFP–, tdT+: 49.8 ± 6.4 Hz, n = 9; P = 0.73; one-way ANOVA; h) and MLIs (GFP+, tdT–: 423 ± 115 MΩ, n = 6; GFP+, tdT+: 397 ± 105 MΩ, n = 6; GFP–, tdT–: 137 ± 2.1 Hz, n = 6; GFP–, tdT+: 14.7 ± 3.5 Hz, n = 6; P = 0.87, paired Student’s test; i) did not differ between conditions. PC data were obtained from four GFP+ animals injected with CRE-DOG, and one injected GFP-negative animal. MLI data were obtained from two injected GFP+ animals. Scatter plots are mean ± s.e.m.
of one versus the other. First, overexpression of transcription activation domains may cause undesirable side effects. We found this to be the case for T-DDOG variants using particularly potent activation domains, and overcame the issue by changing the activation domain. Unlike transcription factors, Cre recombinase does not depend on interactions with endogenous cellular machinery and may be expected to induce fewer complications. Indeed, we did not detect any abnormalities in cells overexpressing CRE-DOGOPT. This is supported by physiology experiments in ChR2-expressing cerebellar cells that failed to detect any deleterious effects (Fig. 7). However, we did encounter cytotoxicity when delivering the components to the cerebellum via serotype 8 capsid, but not serotype 1 capsid (data not shown). This is therefore a serotype-specific issue and not related to CRE-DOGOPT expression. Serotype-specific cytotoxicity has been reported previously and is important to consider when designing experiments. Nevertheless, high levels of native Cre were reported to be toxic in some settings. Second, T-DDOG may be preferred when temporal control is desired, as T-DDOG can be modified to respond to drug application. It is also possible to modify CRE-DOGOPT to confer drug inducibility by using domains such as ERT2 or destabilizing domains. However, this would only enable temporal control at the onset of expression, as Cre-mediated recombination is essentially irreversible.

CRE-DOGOPT, as with T-DDOG, is sensitive to high GFP levels in that GFP in excess of the split components can titrate out each component, thereby reducing activity. Nonetheless, empirically, we found Cre-dependent recombination to be fairly efficient, even in lines with strong levels of GFP expression (Figs. 2, 5 and 6). Also, CRE-DOGOPT was able to detect low GFP levels, as demonstrated by our findings with the relatively weakly GFP-expressing RGC lines, which require GFP immuno-staining to reveal neuronal processes (Fig. 3). In fact, we failed to detect GFP in some cells with evidence of recombination activity, presumably because activity was triggered by GFP expression below the threshold of detection or from transient GFP expression (Figs. 2 and 4). This does point out that the proper control for CRE-DOGOPT is a GFP-negative animal; we detected almost no CRE-DOGOPT activity in GFP-negative tissues. The irreversibility of Cre recombination can be exploited to trigger stable expression of a transgene, but may also lead to accumulation of background, even if there is only low GFP-independent recombination activity. We did not find background activity to be an issue for our incubation period of 2–4 weeks. However, beyond this incubation period, we did observe increased background in rAAV-CRE-DOGOPT–injected GFP-negative animals (data not shown). The accumulation of background may be reduced by optimizing plasmid dose or viral titer, and/or by using tightly drug-regulated CRE-DOGOPT variants that await development. Another contributor to variable background activity may be the differential sensitivity of Cre-responsive gene constructs; in our experience, FLEX constructs showed less background recombination than loxP-STOP-loxP alleles.

Although split intein elements were found to promote GFP-specificity of CRE-DOGO, protein splicing did not seem to be involved. It is unclear why the inclusion of intein elements reduced background activity of CRE-DOGO, but one could speculate that the extra elements influenced the orientation or conformation of the split protein fragments such that non-specific CRE-DOGO assembly was inhibited in the absence of GFP. Whether split intein elements would also aid in reducing background activity of other ligand-inducible split protein systems is an interesting issue to consider in the future.

From a broader perspective, our work demonstrates the utility of artificially derived–binding proteins (for example, nanobodies, intrabodies, etc.) for constructing split enzyme systems inducible by intracellular proteins. The ability to rapidly isolate additional high-affinity nanobodies should facilitate the extension of this approach to other target proteins. Our work thus offers a new approach for engineering protein-inducible systems in addition to providing a practical solution for retrofitting GFP reporter lines for a much broader set of applications.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.C.Y.T. and C.L.C. initiated and coordinated the entire project. J.C.Y.T. originated the idea and carried out all of the in vitro molecular biology, electroporation and proof-of-principle rAAV experiments to validate the concept. S.R. coordinated brain injections, and performed electrophysiological recordings of cerebellar slices, immunohistochemistry, imaging and analysis of cerebellar and cortical data. O.S.D. and A.D.H. performed viral injections into retinas of GFP lines and subsequent tissue processing, imaging and analysis. V.E.A. and S.C. performed viral injections into spinal cords and subsequent tissue processing, imaging and analysis. E.D. and S.W.L. constructed the ChAg-loxP-TagBP-loxP-mCherry construct. S.W.L. performed electroporation into the Tg(ROX1-GFP) line and subsequent tissue processing. I.R.D. performed viral injections into the cortex and cerebellum and provided feedback on experiments. A.D.H. and W.G.R. supervised aspects of the project involving rAAV injection into transgenic GFP retinas and brains, respectively. C.L.C. supervised the entire project. J.C.Y.T. and C.L.C. wrote the paper, with contributions from all of the authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. All animal experiments performed were approved by the Institutional Animal Care and Use Committees at Harvard University and University of San Diego. All animals studied were Mus musculus. Time-pregnant CD1 mice (Charles River Breeding Laboratories) were used for electroporation experiments. Transgenic GFP mouse lines used were Tg(CRX-GFP)43, Tg(ProX1-GFP) [http://genats.org/index.html], Tg(TRHR-GFP)43, Tg(CD3H-GFP)43, Tg(CRLN2-GFP) [http://genats.org/index.html], Tg(GAD67-GFP)43. Strains were kept in C57BL/6 background except Tg(CD3H-GFP), which is in FVB/N and CD1 mixed background.

Molecular biology reagents. pCAG-GFP (Addgene plasmid 11150)39, pCAG-YFP (Addgene plasmid 11180)39, pCAG-CFP (Addgene plasmid 11179)39, pCAG-td-tf (C.L.C., Harvard Medical School), pCAG-mCherry (C.L.C., Harvard Medical School), pCAG-DsRed (Addgene plasmid 11151)39, pRL-TK (Promega, #E2241), pRho-GFP-ires-AP (referred to as Rho-GFP)40, pCAG-nlacZ (C.L.C., Harvard Medical School). pAAV-CAG-FLEX-td was a gift from E. Boyden (Massachusetts Institute of Technology) (Addgene plasmid #28306), pCALNL-DsRed (Addgene plasmid #13769)41.

CRE-DOG chimeric component constructs. Using standard cloning techniques, coding sequences of GBP1, 2, 6 and 7, the sources of which were described previously44, were fused to those of split Cre and split VMA intein fragments in many possible configurations, and the fusion products were inserted into a pCAG vector42. Split Cre fragments were taken from a codon-optimized Cre coding sequence called improved Cre (iCre)41. Two previously described pairs of complementary split Cre fragments were tested: N-split Cre corresponding to residues 19–59 of full-length Cre (iCre19–59), paired with C-split Cre corresponding to residues 60–314 of full-length Cre (iCre60–314), and N-split Cre corresponding to residues 109–140 of full-length Cre, paired with C-split Cre corresponding to residues 106–314 of full-length Cre7. Split VMA intein fragments, termed N-terminal VMA (N-VMA or NVMA) and C-terminal VMA (C-VMA or CVMA) here, were described previously19 and were codon-optimized for mammalian expression by Genewiz.

Notable CRE-DOG fusion plasmids. pCAG-N-CreintG. An Agel-Kozak-NLS-iCre19–59-NVMA-Nhel restriction digested fragment was inserted into the backbone of Agel/Nhel digested pCAG-Gal4DBD-10gly-GBP7 plasmid27, resulting in in-frame fusion to the 10gly GBP7 coding sequence and replacing Gal4DBD in the original vector.

pCAG-CreintG. An Nhel/NotI flanked and restriction digested fragment containing CVMA/Cre60–143 (C-CreintG) was inserted into the backbone of Nhel/NotI digested pCAG-GFP-2P-10gly-VPmimx3 plasmid27, resulting in in-frame fusion to GBP2 coding sequence and replacing 10gly-VPmimx3 in the original vector.

pCAG-N-CreintG. An Agel/Nhel flanked and restriction digested fragment containing fusion of NLS, iCre19–59 and the last 129 bp of NVMA (N-CreintG) was inserted into the backbone of Agel/Nhel digested pCAG-N-CreintG plasmid, resulting in in-frame fusion to the 10gly GBP7 coding sequence and replacing NLS-iCre19–59-NVMA in the original vector.

pAaA-EF1a-N-CreintG. A BamHI/EcoRI flanked and restriction digested fragment containing Kozak-N-CreintG was inserted into the backbone of BamHI/EcoRI digested pAaA-EF1a-FLEX-GTB (Addgene plasmid #26197)44, pAaA-EF1a-CreintG. A BamHI/EcoRI flanked and restriction digested fragment containing Kozak-C-CreintG was inserted into the backbone of BamHI/EcoRI digested pAaA-EF1a-FLEX-GTB (Addgene plasmid #26197)44.

pCAG-LoxP-TagBP loxP-Mcherry. First, pCAG GFP was digested with SpeI and SmaI to excise the CMV enhancer and a ~700 bp Chx10 enhancer element specific for bipolar cells44 was PCR amplified with flanking sequences corresponding to the pCAG backbone. PCR products were cloned in by Gibson assembly. To generate pCAG-Lox^P-TagBP loxP-Mcherry, pCHAG was digested with Agel and NotI. TagBP was PCR amplified using primers that had overlapping sequence with the digested vector, and Gibson assembly was used. To generate pCAG-Lox^P-TagBP loxP-Mcherry, pCHAG-Lox^P-TagBP loxP-Mcherry was linearized by NotI digestion. Digested vector and amplified mCherry PCR product were gel purified and assembled by Gibson ligation.

pCHAG-N-CreintG. An Agel/NotI flanked and restriction digested N-CreintG fragment was inserted into a pCHAG vector as Agel/NotI sites. pCHAG-C-CreintG. An Agel/NotI flanked and restriction digested C-CreintG fragment was inserted into a pCHAG vector as Agel/NotI sites.

CRE-DOG screens. We tested two combinations of N- and C-terminal split Cre fragments previously reported to be suitable for rapamycin-induced dimerization17. A split Cre pair comprising of residues 19–59 and residues 60–314 of full-length Cre, were each fused with the split intein portions from yeast VMA19 in specific orientations. These original constructs were found to give strong GFP-dependent recombinase activity coupled with low background activity, giving CRE-DOG17. This led to additional screens optimizing for CRE-DOG activity, eventually giving CRE-DOGopt17. All screens were assayed with the CALNL-luc2 reporter in 293T cells. CRE-DOG variant components were expressed using the CAG promoter in pCAG. Transfected DNA of the CRE-DOG components were adjusted with filler DNA (pCAGEN) to ensure comparable molar DNA delivery. To assay for GFP-inducibility, pairwise combinations of CRE-DOG component variants were transfected into cells with pCALNL-luc2, pRL-TK, and either pCAG-GFP or an empty CAG vector (pCAGEN). Cells were assayed for luciferase activity at 1 day post-transfection.

In vitro luciferase assays. Plasmids encoding CAG-driven GFP and N- and C-terminal split Cre chimeric variants were transfected via polyethyleneimine (PEI) into 293T cells along with plasmids encoding CALNL-luc2 and pRL-TK. Between 50–70 mg of total DNA were transfected into single wells of 48-well plates. Cells were ~80–100% confluent at time of transfection. Cells were harvested 24 h later for Dual Luciferase Assay (Promega). All transfections, except for the dosage curve, were done at a 1:1:1 (GFP-N-CreintG:C-CreintG) plasmid molar ratio. Both technical (pipetting of lysate into at least 2–3 different luciferase assay wells) and biological (at least 2–3 different transfection mixtures applied to different cell culture wells) replicates were included in each experiment. All experimental results were verified in at least three independent experiments.

Western blot. 293T cells were seeded onto 6 well plates and transfected with CAG-driven N-CreintG and C-CreintG-FLAG, along with CAG-GFP or empty CAG vector. A CALNL-DsRed reporter was included in some cases to confirm GFP-dependent transfection. 24 h post-transfection, transfected 293T cells were lysed in RIPA buffer (150 mM NaCl, 0.1% NP40 (vol/vol), 0.5% sodium deoxycholate (wt/vol), 0.1% SDS (wt/vol), 50 mM Tris, pH 8.0) for lysate supernatant load or 4× Laemmeli buffer plus β-mercaptoethanol. Samples were run on SDS PAGE and then subjected to western blot analysis. Anti-FLAG (F3165, Sigma) was used at 1:1,000 dilution.

In vivo electroportations. For wild type, Tg(CRX-GFP) and GFP-negative littermate retinas, electroporations, postnatal days 0–3 (P0–3) mouse pups were electroporated in vivo as described previously44. DNA solutions (1–1.5 μg μl−1) were injected through the sclera and into the subretinal space of the mouse retina. pCAGEN was used as an empty vector substitute for excluded plasmids. P0 CD1 retinas were electroporated with plasmids encoding CAG-driven CRE-DOG components and CAG-driven nuclear β-galactosidase (n-βgal, an electroporation marker expressed from CAG-nlacZ plasmid) along with 2 mg pCAG-DSRed and different promoter-GFP constructs. Electroporated retinas were harvested at P14, immunostained for n-βgal and then imaged on a Zeiss LSM780 confocal microscope. For Tg(ProX1-GFP) retina electroporations, P2–3 Tg(ProX1-GFP) or GFP-negative littermate retinas were blindly electroporated with plasmids encoding CHAG-driven CRE-DOG components and CHAG-loxP-TagBP loxP-Mcherry; retinas were harvested between 3–4 weeks of age. Wherever applicable, retinas were immunostained with anti-GFP and anti-β-galactosidase.
Slices were superfused with 31–32 °C artificial cerebrospinal fluid (ACSF) equilibrated with 5% CO2 and 95% O2 in the same solution. Parasagittal vermal slices of the cerebellum were cut at 40-μm thickness on a Leica VT1000S vibratome. Free-floating sections were immunostained using standard methods. Slices were then mounted on Superfrost slides (VWR) using Prolong Diamond mounting medium (Invitrogen). Images were acquired with an Olympus FV1000 or FV1200 confocal microscope. Whole brain sections were imaged on an Olympus MVX10 MacroView dissecting scope.

Antibodies and stains. For immunohistochemistry of retinas: rabbit anti-GFP (1:500 dilution, A-6479, Invitrogen), chicken anti-β-galactosidase (1:1,000 dilution, ab9361, Abcam), rabbit anti-TagRFP (1:1,000, AB233, Evrogen). Note that anti-TagRFP also recognizes TagBFP and shows cross-reactivity to mCherry. The mCherry cross-reactivity did not cause problems for our analysis. In fact, it was advantageous in our identification of electroporated cells in the Tg(PROXI-GFP) experiment. Secondary antibodies raised against the relevant species were obtained from Jackson ImmunoResearch or Invitrogen.

For immunohistochemistry of rAAV-infected transgenic GFP retinas: rabbit anti-DsRed (1:200 dilution, 632292, Clontech), chicken anti-GFP (1:2,000 dilution, 1020, Aves Labs).

For spinal cord immunohistochemistry: chicken anti-GFP (1:1,000 dilution, 1020, Aves Labs), rabbit anti-DsRed (1:1,000 dilution, 632292, Clontech), rabbit anti-Cre (1:200 dilution, 69050-3, Novagen), Isolectin GS-IB4 (Alexa Fluor conjugated, Molecular Probes, 1:500).

For cerebellar and cortical immunohistochemistry: chicken anti-GFP (1:200 dilution, ab13970, Abcam), rabbit anti-calbindin (1:500 dilution, AB1779, Millipore), rabbit anti-parvalbumin (1:1,000 dilution, PV25, Swant), rabbit anti-Cre (1:200 dilution, 69050-3, Novagen). Secondary antibodies: goat anti-rabbit and chicken anti-rabbit coupled to Alexa 647 (1:1,000 A-21443 and A21245, Invitrogen).

Slice preparation for electrophysiology. Mice were anaesthetized with isoflurane and decapitated. The brain was quickly removed and immersed in oxygenated ice-cold cutting solution containing (in mM) 110 choline chloride, 25 glucose, 25 NaHCO3, 11.5 sodium ascorbate, 7 MgCl2, 3 sodium pyruvate, 2.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2. Parasagittal cerebellar slices were cut at 270-μm thickness on a Leica VT1000S and subsequently allowed to recover for 30 min at 32 °C in artificial cerebrospinal fluid (ACSF) equilibrated with 95% O2 and 5% CO2, containing in (mM) 125 NaCl, 26 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 1, MgCl2, 2 CaCl2 and 25 glucose. Slices were then kept at 21–23 °C for up to 5 h.

Electrophysiological recordings. Slices were superfused with 31–32 °C warm ACSF at a flow rate of ~3 ml min⁻¹ in a recording chamber heated by an inline heater (Warner instruments). PCs and MLs were visualized using an Olympus BX51WI microscope equipped with differential interference contrast (DIC). GFP+ and ChR2-tdt+ were imaged using a custom two-photon laser-scanning microscope with 750 nm illumination49. Visually guided recordings were performed with ~2 MΩ (PCs) and 3.5 MΩ (MLs) borosilicate glass pipettes (Sutter Instrument). The internal solution for voltage-clamp recordings contained the following (in mM): 140 cesium methanesulfonate, 15 HEPES, 0.5 EGTA, 2 TEA-Cl, 2 MgATP, 0.3 NaGTP, 10 phosphocreatine-tris, and 2 QX 314-Cl (pH adjusted to 7.2 with CsOH). For current-clamp recordings, the internal solution contained 150 potassium gluconate, 3 KCl, 10 HEPES, 0.5 EGTA, 3 MgATP, 0.5 GTP, 5 phosphocreatine-tris, and 5 sodium phosphocarbonate (pH adjusted to 7.2 with KOH). Recordings were performed with a 700B Axoclamp amplifier (Molecular Devices) and were controlled with custom software written in Matlab (generously provided by B. Sabatini, Harvard Medical School). ChR2-tdt+ cells were excited using a 473-nm wavelength blue laser (OptoEngine) coupled through the excitation pathway of the microscope. Laser light was focused onto slices through a 40x water-immersion objective. Brief light pulses (0.5–1 ms) at an intensity of 3–5 mW mm⁻² evoked ChR2-mediated photocurrent and stimulus-locked action potentials.

Data analysis. Imaging data was analyzed and processed with ImageJ (US National Institutes of Health), Imaris (Bitplane), ZEN (Carl Zeiss Microscopy)
and/or Photoshop (Adobe Systems) software. Prism (GraphPad) was used to generate graphs. Electrophysiological data was analyzed with IgorPro (Wavemetrics), AxographX (Axograph Scientific) and Prism (GraphPad). For luciferase assays, two-tailed Student’s t test assuming unequal variance was used for all comparisons. \( P < 0.05 \) is judged as statistically significant.

Quantification of electroporated retinas. Confocal Z-stack images were taken of retinal regions with dense expression of electroporation markers. Electroporated cells were marked by the presence of electroporated GFP, n-\( \beta \)-gal (from CAG-nlacZ) or the combination of TagBFP and mCherry (driven from ChAG-loxP-tBFP-loxP-mCherry cassette; combination is detected by a polyclonal TagRFP antibody that detects both TagBFP and mCherry). Cells expressing a given marker (for example, Y) were selected using ImageJ Cell Counter plug-in, and then evaluated for coexpression of a second marker of interest (for example, X). Counted number of the second marker were divided by selected number of cells expressing the given marker, and multiplied by 100 to get the percentage of X+ cell given Y+ cell (for example, % X+/Y+). This general quantification scheme was also used for rAAV-infected tissues. To avoid counting green fluorescent DsRed molecules in GFP and DsRed colocalization analysis, we imaged for Anti-GFP expression in the blue or far red channel, and occasionally used the native GFP fluorescence as an aid for determining GFP expression.

Electroporated retina cells labeled by either n-\( \beta \)-gal or TagBFP immunostaining were quantified for DsRed or mCherry fluorescence, respectively. Using ImageJ, a mask for the chosen cell body was created on the anti-\( \beta \)-gal or anti-TagBFP channels and transferred to the DsRed or mCherry fluorescence channel, respectively. Measurements of the mean pixel intensity on single confocal slices were taken for the chosen cell bodies. Each measurement was paired with a background fluorescence intensity measurement from the same slice, chosen at a location in the slice where no cells were present. Each measurement was then subtracted from the background to obtain the net fluorescence, in arbitrary units (a.u.). We found mCherry to show slight aggregation in retinal cells, especially INL cell types. Thus, we avoided quantifying cellular regions with strong mCherry aggregation. To quantify fluorescence, we drew a shape circling the soma region with minimal aggregation in the confocal slice corresponding to the strongest mCherry fluorescence.

Quantification of rAAV-infected tissues. Confocal Z-stack images of rAAV-infected retinal, spinal cord, or brain samples were manually analyzed on ZEN (Zeiss) or ImageJ (US National Institutes of Health). The ImageJ plugin, Cell Counter, was used to aid counting.

Quantification of electroporated retinas. This general quantification scheme was assumed when \( P < 0.05 \).