Expanding the Repertoire of Optogenetically Targeted Cells with an Enhanced Gene Expression System

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SUMMARY
Optogenetics has been enthusiastically pursued in recent neuroscience research, and the causal relationship between neural activity and behavior is becoming ever more accessible. Here, we established knockin-mediated enhanced gene expression by improved tetracycline-controlled gene induction (KENGE-tet) and succeeded in generating transgenic mice expressing a highly light-sensitive channelrhodopsin-2 mutant at levels sufficient to drive the activities of multiple cell types. This method requires two lines of mice: one that controls the pattern of expression and another that determines the protein to be produced. The generation of new lines of either type readily expands the repertoire to choose from. In addition to neurons, we were able to manipulate the activity of nonexcitable glial cells in vivo. This shows that our system is applicable not only to neuroscience but also to any biomedical study that requires understanding of how the activity of a selected population of cells propagates through the intricate organic systems.

INTRODUCTION
There are two main approaches that one can take to understand how a complex biological system composed of numerous cells operates. One approach is to record the activities of as many cells as possible and compare these with the behavior of the entire system. By nature, this approach is a correlation study, and the causal relationship of the cells’ activities and the behavior could only be estimated. The other approach is to actively manipulate the cells’ activities and observe how such perturbation influences the system’s behavior. Light-sensitive proteins have recently gained recognition because they can be used to control the cells’ activities by use of light when it is expressed in cells (Nagel et al., 2003; Boyden et al., 2005). The advantage of this method is that, if the expression can be targeted, the physiological role of a selected population of cells in a complex system can be readily assessed.

The quickest way of applying this method is to use viruses, but this approach has problems in the reproducibility of expression patterns and levels. Therefore, we chose to use a transgenic approach, which allows stable expression. We also avoided the conventional transgenic approach, in which separate lines of animals for each promoter-protein combination are generated, because this approach seemed too laborious and because obtaining a line with sufficiently high expression levels would not be trivial.

We instead used a bigenic approach involving a tet-inducible promoter system (Gossen et al., 1995). We generated or received transgenic mice, each with a cell type-specific promoter driving a tetracycline-controlled transcriptional activator (tTA) expressing allele. We also generated transgenic mice with the tTA-dependent promoter (tetO) driving the expression of channelrhodopsin-2 (ChR2), a blue light-gated, nonselective, cation channel (Boyden et al., 2005; Nagel et al., 2003). Once equipped with both sets, we had only to perform crosses between mice from the two sets to obtain animals expressing ChR2 in a wide...
variety of selected cell populations. While ChR2 induction can be turned off by administration of doxycycline, we used the system solely to achieve cell-type specificity and amplification of transgene induction.

We previously attempted to use the tTA-mediated gene induction system to achieve specific and sufficient ChR2 expression. CaMKII-tTA mice (Mayford et al., 1996) were crossed with mice having a bidirectional tTA-dependent promoter (bitetO) driving both ChR2-mCherry and a halorhodopsin (HaloR; a yellow light-driven chloride pump)-EGFP fusion gene that we generated (line BTR6) (Chuhma et al., 2011). The bigenic line, CaMKII-tTA::BTR6, exhibited sparse ChR2 expression in striatal medium spiny neurons, which enabled us to conduct a connectome analysis (Chuhma et al., 2011). However, even though tTA was expressed under the control of the well-known CaMKII promoter, the line hardly showed any ChR2 expression in the hippocampus or dorsal cortex. We have found similarly poor induction using other combinations, indicating a need for an improved system. Here we report on technical improvements that were made to overcome this limitation of the conventional tTA-mediated gene induction.

**RESULTS**

**Improvement of the tet System**

When using the tet system to achieve cell type-specific expression of ChR2, the repertoire of bigenic transgenic lines can be expanded by increasing the numbers of either the tTA or the tetO lines coding variants of ChR2. However, the tet system often fails to produce sufficient expression in the expected pattern or cellular targets. It is likely that, even with sufficient tTA being expressed, the inserted tetO promoter locus may be influenced by suppressor sequences nearby or may not be readily accessible by the increased promoter DNA methylation or by the repressive histone modifications (Oyer et al., 2009; Zhu et al., 2007). We anticipated that knocking in the tetO-Chr2 gene at or near a housekeeping gene would lead to high levels of transactivation because: (1) we empirically noticed that the levels of tet system-mediated gene induction were extremely high when tetO was knocked into the genome by embryonic stem (ES) cell homologous recombination (Tanaka et al., 2010), and (2) the transcriptional machinery is likely to be accessible to the genome around the housekeeping gene, which may permit binding of tTA to tetO site in any cell type (Palais et al., 2009).

Based on the above reasoning and observations, we targeted transgene insertion just downstream of the β-actin gene polyadenylation signal and generated tetO-Chr2(C128S)-EYFP knockin mice by ES cell homologous recombination (knockin; Figures 1A, 1C, and S1). We chose the C128S mutant of ChR2 because it has a much higher effective sensitivity to light than does the wild-type ChR2 due to the very slow dark recovery (Berndt et al., 2009), which potentially allows stimulation of cells with even modest levels of expression. The activation/deactivation rates with blue light are slow (\(t_{on} = 20\) and \(1.7\) ms, \(t_{off} = 108\) s and \(10\) ms, for ChR2(C128S) and wild-type ChR2, respectively) (Berndt et al., 2009); however, \(t_{off}\) can be accelerated up to \(57\) ms by yellow light pulse with the maximum light intensity achievable in our setup. As ChR2(C128S) mutant of ChR2 allows continuous, heightened activity of the expressed cell with minimal blue light illumination, it can be used to mimic the elevated neural activity (upstate), which is often observed in in vivo recordings (Steriade et al., 1993). If the cell’s action potential firing rate does not accommodate, the light-stimulated cell expressing ChR2(C128S) can potentially fire consecutively at its maximum capacity.

To determine whether the method of inserting the transgene (homologous recombination versus random insertion) has any
effect on tTA-mediated transcription, we also generated a bacterial artificial chromosome (BAC) transgenic mouse, in which the identical cassette was inserted into the identical site downstream of the β-actin gene within the BAC (BAC transgenic; Figure 1B). The insertion site of the modified BAC in the mouse genome is random, but, as the tetO-ChR2(C128S)-EYFP transgene is flanked by a large fragment of genomic DNA from the site near the β-actin gene, the chromosomal positional effect, if present, is expected to be small. As a case for random insertion of the sequence starting only from the tetO promoter, we used bitetO-ChR2-mCherry and HaloR-EGFP transgenic mice (line BTR6; transgenic; (Chuhma et al., 2011)).

These tetO lines were crossed with cell type-specific tTA lines (Figure 1D), and the degree of tTA-mediated transcription was examined by immunohistochemistry on fluorescent proteins (Figure 2). First, the tetO lines were crossed with the αCaMKII-tTA mice (Mayford et al., 1996), in which case αCaMKII promoter activity should produce tTA expression in most pyramidal cells in the hippocampus. In the case of the tetO conventional transgenic mice (BTR6; transgenic; Chuhma et al., 2011), tTA-mediated gene expression, as assessed by the EGFP immunohistochemistry, was sparse in the hippocampal CA1, and no expression was found in the dentate gyrus (Figure 2, bottom). The poor expressions of HaloR-EGFP might be explained by the aggregation formation of this protein because the original version of the HaloR-EGFP that we used was often found to aggregate easily. However, we failed to detect even the mRNA of HaloR-EGFP in the dentate gyrus, indicating that tTA-mediated transactivation was limited in tetO conventional transgenic mice. In the case of the tetO BAC transgenic mice, EYFP induction in CA1 was dramatically increased, but expression in the dentate gyrus was still not observed. tetO knockin mice exhibited EYFP induction in both CA1 and the dentate gyrus, an expression profile consistent with that of αCaMKII promoter activity (Mayford et al., 1996).

Crosses with other tTA lines, specific for orexin neurons (Orexin-tTA; A.Y., unpublished data), astrocytes (Mlc1-tTA; Tanaka et al., 2010), oligodendrocytes (PLP-tTA; Inamura et al., 2012), and microglia (Iba1-tTA; Figure S2), exhibited the same tendency: no/rare induction of EGFP in the tetO transgenic, moderate/high levels of EYFP expression in the tetO BAC transgenic, and the highest levels of expression in the tetO knockin mice. These observations indicated that both the genomic position of the tetO cassette and the method of genomic insertion were critical in gaining efficient gene induction. As the transgene

### Figure 2.

Superiority of the Knockin-Mediated ENhanced Gene Expression by Improved tet System over Other Transgene Expression Methods

Five cell type-specific tTA lines (columns) were crossed with three tetO lines (rows) coding light-sensitive protein (ChR2 and/or HaloR) and a fluorescent protein (EYFP or EGFP) fusion. Top, middle, and bottom row panels show EYFP immunohistochemistry of tissues from mice crossed with tetO-Chr2(C128S)-EYFP knockin mice (knockin), EYFP from mice crossed with tetO-Chr2(C128S)-EYFP BAC transgenic mice (BAC transgenic), and EGFP from mice crossed with bitetO transgenic line (BTR6; transgenic). tTA lines specific for neurons (αCaMKII and orexin promoter; first and second columns, respectively), astrocytes (Mlc1 promoter; third column), oligodendrocytes (PLP promoter; fourth column), and microglia (Iba1 promoter; fifth column) were used. The first, fourth, and fifth columns are from tissues of the hippocampus, the second column is from the lateral hypothalamus, and the third column is from the cerebellar lobe. Note that crossing of the cell type-specific tTA lines with tetO knockin mice (KENGE-tet system) provided the highest levels of gene expression in all cell types.

Scale: 200 μm.

See also Figure S2.
insertion would occur randomly in conventional transgenic approach, it is possible to obtain ubiquitous high expression by pure chance. However, by knocking in the tetO cassette downstream of the β-actin gene, we have established the Knockin-mediated ENhanced Gene Expression by improved tetracycline-controlled gene induction (KENGE-tet) system for reliably generating transgenic mice with light-responsive cells.

Repertoire of Optogenetic Control of Neurons and Glial Cells
As we found that the KENGE-tet strategy greatly improved the expression level of the transgene, we next sought to determine whether the expression was high enough to drive cellular activity upon illumination (Figures 3C–3E). Use of the existing cell type-specific tTA lines, along with the tetO knockin mice, enabled...
us to construct a repertoire of mice expressing ChR(C128S)-EYFP in a variety of tissues and cell types. We first prepared bigenic mice (P14–21) with a neuron-specific promoter driving tTA and performed whole cell patch-clamp recordings on cells expressing EYFP. Light-evoked responses of ChR2(C128S) from Purkinje cells in the cerebellum (with GAD67-tTA-mediated induction; generated by J.N. and Y.Y., see Extended Experimental Procedures), neurons in the medial habenula (serotonin receptor 5B-tTA [Htr5B-tTA]; Figure S3), and neurons in the inferior olive (Htr5B-tTA) were examined. In all cases, inward current (74.1 ± 49.7 pA, 16.1 ± 7.1 pA, and 29.9 pA in Purkinje cells, medial habenula neurons, and inferior olive neurons positive for EYFP; n = 4, 4, and 1, respectively) was evoked with blue light, and yellow light shut down the current, a feature of the bistable switch of ChR2(C128S). In most cases, the amount of photocurrent was sufficient to produce action potential firing in current clamp mode. Using slices from older animals (P30), we observed that the photocurrent was an order of magnitude larger in Purkinje cells positive for EYFP (Figure S4; P17–20, 52.9 ± 21.6 pA, P30, 582.7 ± 178.1 pA; n = 6 and 3, respectively). This implies that the ChR2(C128S)-EYFP expression progressively increases with age allowing robust control of cells’ activity, especially in adult animals used for most in vivo studies. We also generated bigenic mice with a glia-specific promoter driving tTA and recorded light responses from the Bergmann glial cells (astrocytes) in the cerebellum (Mic1-tTA) (Figure 3F), from oligodendrocytes in the hippocampal alveus (PLP-tTA) (Figure 3G), and from microglia in the caudal cortex (Iba1-tTA) (Figure 3H). Light-evoked current could also be induced in these glial cells, which are traditionally categorized as nonexcitable (104.5 ± 59.0 pA, 94.6 ± 35.7 pA, and 11.5 ± 2.4 pA for Bergmann glial cells, oligodendrocytes, and microglia positive for EYFP; n = 5, 6, and 5, respectively). The depolarization in astrocytes was relatively moderate, likely due to the low input resistance of these cells and the absence of major voltage-gated conductances.

**In Vivo Optogenetic Control of Neurons**

The benefit of our transgenic method is that we can obtain consistent ChR2(C128S)-EYFP expression patterns and levels within a given line of bigenic animals. In vivo experiments especially are often faced with variability between individual animals, and virus-mediated ChR2 expression would only add to the complexity of interpretation of the data, as the extent and localization of infection would inevitably vary between trials. The choice of the modified ChR2(C128S), which exhibits slow inactivation, seemed also advantageous in in vivo studies, as prolonged activation of cells is possible with only brief illumination with blue light, which should minimize the toxicity caused by the illumination itself. We took advantage of our system for the stable expression of ChR2(C128S)-EYFP and studied the light-evoked responses of neurons in vivo and the effect on the behavior of the animal.

For this study, we crossed a well-established tTA line, the αCaMKII-tTA line, with the tetO-Chr2(C128S)-EYFP knockin line that we generated (Figure 4A). The bigenic mice showed strong expression of ChR2(C128S)-EYFP in the hippocampal pyramidal neurons (Figure 2). Therefore, we first examined the effect of ChR2(C128S) activation on the field potential in the hippocampal CA1 area by in vivo recording using a microelectrode attached to an optical fiber (Figure 4A). Incidents of multi-unit and ripple-like synchronous events were prominently increased in response to blue light illumination (500-ms pulses, every 1 min; Figure 4B). As yellow light was not applied to rapidly terminate the ChR2(C128S) activation, the increase in the event frequency lasted for tens of seconds, but the event frequency came back close to the baseline within 1 min (Figure 4B, bottom), which allowed repetitive stimulation. These data show that the effect of the light stimulation on neuronal activity was clear and consistent (Figure 4B, n = 9 recordings from three animals).

To determine whether such manipulation of cell activity was powerful enough to cause behavioral responses, we implanted an optical fiber in position for illuminating the left, dorsal hippocampal CA1 area and connected it to a laser light source via an optical swivel to avoid tangling of the fiber from the free-moving mouse (Figure 4C). The mouse was kept in its home cage, and the mouse tended to stay in one location prior to the illumination. However, upon illumination (500-ms pulses, every 1 min), an increase in the mouse’s locomotion activity occurred, along with an emergence of behavior resembling thigmotaxis. Seeking behavior, as well as digging of the bedding, was also observed. Interestingly, such behavior started with a delay of approximately 100 s, which contrasts with the direct ChR2 activation of neurons in the motor cortex, which causes muscle movements almost immediately upon illumination (Aylings et al., 2009; Hira et al., 2009). This suggests that the light stimulation in our case was not simply evoking motor behavior; some sort of cognitive response prior to the motor behavior could also have been evoked. Even though light pulses were periodically applied, the increase in locomotion activity gradually settled, which correlate with the moderate rundown of light-evoked neuronal activity with repetitive stimuli (Figure 4B). However, the increase was still observable after 30 min from the onset of illumination.

The mouse was sacrificed at 30 min after the initiation of illumination and processed for histochemical analyses (Figure 4D). It has been reported that increase in c-fos is related to cell activity (Schoenenberger et al., 2009; Sheng and Greenberg, 1990), and by using in situ hybridization, we revealed that unilateral 30 min activation of ChR2(C128S) in the hippocampal CA1 region resulted in bilateral increase in c-fos induction in neurons of the hippocampus. It is possible that diffuse light from the optic fiber directly stimulated the cells in the contralateral hippocampus; however, even though ChR2(C128S) was also expressed in the dorsal cortex through αCaMKII promoter activity, neurons in this region did not show significant increases in c-fos. Therefore, it is likely that excitation of neurons initiated unilaterally by illumination could have spread to the contralateral region by neural activity subsequent to the direct stimulation. In the wild-type mouse, no increase in c-fos expression or change in behavior was observed with illumination through an optic fiber placed similarly, showing that the neural activity was caused not by the possible heat from or the endogenous response to the illumination.

In contrast to the most previous transgenic approach in which only a weak expression was achieved in a small subset of cells, our KENGE-tet system achieves robust and strong...
Figure 4. In Vivo Manipulations of Neurons and Glial Cells

(A) ChR2(C128S)-EYFP was expressed in hippocampal CA1 neurons by using the αCaMKII-tTA line. Dorsal left hippocampus was illuminated with blue light pulses (500 ms pulses, every 1 min for 20–25 min) through an optical fiber inserted into the brain, and neuronal activity was measured by an accompanying microelectrode.

(B) In vivo extracellular recordings of multi-unit and ripple-like activity of hippocampal neurons from an anesthesized transgenic mouse fixed under a stereotaxic apparatus. Representative three traces before and after illumination are magnified in the middle panels. (bottom left) Peri-stimulus time histogram of the ripple-like events from the example recording shown on top. Summary of the frequencies of ripple-like events during the 2 s leading up to (Control) and during the 2 s following (Light) the light illumination are shown in the bottom middle panel (n = 9 recordings from 3 animals; *p < 0.05, paired t test). (bottom right) Half decay time of the ripple-like event frequency from the stimulus onset. Error bars: SEM.

(C) A photo of a freely moving mouse in its home cage with an optical fiber inserted into its brain close to the hippocampus (top left). Representative data of the tracking of the head position (1 s intervals; bottom) during the 6 min period prior to (Control) and the 6 min period after (Light) the onset of a blue light stimulus train (500 ms pulses at 1 min intervals). Locomotion velocity is plotted against time (bin width = 10 s; top right). Blue bars on top represent the timing of the light stimuli. Bottom left panel shows the summary of the total travel distance during the 25 min after the initiation of light illumination train (n = 2 and 3 for transgenic and wild mice, respectively; *p < 0.05, Student’s t test).
ChR2(C128S)-EYFP expression. The above data demonstrate that the expression is high enough that consistent changing of the neuronal firing is possible and artificial manipulation of the circuit can be strong enough to manifest in behavioral response and in c-fos expression.

**Optogenetic Manipulation of Glial Cells**

Stimulation of neurons is often realized by using extracellular electrodes in electrophysiological studies, but what is often overlooked is that such stimulation inevitably stimulates the nearby glial cells as well. To understand how glial cells interact with neurons and participate in information processing in the brain, specific stimulation of glial cells is desirable. To this end, optogenetic manipulation of glial cell activity would be ideal; however, we were uncertain whether current injection through ChR2(C128S) would be sufficient to change glial cell activity status. Depolarization by current injection through ChR2(C128S) by itself is not surprising as any cell should react in this way (Figures 3F and 3G). Therefore, we used our astrocyte-specific ChR2(C128S)-EYFP-expressing mice, the Mlc1-tTA:tetO-ChR2(C128S)-EYFP knockin mice, and examined whether light stimulation could promote c-fos mRNA induction in astrocytes (Figure 4, right).

When studying glial cells, one needs to remember that these cells are very sensitive to injury. For example, just the insertion of an optical plastic fiber resulted in gliosis around the fiber and blue light illumination resulted in c-fos induction in glial cells beneath the fiber in wild-type mice, which have no expression of ChR2(C128S)-EYFP (data not shown). It is possible that local heat produced by the illumination (Yizhar et al., 2011) could have induced the c-fos expression in the injured glial cells (Dragunow et al., 1989). This result indicates that surgical operation should be avoided when studying the physiological roles of glial cell activity; therefore, the use of transgenic expression of ChR2 is preferred over virus mediated gene transfer. Our choice of the modified ChR2, ChR2(C128S), was also ideal because this ChR2 mutant is highly sensitive to light; therefore, the chance of stimulating this ChR2 variant through the skull without optical fiber insertion was high.

An optical plastic fiber was placed on top of the skull and fixed with dental cement, and the brain was illuminated only through the skull (Figure 4E). Such manipulation alone did not produce any injury to the brain or c-fos induction (data not shown). The induction of c-fos mRNA in cortical astrocytes was confirmed at 30 min after the onset of illumination (500-ms pulses, every 10 s; Figures 4G and 4H). c-fos mRNA induction in astrocytes was never observed in wild-type mice after illumination through the skull, and induction was reproducibly observed in the bigenic mice, indicating that this manipulation is specific. We also checked the expression of c-fms mRNA, which gets increased in activated microglial cells, and found that the expression level was not increased surrounding the operated and illuminated area (data not shown). This confirms that our light illumination protocol itself did not induce specific damage to the brain tissue.

Our research shows that c-fos induction in astrocytes is manipulable by optogenetics. Two recent studies showed optogenetic tool expression either in cultured astrocytes (Li et al., 2012) or in astrocytes in vivo using viral delivery of the transgene (Gourine et al., 2010); however, our method is unique, as whole animals expressing ChR2(C128S) were obtained, allowing low-invasiveness in vivo experiments. We have yet to understand the mechanistic pathway leading from ChR2(C128S)-mediated current influx to c-fos induction; however, the results show that ChR2(C128S) activation could be used as a tool to specifically manipulate astrocyte activity and the trace of manipulation can be pursued by c-fos histochemistry.

**DISCUSSION**

Our initial attempts, as well as those of some of our colleagues, to express ChR2 in a cell type-specific manner at sufficiently high levels to accomplish optogenetic manipulation of cell activity using the transgenic approach have largely failed because achievement of both specific and abundant expression was difficult. We have provided a solution to these conflicting requirements by using the improved KENGE-tet system. Specificity in this system was attained by preparing a panel of tissue-specific tTA lines, which allow cell type-specific expression in various cell types. It has also been reported that the bipartite tet system can amplify the level of transgene expression much more than direct connection of the cell type-specific promoter to the transgene (Arenkiewicz et al., 2007; Dhawale et al., 2010; Tomita et al., 2009; Wang et al., 2007). In addition, knocking in our transgene, ChR2(C128S)-EYFP, into a locus just downstream of β-actin allowed sufficiently high induction for light responses.

The keys to our success of achieving high levels of gene induction by the KENGE-tet system were that: (1) the teto was inserted to the genome as “knockin” and (2) the targeted locus of the “knockin” was strategically selected. TetO mice generated by conventional plasmid transgenic approach would cause transgene to insert in more or less random locations and tTA-mediated gene induction in these mice often fails presumably due to chromosomal position effects. We have already shown in our previous study that teto knockin yielded a high level of
transactivation (Tanaka et al., 2010). In the current study, to avoid the chromosomal position effects as much as possible, genomic loci where the tetO promoter is activatable by the tTA produced were searched. Previous studies have reported that several loci, including the TIGRE locus (chr9, between AB124611 and Carm1 genes) (Zeng et al., 2008), the LC-1 locus (chr6, between Vmn1r33 and Vmn1r34 genes) (Schönig et al., 2011), and the HPRT locus (chr X) (Palais et al., 2009), provide high tTA-mediated transactivation when the tetO promoter is inserted. HPRT is generally regarded as a housekeeping gene, motivating us to select another housekeeping gene, in this case the gene for β-actin, for our gene targeting.

Once the targeted locus was selected, the BAC transgenic strategy could be used to avoid a chromosomal position effect (Schönig et al., 2011). However, we assumed that tetO knockin by ES cell homologous recombination would yield an even higher level of transactivation than did the BAC transgenic strategy, according to our experience with tetO knockin mice (Tanaka et al., 2010). In the current study, we performed a direct comparison between BAC transgenic and knockin approaches, and we showed that the knockin of tetO into downstream of housekeeping gene provided improvement to the tet system, although the underlying mechanism of the enhancement of expression was not made clear. We do need to note that in some cases where the endogenous promoter activity is extremely high, such as the orexin and PLP promoters, abundant tTA expression levels are attained. In such cases, BAC transgenic approach with tetO inserted downstream of housekeeping gene within BAC provides high enough levels of ChR2 (Figure 2) to trigger photocurrents (data not shown). However, many endogenous promoters that are specific to a certain population of cells usually do not have such a high activity, and, in such case, only low to moderate tTA levels could be attained, resulting low levels of ChR2 induction. According to numbers of trials that we tested, only the tetO knockin strategy, the KENGE-tet system, reliably produced high levels of gene induction.

In addition to our improved KENGE-tet system for ChR2(C128S)-EYFP expression, there are two other genetic approaches to the induction of cell type-specific ChR2 expression in mice published to date: the ChR2 BAC transgenic approach (Häglund et al., 2010; Zhao et al., 2011) and Cre-loxP-mediated ChR2 expression (Kätzel et al., 2011; Madisen et al., 2012). Maintenance of ChR2 BAC transgenic mice is the easiest among the three approaches, as only a single line of mice needs to be kept. However, unlike the tet system approach, simple BAC transgenic strategy lacks gene amplification modules, and thus this strategy often requires strong endogenous promoter activity. There are five successful, published modules, and thus this strategy often requires strong endogenous promoters that can strongly drive gene induction. Our choice of the modified ChR2(C128S) as a target transgene was also advantageous for optogenetic control of cell activity because of the higher sensitivity to light and the shorter illumination time required for long-term ChR2 activation.

As shown in this study, our KENGE-tet system is applicable to optogenetic manipulation of not only excitable neurons but also “nonexcitable” glial cells. We demonstrated that ChR2(C128S)-mediated inward currents resulted in c-fos mRNA induction in astrocytes. We also observed c-fos mRNA induction in oligodendrocytes in PLP-tTA::tetO-ChR2(C128S)-EYFP knockin mice after illumination (data not shown). These results indicate that ChR2(C128S) activation can be used as a tool to change the status of nonexcitable cells and that the change in their activity can be traced by c-fos induction.

Optogenetic manipulation of nonexcitable cells outside the nervous system could also be used to understand cell type-specific functionality in various tissues. For example, we have noticed that tissue macrophages—osteoclasts in bone, Kupffer cells in liver, and alveolar macrophages in lung—all express ChR2(C128S)-EYFP in Iba1-tTA::tetO-ChR2(C128S)-EYFP knockin mice (data not shown). For driving the activity of these nonexcitable cells, in addition to ChR2(C128S), Ca2+-permeable ChR2 (Kleinlogel et al., 2011) could be used for Ca2+ induction and photoactivated adenylyl cyclase (Schröder-Lang et al., 2007) could be used for cAMP induction. Construction of a panel of tetO knockin lines, including the above-mentioned light-sensitive proteins, will add options for optogenetic manipulation of cell function in both excitable and nonexcitable cells and facilitate research in many fields.

**EXPERIMENTAL PROCEDURES**

**Generation of Transgenic Mice**

Detailed information of generating tetO-ChR2(C128S)-EYFP BAC transgenic mice, tetO-ChR2(C128S)-EYFP knockin mouse, Iba1-tTA mouse, Htr5B-tTA mouse, and QAD67-tTA mouse is available in the Extended Experimental Procedures. A schematic diagram indicating the targeting vector for tetO-ChR2(C128S)-EYFP is given in Figure S1. Basic characterization of Iba1-tTA and Htr5B-tTA mice is shown in Figures S2 and S3. Information regarding Orexin-tTA mouse (generated by A.Y.) and CaMKII-tTA (Mayford et al., 1996), BTR6 (Chuhma et al., 2011), Mlc1-tTA (Tanaka et al., 2010), and PLP-tTA (Inamura et al., 2012) mice are already published. Information regarding Orexin-tTA mouse (generated by A.Y.) is unpublished. Genotyping primers are also given in the Extended Experimental Procedures.
Immunohistochemistry and In Situ Hybridization

Standard procedures were taken for immunohistochemistry and the antibodies used and the reaction times are given in detail in the Extended Experimental Procedures. The in situ hybridization method was as described previously (Ma et al., 2006) and written briefly in the Extended Experimental Procedures.

Acute Brain Slice Preparations and Patch-Clamp Recordings

Brain slices were prepared from mice aged P14–P21, unless otherwise noted. Slices were cut at a thickness of 250–400 μm using a microslicer and the slices were transferred to a submerged-type recording chamber and continuously superfused. All recordings were performed at room temperature (22–25 °C). Visually identified cells were voltage clamped at ~70 mV in whole-cell patch clamp mode. High-power blue LED (470 nm; 8 mW at sample location) was placed underneath the condenser lens of the upright microscope for full-field activation of ChR2(C128S) and yellow light, filtered (560 nm center 14 nm band-pass filter; 0.15 mW at sample location) from a mercury lamp, was directed through the epifluorescence port for closing of the ChR2(C128S).

In Vivo Electrophysiological Recording and Optical Stimulation

Each mouse was anesthetized with ketamine hydrochloride (100 mg/kg body weight, i.p.) and xylazine hydrochloride (5 mg/kg body weight, i.p.). A part of the skull in one hemisphere was removed to access the hippocampus. For recording neural activity while illuminating with blue light, a glass-coated Elgiloy microelectrode (0.5–1.0 MΩ) was inserted perpendicularly into the brain through the dura mater using a hydraulic microdrive. A 50 mW blue laser was coupled to an optical fiber. The target area was 2.0–3.0 mm posterior and 1.5–3.0 mm lateral to bregma and 1.2–1.6 mm deep from the brain surface for the hippocampus (Franklin and Paxinos, 2008).

In Vivo Optical Stimulation in Freely Moving Mice

Violet light was generated by laser diode (445 nm, 400 mW) and applied through plastic optical fibers (0.5 mm diameter). An optical swivel (COME2, Lucir, Tsukuba, Japan) was used for unrestricted in vivo illumination. Violet light power intensity at the tip of the plastic fiber was 6.7 mW/mm². For hippocampal CA1 pyramidal neuron stimulation, a plastic optical fiber was inserted perpendicularly into the brain through the dura mater using a hydraulic microdrive. A 50 mW blue laser was coupled to an optical fiber. The target area was 2.0–3.0 mm posterior and 1.5–3.0 mm lateral to bregma and 1.2–1.6 mm deep from the brain surface for the hippocampus (Franklin and Paxinos, 2008).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.06.011.

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