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**ORIGINAL ARTICLE**

# Identification of cell-type-specific promoters within the brain using lentiviral vectors

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*The development of cell-type-specific mini-promoters for genetic studies is complicated by a number of issues. Here, we describe a general method for the relatively rapid screening of specific promoter activity in cell culture, in acute brain slice preparations and* in vivo. *Specifically, we examine the activity of an* ~3 kb *promoter region from the neuroactive peptide cholecystokinin (CCK) compared to the commonly used cytomegalovirus promoter. We find a high degree of cell-type selectivity* in vivo *using lentiviral approaches in rats and traditional transgenic approaches in mice. Appropriate colocalization of Cre-recombinase and CCK gene expression is found within the hippocampus, when the CCK promoter is driving either the expression of Cre-recombinase or green fluorescent protein. We also demonstrate fluorescent identification of CCK-positive interneurons that allows for cell-type-specific electrophysiologic studies in rats and mice. In conclusion, these studies identify a functional mini-promoter for the CCK gene and outline a novel and sensitive general method to test activity of selective promoters* in vitro *and* in vivo. *This approach may allow for the more rapid identification of specific promoters for use with transgenic animals, in genetically modified viruses, and in the design of targeted, therapeutic gene-delivery systems.*
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## Introduction

The potential for using lentiviral vectors in experimental therapeutics is quite promising, largely owing to their ability to (1) integrate into both dividing and post-mitotic cells, and (2) to achieve stable, long-lasting transgene expression.[1] Additionally, in comparison to adeno-associated-based and sindbis-based vectors, lentiviral vectors allow for the packaging of larger inserts, opening the possibility for the use of larger promoter lengths to achieve gene expression in selected populations of cells.[2–4] Achieving restricted expression would be beneficial to both basic science studies of the different cell types interacting in complex biologic systems, and to the development of therapeutic gene-delivery systems that are active only within specific types of cells.

Despite these potential benefits, the process of determining minimal promoter lengths to achieve biologically appropriate expression is made difficult by the inability to identify *a priori* enhancer and silencer regions within putative promoter regions, and by the possibility that regions of the 5′ untranslated region (5′ UTR) of a given gene may have an important bearing on its expression. In this context, the screening of putative promoters of varying lengths has largely depended on the selective expression of transfected promoter-reporter constructs in cultured cells, based on the notion that selectivity *in vitro* may indicate selectivity *in vivo*. As another option, promoter-reporter constructs have been introduced into embryonic stem cells or pre-implantation embryos to generate transgenic animals (usually mice), which can then be screened for appropriate transgene expression.

In the present study, we outline an alternative method of screening promoters for cell-type-specific expression *in vitro* and *in vivo*. As this method makes use of viral vectors to assess appropriate promoter expression, it offers several advantages over transgenic approaches, including lower cost, more rapid throughput and most importantly, the ability to use the resulting vectors in a variety of different species. We divide our approach into two sections – a high-sensitivity assessment of minimal promoter expression and cell-type specificity, and a lower sensitivity examination assessing the levels of transgene expression that may be expected from a given promoter. As an example of the use of this technique, we describe the screening process for ~3 kb of the promoter and 5′ UTR region of the gene for cholecystokinin (CCK), an abundant neuropeptide of significant interest in psychiatry[5,6] and neuroscience.[7,8]

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## Results

### *Assessment of promoter activity* in vitro

Before production of active viral particles, the viral constructs for CCK-GFP and cytomegalovirus (CMV)-

GFP were transiently transfected into HEK293 cells to determine if the CCK promoter would provide for transgene expression, and determine its level of activity compared to the CMV promoter (Figure 1). Examination of cells 48-h post-transfection indicated that the CCK promoter was indeed able to generate detectable expression of GFP in HEK293 cells. The level of expression attained with the *CCK* promoter appeared to be quite high, although still far less than the expression of the CMV promoter-containing vector.

A similar test of the CCK-Cre-containing vector was performed by transfecting this plasmid along with a reporter plasmid designed to detect Cre-recombinase activity. The reporter plasmid (CX1-LEL-DsRed) was designed with the coding sequence of the GFP gene and its stop codon flanked by loxP sites, upstream of the coding sequence for DsRed, a red fluorescent protein (RFP). In the absence of Cre-recombinase, this reporter plasmid shows high levels of GFP fluorescence with no red fluorescence; in the presence of Cre-recombinase, the GFP coding sequence and its stop codon are excised, resulting in a loss of GFP expression and the emergence of red fluorescence. We observed high levels of DsRed expression in cells co-transfected with CX1-LEL-DsRed and CCK-Cre (Figure 2), demonstrating the CCK-Cre vector produces detectable levels of Cre-recombinase activity *in vitro*.

### *Assessment of the selectivity of promoter activity* in vivo

Minimal promoter activity was assessed by performing microinjections of CCK-Cre viral particles into the hippocampus of RosaLacZ mice.[9] This transgenic strain expresses the LacZ reporter only in cells in which a floxed stop codon has been excised by Cre-recombinase. As excision of the stop codon is possible even in the presence of only one Cre-recombinase molecule, the expression of LacZ in these animals serves as an extremely sensitive measure of Cre expression from the CCK-Cre virus. In addition to being able to detect extremely low levels of expression, this assay system also provides a sensitive measure of the selectivity of expression from a given promoter, as inappropriate expression would also be detectable with a high degree of sensitivity.

In order to determine the selectivity and expression patterns generated by the Cre-expression vector, it was necessary to select a site of injection in which only a subpopulation of cells expressed CCK, and where it might be possible to easily judge the appropriate pattern of expression. To identify a suitable injection site, we performed an *in situ* hybridization study of CCK mRNA expression within the mouse brain (Figure 3). We chose the CA1, CA3 and dentate gyrus regions of the hippocampus as our injection sites, largely owing to the fact that the stereotyped cytoarchitecture of the hippocampus led to areas in which large numbers of CCK mRNA-positive cells were in close apposition to CCK-negative cells. This was especially true in the dentate gyrus where, in agreement with previous studies (e.g. Marsicano and Lutz[10]), CCK mRNA expression was observed to be low or absent in the granule cell layer (comprised of densely packed excitatory cells), but extremely high in the interneurons interdigitating the horns of Ammon (polymorph layer of the dentate gyrus, PoDG, Figure 3b).

Representative sections of LacZ expression in virally infected animals are depicted in Figure 4. In addition to assessing LacZ reporter activity, we assessed the expression of CCK mRNA in the same sections subjected to LacZ staining, allowing us to visually colocalize LacZ with CCK mRNA. *In situ* hybridization rather than double-labeled immunocytochemistry was used to label CCK-positive cells owing to its higher sensitivity, and propensity to yield robust somatic staining (as opposed to the diffuse neuropil staining expected from immunocytochemical staining for the mature CCK-8 peptide). Additionally, identification of CCK-positive cells using hybridization for full-length CCK mRNA avoids the potential complication that distinct cell types may process the immature CCK peptide into different, mature peptide products.

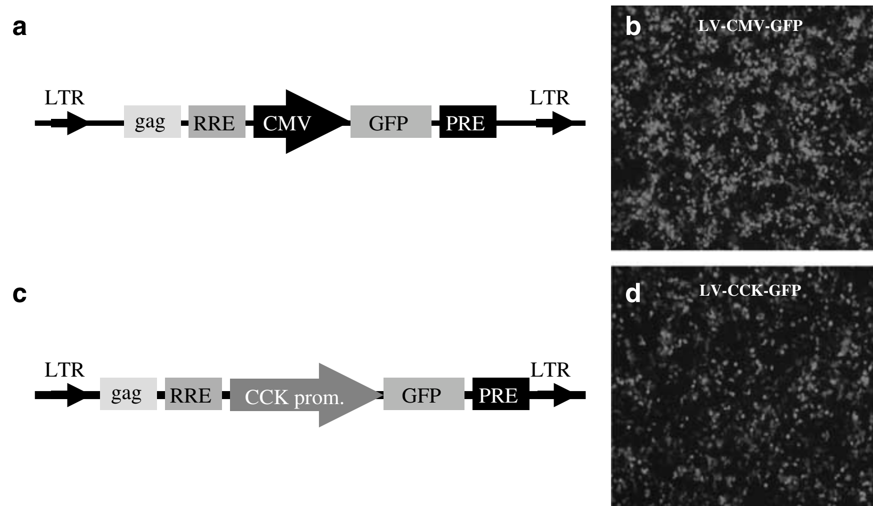


**Figure 1** GFP expression vectors. An initial screen of promoter activity from the CCK promoter was made by constructing a vector in which the CCK promoter was used to drive expression of GFP (**c**), by replacing the CMV promoter of pCMV-GFP-dNhe (**a**). This vector was then transfected into HEK293 cells. GFP expression from the CCK promoter (**d**) was compared to the expression of GFP driven by the CMV promoter (**b**).

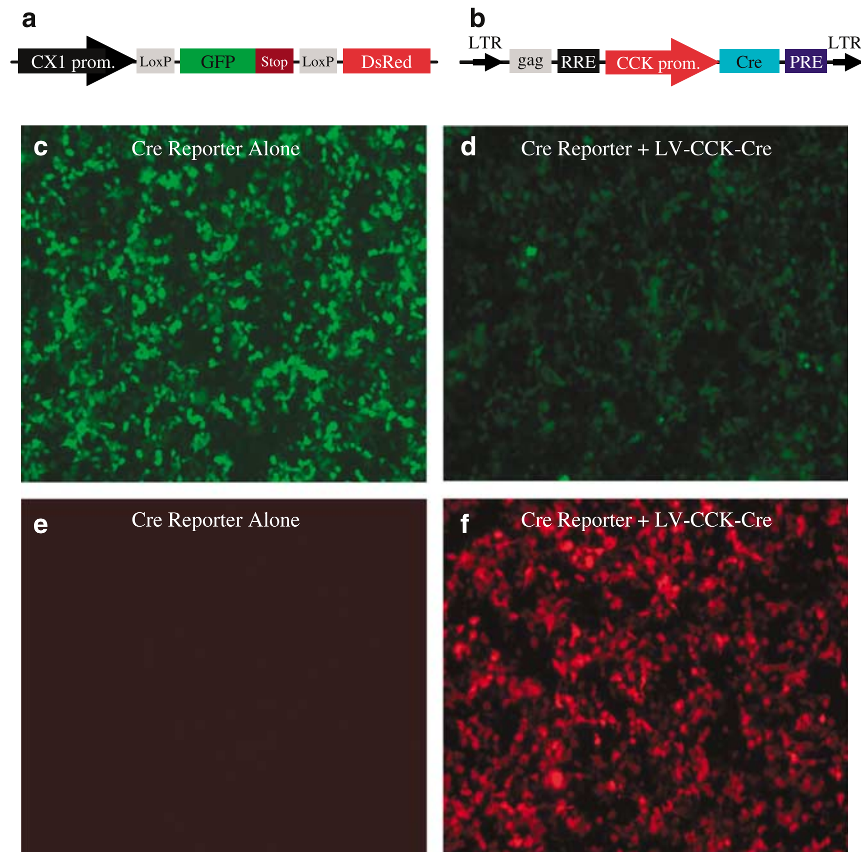


**Figure 2** Cre expression vector, and assays for Cre activity. A Cre-reporter plasmid was constructed in which the coding sequence for GFP (including stop codon) was surrounded by LoxP sites (floxed) and placed upstream of DsRed (CX1-LEL, **a**). A plasmid in which the CCK promoter was used to express Cre-recombinase was generated by replacing GFP from CCK-GFP with the nlsCre coding sequence (CCK-Cre, **b**). Transfection of the Cre-reporter alone generated robust green fluorescence and an absence of red fluorescence (**c**, **e**). Co-transfection of CCK-Cre with Cre-reporter generated a relative suppression of GFP expression (owing to excision of GFP, **d**) and activation of RFP expression (DsRed, **f**).

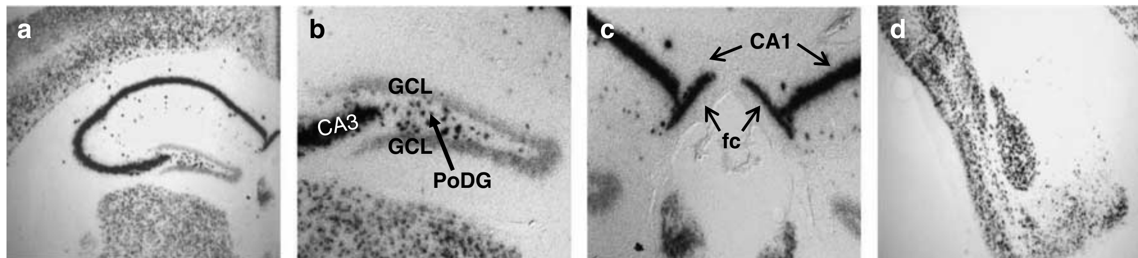


**Figure 3** CCK mRNA expression. CCK mRNA was examined using *in situ* hybridization, demonstrating high levels of expression within the hippocampal formation (low power: **a**; high power: **b**, **c**) and within the basolateral complex of the amygdala (**d**). Intense CCK mRNA expression is present within the CA3 subfield, and within the interneuron-rich region (PoDG) separating the granule cell layers of the dentate gyrus (GCL; **a**, **b**). Notably, the GCL lacks CCK mRNA+ cell bodies. CCK mRNA is highly expressed in the CA1 and fasciola cinereum (fc; **a**, **c**).

We observed that LacZ staining closely paralleled CCK mRNA expression in the dentate, CA1 and CA3 regions of the hippocampus (Figures 3 and 4). Colocalization in the dentate was particularly interesting, as the expression of LacZ was largely absent from the pyramidal (presumably excitatory) cells in the upper and lower blades of the dentate, whereas it was extremely high in the interneuron-rich sections in-between the pyramidal cell layers (Figure 3a and c). Notably, this pattern of LacZ expression closely resembles the expression of CCK mRNA, suggesting that whereas the large number of CCK-negative pyramidal neurons in the dentate gyrus were likely infected, that Cre expression was restricted to the minority of CCK mRNA-positive cells. Indeed, when we performed similar injections of CMV-Cre virus into the dentate, we found that these dentate pyramidal cells could readily be induced to express LacZ (Figure 4f). Taken together, these observations suggest that the *CCK* promoter length used was capable of producing highly selective expression in the appropriate sub-population of

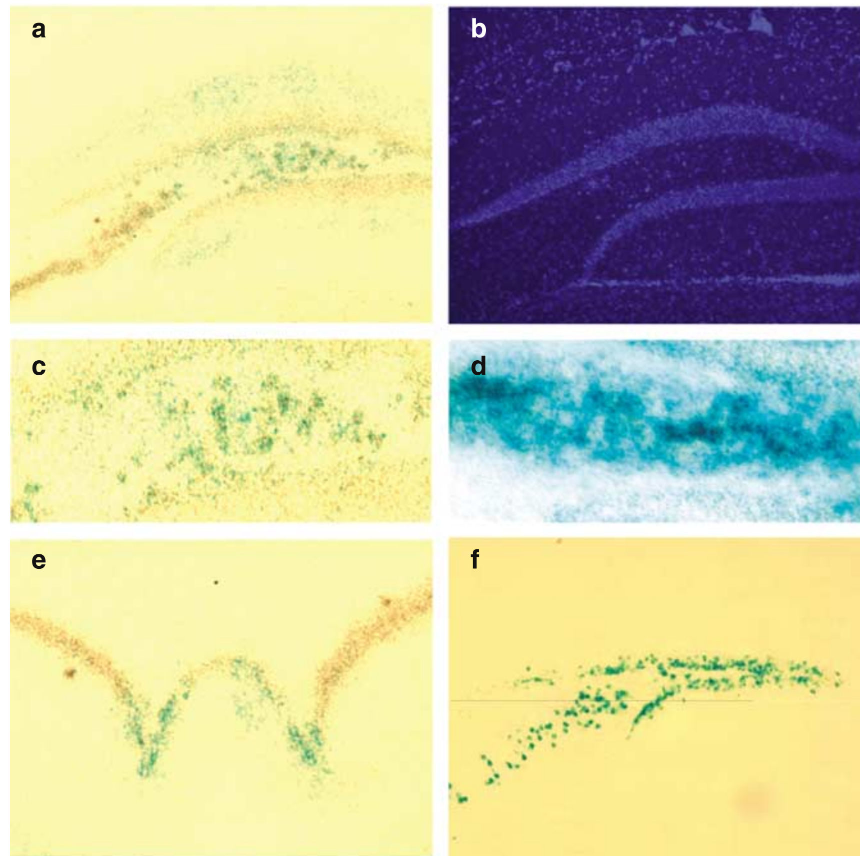


**Figure 4** LacZ expression parallels CCK mRNA expression. RosaLacZ Cre-reporter mice were injected with CCK-Cre virus (2 $\mu$l/side) and killed 10–12 days later. LacZ expression (blue precipitate) and CCK mRNA expression (silver grains) were assessed in the same sections (**a**–**f**). A high degree of overlap in mRNA expression was observed in the polymorph layer of the dentate (low power: **a**; high power: **c**, **d**). Despite the large number of cells within the granule cell layer of the dentate, readily visible in hoescht (bisbenzamide) counter-stained section (**b**), virtually no LacZ expression was noted in the granule cell layer. Panels c and d depict high-power images of low (**c**) and high (**d**) CCK mRNA expression in the polymorph layer of the dentate, with correspondingly low and high numbers of LacZ-positive cells. Similar co-expression was seen in the CA1 and fasciola cinereum (**e**). Notably, injection of CMV-Cre virus into the dentate demonstrates that the cells within the granule layer are readily infected with lentiviral vectors (**f**). This suggests that the pattern seen in (**a**) is likely due to the specificity of CCK promoter activity, rather than innate viral tropism.

cells. Additionally, non-cell-type-specific LacZ expression seen in animals infected with CMV-Cre virus strongly suggested the selectivity of Cre expression was due to the promoter used, rather than to the innate viral tropism.

### *Assessing the potential for transgene expression in vivo*

In the foregoing experiments, following Cre-mediated excision of the stop codon preceding the LacZ coding sequence in the RosaLacZ transgene, the expression of the reporter becomes independent of Cre-recombinase expression driven by the *CCK* promoter. This uncoupling of LacZ expression from promoter-driven transgene expression, although providing high sensitivity, leaves open the question of the amount of transgene expression generated by the *CCK* promoter *in vivo*.

We addressed this question by performing hippocampal injections of viral particles in which the *CCK* promoter drove the expression of GFP (CCK-GFP). We observed that GFP expression in various subfields of the hippocampus was generally similar to the patterns of expression seen in our prior experiments. More specifically, we observed that the expression of GFP in the dentate was contained to the interneuron-rich regions in-between the pyramidal cell layers of the dentate, reflecting the expression patterns seen for CCK mRNA in the dentate and the pattern of LacZ expression in the higher sensitivity CCK-Cre experiments. The high level of GFP expression seen in these experiments suggests that the *CCK* promoter length used here may be suitable to drive selective expression of transgenes *in vivo*. Additionally, the high levels of expression generated by this promoter may make it useful in the generation of transgenic mouse strains.

### *Transgene expression for fluorescence-guided electrophysiologic recordings in rats*

One of the main advantages of virally mediated transgene expression is the potential for using these vectors in a number of different species. Building on this possibility, we examined the suitability of using the CCK-GFP virus in rats, as a means of identifying CCK-positive interneurons for the purpose of electrophysiologic recording (Figure 5). Following on previous work from the laboratory of Donald Rainnie characterizing the

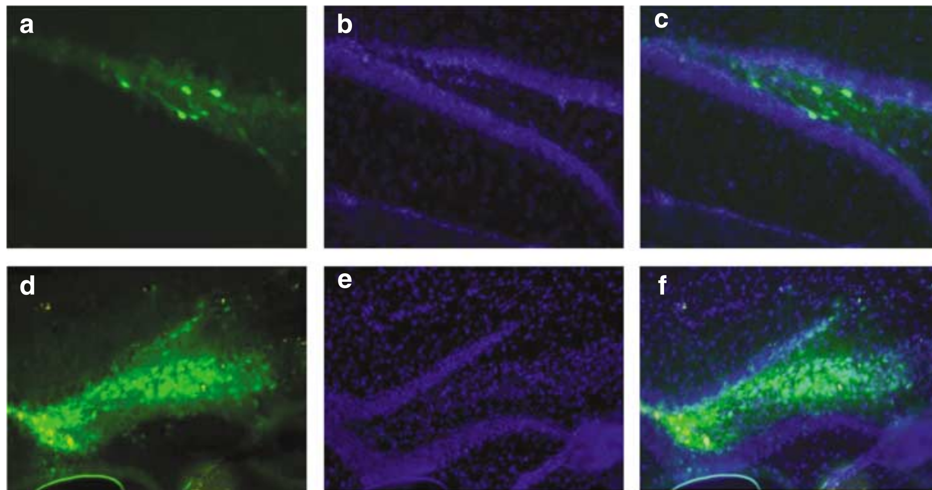


**Figure 5** GFP expression *in vivo*. Virus encoding CCK-GFP was injected into the dentate gyrus of C57/BL6 adult mice (2 μl/side over 10 min). Animals were killed 10 days later and fluorescence was assessed on sectioned, fixed tissue. Examples from sections showing sparse (**a**–**c**) and dense expression (**d**–**f**) are shown above. (**b**, **e**) Hoechst-stained photomicrographs of the same sections shown in (**a**) and (**d**), respectively. (**c**, **f**) Overlays of **a** and **b**, and **d** and **e**, respectively.

electrophysiologic properties of interneurons in the rat brain,[11–13] we explored the use of the CCK-GFP virus as a means to label cells for fluorescently guided electrophysiologic recording *in vitro*. Twelve days following *in vivo* infection, acute brain slices were prepared and electrophysiologic recordings were obtained from visually identified CCK-GFP-infected neurons of the rat hippocampus. An example of such a recording is shown in Figure 6, and demonstrates the viability of using cell-type-selective viral promoters to label and characterize the electrophysiologic properties of sub-populations of neurons. Using such a method to label cell types of interest allows for their targeted recording, which can be particularly advantageous if the desired cell type is present in low numbers or is found in close apposition to other cell types, as is commonly the case. Efforts are currently underway to use this technique to characterize the CCK-containing interneurons of the basolateral amygdala, a region in which routine recordings generally provide a low yield of interneurons in general, and an even lower yield of CCK+ neurons, owing to the fact that CCK+ interneurons represent a modest percentage (<5%) of the total neuronal population of the basolateral amygdala.[14]

### *Identified promoter lengths can be used to generate transgenic animals*

Although the aforementioned viral vectors can be used to induce transgene expression in restricted regions, in many cases widespread transgene expression is desirable. To assess the suitability of using promoter lengths identified in the aforementioned manner for the production of transgenic mice, we subcloned the coding sequence for Cre-IRES-DsRed into the CCK-GFP vector. This new plasmid (CCID) was then linearized and microinjected into C57/BL6 embryos. These injections yielded three founders carrying the CCID gene, and these were subsequently mated with RosaLacZ reporter mice to assess transgene expression. The patterns of Cre-mediated LacZ expression, depicted in Figure 7, suggest that the *CCK* promoter was capable of driving Cre expression in the transgenic construct. The expression of LacZ varied widely among the three founder lines, although ubiquitous, non-selective expression was not observed in any of the lines. The variability of expression suggests that there may be significant silencing of transgene expression depending on the insertion site of the recombinant sequence, a commonly observed phenomenon in neural systems[15] (this was especially true for the DsRed portion of the gene, which appeared to be completely silenced in all three transgenic lines). Animals from CCID line B showed the widest expression of LacZ, which was essentially identical to endogenous *CCK* gene expression (Figure 7a and c), suggesting that they may be suitable for use in generating CCK-selective knockouts when crossed with animals containing floxed transgenes.

## Discussion

We have described the production of a viral vector incorporating a cell-type-specific promoter (*CCK*) that generates high levels of gene expression with a high degree of specificity, and can be used in multiple species. More broadly, given the time and expense involved in the production of transgenic animals (and the sizable chance that an appropriate gene insert will not be found), we outlined an alternative means of screening usable promoter lengths *in vivo*. As part of this screening process, we described high-sensitivity screens for the cell-type specificity of particular promoters by making use of Cre-mediated recombination in reporter mouse strains. This high-sensitivity approach was complemented by using the same promoter to express a fluorescent-reporter gene to assess *in vivo* activity of the promoter length chosen. This technique was also used to label sub-populations of neurons for *in vitro* electrophysiologic characterization in rats. Finally, the identified promoter was used to produce transgenic animals, through injection of purified DNA. Additionally, the possibility

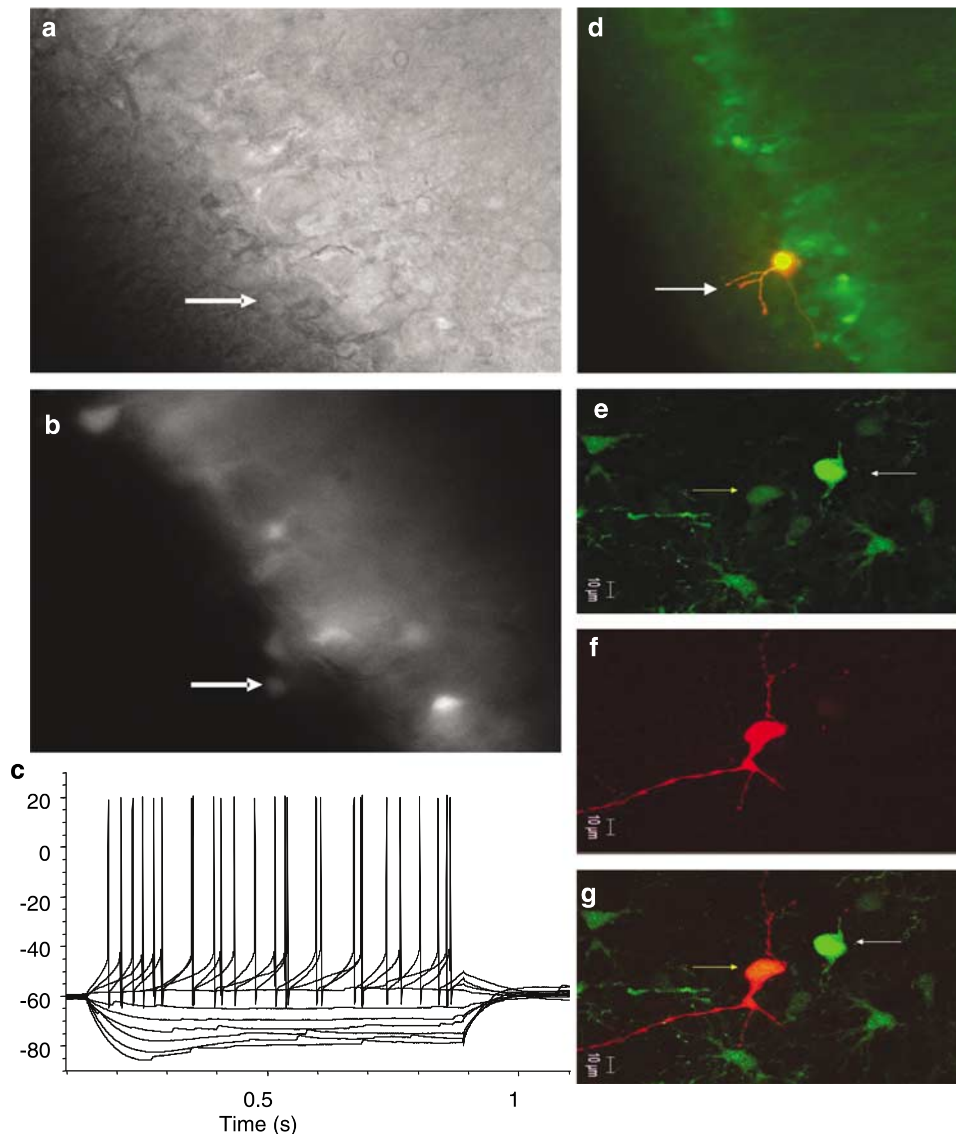


**Figure 6** Example recording from rat interneurons. Acute brain slices were prepared from juvenile rats 12 days post-infusion of CCK-GFP into the CA1 region of the hippocampus (**a**–**d**). GFP fluorescence (**b**) was first used to identify a putative CCK interneuron within the slice (arrow), which was then located using normal brightfield illumination (DIC, **a**). The fluorescently identified cell was then patched and electrophysiologic recordings obtained (example trace shown in panel c). During the recording process, the cell was passively filled with 0.3% biotcytin. Slices were then fixed overnight, resectioned to 75 $\mu$m and visualized using a streptavidin-Texas Red conjugate (**d**, red = biocytin, green = CCK-GFP expression). This neuron exhibited a voltage-dependent depolarizing sag in the voltage response to hyperpolarizing current injection (**c**). The amplitude and rate of onset of this rectification became more pronounced with increasing hyperpolarization, which is indicative of activation of hyperpolarization-activated non-selective cation channels. In the depolarizing direction, this neuron exhibited a rhythmic pattern of action potential firing, and a pronounced fast after-hyperpolarizing potential following each spike, which are properties similar to those previously reported for hippocampal CCK interneurons.[29] The same CCK-GFP virus was also used to label rat BLA neurons, and to identify them for electrophysiologic recording (**e**–**g**). Confocal images of CCK neurons are shown at ×60 magnification with arrows denoting fluorescing neurons. One of these putative CCK neurons (**e**–**g**, yellow arrow) was patched and recorded. During re-recording, this cell was filled with a biocytin, and visualized (**f**, **g**), using the same *post hoc* technique as used in the hippocampus.

exists for using these same vectors to achieve virally mediated transgenesis of oocytes or spermatogonia.[16,17] In addition to these applications, cell-type-selective viral vectors could potentially be used to generate region-specific, cell-type-selective expression of desired transgenes, as well as region-specific, cell-type-selective, post-developmental knockout of floxed genes.

The identification of a functioning *CCK* mini-promoter will be quite useful for studies aimed at understanding the function of the CCK sub-population of interneurons[10,18] within cortical and subcortical regions. CCK has been implicated in a variety of psychiatric disorders,[5,19,20] and the ability to now specifically manipulate activity of CCK-specific neurons *in vivo* will allow a greater understanding of the circuit-specific functions of this population.

Taken together, the method outlined here represents a streamlined technique, which allows for the reliable assessment of promoter specificity *in vivo*, whereas at the same time producing useful viral tools usable in a variety

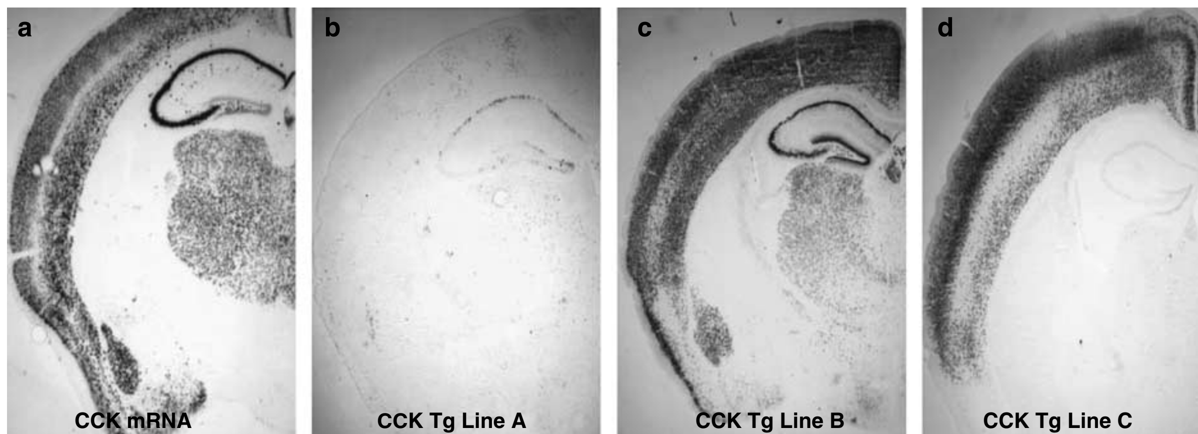


**Figure 7** LacZ expression in transgenic animals. Linearized template DNA in which the coding sequence for Cre-IRES-DsRed was placed under the control of the CCK promoter (CCID) was used to create transgenic mice according to the procedures described in the Materials and methods section. Three founder animals, with distinct transgene insertion sites, were generated and bred to RosaLacZ animals to assess Cre-mediated LacZ expression. Normal patterns of CCK mRNA expression are shown (**a**). LacZ expression in each of the three transgenic lines is shown (**b**–**d**) (each of these animals carries both the CCID and RosaLacZ alleles). Results indicate that animals from line A (**b**) show low levels of Cre expression, limited to the CA1, dentate gyrus and endopiriform regions. Line B animals (**c**) showed relatively widespread expression, which was, with the exception of the dentate, similar to endogenous CCK mRNA expression. Line C (**d**) showed high levels of Cre expression in the cortex, in a manner that was qualitatively similar to observed patterns of CCK mRNA expression. However, Line C animals showed virtually no Cre-mediated LacZ expression in any subcortical regions. Notably, consistent with wild-type CCK mRNA expression patterns, no LacZ expression was seen in the striatum or in the medial or central nuclei of the amygdala in any of the CCID transgenic lines.

of different contexts. This, in turn, may lead to the more rapid development of tools to dissect out the roles of various cell types in complex neural circuits. Eventually, the identification of mammalian promoters that provide this sort of restricted, cell-type-specific expression may lead to the development of more precisely targeted gene delivery vectors for use in clinical settings.

## Materials and methods

### *Plasmid design and construction*

Primers were designed to amplify a region of the *CCK* gene containing the ATG transcription start sequence and upstream stretch of $\sim$3 kb base pairs, using mouse fibroblast DNA as a template. The resulting polymerase chain reaction (PCR) products were then cloned into topo v2.1 (Invitrogen Corp., Carlsbad, CA, USA), and subsequently into a lentiviral packaging construct, pCMV-GFP-dNhe[21] (kind gift of Inder Verma, Salk Institute, La Jolla, CA, USA). Incorporated into the original primers were custom restriction sites used for subcloning this promoter length into the PCMV-GFP-dNhe (5′ *Cla*1: ccatcgattcccagggaagatgaagaaaccatggctac; 3′ *Bam*H1 tag gatccctcttcatggctatgggaagcaaaggcag) vector to generate CCK-green fluorescent protein (GFP). Subsequently, the GFP coding sequence from CCK-GFP was removed (*Bam*H1/*Sal*1 fragment) and replaced with the coding sequence for Cre-recombinase (with an N-terminus nuclear localization sequence; *Bam*H1/*Sal*1 fragment) to generate the CCK-Cre vector.

Viral production procedures have been described in detail[22,23] and follow from procedures initially outlined by Verma and co-workers.[4,24,25] In brief, active viral particles were produced by co-transfecting these lentiviral packaging constructs with plasmids coding for delta8.9 (env) and vesicular stomatitis virus G into HEK293T cells. The packaged, unconcentrated virus was collected over a period of 5 days post-transfection, and then concentrated using ultracentrifugation and resuspension in sterile phosphate-buffered saline (PBS)/1% bovine serum albumin. The resulting titer was assessed in HEK293T cells, and the observed titer of the virus used here ranged from $5 \times 10^8$ to $1 \times 10^9$ infectious particles per ml

### *Animal subjects*

The procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Breeding pairs for transgenic Cre-reporter mice were obtained from Jackson Labs, Bar Harbor, ME, USA. Male animals carrying the RosaB26-floxed LacZ insert (RosaLacZ[9]) and aged 6–10 weeks were anesthetized with a 4:5 mixture of Ketamine and Domitor (given intraperitoneally), and then injected bilaterally into the hippocampus with 2 $\mu$l (per side) of concentrated CCK-Cre virus in a stereotaxic apparatus (0.2 $\mu$l/min; coordinates with respect to bregma: AP: $-1.8$ mm; ML: $\pm 1.0$ mm; DV: $-1.5$ mm). Similar procedures were used for CCK-GFP injections into wild-type, C57-BL6 animals (Jackson Labs), and into juvenile (4–6 weeks old) Sprague–Dawley rats (Charles River, Raleigh, NC, USA) (infusion parameters: 0.2 $\mu$l/min; coordinates with respect to bregma: AP: $-2.0$ mm; ML: $\pm 1.4$ mm; DV: $-2.4$ mm).

### *Tissue preparation*

Animals were killed 10–12 days post-infection with an overdose of chloral hydrate. In experiments where *in situ* hybridization was to be performed, the brains were rapidly dissected, snap-frozen and sectioned at 30 $\mu$M onto SuperFrost Plus slides, which were then stored at $-80^\circ$C until processing. In other experiments, intracardiac perfusion (PBS, 4% paraformaldehyde), a brief

post-fixation step (2 h, 4% paraformaldehyde), and cryoprotection (20% sucrose in PBS, ~16 h at 4°C) preceded sectioning and storage at −80°C.

### *In situ hybridization*

This protocol has been described in detail previously.[26,27] A cDNA clone containing the coding sequence of the mouse gene for CCK was linearized after sequence verification. An antisense riboprobe was generated with T3 RNA polymerase. Slide-mounted sections of snap-frozen rodent brain tissue were post-fixed, proteinase K digested, blocked and followed by overnight hybridization of the tissue at 52°C with 35S-UTP labeled riboprobes. After a stringent wash protocol, slides were dipped in an autoradiographic emulsion for 4 weeks and then developed.

### *LacZ/XGal staining*

Before *in situ* hybridization, slide-mounted tissue was incubated at 37°C overnight in 1 mg/ml XGal dissolved in 150 mM potassium ferricyanide, 150 mM potassium ferrocyanide and 20 mM magnesium chloride, in 1 × PBS. The resulting sections were thoroughly rinsed in 1 × PBS and then processed for *in situ* hybridization.

### *Electrophysiologic methods*

Ten to twelve days after CCK-GFP virus infusion, rats were decapitated under isoflurane anesthesia (Abbott Laboratories, North Chicago, IL, USA), and the brains rapidly removed and placed in ice-cold kynurenic acid-based artificial cerebrospinal fluid containing ($ACSF_{KA}$), which contained (in mM): NaCl (130), KCl (3.5), $KH_2PO_4$ (1.1), $MgCl_2$ (6.0), $CaCl_2$ (1.0), $NaHCO_3$ (30), glucose (10) and kynurenic acid (2) as described previously.[11,12,28] A block of tissue containing the hippocampus was then mounted on the chuck of a Leica VTS-1000 vibrating microtome (Leica Microsystems Inc., Bannockburn, IL, USA), and 350 μm coronal slices were cut. Slices were then hemisected and transferred to a holding chamber containing $ACSF_{KA}$ at room temperature and gassed with a 95–5% oxygen/carbon dioxide mixture for 1 h before being placed in oxygenated control ACSF containing (in mM): NaCl (130), KCl (3.5), $KH_2PO_4$ (1.1), $MgCl_2$ (1.3), $CaCl_2$ (2.5), $NaHCO_3$ (30) and glucose (10). Experiments started a minimum of half an hour following the transfer of slices into the control ACSF.

### *Visual identification of CA1 neurons*

To visualize neurons, slices were placed in a Warner Series 20 recording chamber (Warner Instruments, Hamden, CT, USA) mounted on the fixed stage of a Leica DM-LFS microscope (Leica Microsystems). Slices were fully submerged and continuously perfused at a rate of 1–2 ml per min with heated (32°C) and oxygenated ACSF. Neurons were visualized using either infrared (IR) illumination together with differential interference contrast (DIC) optics or fluorescence with a ×40 water immersion objective (Leica Microsystems). Images were recorded using an IR-sensitive charge-coupled device digital camera (Orca ER, Hamamatsu, Tokyo, Japan) coupled to a Meteor-II video frame grabber (Matrox Electronic Systems Ltd, Dorval, Canada), and displayed on a computer monitor using Simple PCI 6.11 software (Compix Inc., Cranberry Township, PA, USA).

### *Recording procedures*

Thick-walled borosilicate glass patch electrodes (WPI, Sarasota, FL, USA) were pulled on a Flaming/Brown micropipette puller (Model P-97), and had resistances ranging from 4 to 8 MΩ, when filled with a standard patch recording solution that contained 0.3% biocytin and (in mM): K-gluconate (138), KCl (2), $MgCl_2$ (3), phosphocreatine (5), K-ATP (2), NaGTP (0.2), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (10) and 0.3% biocytin. The patch recording solution was adjusted to a pH of 7.3 with KOH and filtered through a 0.2 μm filter (Altech Associates, Inc., Deerfield, IL, USA). Whole-cell patch clamp recordings were obtained with standard techniques, as described previously.[11,12,28] Briefly, recordings were made with an Axopatch-1D amplifier (Molecular Devices, Sunnyvale, CA, USA) using a Digidata 1320A A-D interface and pClamp 8.2 software (Molecular Devices). In cell-attached mode, patch electrode seal resistance was considered acceptable if it was >1.5 GΩ. Whole-cell patch clamp configuration was established in neurons in current-clamp mode, and all data were filtered at 5 kHz. The membrane input resistance ($R_m$) and intrinsic currents, activated at membrane potentials more negative than −60 mV, were assessed by determining the voltage response to transient (750 mS) current injection ranging from −40 to +23 pA.

### *Transgenic production*

The coding sequence for Cre-recombinase was subcloned upstream of the internal ribosomal entry site (IRES) sequence in the pCMV-IRES-DsRed2 vector (Invitrogen). A fragment containing Cre-IRES-DsRed2 was then inserted in place of GFP in the CCK-GFP vector. The resulting plasmid (CCK-Cre-IRES-DsRed2 or CCID) was linearized, purified and microinjected into the pronuclei of one-cell C57/BL6 embryos, which were then implanted into pseudo-pregnant C57/BL6 females. The purification and injections were performed at the Emory University Transgenic Facility. Three founder animals (lines A, B and C) were obtained following these injections. Subsequent to PCR verification of their genotypes, founders were mated to mice hemizygous for the RosaLacZ (see above). $F_1$ animals from these matings were genotyped, and animals carrying both the CCID and RosaLacZ alleles were killed, sectioned and Cre-mediated recombination was assessed through XGal staining, as outlined above. Notably, none of the three lines showed any observable DsRed fluorescence, suggesting that DsRed expression was silenced in all three lines. Furthermore, the low frequency of Cre+ animals from these matings suggested that carriers of the CCID allele may have reduced survival as compared to their CCID-negative littermates.

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