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Electroporation and RNAi in the rodent retina *in vivo* and *in vitro*

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Abbreviation footnote

MLV, Molony murine leukemia virus; CABP5, calcium binding protein 5; CRALBP, cellular retinaldehyde binding protein; GCL; ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segment; VZ, ventricular zone; PR, photoreceptor; RNAi, RNA interference.

Abstract

The large number of candidate genes made available by comprehensive genome analysis requires that relatively rapid techniques for the study of function be developed. Here we report a rapid and convenient electroporation method for both gain- and loss-of-function studies *in vivo* and *in vitro* in the rodent retina. Plasmid DNA directly injected into the subretinal space of neonatal rodent pups was taken up by a significant fraction of exposed cells following several pulses of high voltage. Using this technique, GFP expression vectors were efficiently transfected into retinal cells with little damage to the operated pups. Transfected GFP allowed clear visualization of cell morphologies, and the expression persisted for at least 50 days. DNA-based RNAi vectors directed against two transcription factors important in photoreceptor development led to photoreceptor phenotypes similar to those of the corresponding knock-out mice. Reporter constructs carrying retinal cell type-specific promoters were readily introduced into the retina *in vivo*, where they exhibited the appropriate expression patterns. Plasmid DNA was also efficiently transfected into retinal explants *in vitro* by high voltage pulses.

Introduction

Recent advances in comprehensive expression profiling, such as microarray analysis (1, 2) and serial analysis of gene expression (SAGE, ref.3), have enabled the identification of a large number of genes that might play a role in mammalian retinal development and disease. It is now more important than ever that a rapid and convenient method for the analysis of function be developed, particularly for application in intact tissue *in vivo* or *in vitro*.

We have previously used retroviral vectors, based upon Molony murine leukemia virus (MLV) to deliver genes to the developing retina (4-6). Such vectors can enable expression of virally transduced genes in the retina without the time and expense of transgenic animals. However, there are disadvantages inherent in the use of such vectors. First, the use of an MLV vector is limited to gene transfer into mitotic retinal progenitor cells, as it requires proliferation of the target cells for integration. Second, it is time-consuming to prepare high titer virus stocks to achieve efficient gene transfer. Third, MLV vectors have a size limitation for insert DNA (typically < 7kb). Fourth, such vectors do not readily allow introduction of more than two genes into the same cells. Some of these problems can be bypassed by using other types of viral vectors, such as lentivirus (7) and adeno-associated virus (8), although size and efficiency still limit certain applications and one still must make viral stocks.

To overcome these problems, we have applied a relatively new technique for the introduction of DNA into neonatal mouse and rat retinae, that of *in vivo* electroporation, which has recently been applied to several tissues of various animal species (reviewed in ref. 9). A solution of DNA is directly injected into the subretinal space of neonatal mouse/rat pups, and electric pulses are applied using tweezer-type electrodes. This method is faster than other viral and transgenic gene transfer methods. The gene transduction efficiency of this method is also higher than that of MLV viral vectors. We

have also applied an electroporation technique for gene introduction into retinal explants using a micro electroporation chamber (*in vitro* electroporation).

In this paper, we report several types of experiments performed using these gene transfer methods. These include (i) promoter analysis, (ii) gain-of-function analysis using a bicistronic expression vector, and (iii) loss-of-function analysis using a DNA-based RNAi vector.

MATERIALS AND METHODS

Animals

Timed pregnant Sprague Dawley rats and Swiss Webster mice were purchased from Taconic, and CD1 mice were from Charles River. All the animal experiments in this study were approved by the Institutional Animal Care and Use Committee at Harvard University.

DNA construction

For construction of pCAG-GFP and pCAG-DsRed, cDNAs encoding EGFP and DsRed2 were excised from pEGFP-N1 (Clontech) and pDsRed2-N1 (Clontech), respectively, and cloned into pCAGGS (10) with modified multiple cloning sites. For construction of pCMV-GFP, pEF-GFP and pUB-GFP, the promoter region of pCAG-GFP was replaced by the CMV promoter excised from pEGFP-N1, the human EF1 α promoter excised from pCE-EGFP-1 (11), and human ubiquitin C promoter excised from pFUGW (12), respectively.

For construction of pRho-2.2K-DsRed, pCABP5-4.7K-DsRed and pCRALBP-4K-DsRed, the promoter region of pCAG-DsRed was replaced by bovine rhodopsin promoter (from -2174 to +70), mouse CABP5 promoter (from -4534 to +148, NCBI locus ID. 29865) and mouse CRALBP promoter (from -3932 to +71, NCBI locus ID. 19771), respectively. The bovine rhodopsin promoter was excised from the -2174-lacZ construct (13), and the mouse CABP5 and CRALBP promoters were amplified by genomic PCR. The following PCR primers were used to amplify the promoters. Mouse CABP5 promoter, 5'-CCCTTTGCTAGCACTGAGACCCTTTAATACG-3' and 5'-CCTGGATTGCAATGCTGTCTCTCACACTTGC-3'. Mouse CRALBP promoter, 5'-TCCCTTTCTCCTATGAGAAGCGGGAGGCC-3' and 5'-CCAAGCTCTGATGTCAAGATGGCCCCTCCT-3'. pRho-2.2K-CFP was

constructed by replacing the coding region of DsRed2 in pRho-2.2K-DsRed with ECFP excised from pECFP-N1 (Clontech).

For construction of pCAGIG, an IRES-GFP cassette excised from pMX-IRES-GFP (14) was ligated into the pCAGGS vector. A cDNA encoding mouse Rax (15) was amplified by PCR to trim the 5'- and 3'-untranslated regions, and cloned into pCAGIG to make pCAGIG-mRax. RNAi vectors for mouse Crx, Nrl and GAPDH were constructed by inserting the annealed oligonucleotides into pBS/U6 (16) digested with Apa I (blunted) and Eco RI. Oligonucleotides were as follows. Mouse Crx (coding region 408-428), 5'-

GGCATCTCAGATTCTTACAGAAGCTTCTGTAAGAATCTGAGATGCCCTTTT

G-3' and 5'-

AATTCAAAAAGGGCATCTCAGATTCTTACAGAAGCTTCTGTAAGAATCTGAGATGCC-3'. Mouse Nrl (306-326), 5'-

GGTCCTGTCTCTATGGAAGGAAGCTTCCTTCCATAGAGACAGGACCCTTTT

TG-3' and 5'-

AATTCAAAAAGGGTCCTGTCTCTATGGAAGGAAGCTTCCTTCCATAGAGACAGGACC-3'. Mouse GAPDH (393-413), 5'-

GGTGTGAACCACGAGAAATAAAGCTTTATTTCTCGTGGTTCACACCCTTTT

G-3' and 5'-

AATTCAAAAAGGGTGTGAACCACGAGAAATAAAGCTTTATTTCTCGTGGTTCACACC-3'.

***In vivo* electroporation and virus infection**

Newborn rat or mouse pups were anesthetized by chilling on ice and a small incision was made in the sclera near the lens using a 30-gauge needle. DNA solutions (3~6 μ g/ μ l) in PBS containing 0.1% fast green as a tracer were injected into the subretinal space through the incision using a Hamilton syringe with a 32- or 33-gauge blunt-ended needle

under a dissecting microscope. For newborn rat pups, ~1 μ l of DNA was injected, and for mouse newborn pups, ~0.5 μ l of DNA was injected. After DNA injection, tweezer-type electrodes (BTX, model 520, 7mm diameter) briefly soaked in PBS were placed to softly hold the heads of the pups, and five square pulses of 50 ms duration with 950 ms intervals were applied using a pulse generator CUY21 (Nepagene, Japan), or ECM830 (BTX). For rat newborn pups, 100V pulses were applied, and for mouse newborn pups, 80V pulses were applied. Usually DNA was transfected into only right eyes.

Replication incompetent retroviruses were produced by transfecting pLIA (17) into Phoenix-E packaging cells, concentrated and titrated on NIH3T3 cells. Concentrated virus (1×10^7 cfu/ml) was injected into the subretinal space of newborn rats as described for DNA injection.

***In vitro* electroporation and retinal explant culture**

Dissected retinae were transferred to a micro electroporation chamber (Nepagene, model CUY532, 3mm x 10mm x 5mm) filled with a DNA solution (1 μ g/ μ l in HBSS), and five square pulses (30V) of 50 ms duration with 950 ms intervals were applied using a pulse generator CUY21. Electroporated retinae were cultured at 37°C on Nucleopore polycarbonate filters (Whatman, 0.2 μ m pore size) with Neurobasal Medium (Invitrogen) containing 1x B-27 serum-free supplement (Invitrogen).

Preparation of retinal sections

Electroporated retinae were harvested 2-50 days after electroporation and dissected under a fluorescent microscope (Leica, MZFL III) to select the GFP- or DsRed-positive retinae. Dissected retinae were fixed with 4% paraformaldehyde in PBS for 20min at room temperature, cryoprotected in PBS containing 30% sucrose for several hours at

4°C, and embedded in OCT compound (SAKURA) on dry ice. Cryosections (20 or 30 μm) were cut on a cryostat.

Immunostaining of dissociated retinal cells

Retinae were dissociated into single cells essentially as described previously(18), except that papain (Worthington, final 50ng/μl) was used instead of trypsin, and stained with following antibodies: Anti-rhodopsin (Rho4D2 obtained from R. Molday, ref. 19), anti-Crx (obtained from S. Chen, ref. 20), anti-Nrl (obtained from A. Swaroop, ref. 21), anti-GAPDH (Ambion #4300), anti-Chx10 (developed by our lab, unpublished), anti-protein kinase C α (Oncogene #OP74), anti-glutamine synthetase (Chemicon #MAB302), anti-HPC-1 (SantaCruz #sc-12736), anti-Calbindin (Sigma #C9848), anti-Gt2 α (SantaCruz #sc-390) and anti-Thy-1 (SantaCruz #sc-19614).

RESULTS

In vivo electroporation

DNA constructs were injected into the subretinal space of newborn mouse or rat pups (for detailed protocol, see Figure 8, available as supporting information on the PNAS website, www.pnas.org). Tweezer-type electrodes were then placed on the heads of pups, and five electric pulses of 50 ms duration (100 V for rats, 80 V for mice) were applied to the eyes in the direction shown in Fig.8A using a square wave electroporator. The DNA was transduced into the scleral side of the retina, where undifferentiated mitotic and newly postmitotic cells exist. Almost all operated pups survived and were apparently healthy after electroporation. When a GFP expression vector driven by the CAG (chicken β -actin promoter with cytomegalovirus enhancer, ref. 10) promoter, a strong ubiquitous promoter, was electroporated into the retinae on postnatal day 0 (P0), an average of ~80% rat retinae and ~50% mouse retinae expressed GFP. In a good transfection, GFP expression was observed in a wide area of the retina (Fig.1A).

A GFP expression vector transfected at P0 allowed clear visualization of the morphologies of retinal cells throughout development (Fig.1B). At P2, GFP was detected in retinal progenitor cells in the ventricular zone (panels d-f). At P5, GFP was observed in two different cell populations, one forming the future outer nuclear layer (ONL) and the other forming the inner nuclear layer (INL), seen as beginning to segregate in the former ventricular zone area (panels g-i). At P8, GFP labeled immature rod photoreceptors (PR) in the ONL, and immature bipolar cells, differentiating Müller glial cells and/or progenitor cells, whose cell bodies were located in the INL (panels j-l). At P14, the majority of the GFP-positive cells were differentiated into rod PR located in the ONL, and the rest became bipolar cells and Müller glial cells (panels m-o). Rod outer segments (OS) were also clearly labeled by GFP. The GFP expression was observed at the latest time point examined (P50, panels p-r). However, it appeared that the GFP expression level was gradually decreasing by 3-4 weeks after electroporation.

The distribution of GFP-positive cells in the P14 differentiated rat retinae was examined following DNA introduction at P0 (Fig.2). Judging based on cell morphology and location, approximately 80 % of the GFP-positive cells were rod PRs, ~15% were bipolar cells, ~3% were Müller glial cells and <1% were amacrine cells (Fig.2A). These values are largely comparable to those of retinal cells infected with a replication-incompetent MLV retrovirus (LIA) carrying an alkaline phosphatase reporter (Fig.2A purple bars). This result was further confirmed by staining the dissociated retinal cells with several retinal cell type-specific antibodies. ~75 % of the GFP-positive cells expressed rhodopsin (a marker for rod), ~20% expressed Chx10 (a marker for bipolar), ~4% expressed glutamine synthetase (a marker for Müller glia) and <1% expressed HPC1 (a marker for amacrine) (Fig. 2B). Very few GFP-positive (<1%) cells were positive for Gt2 α (a marker for cone), but we could not detect GFP-positive cells expressing calbindin (a marker for horizontal) or Thy-1 (a marker for ganglion).

Several ubiquitous promoters, including cytomegalovirus (CMV, ref. 22), human elongation factor (EF) 1 α (11) and human ubiquitin C (23) promoters, were also tested in the developing rat retina. EF1 α and ubiquitin C promoters exhibited higher GFP expression than CMV or CAG. When sectioned at P10, CMV and EF 1 α promoters appeared to label the cells whose cell bodies were in the INL with processes extending to the ONL and GCL, more than those in the ONL, suggesting a relative lack of activity, perhaps due to silencing of these two promoters in PR (Fig.3B, C). The GFP expression pattern driven by the ubiquitin C promoter was similar to that by the CAG promoter (Fig.3D), and the cell type composition labeled by the ubiquitin promoter, determined by immunostaining of dissociated cells, was comparable to that by the CAG promoter (data not shown). These results indicate that some ubiquitous promoters are not suitable for the studies of mammalian retinal development when PR need to be labeled.

***In vitro* electroporation**

In order to label ganglion cells (GC), which line the surface of the retina facing the vitreous body, a GFP expression vector (pCAG-GFP) was injected into the vitreous of P0 rat eyes and electric pulses were applied in the direction opposite to that used for Fig.1. However, few GFP-positive cells were detected (data not shown), suggesting that unlike progenitor cells in the ventricular zone, GCs are not highly transfectable. To further test this possibility, DNA was electroporated into CD1 mouse retinae *in vitro* from the scleral side or from the vitreal side, using a micro chamber (shown in Fig.8C, supporting information). A significant number of retinal cells (5-20% of total cells) became GFP-positive (Fig.4A-a) when electroporated from the scleral side, with the distribution in sections (Fig.4B) similar to that of *in vivo* electroporated retinae (Fig.1). In contrast, only a few cells became GFP-positive when the vector was transfected from the vitreal side (Fig.4A-b). Similar result was observed when the ubiquitin C promoter was used to express GFP (data not shown).

Using the *in vitro* electroporation technique, we examined if DNA could be electroporated into adult mouse retina. In the normal retina of adult CD1 mice, few GFP-positive cells were observed even if the vector was transfected from the scleral side (Fig.4A-c). DNA was also not efficiently transfected from the vitreal side (Fig.4A-d). Interestingly, many GFP-positive cells were detected when adult Swiss Webster mouse retinae, having a retinal degeneration (rd) mutation, were transfected from the scleral side (Fig.4A-e). Immunostaining with anti-glutamine synthetase antibody showed that most of GFP-positive cells were Müller glial cells (data not shown). These results suggest that it is not easy to transfect DNA into mature rod PR, as well as GC, by electroporation.

Promoter analysis

One application of *in vivo* electroporation is the mapping of transcriptional regulatory elements that can be influenced by viral elements and also frequently need to be larger

than the allowance of viral vectors. To evaluate this application, we chose a characterized, as well as two uncharacterized, regulatory regions. The 2.2k fragment (from -2174 to +70) of bovine rhodopsin promoter has been shown to direct photoreceptor specific expression in transgenic mice (13). The bovine rhodopsin 2.2K promoter, used to express DsRed, was co-transfected into P0 rat retinae with pCAG-GFP. As shown in Fig.5A, at P5, the expression of DsRed was detected only in a small population of cells located at the upper part of the ventricular zone (panels a-c). At P8, the DsRed expression was exclusively detected in the ONL, and most of the GFP-positive cells in the ONL co-expressed DsRed (panels d-f). At P14, the expression of DsRed in the ONL became much stronger (panels g-i), consistent with rhodopsin expression profiles (3, 13).

To determine if other retinal cell types could be labeled by this technique, we tested an uncharacterized sequence from the promoter region of mouse calcium binding protein 5 (CABP5, from -4534 to +148) and a partially characterized promoter sequence of cellular retinaldehyde binding protein (CRALBP, from -3932 to +71, ref. 24) obtained by genomic PCR. CABP5 is expressed by rod bipolar cells and a subset of cone bipolar cells (25, 26), while CRALBP is expressed by Müller glial cells. The CABP5 promoter led to expression of DsRed only in the cells in the INL, consistent with labeling of bipolar cells (Fig.5B). On the other hand, CRALBP promoter led to expression of DsRed only in Müller glial cells (Fig.5C).

Gain-of-function analysis

Using a bicistronic expression vector containing the CAG promoter and an IRES-GFP cassette (pCAGIG, Fig.6A), we tested if the electroporation technique is useful for functional analysis of candidate genes. We focused on a homeobox transcription factor Rax (15) (also called Rx (27)) whose functions had been characterized in the developing rat retina using a replication-incompetent retrovirus vector (28). Rax is expressed by

proliferating retinal progenitor cells as well as differentiating Müller glial cells, and its forced expression in the P0 rat retina using an MLV vector leads to the generation of cells resembling Müller glia (28).

When P0 rat retinæ electroporated with the pCAGIG-mRax expression vector were analyzed at P21, almost all the GFP-positive cells had cell bodies in the INL, with processes extending to the outer limiting membrane (OLM) and/or to the GCL (Fig.6B, panels c-d). This morphology is characteristic of differentiating Müller glial cells or retinal progenitor cells, confirming the previous results obtained using a retrovirus vector (28). In contrast, in the rat retina transfected with the pCAGIG empty vector, most GFP-positive cells were rod PR in the ONL, and a few cells were bipolar and Müller glial cells (Fig.6B).

Loss-of-function analysis

Recently, several groups have developed DNA-based RNAi vectors that stably produce double-stranded small interfering RNAs in mammalian cells (16, 29-34). Using such RNAi vectors, we tested whether the *in vivo* electroporation technique would be useful for loss-of-function analyses in the retina. In this experiment, we chose two retinal transcription factors, Crx (35-37) and Nrl (38), as their loss-of-function phenotypes were known through studies of conventional knock-out (KO) mice (39, 40). Both of these mutant mice have a normal complement of retinal cell types other than PR. The Crx KO mice initially have approximately normal levels of PR, but lack outer segments, while the Nrl KO mice lack rods and have more than the normal number of cones, with abnormal outer segments. Significant reduction of many rod PR-specific genes, including rhodopsin, was reported for both mutant mice.

Several RNAi vectors were designed based on the mouse Crx and Nrl nucleotide sequences. We wished to have as a control an RNAi vector that would target a retinal RNA, rather than an ineffective double-stranded RNA, in the event that a successful targeting vector would trigger a cellular reaction that would non-specifically alter PR

development. To this end, a control RNAi vector that suppressed the expression of mouse GAPDH was designed. We found that vectors producing the double-stranded RNAs corresponding to the coding regions of mCrx (408-428), mNrl (306-326) and mGAPDH (393-413) efficiently and selectively suppressed the expression of mCrx:GFP, mNrl:GFP and mGAPDH:GFP fusion proteins, respectively, in 293T cells (Fig.9 available as supporting information on the PNAS website).

To determine if these RNAi vectors could work in the retina, they were co-electroporated *in vivo* with pCAG-GFP into CD1 mouse retinæ at P0. When electroporated retinæ were dissociated into single cells at P10, and stained with antibodies, the numbers of Crx-, Nrl- and GAPDH-positive cells were significantly and specifically reduced in the GFP-positive fractions of the retinæ transfected with Crx-, Nrl- and GAPDH-RNAi vectors (U6-Crx, U6-Nrl and U6-GAPDH), respectively (Fig.7A). In the retinæ transfected with U6-Crx and U6-Nrl, the numbers of rhodopsin-positive cells were also significantly reduced. An empty RNAi vector (U6) had no apparent effects.

When the sections of electroporated retinæ were analyzed at P20, the ratios of GFP-positive cells in the ONL to those in the INL were almost comparable among the retinæ transfected with U6-empty, U6-Crx, U6-Nrl and U6-GAPDH vectors (Fig.7B). However, in the retinæ transfected with U6-Crx and U6-Nrl, most GFP-positive cells in the ONL did not have clear outer segment structures (panels b, c), consistent with the reported phenotypes of KO mice. Moreover, in the retinæ transfected with U6-Nrl, the nuclei of most GFP-positive cells in the ONL were localized right below the outer limiting membrane, as are cone PR. A similar tendency was observed in the Crx-RNAi vector transfected retinæ, although it was not as significant. In the retinæ transfected with U6-empty or U6-GAPDH vectors, the GFP-positive cells in the ONL had clear outer segments (panels a, d).

DISCUSSION

We demonstrate that DNA can be readily introduced into retinal cells of newborn mouse and rat by electroporation *in vivo* and *in vitro*. *In vivo* electroporation has been applied to a variety of tissues of various animal species including mouse, chicken and frog (9). For the mammalian central nervous system, this technique has been used to introduce genes into the fetal mouse brain, targeting the neural progenitor cells in the ventricular zone (41-43). It has also been used for the retina in that Dezawa *et al.* recently reported the introduction of DNA into retinal GC of the adult rat from the vitreous (44). However, at least in our experiments, we could see only a few GFP-positive cells when the GFP expression vector was injected into the vitreous of newborn rat eyes, followed by electroporation. Moreover, we could detect few GFP-positive cells when the vector was electroporated into the vitreal side of newborn and adult mouse retinae *in vitro*. It is likely that electroporation from the vitreal side is less efficient, requiring further improvement of the transfection conditions. The presence of a basal laminar at the inner limiting membrane might impede vector penetration. Nonetheless, this could also be a very useful technique for the introduction of genes into GC, where it is difficult to transduce using other methods.

The electroporation technique described here has several advantages over conventional methods to deliver genes into the rodent retina. First, this method is rapid and safe. Second, the DNA transduction efficiency can be remarkably high when the injection of DNA is performed accurately. Third, it is possible to introduce various types of DNA constructs with a size limitation that is significantly larger than that of viral vectors. These constructs include cell type-specific promoters used to express reporter genes as well as recently developed DNA-based RNAi vectors. Fourth, multiple DNA constructs can be introduced into single retinal cells. Recently, it has been reported that a combination of multiple transcription factors, rather than a single transcription factor, is important for retinal cell type specification (45, 46). For example, co-expression of Math1 or Math3 with Chx10 efficiently induces bipolar cell genesis in retinal explants, while

misexpression of either Math1/Math3 or Chx10 alone can not (45). Thus, the fourth point would be especially helpful to analyze the functions of retinal transcription factors

Unlike retrovirus vectors that integrate into the host genome and stably express foreign genes for a long time period, gene expression from DNA constructs introduced by *in vivo* electroporation should not be so stable. Although we did not determine exactly how long expression of introduced genes is maintained in the retina, it is unlikely that the gene expression persists for more than several months. For this reason, *in vivo* electroporation may not be suitable for therapeutic uses, such as gene therapy of inherited retinal diseases. Nonetheless, we did find that the GFP expression is visible for at least 50 days, enough for the study of retinal development, including maturation of differentiated retinal cells (*e.g.* elongation of rod outer segment).

Recently, we have identified a large number of candidate genes involved in retinal development and disease, by microarray and SAGE analyses (1, 3). The *in vivo* and *in vitro* electroporation techniques, together with the conventional virus-mediated gene transfer system, would greatly contribute to elucidate the functions of these candidate genes.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. *In vivo* electroporated rat retinae harvested at various developmental stages.

(A) Whole mount preparation of rat retina *in vivo* electroporated with pCAG-GFP at P0 and harvested at P21. Pictures were taken from the scleral side.

(B) Rat retinae *in vivo* electroporated with pCAG-GFP at P0 were harvested at P2 (d-f), P5 (g-l), P8 (j-l), P14 (m-o) or P50 (p-r), and cryosections were prepared.

Figure 2. Cell type composition of retinal cells labeled by *in vivo* electroporation.

(A) Cell type composition determined based on morphologies and locations in the retina. Rat retinae electroporated *in vivo* with pCAG-GFP, or infected *in vivo* with the replication-incompetent LIA retrovirus at P0, were harvested at P14, and sectioned. The LIA-infected retinae expressing alkaline phosphatase (AP) were stained histochemically for AP activity. Green bars represent the retinal cells electroporated with the pCAG-GFP plasmid. Purple bars represent the retinal cells infected with the LIA-retrovirus.

(B) Cell type composition determined by immunostaining. Rat retinae electroporated *in vivo* with pCAG-GFP at P0 were harvested and dissociated into single cells at P14. Dissociated cells were stained with anti-rhodopsin (rod PR), anti-Chx10 (bipolar), anti-protein kinase C α (rod bipolar), anti-glutamine synthetase (Müller glia), anti-HPC-1 (amacrine), anti-calbindin (horizontal), anti-Gt2 α (cone PR) or anti Thy-1 (GC), and the numbers of positive cells were scored. Both GFP-positive (green bars) and -negative cells (yellow bars) were analyzed.

Figure 3. Comparison of ubiquitous promoters in the developing rat retina.

Rat retinae were electroporated *in vivo* at P0 with the GFP expression vectors driven by CAG promoter (A), CMV promoter (B), human EF1 α promoter (C) or human ubiquitin C promoter (D), and sectioned at P10.

Figure 4. *In vitro* electroporated mouse retinal explants.

(A) Mouse retinæ of P0 CD1 (a, b), adult CD1 (c, d) or adult Swiss Webster having a retinal degeneration mutation (e, f) were *in vitro* electroporated with pCAG-GFP from the scleral side (a, c, e) or from the vitreal side (b, d, f), and cultured for 5 days. Similar results were observed 16 hours after electroporation. Pictures a, c, e were taken from the scleral side, and pictures b, d, f were taken from the vitreal side.

(B) A section of CD1 mouse retina *in vitro* electroporated with pCAG-GFP at P0, and cultured for 10 days.

Figure 5. Cell type-specific labeling using rhodopsin-, CABP5- and CRALBP-promoters

(A) Rat retinæ were co-electroporated *in vivo* with pCAG-GFP (3 μ g/ μ l) and bovine rhodopsin promoter 2.2K-DsRed (pRho 2.2K-DsRed, 3 μ g/ μ l) at P0, harvested at P5 (a-c), P8 (d-f) or P14 (g-i), and sectioned.

(B) Rat retinæ were co-electroporated *in vivo* with pRho 2.2K-CFP (3 μ g/ μ l) and mouse CABP5 promoter 4.7K-DsRed (pCABP5 4.7K-DsRed, 3 μ g/ μ l) at P0, harvested at P14 and sectioned.

(C) Rat retinæ were co-electroporated *in vivo* with pCAG-GFP (3 μ g/ μ l) and mouse CRALBP promoter 4.0K-DsRed (pCRALBP4.0K-DsRed, 3 μ g/ μ l) at P0, harvested at P10 and sectioned. Some DsRed-positive Müller glial cells were truncated in the process of cutting sections.

Figure 6. Ectopic expression of Rax using the IRES-GFP vector

(A) Structures of pCAGIG and pCAGIG-mRax vectors.

(B) Rat retinae *in vivo* electroporated with pCAGIG (a, b) or pCAGIG-mRax (c, d) at P0, were harvested at P21 and sectioned. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

Figure 7. Knockdown of Crx and Nrl expressions in the mouse retina using RNAi vectors

(A) Specific down-regulation of the expression of Crx, Nrl, GAPDH and rhodopsin in the RNAi-vector transfected mouse retinae. CD1 mouse retinae were co-electroporated *in vivo* with pCAG-GFP (2 μ g/ μ l) and an RNAi vector (U6, U6-Crx, U6-Nrl or U6-GAPDH; 4 μ g/ μ l) at P0, harvested at P10 and dissociated into single cells. The dissociated cells were stained with anti-Crx, anti-Nrl, anti-GAPDH or anti-rhodopsin antibody, and the numbers of positive cells were scored. Both GFP-positive (green bars) and -negative cells (yellow bars) were analyzed.

(B) Morphology of the retinal cells transfected with RNAi vector. CD1 mouse retinae were co-electroporated *in vivo* with pCAG-GFP and an RNAi vector at P0, harvested at P20 and sectioned. Sections were stained with anti-rhodopsin antibody (red) and DAPI (blue), and images were taken using a confocal microscope (Zeiss LSM 510). Inset: higher magnification views of outer segments.

Figure 8. (supporting figure) Procedure and apparatus for *in vivo* and *in vitro* electroporation.

(A) Schematic illustration showing the *in vivo* electroporation method.

(B) The electrodes used in this study are shown. Tweezer-type electrodes (a) are placed to hold the head of newborn (P0) rat or mouse (b).

(C) A micro-chamber used for *in vitro* electroporation is shown.

Figure 9. (supporting figure) Evaluation of RNAi vectors in 293T cells using GFP-fusion proteins.

293T cells (8×10^5 cells in 60 mm dish) were co-transfected with three DNA constructs, a GFP-fusion protein expression vector (1 μ g), an RNAi vector (3 μ g) and pCAG-HcRed (1 μ g) as a control, and analyzed 72 hours after transfection. The GFP-fusion proteins, Crx:GFP(a-d), Nrl:GFP (i-l) and GAPDH:GFP (g-t) were expressed under the control of the CAG promoter. Note that Crx:GFP and Nrl:GFP fusion proteins were localized in the cell nucleus, while GAPDH:GFP fusion protein was localized in the cytosol. The RNAi vector was a pBS/U6 empty vector (U6; a, e, l, m, q and u) or pBS/U6 carrying a DNA template that directs synthesis of mCrx siRNA (b, f, j, n, r and v), mNrl siRNA (c, g, k, o, s and w) or mGAPDH siRNAs (d, h, l, p, t and x). For construction of cDNAs encoding Crx:GFP, Nrl:GFP and GAPDH:GFP fusion proteins, AgeI sites were created by PCR at the end of coding regions of mCrx, mNrl and mGAPDH, respectively, and ligated with the GFP cDNA excised from pEGFP-N1 (Clontech) with Age I and Not I. These cDNAs were cloned into pCAGGS with modified multiple cloning sites. For construction of pCAG-HcRed, a coding region of HcRed was excised from pHcRed1-N1 (Clontech) and cloned into pCAGGS with modified multiple cloning sites.

Figure 1

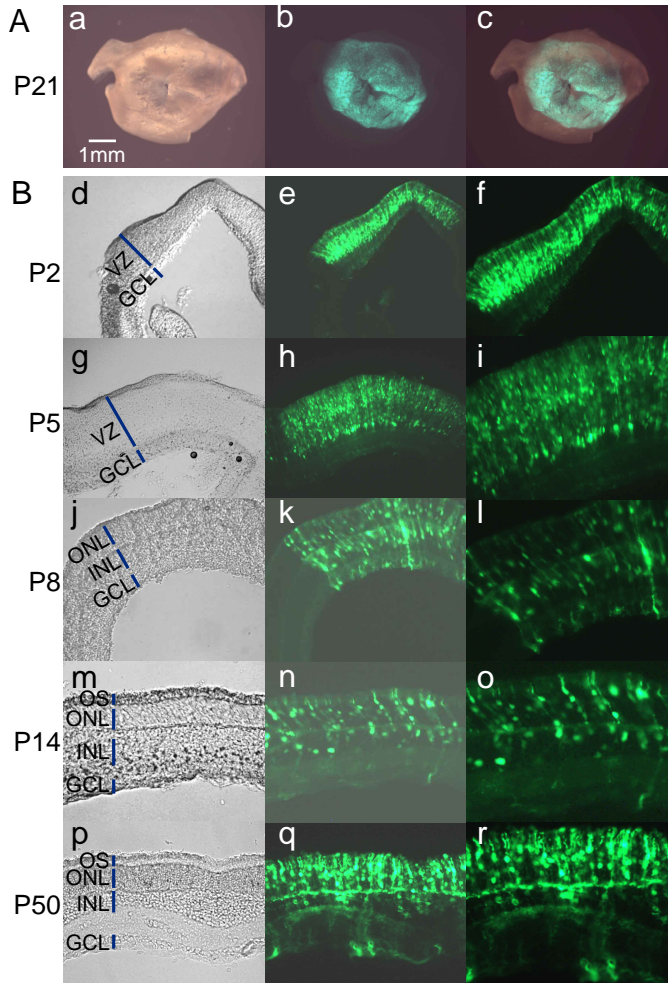
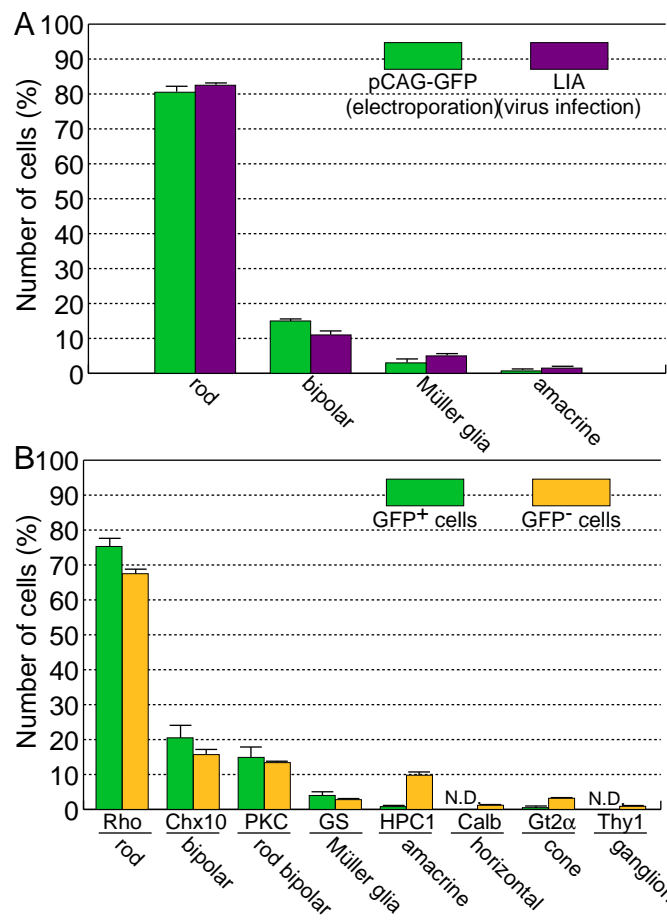
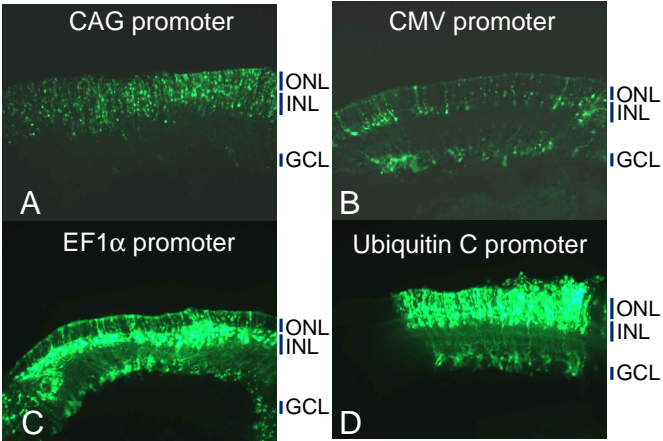
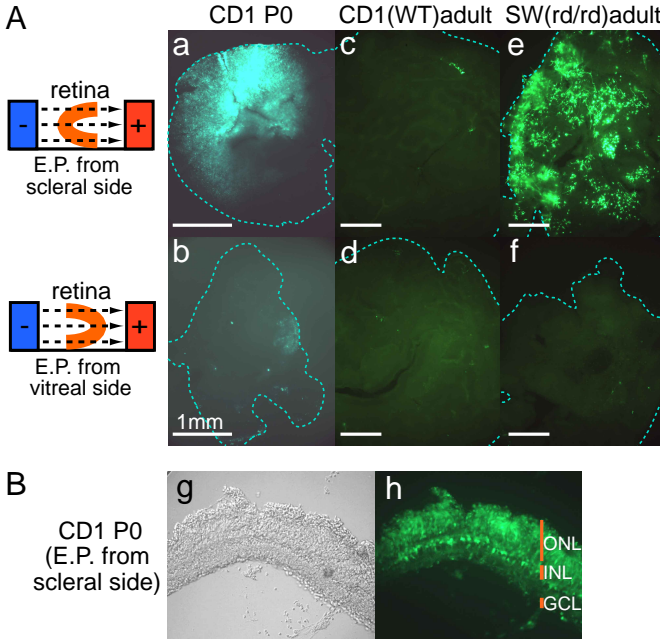
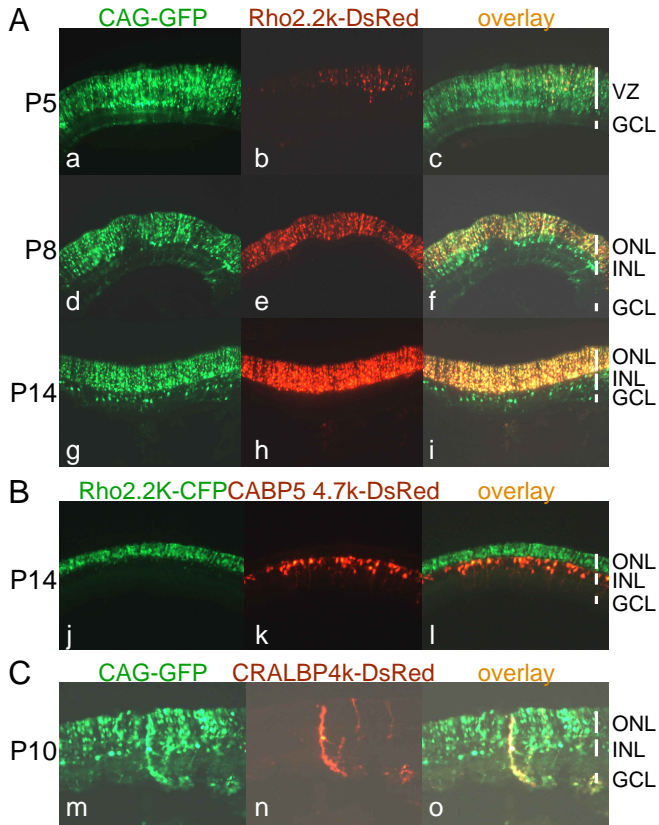


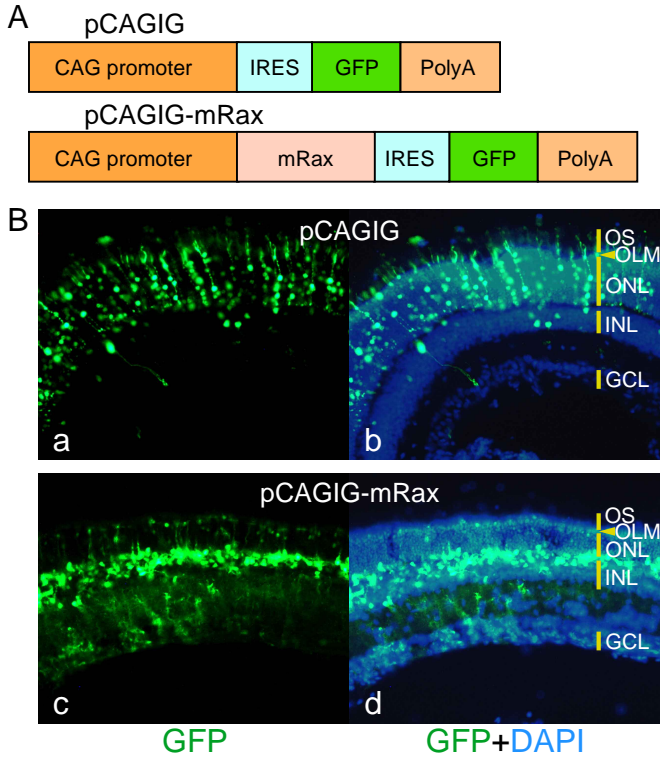
Figure 2

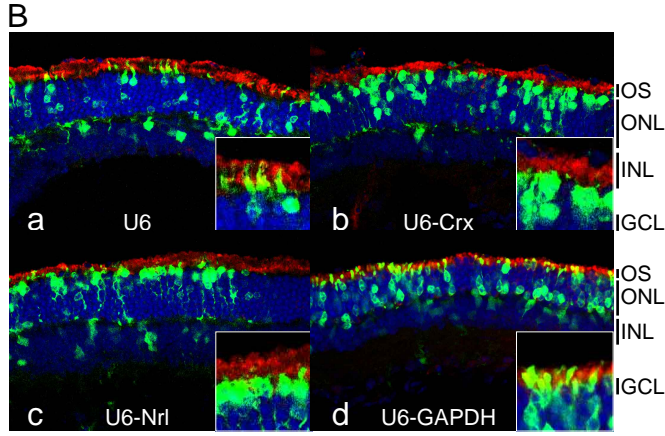
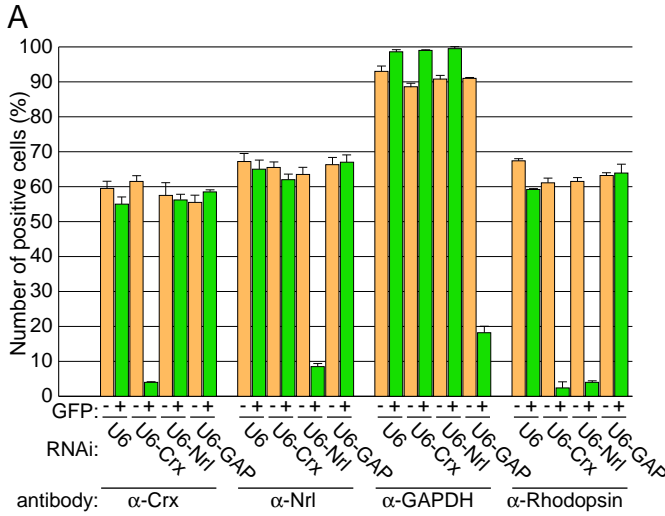




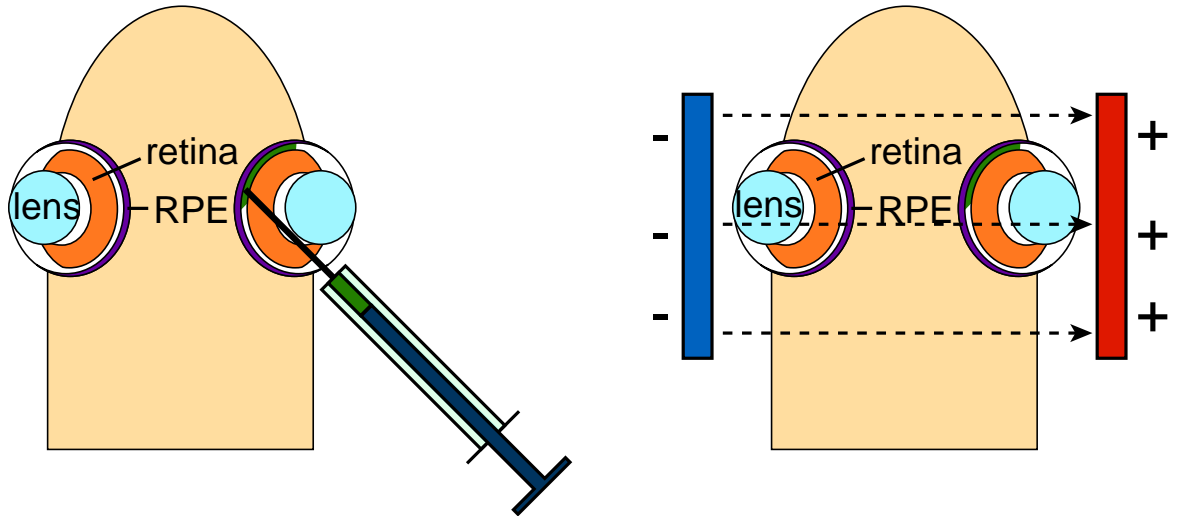






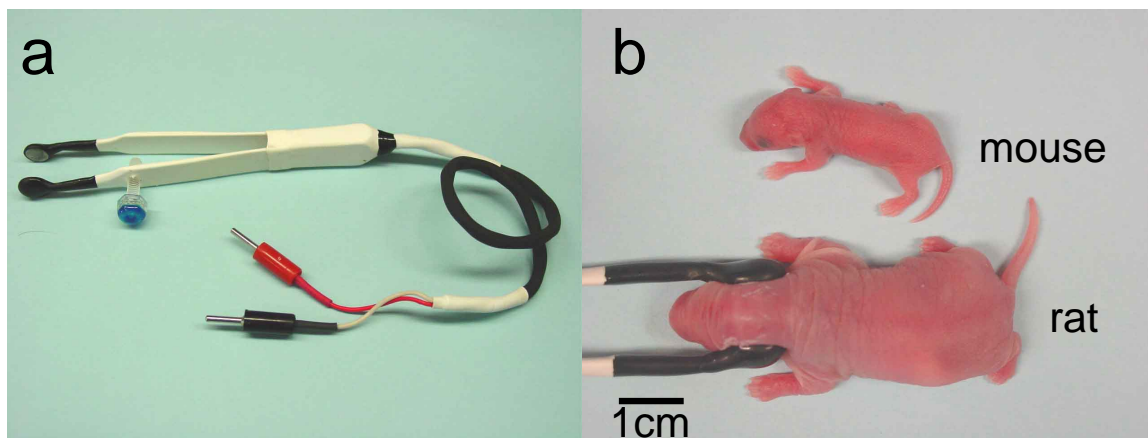


A



(1) DNA injection into subretinal space (2) electroporation

B



C

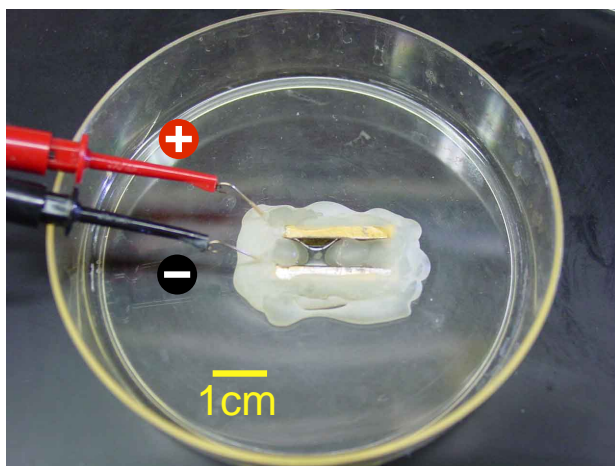


Figure 9 (supporting figure)

