Short Communication

Coupling ex vivo electroporation of mouse retinas and luciferase reporter assays to assess rod-specific promoter activity

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Abstract

Ex vivo electroporation of mouse retinas is an established tool to modulate gene expression and to study cell type-specific gene expression. Here we coupled ex vivo electroporation to luciferase reporter assays to facilitate the study of rod-photoreceptor-specific gene promoters. The activity of the rod-specific proximal bovine rhodopsin promoter was significantly increased in C57BL/6J wild-type retinas at postnatal days 1 and 7 by 3.4-fold and 8.7-fold respectively. In C57BL/6J *Nr2e3^{rd7/rd7}* retinas, where the rod photoreceptor-specific nuclear receptor Nr2e3 is not expressed, a significant increase by 2.5-fold was only observed at postnatal day 7. Cone-specific S-opsin promoter activity was not modulated in C57BL/6J wild-type and *Nr2e3^{rd7/rd7}* retinas. Taken together, we describe an easily implementable protocol to assess rod-specific promoter activity in a physiological context resembling that of the developing postnatal mouse retina.

In the adult mouse retina photoreceptors constitute approximately 75% of all cells, and 97% of these are dim light-sensitive rods mediating night vision. Rod generation starts at embryonic day 13 (E13), peaks between birth and postnatal day 2 (P2), and ends between P6 to P10 (Akimoto et al., 2006; Carter-Dawson and LaVail, 1979). Rod-specific rhodopsin (*Rho*) mRNA expression starts to be detected at P2 and increases to high levels at P4 that persist throughout adulthood (Akimoto et al., 2006). Bright light-sensitive cones mediating color vision and visual acuity represent the remaining 3% of photoreceptors and are generated from E10.5 to E16 (Carter-Dawson and LaVail, 1979). Cones are divided into short (S) wavelength-sensitive cones expressing S-opsin (*Opn1sw*) from E18 on and middle (M) wavelength-sensitive cones expressing M-opsin (*Opn1mw*) from P10 on (Roberts et al., 2006). The transcriptional network regulating the development of rod photoreceptors includes CRX (cone-rod homeobox) regulating photoreceptor development, NRL (neural retina leucine zipper) and ROR (retinoid orphan receptor) promoting rod precursor differentiation, and NR2E3 (nuclear receptor class 2 family E member 3) repressing cone-specific gene expression in rods (Swaroop et al., 2010).

The technically demanding *in vivo* electroporation of neonatal rodent retinas greatly contributed to the identification of cell type-specific regulatory regions underlying the variety of retinal neurons (Matsuda and Cepko, 2004, 2007, 2008). Subretinal injection and *in vivo* electroporation of a plasmid where the rhodopsin promoter drives fluorescent protein expression, resulted in increasing fluorescence levels in the outer retina, where rods reside, at P5, P8 and P14 (Matsuda and Cepko, 2004). The technically less challenging *ex vivo* electroporation of neonatal mouse retinas and subsequent explant culture also resulted in efficient expression of transfected plasmids in rods (Matsuda and Cepko, 2004, 2008). Consequently, *ex vivo* electroporation was used to characterize rod-specific regulatory regions (Corbo et al., 2010; Hsiao et al., 2007). To assess the activity of synthetic rhodopsin promoters (Lee et al., 2010), a fluorescence-based quantitation was

developed (Montana et al., 2011). Co-electroporation of pCAG-GFP ubiquitously expressing green fluorescent protein (GFP) with plasmids where regulatory regions drive dsRed fluorescent protein expression, allows subsequent quantification of micrographs acquired in the red and green channels, by normalizing dsRed levels to GFP levels (Montana et al., 2011). An alternative quantitation method based on next-generation sequencing of barcoded plasmid libraries was used for a high-throughput mutational analysis of the rhodopsin promoter (Kwasnieski et al., 2012) and for the analysis of photoreceptor-specific regulatory regions (Shen et al., 2016; White et al., 2013).

The objective of the present work was to facilitate quantitation of rod-specific promoter activity by coupling the existing protocol of retinal ex vivo electroporation (Matsuda and Cepko, 2004, 2008) to luciferase assays (Fig. 1A). As a prototypical rod-specific promoter we tested the proximal -225/+74 region of the bovine rhodopsin promoter (NCBI gene ID:509933) harboring two CRX and one NRL binding sites (Zack et al., 1991) subcloned into MluI/BglII sites of the pGL3-basic (Promega) firefly luciferase reporter plasmid (Fig. 1B). To assess cone-specific promoter activity, we amplified a mouse S-opsin -2465/+77 promoter fragment (NCBI gene ID:12057) from C57BL/6J mouse genomic DNA with primers 5'-GCGCACGCGTTGGATGGGACACACACTTCC-3' and 5'-GCGCCTCGAGTCATCCTCTCCTGACATCTCC-3', and subcloned it into KpnI/BglII sites of pGL3-basic. This S-opsin promoter fragment contained two regulatory regions r1 (-195/-47) and r2 (-2275/-2120) (www.mouseencode.org). The proximal r1 harbors a CRX binding site 5'-CTAATC-3' at -88/-83 and a 5'-extended ROR binding site 5'-TGACCTCAATCT-3' at -170/-159 (Fig. 1B). The pGL3-control plasmid (Promega) was used as a positive control.

Experiments were performed on retinas of wild-type C57BL/6J and B6.Cg-*Nr2e3^{rd7}/J* (*Nr2e3^{rd7/rd7}*; Jackson Laboratory) mice. In the *Nr2e3^{rd7/rd7}* retinas, the rod-specific nuclear receptor NR2E3 is not expressed: early-born rod precursors are misspecified towards S-

opsin expressing cones and late-born rod precursors result in non-functional hybrid photoreceptors, expressing both rod- and cone-specific genes (Corbo and Cepko, 2005; Haider et al., 2001). Consequently, a 2- to 3-fold increase of S-cones is observed, and a delay in the onset of rhodopsin expression, with barely detectable levels at P6 and subnormal levels at P14 (Corbo and Cepko, 2005; Haider et al., 2001).

For ex vivo electroporation, mice were sacrificed at postnatal days 1 and 7 (P1 and P7), eyes enucleated, and up to 4 retinas folded around the lens transferred to a microelectroporation chamber with a 5 mm-gap between electrodes (CUY535, Nepa Gene, Ichikawa, Japan), the posterior retina (scleral side) facing the negative electrode chamber wall (Matsuda and Cepko, 2004)(Fig. 1A). Animal handling was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the local Veterinary Service (Authorization VD3090). The microelectroporation chamber was filled with 150 μ l of a 1.5 μ g/ μ l DNA solution in Hanks' balanced salt solution, with an equal amount (112.5 μ g) of rhodopsin or S-opsin pGL3 firefly luciferase reporter constructs and of the pRL-CMV vector in which the cytomegalovirus promoter directs Renilla luciferase expression (Promega). The CMV promoter is expected to drive expression in inner retinal neurons rather than in photoreceptors, and is therefore appropriate to normalize values between mouse strains where photoreceptors are genetically affected. Five square pulses of 50 msec duration at 30 V with 950 msec intervals were applied (CUY21SC in vivo electroporator, Nepa Gene), resulting in output currents of 120-140 mA. DNA solution was stored at -20°C to be reused for a second series of experiments. Electroporated retinas were separated from the lens, flattened by four incisions in the cardinal directions, transferred on 0.2 μ M polycarbonate filters (25 mm Nucleopore Track-Etch Membrane, Whatman GE Life Sciences) and cultured in 6-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 μ g/ml Normocin at 37°C and 5% CO₂ for 7 days (Kwasnieski et al., 2012). Then,

each retinal explant was rinsed in 200 μ l of Dulbecco's Phosphate-Buffered Saline and manually disrupted in 100 μ l of Renilla Luciferase Assay Lysis Buffer (Promega) with a microtube-fitting Potter homogenizer. After centrifugation at 4°C for 5 min at 13'000 rpm, 5 μ l supernatant was added to 20 μ l Firefly Luciferase reagent (Promega) in white 384-well microplates (Optiplate) for assay, and to 20 μ l Renilla Luciferase reagent (Promega) for normalization. Relative luciferase activity was determined by dividing the luminescence values of firefly luciferase activity by those of the co-transfected Renilla luciferase activity.

When compared to C57BL/6J pGL3-basic levels, the pGL3-control levels were significantly increased in C57BL/6J and *Nr2e3^{rd7/rd7}* retinas by about 2.5-fold at P1 and P7 (Fig. 1C). Rhodopsin promoter-driven luciferase activity was significantly increased by 3.4-fold at P1, and by 8.7-fold at P7 in C57BL/6J retinas (Fig. 1C). This increase measured in wild-type P7 versus P1 retinal explants, parallels the reported developmental increase in rhodopsin expression between P2 and P4 (Akimoto et al., 2006). The 2.5-fold increase in luciferase expression measured in *Nr2e3^{rd7/rd7}* retinas at P7 was significant, when compared to C57BL/6J or to *Nr2e3^{rd7/rd7}* pGL3-basic levels. This low luciferase activity in P7 *Nr2e3^{rd7/rd7}* retinas fits the delayed onset of rhodopsin expression assessed by in situ hybridization in *Nr2e3^{rd7/rd7}* retinas, where rhodopsin mRNA was detected at P14, but barely at P6 (Corbo and Cepko, 2005). These results with a prototypical rod-specific gene provide circumstantial evidence that i) the transcriptional network necessary for rod-specific gene regulation is conserved in explant cultures, ii) a differential promoter activity between P1 and P7 is indicative of rod-specific gene expression, and, iii) rod precursors are readily electroporated at P1 and P7.

The S-opsin luciferase construct showed no significant expression in C57BL/6J and *Nr2e3^{rd7/rd7}* retinas compared to pGL3-basic levels at P1 and no increase in S-opsin activity was observed in *Nr2e3^{rd7/rd7}* versus C57BL/6J retinas (Fig. 1C), despite the fact that

Nr2e3^{rd7/rd7} retinas exhibit a 2- to 3-fold increase in S-opsin expressing cells (Corbo and Cepko, 2005; Haider et al., 2001), and the S-opsin promoter construct contained both the proximal promoter and the upstream looping organizer necessary for CRX-dependent regulation of the S-opsin expression (Peng and Chen, 2011). We hypothesize that the limited number of cones in the mouse retina and the inherent limitation of ex vivo electroporation to transfect 'early-born' retinal cell types like cones (Matsuda and Cepko, 2004) hamper cone promoter analysis. This obstacle might be overcome by using the highly sensitive NanoLuc luciferase system (Promega) (Hall et al., 2012), which has been shown to generate on average an 80-fold stronger signal than the Firefly luciferase system and might therefore allow detection from a few electroporated cells.

In conclusion, ex vivo electroporation coupled to luciferase assays is restricted to accurate quantitation of early postnatal rod-specific promoter activity. Compared to the previously reported fluorescence- or next-generation-sequencing-based quantitation of rod-specific promoter activity (Kwasnieski et al., 2012; Montana et al., 2011), quantitation by measuring luciferase activity is cheap and rapid. Requiring no technical knowledge in either fluorescence microscopy or library construction, and given the broad use of cell-based luciferase reporter assays in many labs, this technique can be easily implemented, for example as a primary screen for the activity of potential promoter regions identified by chromatin immunoprecipitation of rod-specific transcription factors. Furthermore, the luciferase promoter constructs routinely used in cell-based transient transactivation assays can directly be used in ex vivo electroporation without the need of subcloning them into plasmids where the regulatory regions drive fluorescent protein expression. The lack of information about which retinal cell types other than rods express the electroporated plasmids is compensated for by a reproducible quantitation of the promoter activity.

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Figure legend

Figure 1. Ex vivo electroporation coupled to luciferase assays in mouse retinas. **(A)** Retinal explant culture lasts for 7 d before homogenization for luciferase assay (microtube from clker.com). **(B)** Schematic drawings of rhodopsin and S-opsin promoter constructs with transcriptional start site (arrow), firefly luciferase cassette (Luc), CRX (open oval), NRL (grey triangle) and ROR (black rectangle) binding sites, repetitive sequences (open rectangle), and DNaseI hypersensitive regulatory regions r1 and r2 (grey rectangles). **(C)** Luciferase activities of ex vivo electroporated C57BL/6J (grey bars) and *Nr2e3^{rd7/rd7}* (black bars) retinas at P1 and P7. Means of at least 4 independent experiments $\pm$ SD (n=4 eyes with luciferase activities measured in duplicate; n=6 eyes for pGL3_basic and Rho-225/+74) normalized to Renilla luciferase and to P1 pGL3-basic levels are shown. Unpaired t-test with equal SD (Prism 4.0.2, GraphPad Software) determined significant changes with the activity of the control pGL3-basic vector in wild-type C57BL/6J retinas at P1, or between rhodopsin samples at P1 and P7 (*: p<0.05; **: p<0.01; ****: p<0.0001).

Figure 1