Optogenetic tools for *in vivo* applications in neonatal mice

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ABSTRACT

Spontaneous neural activities exist early in development and their spatiotemporal patterns play important roles in the development of sensory maps such as maps of retinotopy in the visual system. We summarized different optogenetic tools, including transgenic mouse lines, viral-mediated transfection and electroporation methods to enable the expression of light-gated channelrhodopsin (ChR2) in retinal ganglion cells (RGCs) before the onset of vision. Patch-clamp and extracellular recording experiments verified that activities of ChR2-expressing cells were precisely manipulated by the patterns of optical stimuli. In chronic stimulation experiments, light-emitting diodes controlled the activity patterns of ChR2-expressing RGCs *in vivo*. Changes in the retinotopic map in Superior Colliculus (SC) were examined by quantifying the relative sizes of fluorescently labeled target zones. Our results revealed that various optogenetic and optical tools can manipulate retinal activities with precise temporal patterns. These techniques can be readily used in studying the development of the central nervous system of neonatal rodents.

Keywords: optogenetics; neural stimulation; neonatal mice

1. INTRODUCTION

Optogenetics combines genetic targeting of specific neuron types with optical technology for the control of neuronal activity at millisecond timescales in intact and living neural circuits. This technology uses a gene derived from the algae *Chlmydomonas reinhardtii* that encodes a blue light-gated cation channel *Channelrhodopsin-2* (ChR2) [1] as well as a yellow light-gated cation channel *Volvox Channelrhodopsin-2* (VChR2) [2]. Delivery of millisecond-scale pulses of blue light to ChR2 expressing neurons triggers trains of spiking activity *in vivo* [3, 4]. Technical advantages of using the optogenetics approach include the ability to produce highly consistent and sustained activity patterns over long periods of time (in comparison to pharmacological induction of neuronal activity), precise spike timing control (in comparison to optical uncaging), and cell type specificity (in comparison to electrical stimulation).
The vertebrate brain organizes visual information about the sensory world into remarkably precise maps. Neighboring Retinal Ganglion Cells (RGCs) in the retina project to neighboring cells in the brain, forming feature maps such as retinotopy in the lateral geniculate nucleus (LGN), superior colliculus (SC) and visual cortex (VC). Remarkably,

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anatomically and functionally precise maps emerge before the onset of vision [5]. It is widely hypothesized that molecular cues are responsible for the establishment of coarse map structure in the SC, and activity dependent processes subsequently refine these sensory circuits to functional precision [6]. RGCs exhibit spontaneous activity from late embroynic ages (~E17 in the mouse) until eye opening (~P13 in the mouse) [7]. This spontaneous retinal activity consists of spatiotemporally correlated slowly propagating waves [8] that are thought to be the responsible for the activity dependent refinement of visual maps. Mutant mice lacking the β2 subunit of the nicotinic acetylcholine receptor have disrupted spontaneous retinal activity [9] and the projection pattern of RGCs to the colliculus is left in an unrefined, immature state [10]. While these results provide some evidence for an instructive role of retinal activity in map refinement, it is not clear how the spatiotemporal activity pattern conveys information necessary for the development of visual maps. A fundamental difficulty in examining the role of activity-dependent 'instruction' in map development is that we have poor experimental control of the spatiotemporal retinal activity patterns *in vivo*. Since the retinocollicular map matures before the onset of vision, it is impossible to directly manipulate retinal activity using 'normal' visual stimuli. Similarly, electrical stimuli applied directly to the optic nerve *in vivo* also disturbs aspects of visual map development in ferrets [11], but this manipulation is also very crude and difficult to control in early postnatal mice. We present here a collection of optogenetics tools for ChR2 expression in RGCs, as well as chronic optical manipulation of retinal activities. These techniques can be readily used in studying the development of the central nervous system of neonatal rodents.

2. METHODS

2.1 Retina section and immunohistochemistry

Thy1-ChR2-eYFP mice were obtained from Guoping Feng's lab at MIT. Animals were genotyped according to protocols from the Jackson's Laboratory (stock number 007612). Retinas of Thy1-ChR2 mice were postfixed for 6 hr in 4% paraformaldehyde after dissection and then immersed in 30% sucrose for 10 hr. The retina samples were frozen in embedding medium (Jung, Leica Microsystems) in -80°C followed by sectioning at 14μm on a freezing microtome (Leica CM 1850). Retina sections were rinsed with 0.5% Triton-X100 in PBS for 20 min and blocked with 2% donkey serum and 2 mg/ml BSA in PBS containing 0.05% Triton-X100 overnight at 4°C. Primary antibody (anti-GFP,1:100, Abcam) incubation was overnight at 4°C followed by five washes in PBS, and secondary antibody (Alexa 488-donkey anti-chicken,1:200, Jackson ImmunoResearch) incubation was overnight at 4°C. After three washes in PBS, retina sections were mounted for imaging.

2.2 Virus injection

P1 ChaT-CRE, Pax6-CRE or C57 pups were anesthetized by hypothermia for 4-6 min before injection surgery. AAV-hSyn-hChR2-mCherry (Neuron Biotech, Shanghai) or AAV-DIO-ChR2-mCherry (North Carolina Core Facility) virus was filled into pulled glass pipettes (tip diameter ~2–3 μm); eyelid of the anaesthetic mice was opened and the eyeball protruded; viral solution was injected intravitreously in the dorsal, ventral, temporal and nasal region of each eye (350 nl in total) using the Nanoject II system (Drummond Scientific Company) at its original titer.
For cortical virus injection, P14 mice were anesthetized by intraperitoneal injections of ketamine(70mg/kg) and Dorminor (0.5mg/kg) cocktail. A small craniotomy was made at bregma (lateral 3 mm, ventral 0.85mm). 0.5 μl of lentivirual solution was injected using a programmable pump (PHD2000, Harvard Apparatus). Animal was recovered and returned after suturing the skins.

2.3 Electroporation

P1-2 C57 pups were anesthetized by hypothermia for 4-6 min before surgery. Total amount of 0.6μl plasmid DNA solutions containing CAG-ChR2-Venus (Addgene) and CAG-tdtomato were injected intravitreously into each eye. Setup for electroporation was made as previously described [12]. Briefly, electrodes were made from stainless steel forceps. Isolated pulse stimulator (AM systems Model 2100) generated 3-5 pairs of bipolar square pulses (25V-pp, 50 msec, 1sec interval) that were applied through the electrodes across the eyeballs. Pups were placed on a temperature-controlled heat pad after surgery and returned to their mothers upon recovery. Fluorescent images were taken at P3-4. Chronic optical stimulation experiments started at P3-4. Detailed stimulation protocols can be found in 2.5. Animals were terminated at P7-8 for retinotopic mapping.

2.4 *In vitro* electrophysiology

350 μm cortical slices were prepared using a vibratome (VT1000, Leica) in ice cold artificial cerebrospinal fluid (in mM: NaCl 126, KCl 3, NaH_2PO_4 1.25, $MgSO_4$-$7H_2O$ 2, $NaHCO_3$ 26, dextrose 10 and $CaCl_2$ 2, equilibrated with 95% O_2 and 5% CO_2). Intracellular recordings were performed at current clamp mode using Axoclamp 2B (Axon Instrument) at room temperature. Cells were visualized under a Differential Interference Contrast microscope. A 473nm blue laser (World Star tech) was coupled to the back of the DIC microscope to provide optical stimulation.

The retina of Thy1-ChR2 mice at P10 was dissected in Ringer's solution containing (in mM) NaCl 124, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 26 and glucose 22, pH 7.35 and oxygenated with 95% O_2 and 5% CO_2. RGC responses were recorded using a multielectrode array (100 μm apart, multichannel systems). The retina was stimulated by a blue LED (Luxeon K2 blue, Philips). Action potentials were thresholded offline (40 μV) and filtered between 100 Hz and 3 kHz. Offline data were analyzed using Offline Sorter (Plexon), Neuro Explorer (Nex Technologies) and a custom program.

2.5 Chronic stimulation

Mice were electroporated with CAG-ChR2-Venus and CAG-tdtomato at P1 and chronic stimulation started P3 – P4 in an isoflurane chamber (0.7%). Eyelids of the pups were cut and glued open with atropine application before stimulation. Blue light stimulation (1 sec duration, 10 sec interval) was provided by a blue LED (connected to Master 8 stimulator). The stimulation lasted for 2-3 days, with12 hr stimulation everyday before the pups were recovered and returned to the cage.

2.6 Fluorescence image acquisition and analysis

Fluorescence images were acquired using Olympus BX51 and Zeiss Imager Z under automatic exposure mode. For the analysis of target zone sizes in the chronic stimulation experiment, the images were processed using a homemade Matlab program[13]. Briefly, the outline of the SC was drawn manually and background fluorescence was subtracted. The fraction of SC with fluorescence intensity above half maximum was calculated for each animal. Statistical significance was calculated using student's t-test.

3. RESULTS

3.1 ChR2 expression in the retinas of neonatal mice

RGCs convey the output of retina to the central visual area using trains of action potentials. The onset of light responsiveness happens around P12. In order to manipulate retinal input in neonatal mice during the development of visual maps, expression of ChR2 in RGCs is required to start before P12. Ideally, subtypes of RGCs with specific anatomical or functional features[14] should be driven to express ChR2.

Various transgenic mouse lines have been generated to carry ChR2 gene [15], including cre-dependent mouse lines that provides convenient tools for expression in specific cell types [16]. RGCs are neurons with long projecting axons, suggesting that specific expression of ChR2 in RGCs is likely to be found in transgenic lines with axonal projection-specific promoters such as Thy1. Fig. 1 demonstrated the expression of ChR2 in the retina of Thy1-ChR2 mice. The expression starts around P9. Cell bodies of the ChR2-expressing neurons lied in the RGC layer, and their dendrites stratified in the ON and OFF sublayers of the inner plexiform layer (Fig.1B). Axons of the ChR2-expressing RGCs exit the retina through the optical disc. Transgenic mouse lines have relatively stable expression level and pattern between generations. Once the cre-lox lines for optogenetics have been established, manipulation of activities from subtype RGCs such as direction-selective RGCs could provide insights into the development of different visual functions.

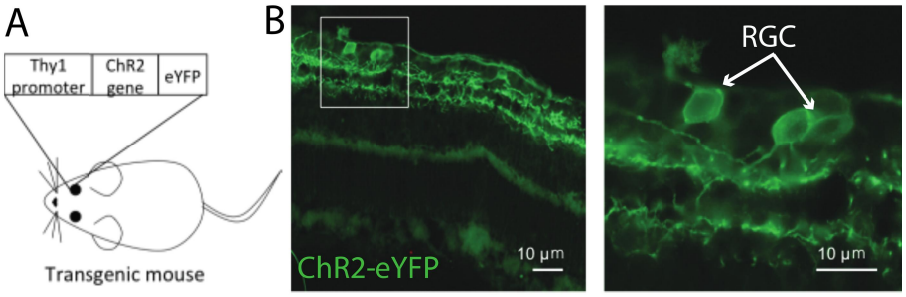

Figure 1. ChR2 transgenic mice with RGC expression in the retina. (A) Thy1 transgenic mice that carry ChR2-eYFP gene; (B) Retina sections of Thy1 transgenic mice stained for YFP at P26. The white arrows show cell bodies of RGCs. Dendrites of RGCs form two layers in inner plexiform layer.

Viral mediated transfections in the retina could also induce ChR2 expression in neonatal mice. Bi et al first used Adeno-Associated Virus (AAV) for ectopic expression of ChR2 in the retina of adult mice three to four weeks after viral injection [17]. AAV is a single-stranded DNA parvovirus, which became a strong candidate vector for gene therapy because of its infection efficiency, safety and success in preclinical and clinical studies [18]. We injected AAV virus (serotype 8) carrying hChR2(H134R) under synapsin (hSyn) promoter intravitrously into the eyes of P1 pups. Expression of ChR2 started as early as P6 in a large population of retinal neurons (Fig. 2B). Axons from these neurons were visible, suggesting that a fraction of these ChR2-expressing neurons are RGCs. Moreover, using cre-lox strategy, cell-type specific expression of ChR2 is possible in neonatal retinas. Figure 2C showed that AAV virus with doublefloxed Inverse Orf(DIO)-hChR2 (serotype 5) induced ChR2 expression in ChaT-CRE positive starburst amacrine cells (SACs). SACs had intrinsically bursting activities, which were thought to underlie spontaneous retinal waves [19]. The distribution of these ChR2-expressing SACs were sparse, whose dendritic trees could be isolated from each other. Similarly, Pax-6 is a critical gene for eye development[20]. A small fraction of Pax6-CRE positive cells expressed hChR2 after injection of AAV-DIO-ChR2 virus. As cell identities of retinal cells are mapped in more detail[21], manipulation of different subtypes of retinal cells becomes possible.

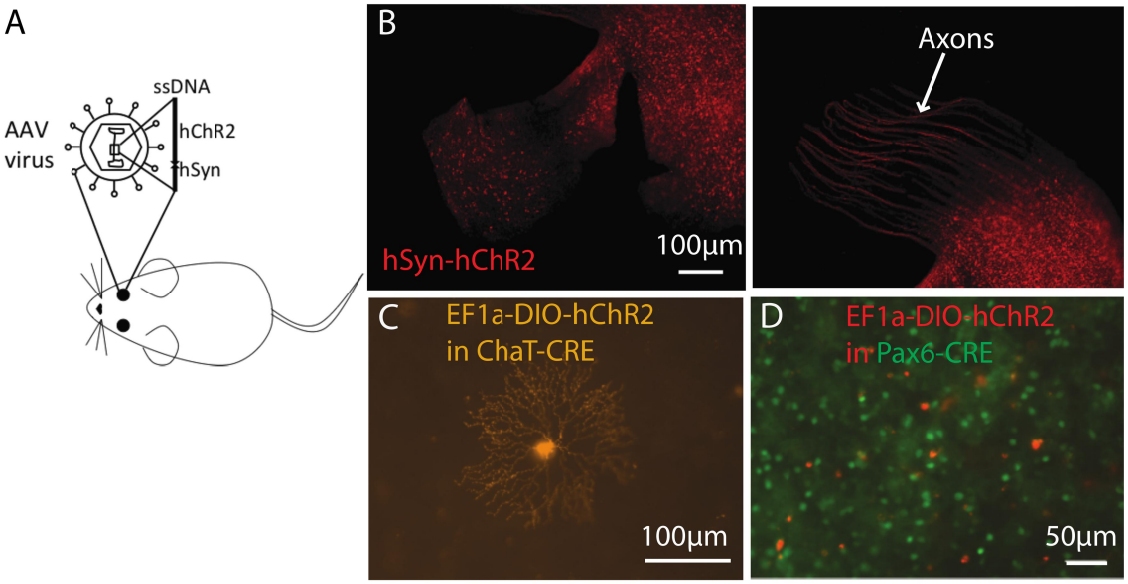

Figure 2. AAV-driven ChR2 expression in the retina. (A) Schematic of AAV virus carrying hChR2 gene; (B) Whole mount of P6 mice injected with AAV-hSyn-hChR2-mCherry. The white arrow indicated axons of transfected RGCs; (C) Whole mount of P9 ChaT-CRE mice injected with AAV-EF1a-DIO-hChR2-mCherry. The bright fluorescent dot were cell bodies of SACs, with surrounding dendritic trees. (D) Whole mount of P9 Pax6-CRE-IRES-GFP mice injected with AAV-EF1a-DIO-hChR2-mCherry. Pax6-CRE neurons were fluorescent in green color.

As a convenient and straightforward way of delivering plasmid DNAs, electroporation in prenatal mice (*in utero*) enables regulation of target genes or expression of proteins at specific developmental stage[22]. The Cepko lab developed *in vivo* electroporation techniques for transfecting retinal cells of neonatal mice[23]. The advantages of *in vivo* electroporation including the temporal and spatial regulation of target protein expression and the short turn-on time for expression[12, 24]. While AAV mediated expression starts at least 5 days after injection, expression can be found as early as one day after electroporation. In our experiments, RGCs were examined two days after electroporation surgery. Fig. 3 demonstrated that over 80% of RGCs were co-transfected by two different plasmids and expressed both ChR2-Venus and tdtomato. Activities of ChR2 RGCs can be optically manipulated, and their axonal projection into the LGN and SC can be visualized by the fluorescence from tdtomato.

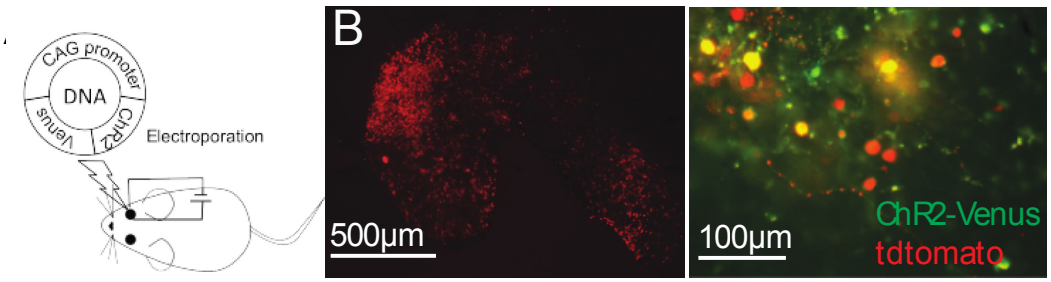

Figure 3. ChR2 expression in RGCs by retinal electroporation of ChR2-carrying DNA plasmids. (A) Schematic of setup for RGC electroporation; (B) Whole mount of P6 mice electroporated with CAG-ChR2-Venus and CAG-tdtomato. RGCs were co-transfected by both plasmids.

3.2 Optical responses in ChR2-expressing cells

It is already well established that light from laser, LED or bandpass filtered mercury lamps can reliably trigger spike activities in ChR2-expressing cells, as long as their wavelength(s) lies within the range of the activation spectrum[1]. Here we made optical response recordings in both cortical neurons and RGCs with ChR2 expression. Cortical neurons with regular spiking patterns (data not shown) were identified through intracellular recordings, indicating that it was a pyramidal neuron. Fig. 4A showed that laser light was focused onto the soma of the recorded neuron. 5 msec stimulation triggered single spike activities with 91.6% firing fidelity (Fig. 4B). Repetitive 5 msec stimulations also triggered spike responses in RGCs (Fig. 4C). These experiments confirmed that optical stimulations can precisely manipulate activities of ChR2-expressing cells.

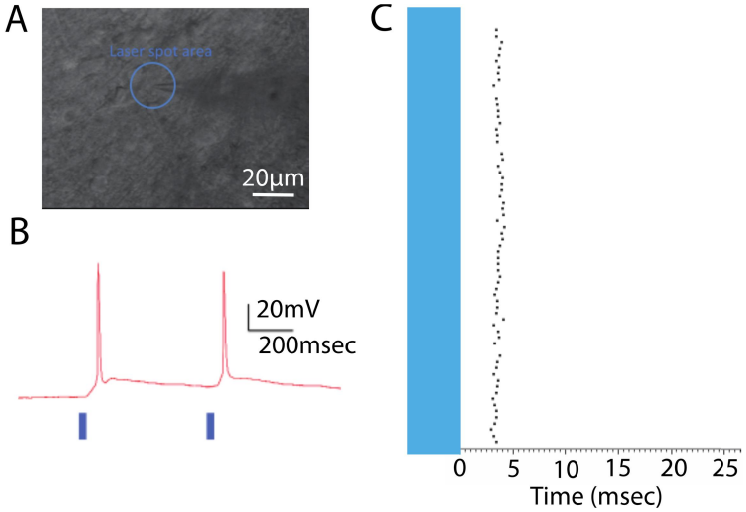

Figure 4. Responses of ChR2-expressing cells *in vitro*. (A) Intracellular recording of a cortical neuron. Blue circle represents the area of laser spot. (B) Sample traces for the spike responses of the cortical neuron to 5 msec stimulation. (C) Raster plot for the spike responses of a RGC to 5 msec stimulation.

3.3 Chronic stimulation induces decrease in target zone size

Axons of neighboring RGCs project to corresponding neighboring cells in the SC, forming terminal zones of the retinal projections. The Ephrin family and other axon guidance molecules lay out the topographical positions of the terminal zones, whereas activities of RGCs participate in the refinement of the terminal zone sizes [6]. Genetic silencing of RGC activities or blocking of neurotransmitter release regulates RGC axon growth in the tectum in zebrafish, though the exact competition effect is not clear yet [25, 26]. As in Fig. 3, we electroporated retinas with both ChR2 and tdtomato plasmids for RGC activity control and axon arbor labeling/visualization in the SC. Fig. 5 showed that after chronic stimulation, the size of the target zones for ChR2-expressing RGCs was statistically smaller than that of control (with tdtomato electroporation only). This result indicated that axon arbor territory of RGCs decreases with increasing amount of activities. Since disruption of retinal activities resulted in the increase of terminal zone areas, the smaller terminal zones in the chronically stimulated mice might lead to increased visual acuity. The number of GFP-expressing cells in Thy1-ChR2 mice did not change before and after stimulation (Fig. 6), indicating that that chronic stimulation did not cause damage to the retinal cells.

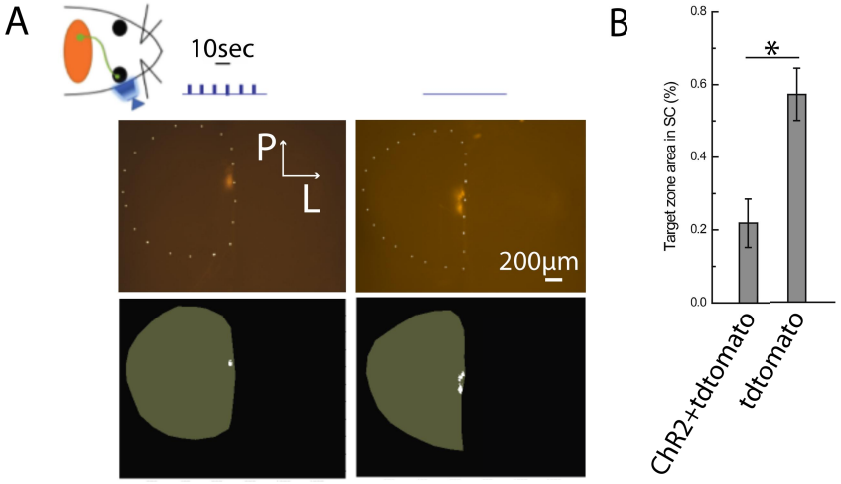

Figure 5. Chronic stimulation of ChR2-expressing RGCs in ChR2-Venus and tdtomato electroporated mice induced target zone shrinkage. (A) Top row showed schematic of stimulation protocols for electroporated mice. Middle and bottom rows showed RGC projections in the whole mount of SC. The white dots represented outlines of one hemisphere of the SCs. P: posterior; L: lateral. (B) Average sizes of the target zone area in the SC for chronically stimulated mice with electroporation of ChR2-Venus + tdtomato (n = 5) and tdtomato only (n = 4). *P<0.05.

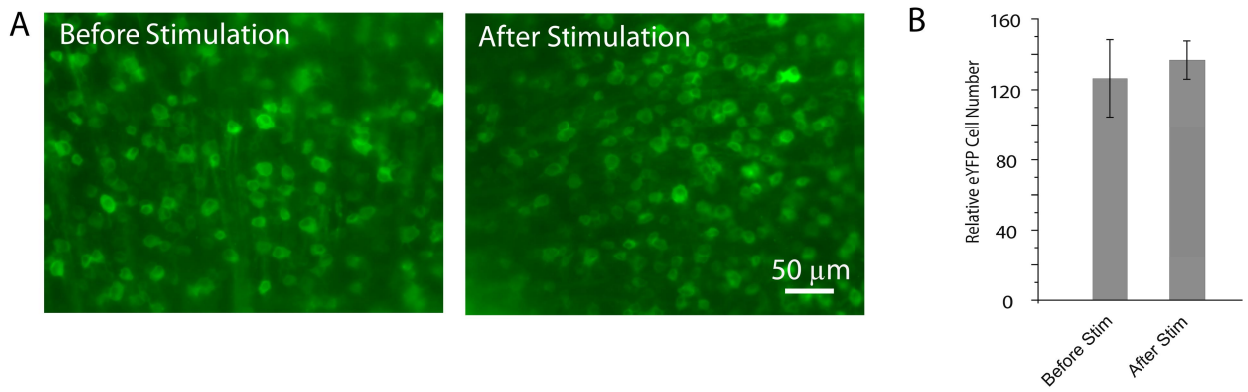

Figure 6 RGC cell number didn't change after the chronic in vivo stimulation. (A) Fluorescence images for eYFP labeled ChR2 expressing RGCs before and after chronic stimulation in Thy1-ChR2 mice. (B) eYFP labeled RGC cell densities were not affected by the chronic stimulation (126 ± 21before stimulation, n = 5; 136 ± 11 after stimulation, n = 4).

4. CONCLUSIONS

Neural circuits in neonatal mice develop through a complex process that is regulated by many factors including genetic background and environmental modifications. Neural activities play important roles in neural circuit development, in the form of both spontaneous patterned activities that are programmed genetically [27] and environmentally triggered sensory responses. Studies of whether and how neural activity and experience regulate the developmental neurogenesis process are under investigation. Neural activity and experience can also affect the activation and self-renewal mode of adult neural stem cells through a local circuit mechanism[28]. In these studies, being able to manipulate the activity of specific cell types is a crucial step for understanding the mechanisms of neural circuit development, plasticity and possibly regeneration.

Here, we summarized the optogenetic tools suitable for the study of visual map development in neonatal mice. These tools are readily available with minimal requirements for modification, and can be easily applied to other sensory systems. For more advanced applications in neural circuits such as development of functional networks, integration of more advanced equipment such as random-access mapping of laser stimulation and two-photon imaging systems are necessary.

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REFERENCES

[1] Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G., & Deisseroth, K., Millisecond-timescale, genetically targeted optical control of neural activity. *Nat Neurosci* 8, 1263-8 (2005).

[2] Zhang, F., Prigge, M., Beyrière, F., Tsunoda, S. P., Mattis, J., Yizhar, O., Hegemann, P., & Deisseroth, K., Red-shifted optogenetic excitation: a tool for fast neural control derived from Volvox carteri. *Nat Neurosci* 11, 631-3 (2008).

[3] Aravanis, A. M., Wang, L.-P., Zhang, F., Meltzer, L. A., Mogri, M. Z., Schneider, M. B., & Deisseroth, K., An optical neural interface: in vivo control of rodent motor cortex with integrated fiberoptic and optogenetic technology. *J Neural Eng* 4, S143-56 (2007).
[4] Huber, D., Petreanu, L., Ghitani, N., Ranade, S., Hromádka, T., Mainen, Z., & Svoboda, K., Sparse optical microstimulation in barrel cortex drives learned behaviour in freely moving mice. *Nature* 451, 61-4 (2008).
[5] Hubel, D. H. & Wiesel, T. N., Receptive Fields of Cells in Striate Cortex of Very Young, Visually Inexperienced Kittens. *J Neurophysiol* 26, 994-1002 (1963).
[6] Huberman, A. D., Feller, M. B., & Chapman, B., Mechanisms underlying development of visual maps and receptive fields. *Annu Rev Neurosci* 31, 479-509 (2008).
[7] Galli, L. & Maffei, L., Spontaneous impulse activity of rat retinal ganglion cells in prenatal life. *Science* 242, 90-1 (1988).
[8] Wong, R. O., Meister, M., & Shatz, C. J., Transient period of correlated bursting activity during development of the mammalian retina. *Neuron* 11, 923-38 (1993).
[9] McLaughlin, T., Torborg, C. L., Feller, M. B., & O'Leary, D. D. M., Retinotopic map refinement requires spontaneous retinal waves during a brief critical period of development. *Neuron* 40, 1147-60 (2003).
[10] Chandrasekaran, A. R., Plas, D. T., Gonzalez, E., & Crair, M. C., Evidence for an instructive role of retinal activity in retinotopic map refinement in the superior colliculus of the mouse. *J Neurosci* 25, 6929-38 (2005).
[11] Weliky, M. & Katz, L. C., Disruption of orientation tuning in visual cortex by artificially correlated neuronal activity. *Nature* 386, 680-5 (1997).
[12] Dhande, O. S., Hua, E. W., Guh, E., Yeh, J., Bhatt, S., Zhang, Y., Ruthazer, E. S., Feller, M. B., & Crair, M. C., Development of single retinofugal axon arbors in normal and β2 knock-out mice. *J Neurosci* 31, 3384-99 (2011).
[13] Xu, H.-p., Furman, M., Mineur, Y. S., Chen, H., King, S. L., Zenisek, D., Zhou, Z. J., Butts, D. A., Tian, N., Picciotto, M. R., & Crair, M. C., An instructive role for patterned spontaneous retinal activity in mouse visual map development. *Neuron* 70, 1115-27 (2011).
[14] Kim, I.-J., Zhang, Y., Meister, M., & Sanes, J. R., Laminar restriction of retinal ganglion cell dendrites and axons: subtype-specific developmental patterns revealed with transgenic markers. *J Neurosci* 30, 1452-62 (2010).
[15] Zhao, S., Ting, J. T., Atallah, H. E., Qiu, L., Tan, J., Gloss, B., Augustine, G. J., Deisseroth, K., Luo, M., Graybiel, A. M., & Feng, G., Cell type--specific channelrhodopsin-2 transgenic mice for optogenetic dissection of neural circuitry function. *Nat Methods* 8, 745-52 (2011).
[16] Madisen, L., Mao, T., Koch, H., Zhuo, J.-m., Berenyi, A., Fujisawa, S., Hsu, Y.-W. A., Garcia, 3rd, A. J., Gu, X., Zanella, S., Kidney, J., Gu, H., Mao, Y., Hooks, B. M., Boyden, E. S., Buzsáki, G., Ramirez, J. M., Jones, A. R., Svoboda, K., Han, X., Turner, E. E., & Zeng, H., A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. *Nat Neurosci* 15, 793-802 (2012).
[17] Bi, A., Cui, J., Ma, Y.-P., Olshevskaya, E., Pu, M., Dizhoor, A. M., & Pan, Z.-H., Ectopic expression of a microbial-type rhodopsin restores visual responses in mice with photoreceptor degeneration. *Neuron* 50, 23-33 (2006).
[18] Grieger, J. C. & Samulski, R. J., Adeno-associated virus vectorology, manufacturing, and clinical applications. *Methods Enzymol* 507, 229-54 (2012).
[19] Zheng, J., Lee, S., & Zhou, Z. J., A transient network of intrinsically bursting starburst cells

underlies the generation of retinal waves. *Nat Neurosci* 9, 363-71 (2006).
[20] Jordan, T., Hanson, I., Zaletayev, D., Hodgson, S., Prosser, J., Seawright, A., Hastie, N., & van Heyningen, V., The human PAX6 gene is mutated in two patients with aniridia. *Nat Genet* 1, 328-32 (1992).
[21] Siegert, S., Cabuy, E., Scherf, B. G., Kohler, H., Panda, S., Le, Y.-Z., Fehling, H. J., Gaidatzis, D., Stadler, M. B., & Roska, B., Transcriptional code and disease map for adult retinal cell types. *Nat Neurosci* 15, 487-95, S1-2 (2012).
[22] Han, W., Kwan, K. Y., Shim, S., Lam, M. M. S., Shin, Y., Xu, X., Zhu, Y., Li, M., & Sestan, N., TBR1 directly represses Fezf2 to control the laminar origin and development of the corticospinal tract. *Proc Natl Acad Sci U S A* 108, 3041-6 (2011).
[23] Matsuda, T. & Cepko, C. L., Electroporation and RNA interference in the rodent retina in vivo and in vitro. *Proc Natl Acad Sci U S A* 101, 16-22 (2004).
[24] Matsuda, T. & Cepko, C. L., Controlled expression of transgenes introduced by in vivo electroporation. *Proc Natl Acad Sci U S A* 104, 1027-32 (2007).
[25] Ben Fredj, N., Hammond, S., Otsuna, H., Chien, C.-B., Burrone, J., & Meyer, M. P., Synaptic activity and activity-dependent competition regulates axon arbor maturation, growth arrest, and territory in the retinotectal projection. *J Neurosci* 30, 10939-51 (2010).
[26] Hua, J. Y., Smear, M. C., Baier, H., & Smith, S. J., Regulation of axon growth in vivo by activity-based competition. *Nature* 434, 1022-6 (2005).
[27] Blankenship, A. G. & Feller, M. B., Mechanisms underlying spontaneous patterned activity in developing neural circuits. *Nat Rev Neurosci* 11, 18-29 (2010).
[28] Song, J., Zhong, C., Bonaguidi, M. A., Sun, G. J., Hsu, D., Gu, Y., Meletis, K., Huang, Z. J., Ge, S., Enikolopov, G., Deisseroth, K., Luscher, B., Christian, K. M., Ming, G.-l., & Song, H., Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. *Nature* 489, 150-4 (2012).