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Non-invasive activation of optogenetic actuators

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ABSTRACT

The manipulation of genetically targeted neurons with light (optogenetics) continues to provide unprecedented avenues into studying the function of the mammalian brain. However, potential translation into the clinical arena faces a number of significant hurdles, foremost among them the need for insertion of optical fibers into the brain to deliver light to opsins expressed on neuronal membranes. In order to overcome these hardware-related problems, we have developed an alternative strategy for delivering light to opsins which does not involve fiber implants. Rather, the light is produced by a protein, luciferase, which oxidizes intravenously applied substrate, thereby emitting bioluminescence. In proof-of-principle studies employing a fusion protein of a light-generating luciferase to a light-sensing opsin (luminopsin), we showed that light emitted by *Gaussia* luciferase is indeed able to activate channelrhodopsin, allowing modulation of neuronal activity when expressed in cultured neurons. Here we assessed applicability of the concept *in vivo* in mice expressing luminopsins from viral vectors and from genetically engineered transgenes. The experiments demonstrate that intravenously applied substrate reaches neurons in the brain, causing the luciferase to produce bioluminescence which can be imaged *in vivo*, and that activation of channelrhodopsin by bioluminescence is sufficient to affect behavior. Further developments of such technology based on combining optogenetics with bioluminescence - i.e. combining light-sensing molecules with biologically produced light through luciferases - should bring optogenetics closer to clinical applications.

Keywords: bioluminescence, *Gaussia* luciferase, coelenterazine, channelrhodopsin, viral vectors, *in vivo*, imaging, transgenic mice

1. INTRODUCTION

Manipulating the activity of defined neuronal populations in the living organism is critical for understanding of how neural circuits process information, and, ultimately, for applying efficient therapies to numerous neurological and psychiatric disorders. The past several years have shown an exciting development of tools for probing neural circuits *in vivo* with light (optogenetics) or with chemical compounds (chemical genetics)¹. These approaches make use of one genetically targeted component, typically a receptor or ion channel, and one externally administered component, such as a small molecule or light. In chemical genetic approaches, for example DREADDs, activation is mediated by a diffusible molecule that can be delivered by non-invasive systemic application. DREADDs are G-protein coupled receptors and thus pose restrictions on the neuronal populations that can be manipulated as well as on the time scales of manipulation². Optogenetic actuators are attractive as they are independent of second messenger systems and thus can be employed more generally. In addition, the optogenetic principle is applicable not only to opsins (channels and pumps, such as channelrhodopsins³⁻⁵, halorhodopsins⁶⁻⁸ or archaerhodopsin⁹), but to a broader and rapidly expanding arsenal of tools for light-based cellular manipulation, such as plant phytochromes, cryptochromes, and light-oxygen-voltage (LOV) domain proteins for controlling protein localization, signaling pathways, and DNA recombination and transcription¹⁰⁻¹². However, the hardware requirements for light delivery *in vivo*, i.e. chronically implanted fiber optics, poses significant limitations in experimental animals and even more so in potential clinical applications. First, in order to activate the same number of neurons in the human brain as in mice, the optical fibers will have to be scaled up in diameter, which leads to considerable neuronal damage along the path of the fiber. Second, inflammatory reactions to the foreign fiber material, especially during chronic insertion, can cause the tissue to become so opaque that the light cannot even reach

the target neurons. Third, to modulate neuronal activity at a corrective level, more than just a few neurons of the affected pathway may have to be modulated, which would necessitate multi-array grids of fibers, multiplying the problems listed above. Fourth, many neurons are located in brain areas which are simply not accessible to fibers. While optogenetic approaches provide control of neuronal activity with rapid, millisecond time scales¹³⁻¹⁸ and with high spatial precision, comprehensive interrogation of neuronal circuits requires acute as well as chronic manipulations of spatially defined subpopulations as well as entire populations dispersed over the brain. In fact, millisecond-level temporal precision, which is a major benefit of optogenetics, is not necessary in many applications. An example would be the temporary activation or inactivation of a brain structure during behavioral testing or long-term therapy; in such cases, there is no need for instantaneous onset or cessation of the effect.

In order to overcome these hardware-related problems, we are developing an alternative strategy for delivering light to opsins that does not involve fiber implants. Rather, the light required for activation of the optogenetic probe is produced by a protein, luciferase, that oxidizes intravenously applied substrate, thereby emitting bioluminescence. In our earlier proof-of-principle studies we showed that luciferase-emitted light is indeed able to activate opsins¹⁹. In this study we present our initial progress on translating this concept from cultured neurons to behaving animals.

Figure 1. Light sources for opsins. Optogenetic approaches to control of neuronal activity utilize light-activated photosensitive proteins (microbial opsins; here: channelrhodopsin, ChR), with a fluorescent protein fused to the C-terminus of ChR to allow visualization by fluorescence microscopy (here: yellow fluorescent protein, YFP). The opsin is activated by applying light via fiber optics from an external light source. Fusion of a luciferase (here: *Gaussia* luciferase, GLuc) to the N-terminus of ChR creates a luminescent opsin, or luminopsin. Application of the GLuc substrate coelenterazine (CTZ) leads to an enzymatic reaction resulting in light (photon) production and opening of the channel. Utilizing this “biological” light source allows non-invasive activation of the opsin.

2. MATERIALS AND METHODS

2.1 Constructs

The generation of plasmids LMO1 and LMO2 is described in detail in reference¹⁹. Briefly, the DNA sequence for *Gaussia* luciferase was fused to that of ChR2-EYFP to generate LMO1 and to that of VChR1-EYFP to generate LMO2. The coding sequence for each luminopsin was cloned into a lentiviral vector, derived from the FUGW promoter (ubiquitin, U)-reporter (GFP, G) construct²⁰ by replacing the ubiquitin promoter with the CAG sequence (C) and replacing the fluorescence reporter with the respective LMO, generating FCLMO1W and FCLMO2W. The ROSA26 targeting vector was based on the Ai9 construct²¹, which includes a loxP-stop-loxP sequence for Cre-activation, allowing conditional expression of the respective insert from the strong CAG promoter/enhancer sequence. The fluorescence reporter of Ai9 was replaced by LMO2, generating ROSA26-LMO2.

2.2 Virus

Lentivirus vectors were made by transfecting 6×10^6 293FT cells with 5 μg of the vesicular stomatitis virus glycoprotein (VSVg) envelope encoding plasmid, 15 μg of the delta-8.9 packaging plasmid, and 20 μg of LMO plasmid using Lipofectamine. After 72 h, supernatant was harvested from three 10 cm culture plates, filtered at 0.45 μm , and pelleted by ultracentrifugation at 26,000 rpm for 2 h at 4°C. After resuspension, serially diluted lentivirus was used to transduce 293FT cells; 72 h later, labeled 293FT cells were counted to calculate the viral titer. Lentiviruses with titers ranging from 1×10^7 to 1×10^{10} TU/ml were used in this study.

2.3 Mice

All experimental procedures were conducted in accordance with National Institutes of Health guidelines and reviewed by Duke University's Institutional Animal Care and Use Committee.

For virus injections mice at postnatal day 1 (P1) were anesthetized by hypothermia. Injections were made into the cortex through the translucent skull using a glass pipette attached to a Hamilton syringe. Sham-injected mice received PBS without virus. Mice were returned to their home cage until weaning at P21 – P28. Adult mice between P30 and P45 were used for imaging and behavioral experiments.

For injection of the luciferase substrate, coelenterazine (CTZ), mice were briefly anesthetized with isoflurane. CTZ of high purity (NanoLight™ Technology, Pinetop, AZ) was injected into the retroorbital venous plexus, dispensing 50 μl per mouse with a 0.5 ml Insulin syringe, for a final concentration of 200 μM . In *in vivo* imaging experiments, mice were kept under continuous isoflurane anesthesia. In behavioral experiments, after recovery from anesthesia, mice were put in a chamber where they were allowed to move freely under video recordings.

2.4 Histology

Mice were killed by an overdose of isoflurane and transcardially perfused with sodium phosphate buffer (PBS), followed by 4% paraformaldehyde (PFA) in PBS. The brain was removed and postfixed in 4% PFA overnight, followed by 30% sucrose overnight. Brains were sliced into transverse or coronal sections on a freezing microtome at 40–50 μm , directly mounted onto glass slides, and cover-slipped using Fluormount-G (Electron Microscopy Sciences, Hatfield, PA). Wide-field fluorescence microscopy images were acquired with an Olympus BX61 upright microscope using a 40x Olympus objective, a GFP filter cube (U-MWIB, Olympus), a high-resolution cooled CCD camera (CoolSNAP fx, Photometrics, Tucson, AZ) and acquisition software (ISce Imaging Systems, Raleigh, NC).

2.5 Bioluminescence imaging *in vivo*

Immediately after retroorbital injection of CTZ, mice were imaged using a Xenogen IVIS 100 system and Living Image 3.0 software (Caliper Life Sciences). Images were displayed as a pseudo-color photon count image. Where applicable, regions of interest were defined using an automatic intensity contour procedure to identify bioluminescent signals with intensities significantly greater than background. The sum of the photon counts in these regions was then calculated.

2.6 Fluorescence and bioluminescence imaging *ex vivo*

Hippocampal slices were prepared using conventional methods²². In brief, the mice were anesthetized with isoflurane and then decapitated. Their brains were removed and placed in cold artificial cerebrospinal fluid (ACSF), containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 26 NaHCO_3 , 20 D(+)-glucose, 2 CaCl_2 and 1.3 MgCl_2 (pH 7.4 after bubbling with 95 % O_2 / 5 % CO_2 , v/v). A vibratome was used to make 250 μm thick coronal sections. The slices were then incubated at 36 °C for 30 min prior to use.

For fluorescence and bioluminescence imaging of slices, an upright epifluorescence microscope (Eclipse FN-1, Nikon, Melville, NY) was used with a 40x 0.8 NA water immersion objective, a metal-halide arc lamp, an electronic shutter (Uniblitz, Vincent Associates, Rochester, NY), a GFP filter cube (B-2E/C, Nikon), a cooled CCD camera (CoolSNAP fx, Photometrics), and acquisition software (Micro-Manager, www.micro-manager.org) on a PowerMac G5 (Apple, Cupertino, CA). Bioluminescence was imaged without any filter cube with 4 by 4 binning and 5-s exposure. CTZ (100 μ M in ACSF) was superfused to the chamber. For imaging brain slices *ex vivo* in the IVIS system, CTZ was added to the dish containing the slice in ACSF.

3. RESULTS

Bioluminescence from *Gaussia* luciferase has been imaged *in vivo* with transfected tumor cells^{23,24} and stem cells²⁵ injected into the brain, respectively. We wanted to know the level of bioluminescence produced in endogenous cells in the brain. We thus transduced neonatal mice with lentivirus carrying luminopsin-1 (GLuc-ChR2-EYFP). We injected viral preparations into the right hemisphere and imaged bioluminescence in the mice 4 – 5 weeks later (Fig. 2A). As LMOs were tagged with EFYP, we could verify their expression in the cerebral cortex by fluorescence imaging in fixed brain sections (Fig. 2B). *In vivo*, the intensity of the bioluminescence signal generated in response to injection of CTZ was proportional to the amount of cells infected with LMO1 (Fig. 2C). We further quantified the photons emitted by determining the radiance over the regions of interest (ROIs; Fig. 2D). These results demonstrate that intravenously administered CTZ reaches the brain, and that GLuc expressed on neuronal membranes produces bioluminescence which can be imaged and quantified.

Figure 2. *In vivo* imaging of luminopsins. A. Experimental scheme: newborn pups (postnatal day 1) were injected into the right cortex with a glass micropipette, delivering lentivirus containing LMO1 (GLuc-ChR2-EYFP) under control of a CAG promoter. Mice were returned to the nest. After weaning, mice were subjected to *in vivo* bioluminescence imaging (postnatal day 30). B. Localized infection of cortical neurons was verified by fluorescence microscopy of brain sections of injected mice at the end of the experiments. As LMOs are fused to EYFP, expression can be readily visualized. C. Bioluminescence images obtained from four different mice after intravenous injection of CTZ (200 μ M final concentration). Mice were shaved to avoid scattering of light from hair. D. Quantification of photons emitted from the four mice shown in C. Radiance over the regions of interest was calculated.

Using an improved version of luminopsin (LMO2; GLuc-VChR1-EYFP¹⁹), we targeted lentiviral injections to the motor cortex in the right hemisphere in neonatal animals. Upon weaning, injected mice (either virus-injected experimental animals or sham-injected controls) were tested for changes in their behavior in response to intravenous injection of CTZ. Both animals showed exploratory behavior upon introduction to a novel chamber (Fig. 3, left). CTZ injection induced repetitive rotations to the left in LMO2-transduced animals, indicating activation of the contralateral premotor cortex (Fig. 3, right). CTZ injection in sham controls induced no apparent change in behavior.

Figure 3. *In vivo* behavioral effects of luminopsins. Neonatal mice were injected into the right motorcortex with lentivirus containing LMO2 (GLuc-VChR1-EYFP) under control of a CAG promoter. Control animals received injections without virus in the solution. Mice were returned to the nest and tested 4-6 weeks later. For behavioral testing, mice were placed in a rectangular bin (14 in x 11 in) and allowed to move freely. After intravenous injection of CTZ, mice were placed back in the bin and allowed to continue moving. Trajectories of their movements were captured over ~ 15 seconds with a video camera, and traced manually. There was no apparent difference in movements of the control mouse (blue traces) before and after CTZ. However, mice which received LMO2-expressing virus in the right motor cortex (red traces) displayed repetitive left (contralateral) turns.

To generate a versatile mouse model that allows the inducible expression of LMO2 in any cell type for which an appropriate Cre-driver can be obtained, we engineered transgenic mice carrying LMO2 as a conditional knock-in allele in the ROSA26 locus (Fig. 4A). Brain sections from offspring of crosses of these mice with a CaMKII α Cre driver line²⁶ were used for fluorescence and bioluminescence imaging. As expected from expression of CaMKII α in various cell types in the brain, at low magnification LMO2 bioluminescence was observed over much of the brain, with largest signals – reflecting highest levels of expression – observed in the hippocampus and in cortical areas (Fig. 4B). To further verify localized expression of LMO2 in neurons, a hippocampal slice was examined under a fluorescent microscope. EYFP expression was observed in dentate gyrus granule cells of the hippocampus (Fig. 4C and D). Upon application of CTZ, these cells emitted bioluminescence, particularly in mossy fibers in the hilus (Fig. 4E and F). These mice should be suitable for behavioral studies, specifically assessing locomotor behavior and symptoms of behavioral seizure classes.

Figure 4. Conditional LMO2 mouse. A. The sequence of LMO2 (GLuc-VChR1-EYFP, colored boxes) was inserted in place of the original reporter in the Ai9 vector²¹ (gray shapes), creating the LMO2-Rosa targeting vector. A mouse line carrying this allele in the Rosa locus was generated by ES cell targeting and blastocyst injection. Mice carrying this conditional LMO2 allele were subsequently mated to CaMKII α Cre-expressing mice. Offspring were genotyped for the presence of both the LMO2-Rosa allele and the CaMKII α Cre transgene. B. Acute horizontal brain slices from mice both positive for CaMKII α Cre and LMO2 were placed in ACSF in a well of a 24-well plate. Bioluminescence images (pseudo color) overlaid on bright-field images (gray scale) were taken before (upper panel) and after (lower panel) addition of CTZ (100 μ M final concentration). C. Fluorescent image showing CaMKII α -directed expression of LMO2 in dentate gyrus granule cells in the hippocampus. ML: molecular layer; GCL: granule cell layer. The dotted rectangle indicates the region shown in high magnification in D and E. D. Higher magnification image of the same slice. E. Bioluminescent image of the same region shown in D, after addition of CTZ to the chamber. F. Time course of bioluminescence. 50 μ M of CTZ was superfused during the time indicated by the horizontal bar.

4. CONCLUSIONS

Here we present preliminary data on the efficacy of *in vivo* expression and functionality of luminopsins. Mice expressing LMOs in cortical neurons transduced by lentivirus showed bioluminescence emission upon intravenous application of the substrate, CTZ. The signal was robust enough to be imaged through the intact skull and skin. Furthermore, activation of LMO-transduced neurons in the secondary motor cortex by intravenous application of substrate caused behavioral changes in mice. Lastly, mice genetically engineered to conditionally express LMO2 under control of a strong promoter produced bioluminescence in targeted cells.

There are two major challenges to translating optogenetics to clinical applications: (1) placing the optogenetic probe into the target cell population; and (2) delivering a sufficient number of photons to activate the optogenetic probe. Viral delivery of optogenetic actuators currently has the highest potential as a method for gene delivery, with non-viral strategies continuously evolving²⁷. Currently, viral vectors are being tested in clinical trials for gene therapies and will pave the way as potential vehicles for delivering opsins to the nervous system. To have the most efficient activation of opsins, both sensitivity of the opsins to the light and intensity of the light source must be improved. Many laboratories are intensely working to improve the light sensitivity and excitation spectra of opsins²⁸⁻³². However, the greatest challenge in translating optogenetics to clinical use is the generation and delivery of light to the target tissue. This challenge is currently being tackled mainly by engineers designing optogenetic hardware: light production devices that meet the energy production requirements^{33,34}, and light delivery devices, such as fiber optics, multi-fiber arrays, 3D multi wave guide, and miniature LED devices³⁵⁻⁴⁰.

Our concept of using biological light eliminates the requirement for invasive fiber implants. The technology is specifically aimed at long-term, chronic manipulations of neuronal populations; however, the ability of acute and millisecond time scale activation is also maintained in these tools by using conventional optogenetic hardware. We are now increasing the efficiency of luminopsins by employing luciferases with higher light emission and opsins with increased light sensitivity. Further improvements of luminopsins will lower dosage requirement of the substrate and will facilitate clinical use.

Our approach will open new avenues for basic research by overcoming the limitations of implanted fiber optics in animal studies. Moreover, our work lays the foundation for technologies enabling minimally invasive and highly efficient diagnostics and therapies for clinical applications.

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