Optical control of zebrafish behavior with halorhodopsin

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Edited by Lily Y. Jan, University of California, San Francisco, CA, and approved August 19, 2009 (received for review June 8, 2009)

Expression of halorhodopsin (NpHR), a light-driven microbial chloride pump, enables optical control of membrane potential and reversible silencing of targeted neurons. We generated transgenic zebrafish expressing enhanced NpHR under control of the Gal4/UAS system. Electrophysiological recordings showed that eNpHR stimulation effectively suppressed spiking of single neurons in vivo. Applying light through thin optic fibers positioned above the head of a semi-restrained zebrafish larva enabled us to target groups of neurons and to simultaneously test the effect of their silencing on behavior. The photostimulated volume of the zebrafish brain could be marked by subsequent photoconversion of co-expressed Kaede or Dendra. These techniques were used to localize swim command circuitry to a small hindbrain region, just rostral to the *commissura infima Halleri*. The kinetics of the hindbrain-generated swim command was investigated by combined and separate photo-activation of NpHR and Channelrhodopsin-2 (ChR2), a light-gated cation channel, in the same neurons. Together this “optogenetic toolkit” allows loss-of-function and gain-of-function analyses of neural circuitry at high spatial and temporal resolution in a behaving vertebrate.

central pattern generator | channelrhodopsin | *Danio rerio* | reticulospinal

Technology for the inactivation of specific neurons within an otherwise intact circuit promises to accelerate research into the neural basis of behavior (1–8). The light-gated chloride pump halorhodopsin (NpHR) from the archaeobacterium *Natronomonas pharaonis* has recently been introduced into neuroscience along with enhanced derivatives (9–14) and enables superior temporal and spatial control. Other light-controlled silencing methods are being developed (15–17), but require covalent attachment of a photo-switchable affinity label. NpHR silencing has been demonstrated electrophysiologically (10, 12) and has been used to reversibly paralyze *Caenorhabditis elegans* expressing NpHR in motor peripheries (12). Despite its promise, however, NpHR has so far found only limited applications for circuit analysis in vivo. In this study, we have devised a versatile and cost-effective optical stimulation strategy for manipulation of animal behavior with this tool. These advances were made possible by our choice of zebrafish as the experimental system.

Zebrafish are ideal models for testing and applying light-controlled channels and pumps in vertebrates, since they are translucent and display a number of quantifiable behaviors during the first 2 weeks of larval development (18–21). Accordingly, Wyart et al. (22) used a re-engineered, light-gated glutamate receptor (LiGluR), to induce swimming by photostimulation of a rare type of spinal neuron. Douglass et al. (23) succeeded in triggering escape responses by activating ChR2 in single zebrafish mechanosensory cells. The adaptation of the Gal4/UAS method from *Drosophila melanogaster* (24) to zebrafish enables targeting transgene expression to specific brain areas and cell types (25–29) and will further contribute to the refinement of an optogenetic toolkit in this system.

Here we report on the generation of UAS:NpHR transgenic zebrafish lines. Using a Gal4 line that drives NpHR broadly in neurons, we show that enhanced NpHR (eNpHR) is targeted efficiently to the surface of neurons in vivo and mediates light-induced suppression of spikes. We then use a non-invasive fiber

optics approach to stimulate small (ca. 30 μ m) CNS areas, while simultaneously monitoring the fish's behavioral responses. We combine NpHR silencing with ChR2-mediated excitation, to identify a critical role for a small cell group in the caudal hindbrain in the control of forward swimming. The ability to selectively silence neurons in vivo with precise temporal and spatial control is likely to have broad applications for the study of functional neuroanatomy and neuronal plasticity.

Results

Enhanced Halorhodopsin (eNpHR) Is Targeted to the Cell Surface of Zebrafish Neurons In Vivo. Different versions of NpHR have been reported to vary in their intracellular distribution and surface localization. To compare their properties in zebrafish, we generated four transgenic lines, *UAS:NpHR-eYFP*, *UAS:NpHR-mCherry*, *UAS:eNpHR-eYFP*, and *UAS:eNpHR-mCherry*. Transgenic fish were crossed to carriers from the enhancer trap line *Gal4^{sl101}*, which expresses the transcriptional activator Gal4-VP16 broadly in most neurons (29) (Fig. 1A). eNpHR was engineered to have improved surface targeting properties compared to NpHR (10). As expected, eNpHR-eYFP proteins (Fig. 1B) trafficked to the cell surface and did not form the intracellular blebs that were observed for NpHR-eYFP (Fig. S1) and NpHR-mCherry (Fig. 1B).

Two lines (NpHR-mCherry and eNpHR-eYFP) were further investigated for surface localization by co-expressing membrane-targeted fluorophores, Dendra-kras or mCherry-kras, respectively (Fig. 1C). While most of the NpHR-mCherry protein remained intracellular, a fraction co-localized with Dendra-kras at the cell surface. In contrast, virtually all eNpHR-eYFP signal was co-localized with mCherry-kras at the plasma membrane. Membrane targeting was also demonstrated by the crisp and intense labeling of cell morphologies including neurites (Fig. S2 shows radial glial cells in the optic tectum). For unknown reasons, fusion proteins containing the identical opsin but different fluorescent tags were sometimes distributed in apparently different cellular compartments (Fig. S1 and Fig. 1B). Together, eNpHR-eYFP appeared to be superior, as it combined excellent surface localization with effectiveness in suppressing spikes (see below).

Single-Unit Electrophysiology Confirms Silencing in Vivo. To determine whether NpHR stimulation suppressed spiking we performed loose-patch recordings in hindbrain neurons. To activate NpHR, a bandpass filter centered on its activation spectrum maximum (HQ 585/70, Fig. 2A) was used. The neuron in Fig. 2B (top two traces) was silenced during illumination periods, and no spikes were generated. After stimulation, the cell resumed firing at a rate comparable to the average firing rate before stimulation. This experiment suggested that NpHR was an effective and reversible

Author contributions: A.B.A. and H.B. designed research; A.B.A. performed research; F.D.B. contributed new reagents; A.B.A. analyzed data; and A.B.A. and H.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0906252106/DCSupplemental.

Fig. 1. Expression of NpHR in zebrafish. (A) Expression pattern of *Gal4^{5101t}; UAS:Kaede* transgenic animals. Dorsal view (i), horizontal optical slice through the hindbrain (ii) and transverse section through the eyes and midbrain (iii) of 5 dpf animals. (B) Expression of NpHR in *Gal4^{5101t}; UAS:(e)NpHR-XFP* animals. (i and ii) NpHR-mCherry and eNpHR-eYFP, respectively. Intracellular blebs (arrows) are labeled in (i). (C) Surface targeting of NpHR. Co-expression of membrane-bound Dendra-kras and NpHR-mCherry in *Gal4^{51013t}; UAS:Dendra-kras; UAS:NpHR-mCherry* animals (i) reveals suboptimal surface targeting. Co-expression of eNpHR-eYFP and membrane-bound mCherry-kras in *Gal4^{51013t}; UAS:eNpHR-eYFP; UAS:mCherry-kras* animals, shows complete surface targeting of eNpHR-eYFP. [Scale bars, 100 μ m in (A); 5 μ m in (B and C).]

silencer of neuronal activity in larval zebrafish. Conversely, the activation of the light-gated cation channel ChR2 (ChR2-H134R) in *Gal4^{sl101t}; UAS:ChR2-eYFP* animals induced firing rates up to 130 Hz for many seconds (Fig. 2*B* bottom trace).

We next assessed the magnitude of the silencing effect across the population of recorded hindbrain neurons. NpHR expressing cells had much lower firing rates during illumination (F2) than without illumination (F1; Fig. 2C). For quantification, the distributions of firing rate ratios (F2/F1) of NpHR expressing cells and NpHR non-expressing cells (wild-type, Fig. 2D and Fig. S3) were compared. The silencing effect in NpHR-expressing cells was highly significant ($P < 0.0001$ for both eNpHR-eYFP and NpHR-mCherry, Ranksum and KS test). Furthermore, the median firing rate ratio (F2/F1) was 0.2 for both eNpHR-eYFP and NpHR-mCherry (see Fig. S4 for the light intensity dependence of the effect). This means that NpHR photostimulation suppressed, on average, 80% of all spontaneous spikes. A fraction of cells ($\approx 15\%$) were not significantly inactivated; very few even increased their spike rate (permutation test with $\alpha = 0.01$, Fig. S5).

In control experiments with wildtype cells, we noted that illumination had a small effect on firing rate in 26% (8/31) of the cells (permutation test, $P < 0.01$). These light responses could be due to synaptic input from the visual system. We therefore recorded from blind *lakritz/atox7* mutant fish, in which retinal ganglion cells do not form (30), and found that the fraction of cells reproducibly responsive to light decreased to 14% (3/23; Fig. 2D). Since the hindbrain of *lakritz/atox7* mutants receives no input from the retina, the significant firing rate change in the few light-responsive cells could stem from inputs from the pineal organ, from intrinsic photosen-

sitivity of the recorded cells, or from a thermal effect of the illumination.

Swimming Behavior Is Inhibited by NpHR Stimulation and Triggered by Rebound from Inhibition. The *Gal4^{UAS101}* transgene drives *NpHR-mCherry* broadly in CNS neurons. We therefore expected an extensive and almost instantaneous inhibition of movements upon global activation of NpHR in the entire animal. Indeed, when a dish containing zebrafish larvae was illuminated with a high-power LED (627 nm), larvae frequently stopped moving and lost coordination ([Movie S1](#) for NpHR-expressing animals and [Movie S2](#) for their non-expressing siblings). Strikingly, the offset of illumination triggered a single bout of forward swimming (swim scoot) synchronized across the population ([Movie S1](#)). A similar response could be elicited in agarose-embedded larvae whose tails were left free to move (Fig. 3A and [Movie S3](#)). The frequency and amplitude ($\approx 55^\circ$) of the induced tail movement were in the range of those previously reported for routine swim scoots (31).

Since the forward swims were induced by switching the illumination off, it seemed likely that cells rebounding from inhibition triggered the behavior. Indeed, a heightened firing rate (up to 10-fold) in the first second after light offset was observed in the majority (60%) of the recorded hindbrain cells (Fig. 3B; the remaining 40% of cells did not show detectable changes). While we did not attempt to relate the neuronal rebound kinetics to the onset of the behavioral response in the same animal, recordings similar to those in Fig. 2 showed that rebound spiking could start within tens of milliseconds after light offset, which is in agreement with the fast NpHR kinetics (12) ($\tau_{\text{OFF}} = 40$ ms). The forward swimming could last up to 5 s, unless the illumination was turned back on, which again blocked all swimming movements (see below; [Movie S3](#)).

Fiber Optics Enables Targeted, High-Resolution Silencing of Neuronal Activity. To investigate which neural structure was triggering the rebound swim behavior, we decided to map its origin in the zebrafish CNS using fiber optics, a method we termed “optogenetic scanning.” First, we needed to determine the spatial resolution achievable with this method. For local activation of NpHR we used multimode optical fibers that were coupled to lasers. To verify that the light was confined to a small cone exiting the fiber we moved the fiber (10 or 50 μm in diameter) across a recorded cell and measured the firing rate ratio F2/F1 for each position (Fig. 3*D* and *E*). In three of eight cells recorded in this manner, silencing only occurred when they were directly illuminated. (In the remaining five cells, firing rates were influenced by illuminating neighboring positions away from the cell bodies, possibly due to local interconnectivity.) Aiming the optic fiber to a position just 30 μm away from the tip of the recording pipette had no influence on the firing rate of the cell in Fig. 3*E*. This observation suggests that non-invasive fiber optics used for silencing has a spatial resolution of 30 μm or better in vivo.

To determine the approximate penetration depth and the extent of light scatter within the tissue as the stimulus light passes through the tissue, we used a photoconvertible fluorescent protein. Fish carrying both *UAS:Dendra-kras* and *Gal4^{ts1/01t}* expressed the membrane-targeted Dendra protein in most neurons. These larvae were embedded in agarose and illuminated with an optic fiber (50 μm) coupled to a blue laser for several minutes. Selective-plane illumination microscopy (32) (SPIM) was used to image the distribution of green-to-red photoconverted Dendra in the hindbrain from two orthogonal directions (Fig. S6). As expected, red cells formed a narrow column whose width was 50 μm at the surface and widened slightly with increasing depth. The divergence angle of the cone of converted cells matched the theoretical value for low numerical-aperture optic fibers (12°, NA = 0.22). This experiment shows that blue light emitted from thin optic fibers penetrates the entire depth of the zebrafish brain with little scattering.

Fig. 6. NpHR and ChR2 can be combined and activated separately. (A) Animals transgenic for *Gal4^{S1101t}; UAS:NpHR-mCherry; UAS:ChR2-eYFP* were illuminated with red or blue light, or both. Illumination with blue light induced locomotion (i), which could be blocked when red light was added (ii). When red light was followed by blue and red light, no locomotion was induced (iii). In (iv), blue light evoked locomotion was blocked with red light three consecutive times. (B) Experiments in (A) were highly reproducible. The probability of locomotion is plotted for four different genotypes (divided by vertical lines) and four different stimulation protocols. Error bars are 95% confidence intervals for binomial distributions (Wilson score, $n = 238$ trials in total, $n \geq 2$ animals for each genotype). For triple transgenic animals, the blue-evoked locomotion probability was significantly different between trials with (A iii) and without (A i) red illumination ($P < 0.0004$, z-test for proportions). The inset shows the activation spectra of ChR2 and NpHR (after ref. 12) and the laser lines for ChR2 (488 nm) and NpHR (633 nm).

response; in rare cases, the firing rate ratio ($F2/F1$) may even be >1 if overall reduction of the cell's inputs leads to a net increase in excitation. We confirmed this intuition by modeling the silencing of a random network of neurons receiving external inputs of varying magnitude (Fig. S9). Global hyperpolarization of all network neurons produced variable changes in firing rates, suggesting that intrinsic network properties alone should produce a broad range of responses.

A requirement for the dissection of behavioral circuitry with NpHR is the spatial restriction of activated NpHR. This can in principle be achieved by genetic targeting (e.g., using cell-type specific Gal4 lines) (25, 27–29) in combination with global illumination. However, Gal4 expression patterns are rarely limited to one defined cell population. Moreover, global illumination inadvertently activates the visual and other photosensitive systems and influences behavior. We therefore relied on optical targeting to restrict the light-activated volume. This was done initially in a broadly expressing line, but could be combined in the future with specific Gal4 drivers to accomplish additional levels of spatial control. Our approach is particularly useful for behavioral studies, because, (i) the position of the light beam can be varied independently from the imaging region; (ii) optic fibers are inexpensive and provide great flexibility in behavioral setups; (iii) light application can be controlled and synchronized with simple electronics; and (iv)

the size of the brain region that is stimulated can be large or small depending on the fiber diameter (10 to 1,500 μm). Notably, alternative light application methods with superior spatial resolution (43) exist, for example, digital micromirror devices (44) and laser-scanning units (45) and will be used to expand this approach further.

We used Kaede and Dendra to mark the photostimulated cells and estimate light scatter and penetration depth. Light delivered by the optic fiber photoconverted a narrow column, whose diameter increased with depth and extended several 100 μm into the brain tissue. The divergence angle of the cone of converted cells matched the theoretical value for low numerical-aperture optic fibers (12° , $\text{NA} = 0.22$). Thus, light scatter appears to be negligible. One caveat is that the photoconverted volume is not identical to the photo-stimulated volume, because Kaede and NpHR require different wavelengths and intensities. The radial spread of light application, however, will be slightly larger for Kaede than for NpHR, because scattering is stronger for shorter wavelengths, for example, by a factor of 5 for 405 compared to 633 nm (Mie theory of scattering). We calculated that the divergence angle difference is small ($<1\%$ for 405 compared to 633 nm). The photoconverted volume is therefore expected to provide a very close, upper-bound estimate for the radial extent of photostimulation.

To demonstrate the utility of this toolkit for functional neuro-anatomy, we used an unbiased “optogenetic scanning” approach. A thin optic fiber was positioned above the head of a semirestrained animal, expressing NpHR in almost all neurons. By moving the fiber across the surface of the brain, we photostimulated various regions, while simultaneously monitoring the animal's behavior. Using this method, we identified a small region in the caudal hindbrain, just rostral to the *commissura infima Halleri*, that initiates a locomotor command when released from inhibition. The swim-inducing region identified by this approach contains bilaterally symmetric groups of neurons called IC, CC, and T that project axons into the spinal cord (38, 39). Because photostimulation of the region immediately rostral to the maximally responsive position showed no effect, we consider it extremely unlikely that the behavior was triggered by activity of *en passant* axons originating from more rostrally positioned cells. Identified reticulospinal cells have been assigned functions in specific behaviors, including escape, turning, and pursuit of prey (46–51). Here we add to this emerging map of behavioral functions in the zebrafish reticular formation a role for caudal cells in the control of forward swimming. The observation that activity in the brain region containing these neurons is necessary and sufficient for swim scoots makes them prime candidates for locomotor command neurons.

We made use of the temporal resolution of NpHR and ChR2 to study the kinetics of rebound-induced swimming. NpHR-assisted “spinalization” of animals in a reversible manner showed that CPGs in the spinal cord are under tight control of descending projections. After light offset, swimming starts with a delay of a little less than 300 ms (267 ms in the animal shown in Results). This latency is the composite of several processes that occur in the hindbrain, spinal cord, and muscle. In the hindbrain, NpHR needs to cease its chloride pump activity, the neurons need to rebound from hyperpolarization and generate spikes, and enough neurons need to be recruited to form a “swim command.” To determine the sum of all activities localized in the hindbrain, we turned the light off and then back on after various intervals, while monitoring the animal's behavior. We found that up to a certain interval length (190 ms in the tested animal), the swim command appeared to be completely dependent on hindbrain activity; i.e., the fish never started swimming when the light was turned back on within 190 ms. After this time, for intervals of 190–300 ms, enough activity appeared to be building up to drive the CPGs, that is, the fish swam. Longer intervals (>300 ms) did not lead to more vigorous swimming. This suggests that the rebounding swim command builds up over roughly 300 ms and for the first 200 ms is completely hindbrain-dependent.