Report

Escape Behavior Elicited by Single, Channelrhodopsin-2-Evoked Spikes in Zebrafish Somatosensory Neurons

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Summary

Somatosensory neurons in teleosts and amphibians are sensitive to thermal, mechanical, or nociceptive stimuli [1, 2]. The two main types of such cells in zebrafish—Rohon-Beard and trigeminal neurons—have served as models for neural development [3–6], but little is known about how they encode tactile stimuli. The hindbrain networks that transduce somatosensory stimuli into a motor output encode information by using very few spikes in a small number of cells [7], but it is unclear whether activity in the primary receptor neurons is similarly efficient. To address this question, we manipulated the activity of zebrafish neurons with the light-activated cation channel, Channelrhodopsin-2 (ChR2) [8, 9]. We found that photoactivation of ChR2 in genetically defined populations of somatosensory neurons triggered escape behaviors in 24-hr-old zebrafish. Electrophysiological recordings from ChR2-positive trigeminal neurons in intact fish revealed that these cells have extremely low rates of spontaneous activity and can be induced to fire by brief pulses of blue light. Using this technique, we find that even a single action potential in a single sensory neuron was at times sufficient to evoke an escape behavior. These results establish ChR2 as a powerful tool for the manipulation of neural activity in zebrafish and reveal a degree of efficiency in coding that has not been found in primary sensory neurons.

Results

Photoactivation of ChR2 in Touch-Sensitive Neurons Triggers Escape Behaviors

We expressed Channelrhodopsin-2 (ChR2) fused to enhanced yellow fluorescent protein (EYFP) in a subset of Rohon-Beard

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and trigeminal neurons by using an enhancer element from the *isl1* gene [10]. Because the fluorescence signal was relatively weak, a *UAS::EGFP* construct was used to enhance visualization of expressing cells (Figures 1A and 1B). The majority of neurons appeared to develop normally and by 24 hr postfertilization showed robust elaboration of sensory axons throughout the skin (Figure 1B).

Between 24 and 40 hr postfertilization (hpf), embryos were assayed for ChR2 responsiveness under a standard dissecting microscope with whole-field light pulses delivered by shuttering of the fluorescence-illumination source. A majority of ChR2-expressing fish exhibited robust behavioral responses to blue light (79% ± 4%, mean ± standard error of the mean [SEM]; n = 3 clutches of 29–79 fish) that appeared to mimic natural escape responses driven by tactile and other stimuli (Figure 1D; Movie S1 available online). Behavioral responses occurred with a latency of less than 30 ms, which is similar to that occurring after tactile stimulation [11, 12] (Movie S2), and showed kinematics similar to touch-evoked escapes (Figure 1C). Remarkably, the direction of tail movement was usually observed to be constant for a given animal across several repetitions, suggesting that small imbalances in the lateral distribution of ChR2-positive cells were sufficient to weight the response in a particular direction. The response to blue light began to disappear by 48 hpf and was never detected later than 72 hpf, correlating with an overall decline in ChR2 levels generated by the transient expression system.

Three experimental lines of evidence indicate that the observed behaviors were driven directly by ChR2-expressing sensory neurons. First, embryos expressing only EGFP in the same neurons failed to show behavioral responses to blue-light illumination (Figure 1D; 1% ± 1%, mean ± SEM; n = 2 clutches of 20–46 fish). Second, the success of eliciting escape behaviors was highly dependent on the wavelength of the illuminating light, with 550 nm light being far less efficient at evoking escapes than 488 nm light (near the peak of the ChR2 excitation spectrum) and 630 nm light being completely ineffective (data not shown). Third, coinjection of a *neurogenin-1* antisense morpholino oligonucleotide, which has been shown to eliminate Rohon-Beard and trigeminal neurons [13, 14], drastically reduced the number of ChR2-expressing cells and abolished the behavioral response to light (*Ngn-1* knockdown: 2.7% ± 1.3% of embryos responded; ChR2-only control: 32% ± 10%. Values are mean ± SEM; n = 3 clutches of 35–84 fish per each condition).

Electrophysiological Characterization of ChR2-Evoked Activity in Trigeminal Neurons

We next determined whether ChR2 has the same electrophysiological properties in fish as in mammalian cells. These experiments also provided the first characterization of the basal-firing activity of trigeminal neurons in larval zebrafish. Extracellular recordings were made from ChR2-expressing trigeminal neurons in intact, paralyzed embryos (Figure 2A). The basal level of spontaneous activity in these neurons was found to be virtually zero—only a single, spontaneous firing event was observed over several hours of recording time in twelve cells (Figure 2C and data not shown). Probability and timing
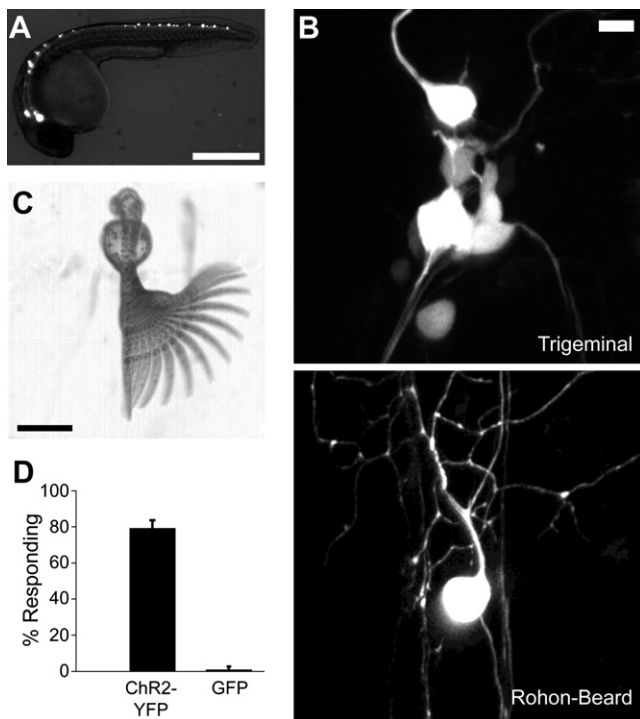
Figure 1. Photoactivation of ChR2 in Zebrafish Somatosensory Neurons Triggers Escape Behaviors

(A) Lateral view of a 24 hpf embryo expressing ChR2-YFP and EGFP in touch-sensitive Rohon-Beard and trigeminal neurons. Anterior is at left, and dorsal is at top. The scale bar represents 100 μm.

(B) Maximum-intensity Z projections of two-photon stacks showing ChR2-YFP and EGFP expression in trigeminal (top panel) and Rohon-Beard (bottom panel) neurons at 24 hpf. The scale bar represents 10 μm.

(C) Time-series projection of a ChR2-YFP-expressing embryo performing an escape in response to illumination at 488 nm. Images were acquired at 500 fps. The scale bar represents 100 μm.

(D) Percentage of experimental (Isl1::Gal4-VP16::UAS-E1b::ChR2-YFP, UAS::GFP) and control (Isl1::Gal4-VP16, UAS::GFP) embryos showing light-evoked escape behaviors. Data are mean ± SEM across three (ChR2) and two (control) clutches of 50–100 injected embryos. Experimental: 79% ± 4%, n = 149 embryos; control: 1% ± 1%, n = 66 embryos.

of light-evoked responses were strongly dependent on both pulse duration and frequency. Pulses of long duration typically evoked trains of action potentials (Figure 2C), with an eventual attenuation starting 50–100 ms after onset of constant illumination. The average spike latency of 20 ± 3 ms (mean ± SEM; n = 9 cells) was longer than that reported in various mammalian cell preparations [9, 15–17], but in some cells, spikes occurred rapidly after pulse onset (Figure 2B). At high stimulation frequencies, spike latency increased over successive repetitions (Figure 2D) whereas spike probability decreased (Figures 2D and 2E). The relationship between spike failure and frequency varied substantially from cell to cell (Figure 2E), but in some cells, reliable responses were observed at up to 10 Hz. Similarly, the dependence of spiking probability on pulse duration at 0.2 Hz was also variable between cells (Figure 2F).

Single Spikes in Single Trigeminal Neurons Can Drive Escape Behaviors
We observed several animals with very low numbers of ChR2-expressing cells that still showed light-evoked escape

behaviors, suggesting that the hindbrain networks downstream of these neurons require very little input to drive a behavioral response. We tested the limit of this requirement by restricting the stimulation to individual neurons. To that end, the field aperture of the fluorescence-illumination port of an upright Olympus BX50WI was closed down to illuminate small regions containing only a single Rohon-Beard or trigeminal neuron (Figure 3A), and local muscle contractions were monitored visually (Movie S3). In 33% of these cells, 100 ms light pulses triggered muscle contractions (n = 33 cells in five fish), which were verified to be a reliable indication of escape behavior with a second low-magnification objective custom mounted in inverse configuration (Movie S4). To rule out the possibility that scattered light or neighboring cells sending processes into the vicinity of the target cell were involved in the behavior, we moved behaviorally responsive regions into and out of the illumination spot along the anterior-posterior axis of the embryo in 20–40 μm steps (Figure 3B). Responses were only observed with the target-cell soma in the illumination spot (Figures 3B and 3C), which demonstrates that single touch-sensitive neurons can drive a full escape behavior.

We also examined the influence of pulse duration—and, indirectly, spike number—on the ability of these cells to elicit a behavior. The dependence of escape probability on pulse length varied greatly between cells, but some neurons (n = 7; 64% of cells that drove a behavior in isolation) were found to drive behaviors with 2–10 ms pulses (Figure 3D). Light pulses of such short duration could be remarkably reliable in triggering an action potential (Figure 3F), but they never elicited more than one spike (n = 9 cells, ∼500 pulses, Figure 3E). This demonstrates that even a single spike in one cell can drive a pronounced escape behavior. This was confirmed in a single cell recorded from a fish that was not paralyzed (Figure S1). These results suggest that zebrafish somatosensory neurons use an extremely high signal-to-noise ratio, derived in part from an extremely low level of spontaneous firing, to efficiently couple small tactile inputs to a robust escape response.

To determine whether actual mechanical stimuli can be encoded with single action potentials, we also monitored trigeminal neuron activity in response to pulses of water directed at the head (Figure S2). In four out of five cells, stimulus intensities were found that reliably elicited either zero or one spike when applied at 0.2 Hz (Figures S2A and S2B). Increasing the stimulus intensity resulted in larger numbers of spikes (Figure S2A). The behavioral response we observed to single, ChR2-evoked spikes thus falls within the normal range of coding properties in this system. Interestingly, we also observed a higher incidence of response failures at higher stimulus frequencies (Figure 2C), just as with ChR2 stimulation, suggesting that these shortcomings of our photoactivation strategy may be imposed by the intrinsic properties of the cells.

Discussion

Studying Zebrafish Behavior and Neurophysiology with ChR2
ChR2-based photoactivation strategies have been implemented in a variety of model systems, including Drosophila [18, 19], C. elegans [15, 20], and mammals [9, 15–17]. The only photoactivation method that had previously been used in the zebrafish—involving a receptor-tethered glutamate mimic [21]—is a promising strategy but gave behavioral results that were not predicted from its expression pattern. Our data

A

B

50 pA

5 ms

C

250 pA
10 ms

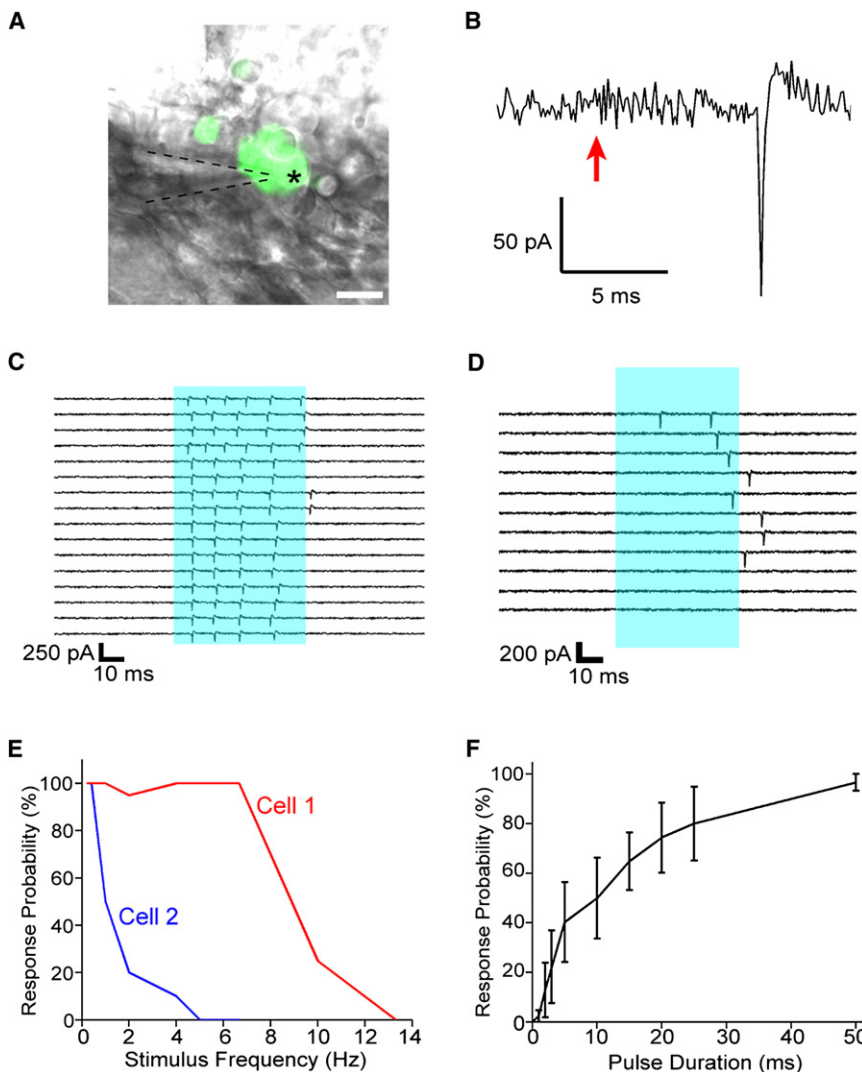**D**

200 pA
10 ms

E

F

Figure 2. Extracellular Recording of ChR2-Evoked Spiking Activity in Trigeminal Neurons

(A) Loose-patch recordings were made by targeting GFP-positive cells (asterisk) with a glass microelectrode (dashed lines) and forming a loose seal of ~50–100 MΩ. The scale bar represents 20 μm.

(B) Current traces from ChR2-expressing neurons showed light-evoked spikes occurring after stimulus onset (arrow).

(C) Prolonged stimuli of 50 ms trigger repetitive firing. Sixteen stimuli of 50 ms (blue bar) were delivered to the target cell at 0.2 Hz.

(D) Failures occur more often with high-frequency stimulation and after many stimulus repetitions. Light pulses of 50 ms (blue bar) were delivered at 1 Hz for 100 s; every tenth trial in the series is shown.

(E) The dependence of failure rate on stimulation frequency varies between cells. Two neurons in two fish were exposed to 20× 50 ms pulses at varying frequencies, and the probability of showing one or more spikes per stimulus was plotted as a function of frequency.

(F) Response probability increases with pulse duration (n = 9). Data are mean ± SEM.

demonstrate the feasibility of using ChR2 in the zebrafish nervous system. Conveniently, and unlike invertebrate model systems [15, 18–20], endogenous stores of the all-*trans* retinal required for ChR2 activity [8, 9] are sufficient for photoactivation of zebrafish sensory neurons. Importantly, the information presented in this study could not have been obtained with the same degree of confidence without a photoactivation strategy, because traditional methods of electrically stimulating a single, defined cell require immobilization of the animal, making it difficult to correlate cell firing with behavior.

Several potential complications of using ChR2 in zebrafish are apparent in this work. One of these is the attenuation of ChR2-driven spiking at high frequencies. Although ChR2-evoked spikes occurred reliably at up to 10 Hz in some trigeminal neurons, this is roughly one-half to one-third of the stimulus frequencies that are known to work in mammalian cells [9, 16, 22]. Given that moderately high-frequency (4 Hz) mechanical stimulation also resulted in attenuation of the response, it seems likely that ChR2 activity is limited by the intrinsic properties of the trigeminal neurons, rather than the channel itself. Further investigation of ChR2 activity in other cell types should clarify this issue.

ChR2-evoked behaviors showed a sharp decline by 48 hr of development. Because *Islet-1* enhancer-driven transient expression fades over developmental time, overall ChR2 expression levels might contribute to this phenomenon. Furthermore, Rohon-Beard neurons begin to disappear at around 48–72 hpf as they are replaced by the dorsal root ganglia [23]. However, because trigeminal cells do not show such downregulation during development, this cannot fully explain the loss of ChR2 responsiveness. Comparisons with other cell types, as well as electrophysiological recordings at later developmental stages and experiments using stably transgenic fish, are necessary to answer these open questions.

Driving Escape Behaviors with Low Levels of Activity in Somatosensory Neurons

Surprisingly, spiking patterns in trigeminal neurons can be as minimal as those in the hindbrain neurons that they innervate. In particular, Mauthner cells can drive escape behaviors by using single action potentials [7], a property thought to be important for rapidly triggering escapes in response to aversive tactile [24] and auditory [25] stimuli. Since the Mauthner cell is one of the downstream targets of the trigeminal neurons [26], our results suggest that single spikes in trigeminal sensory neurons might be translated into single spikes in Mauthner neurons. Functional imaging or electrophysiology in the hindbrain escape network will be necessary to determine whether this is the case and to further characterize the transfer of information from the trigeminal neurons to other downstream targets.

Recently, two groups have independently demonstrated that sparse stimulation of single or very small populations of neurons in the somatosensory barrel cortex of rodents can be used to drive learning and behavior [27, 28]. In no case, however, has it been conclusively shown that single spikes in primary sensory neurons can elicit behavior. Work in
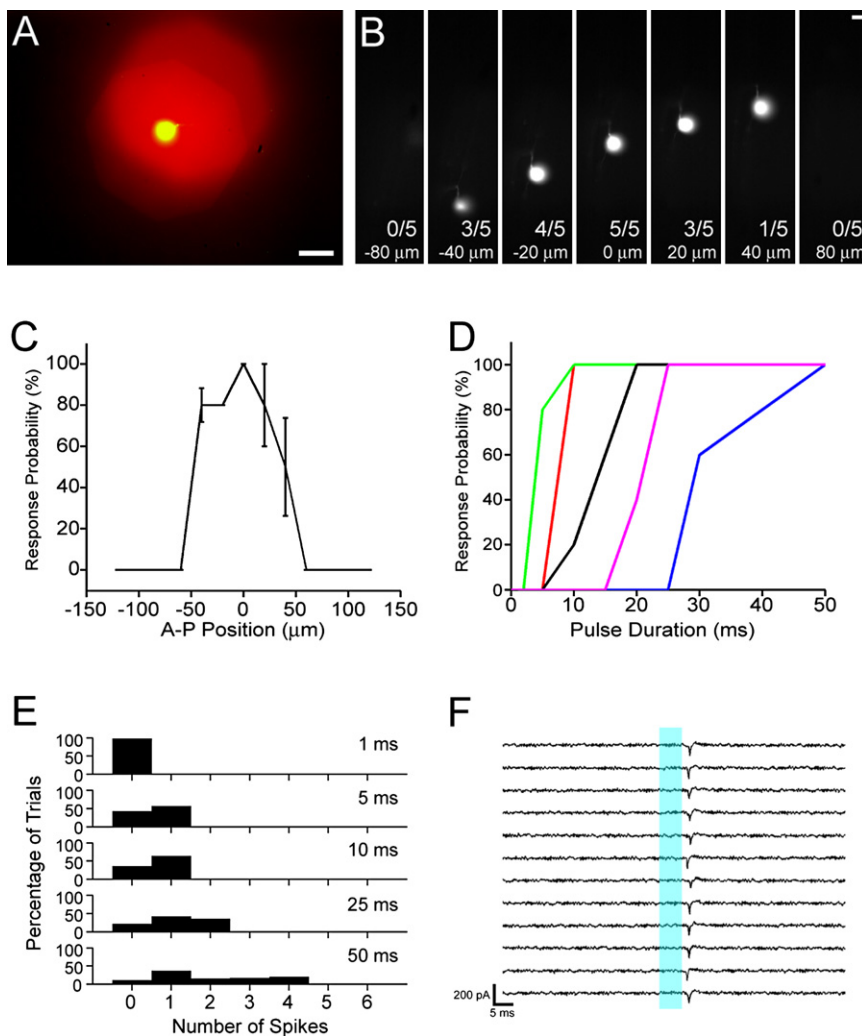
Figure 3. Single Spikes in Single Somatosensory Neurons Can Trigger Escape Behaviors

(A) By closing the microscope's epifluorescence-field aperture, illumination could be restricted to single cells in sparsely labeled embryos. Red channel: illumination light; green channel: ChR2-expressing Rohon-Beard neuron. The scale bar represents 20 μm.

(B) Stimulation of a single cell reliably evokes a behavioral response. One hundred millisecond stimuli were delivered at different positions along the A-P axis of the fish, and behavioral responses were monitored by watching the local contraction of the muscle. A-P displacements are given relative to the field in which the cell was located in the center of the illumination spot (0 μm). The number of successful trials (out of five) at each field is given at the bottom of each panel, above the displacement value. At −80 and +80 μm, the cell has moved completely out of the illumination spot. The scale bar represents 10 μm.

(C) Data pooled for five cells in five different fish that showed single-cell responsiveness. Mean ± SEM response probabilities are shown at several A-P displacements.

(D) Dependence of behavior probability on pulse duration. Cells identified as being sufficient to elicit a behavior were stimulated five times at each of several different pulse durations, and the behavioral output was monitored. Differently colored traces represent different cells (n = 5 cells in four fish; includes three trigeminal and two Rohon-Beard neurons).

(E) Number of spikes evoked by light pulses of different durations. Data show the probability of a given number of spikes occurring at a given pulse length and are pooled from six neurons. Pulses of 10 ms or less never evoked more than a single spike.

(F) Single spikes are reliably evoked by 5 ms light pulses (blue bar) in a trigeminal neuron. Stimuli were delivered at 0.2 Hz.

humans suggested that single spikes in somatosensory neurons might be perceptible [29], but the recording methods used in those studies allowed for ambiguity because they only monitored activity in a small subset of cells. Similarly, studies by Clarke et al. [2] raised the possibility that single spikes in *Xenopus* Rohon-Beard neurons might be sufficient to drive motor networks, but the "fictive swimming" preparation used in these experiments did not allow correlation of Rohon-Beard spiking activity with an actual behavior. Extremely weak stimuli can also be perceived by the human visual system [30] and the insect chemosensory system [31], but in both cases multispike events are likely to occur at the earliest levels of processing. Our data directly demonstrate the efficient coupling between single-spike activity in single sensory neurons and behavioral output.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, two figures, and four movies and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/15/1133/DC1/.

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