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Hearing the Light: A Behavioral and Neurophysiological Comparison of Two Optogenetic Strategies for Direct Excitation of Central Auditory Pathways

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Abstract

For deaf individuals with absent/damaged cochleae or auditory nerves, the auditory brainstem implant (ABI) is the only option to restore hearing. However, most ABI users have only sound awareness without meaningful speech comprehension. These electrical implants are limited by crosstalk between neighboring electrodes that indiscriminately activates large groups of neurons. In contrast, optogenetics provides a means to manipulate neural circuits with temporal and spatial precision by using light to activate genetically modified neurons expressing light-gated ion channels called channelrhodopsins. However, central auditory neurons fire at speeds that exceed the limits of most available channelrhodopsins. In this study, we explored the feasibility of an optogenetic auditory prosthesis by infecting neurons of the murine inferior colliculus (ICc) with viruses expressing standard channelrhodopsin-2 (ChR2) and Chronos, a newly discovered opsin with ultra-fast channel kinetics.

Through extracellular *in vivo* recordings in the ICc, we found that while ChR2-driven neurons can synchronize stimulation rates up to nearly 80 Hz, neurons infected with Chronos entrained pulses as fast as 200 Hz, approximating the synchronization limit for natural acoustic input in the midbrain. Optical stimulation of Chronos at rates as high as 300 Hz evoked minimally-adapting responses, although spikes were no longer fully synchronized. Chronos mediated responses support a superior code for the detection and discrimination of high pulse rates as compared with ChR2.

It was hypothesized that this improved temporal fidelity might translate into better behavioral detection of optogenetic stimulation. After unilateral ICc injections of saline or viral constructs, mice were trained to perform an auditory avoidance task. An optic fiber assembly was implanted into the injected ICc and the detection task was repeated with photostimulation in the place of acoustic input. Chronos and ChR2 expressing mice exhibited similar detection slopes, while saline injected animals performed at chance.

These findings suggest that while Chronos can transform a range of stimulation patterns with higher accuracy compared with ChR2, this does not translate into a perceptual advantage. This project has implications for both the future design of auditory prostheses and our understanding of signal processing in central auditory pathways.

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__Glossary of abbreviations__

AAV – adenoassociated virus

ABI – auditory brainstem implant

AMI – auditory midbrain implant

ChR2 – channelrhodopsin 2

ICc – inferior colliculus

NBN – narrow band noise

NF2 – neurofibromatosis type 2

PSTH – peristimulus time histogram

Introduction

The history of auditory implants

The World Health Organization estimates that 360 million people, representing nearly 5% of the world's population, suffer from disabling hearing loss with functional, social, and economic consequences (1). For the treatment of severe hearing loss, the development of auditory implants such as cochlear and brainstem implants has enabled patients worldwide to regain partial restoration of hearing (2).

The history of the first electrically stimulating auditory implant dates back to the 18th century, shortly after Alessandro Volta invented the first electrolytic cell. During one experiment, Volta connected a battery to two metal rods and discovered that when he inserted the rods into his ear canals he felt a jolt and heard a crackling noise (3). In 1957, Djourno and Eyries electrically stimulated the auditory nerve of a patient during surgery, causing the previously bilaterally deaf patient to report sounds (4). Subsequent experiments by House, Doyle and Simmons in the 1960's spurred the development of the first auditory implant in 1972 wherein a single electrode was placed into the cochlea of a patient with damaged cochlear hair cells. The first implant was simple but provided users with significant speech reading enhancements (5). Over time, implants came to have as many as 22 electrodes. In the cochlea, hair cells and their respective nerve fibers in the cochlea are maximally stimulated by frequencies of decreasing magnitude as sound travels from the base to the apex. Thus, multiple electrodes placed along this tonotopic map in the cochlea can stimulate spiral ganglion cells tuned to different sound frequencies.

Just years after the invention of the cochlear implant, in 1979, patients with absent/damaged cochleae or auditory nerves inspired the first auditory brainstem implant (ABI). These patients include those with auditory nerve aplasia from narrow internal auditory canals, traumatic injury to the auditory nerve, or congenital malformations of the cochlea. A subset of patients has a rare genetic syndrome called neurofibromatosis type 2 (NF2) that is found in 1 in 33,000 newborns (6). These individuals develop bilateral skull

base tumors called vestibular schwannomas; the surgical resection of these tumors results in post-lingual severe to profound hearing loss in both ears. Because the auditory nerve is damaged or severed, cochlear implantation is not a therapeutic option.

The first ABI was pioneered at the House Ear Institute to treat a patient with NF2 (7). It was a simple contraption with two ball electrodes placed on the surface of the patient's cochlear nucleus. Penetrating electrodes were later developed and organized in arrays of 8-16 channels to increase the complexity of stimulation and better harness the tonotopic organization of the cochlear nucleus (8).

Modern auditory prostheses: cochlear implants, ABIs, AMIs

The modern auditory implant has 5 functional components (Figure 1a). Externally, there is a microphone, a speech processor and a transmitter. The microphone curves around the ear and serves to gather sound information and deliver it to the speech processor. The speech processor may be housed separately from the ear piece or, in newer models, can be incorporated into the hardware that sits behind the ear. The speech processor is a computer that separates sounds into different frequency spectrums and organizes the information into an electrical code. The processor transmits this spectral and temporal information about incoming sound to a transmitter that delivers the signal to the internal components of the implant: the receiver and the stimulating electrode array. The transmitter sends signals to the receiver that is implanted just underneath the skin above the ear. In cochlear implants, the receiver passes the coded signals to the electrode array that wraps into the cochlea and the resultant electrical stimulation from specific electrodes causes groups of auditory nerve fibers to fire, delivering auditory sensations (Figure 1b). In auditory brainstem implants, the final electrode array stimulates not the cochlea but higher order auditory neurons of the brainstem (Figure 1c).

Worldwide, approximately 324,200 people have undergone cochlear implantation (9) and more than 1,000 patients have benefited from ABIs (10). The results of cochlear implantation can be life changing: adults report substantial improvements in speech understanding as soon as 3 months after surgery (11,12). Children with cochlear implants benefit from dramatic gains in speech and language development. In one study following

pediatric patients 10-14 years after initial implantation, 77% of patients had speech intelligible to an average listener and all patients were in school or employed and actively engaged in their communities (13).

The outcomes of ABI users are more varied: follow-up studies indicate that this device has a range of audiological responses in implanted patients, from modest gains in sound appreciation to the acquisition of excellent speech recognition in a minority of people, enabling even telephone conversations (14). Most studies conclude that ABIs allow 85% (15) to 96.2% (16) of patients to hear environmental sounds. ABIs have also been shown to enhance lip reading (16–18). However, estimates suggest that as many as 18% (19) of patients do not perceive benefits from their ABIs. Moreover, patients with NF2 have been shown to have particularly poor results, perhaps stemming from tumor- or surgery-related damage to the cochlear nucleus or surrounding brain tissues (20–22).

To improve NF2 patient outcomes and bypass any surgical or tumor-mediated damage to the cochlear nucleus, a different type of brainstem implant called the auditory midbrain implant (AMI) was developed to stimulate hearing at an even more proximal structure: the inferior colliculus (ICc). To date, three patients have been implanted in initial clinical trials, demonstrating improved hearing responses in all three patients (22). The patient with the most optimally positioned AMI gained sound perception and significant improvements in consonant and number recognition, as well as enhancements in lip reading accuracy (22).

The limitations of an electrical implant

Despite these ongoing advances in the hardware and software components of cochlear implants and ABIs, specific challenges remain for patients in search of hearing restoration. One key limitation of all auditory prostheses (cochlear implants, ABIs, AMIs) remains: the use of electricity restricts the density of electrode stimulation. When multiple electrodes are simultaneously active, electrical field interactions can disrupt individual waveforms and this spread of current decreases hearing sensitivity and accuracy (23–26). For example, while modern cochlear implants employ 1-2 dozen electrodes, studies suggest that no more than 4-8 distinct sites should be active at any one

time because of the substantial overlaps from nearby electrodes as they sit in the conductive fluid of the perilymph (Fishman, Shannon, & Slattery, 1997; Friesen, Shannon, Baskent, & Wang, 2001; Garnham, Os'Driscoll, Ramsden And, & Saeed, 2002). This makes poor use of the nearly 30,000 tonopically organized spiral ganglion neurons that are fine tuned to specific frequencies. As such, current cochlear implant technology enables open speech comprehension, but hearing in noisy environments and music appreciation is limited (22,23).

Similarly, studies of multi-channel ABIs suggest that increasing the number of electrodes beyond 5 does not significantly improve perceptual outcomes (31). In auditory brainstem implants, these spreading fields of electrical stimulation may be even more important as central auditory systems consist of complex networks of excitatory and inhibitory interneurons as well as supporting glial cells that may all be affected by unfocused electrical stimulation. Moreover, some ABI users experience side effects such as pain and dizziness as non-auditory neurons are activated (32).

New technology: optogenetics

Due to the aforementioned limitations of electricity-based implants, other strategies for neuronal stimulation are being explored for use in auditory prostheses, including multipolar stimulation (33), intraneural stimulation (34) and optical stimulation. Light energy has been proposed as an exciting alternative to electrical stimulation in auditory prostheses specifically because its resolution is not so limited by spreading field interactions (35,36) and can thereby better harness the fine grained spatial-temporal resolution of auditory centers.

Two optical strategies are under investigation for the use in auditory implants: infrared stimulation and optogenetic stimulation. In the former, infrared light has been shown to depolarize unmodified neurons with less spread of neural activation in the cochlea. However, the long term repercussions of this direct stimulation on sensitive neural tissue is not understood. Infrared light generates thermal gradients to depolarize neurons, which results in the accumulation of heat energy that may be harmful to tissues (37,38). Moreover, the energy required for infrared stimulation exceeds what is practical

for use in portable prostheses (39). Recently, the use of infrared light has even been challenged by the finding that infrared stimulation does not trigger auditory activity in completely deafened cochleae, suggesting that neuronal responses could be an artifact of optoacoustic stimulation by the high energy laser pulses (40).

Thus, research has turned optogenetic technology, an alternative technique that employs genetically modified neurons that express light-gated ion-channels to manipulate specific neural circuits. Through using these light-sensitive opsins, lower levels of light energy are required for optogenetic depolarization of neurons as compared with infrared stimulation (41). Ten years ago, the first opsin "Channelrhodopsin2" (ChR2) was isolated from the green algae *C. reinhardtii* and virally expressed on the surface of neurons (42,43). It was discovered that, in the presence of blue light, channelrhodopsins depolarize neurons by conducting cations into cells. It has been shown since that ChR2-expressing neurons can be activated by focused beams of visible light with spatial selectivity on the scale of microns in the murine brain (44).

Only a few studies to date have applied optogenetics to the auditory nervous system. A pilot investigation of ChR2-expressing transgenic mice has shown that optical stimulation of spiral ganglion cells evokes auditory brainstem responses and local field potentials upstream in the inferior colliculus (ICc) (41). Others have shown that channelrhodopsins introduced with adenoassociated viruses (AAVs) could be used to drive neuronal activity throughout the auditory nervous system, from the cochlea to the auditory cortex (41,45). AAVs do not integrate into the genome, but enable lasting expression of opsins (up to at least 18 months in murine neurons) without causing cellular toxicity (46).

A range of ethical and medical hurdles stand between current studies that manipulate virally delivered opsins in the basic science lab and the future use of optogenetic technologies in human patients. For specific application in the auditory nervous system, one fundamental problem remains: most existing channelrhodopsins have inherently slow channel kinetics, which reduces their ability to deliver long lasting, high-frequency neural stimulation above 40-50 Hz (42,47). In contrast, central auditory

neurons are known to fire at several hundred hertz with exceptional temporal precision to encode the rapidly changing characteristics of human speech (48,49). The ability to faithfully encode neural representations of sound requires an opsin that could reproduce this level of precise, non-adapting neuronal activity.

Recently, a novel channelrhodopsin named "Chronos" was isolated from the algal species *Stigeoclonium helveticum*. Patch clamp recordings from cultured neurons and brain slices initially demonstrate that Chronos has the fastest channel kinetics of any channelrhodopsin described to date combined with high light sensitivity (50). The speed of this new opsin has been attributed to its ultra-fast deactivation time of approximately 3ms, compared with ChR2 at 20 ms. Recent *in vivo* studies have found that Chronos is able to drive neurons of the murine cochlear nucleus at high stimulation rates (51). The discovery of Chronos begs application in the field of auditory prostheses as a way to use optogenetics to bypass the longstanding limitations of electrical devices.

In this thesis project, I compared Chronos and standard ChR2 in the first murine model of an optogenetic auditory brainstem prosthesis. I first determined whether Chronos has superior temporal fidelity compared with ChR2 *in vivo* in the auditory midbrain. I then explored whether optogenetic stimulation of the murine ICc with Chronos or ChR2 translates into differences in perception in a murine behavioral detection task. These experiments demonstrate how Chronos opens new doors to a future optogenetic auditory implant, while highlighting the technical challenges in replicating the complex neuronal circuits that encode sound. Moreover, the development of the first mouse model of a midbrain optogenetic implant represents a foundational step for the future design of optically stimulating auditory implants. The data presented hereon have been published by Guo et al. as of May 2015 (52).

Methods

Reproduced with minor alterations from Guo et al. (52)

Ethics

All procedures were approved by the Massachusetts Eye and Ear Infirmary Animal Care and Use Committee and followed guidelines established by the National Institute of Health for the care of laboratory animals. 20 CBA/CaJ mice were used in this study (10 for *in vivo* ICc recordings; 10 for behavioral assessments and *in vivo* auditory cortex recordings).

Virus injection

Live adeno-associated viruses (AAV) encoding AAV-CAG-ChR2-mCherry or AAV-Synapsin-Chronos-GFP were obtained courtesy of collaborators from the Edward Boyden lab at MIT. Adult male CBA/CaJ mice aged 8–10 weeks were sedated with isoflurane (5% in oxygen), then anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). A surgical plane of anesthesia was maintained with supplements of ketamine (50 mg/kg) as needed. Throughout the procedure, the animal's body temperature was kept near 36.5 °C with a homeothermic blanket system. After numbing the scalp with 0.5% lidocaine, an incision was made along the midline, exposing the skull around the lambdoid suture. A small craniotomy (0.2 × 0.2 mm, with the medial-rostral corner positioned at 0.4 mm lateral and 0.1 mm caudal to lambda) was made with a scalpel to expose the right inferior colliculus. The dura mater was left intact. Electrophysiological recordings were made to identify the location of the central nucleus (ICc) before virus injection (See acute electrophysiology in the IC). Glass capillary pipettes were pulled and back filled with mineral oil before loading with virus. A motorized stereotaxic injector (Stoelting Co.) was used to inject 0.3–0.5 μl of virus expressing Chronos or ChR2 into the right ICc of the mouse, approximately 700 μm below the brain surface at a rate of 0.05 μl/min. The pipette was left in place for an additional 10 minutes before withdrawal. The craniotomy was covered with high viscosity silicon oil, and the skin was sutured

closed. Mice were allowed to recover for 48 hours before behavioral training with NBN and at least 3 weeks before detection was measured with laser stimulation.

Acute electrophysiology in the ICc

The surgical procedure was similar as described in the previous section. Mice were anesthetized and craniotomies were performed over the right ICc. Single-shank multi-channel silicon optrodes (NeuroNexus Technologies) were used to deliver laser pulses and record neural activity (sampled at 24 kHz, digitized at 32 bit, and then band-pass filtered between 300 to 5000 Hz with second-order Butterworth filters). Multiunit spike events on each channel were time stamped at threshold crossing (4.5 s.d. above a 10 s running average of the baseline activity, SpikePac, Tucker-Davis Technologies). All recordings were performed in a double-walled sound-attenuating chamber. The ICc was identified according to the dorsal-ventral low-high tonotopic organization as defined by a pseudorandom series of pure tone pips (4–64 kHz in 0.1 octave steps, 0–60 dB SPL in 5 dB steps, 50 ms duration with 5 ms cosine ramps at the onsets and offsets, 500 ms inter-trial intervals) presented to the contralateral ear with a custom-built, calibrated in-ear acoustic system.

Laser pulses (473 nm, 1 ms pulse width, 1 s total duration, LaserGlow Co.) were presented at various rates (20 to 300 Hz, 20 Hz steps) to the IC via the optic fiber on the optrode, which was positioned 0.2 mm above the topmost recording site. To avoid potential contamination through photoelectric artifacts, threshold crossings during the laser pulse were disregarded. Laser powers were selected to generate suprathreshold responses in the infected tissue on a case-by-case basis, and were generally in the range of 5 to 7 mW. For comparison, narrowband noise bursts (filtered from broadband noise stimuli using fourth-order Butterworth filters, 20 kHz center frequency and 0.25 octave bandwidth, 1 ms duration, 60 dB SPL) were presented at the same rates via the contralateral in-ear acoustic system. Laser and acoustic stimulation were presented in a pseudorandom order and repeated 20 times each.

Optic fiber implantation

Following 2–6 weeks of behavioral testing with acoustic stimuli, mice were anesthetized with ketamine and xylazine, as described previously. An implantable 4 mm optic fiber assembly (NeuroNexus NNC fiber) was advanced 0.35 mm into the ICc along the previous injection site. The implant was then cemented to the skull (C&B Metabond). Mice were allowed to recover for at least 48 hours prior to the continuation of behavioral testing.

Behavioral testing

Behavioral training was carried out in an acoustically transparent enclosure (8 × 6 × 12 inch, L × W × H) bisected into two virtual zones resting atop electrified flooring (8 pole scrambled shocker, Coulbourn Instruments). Mouse position was tracked with a commercial PC webcam. Auditory stimuli were delivered through a free-field speaker positioned above the apparatus to provide a relatively homogenous sound field (Tucker-Davis Technologies). Mice were given at least five minutes to acclimate to the apparatus before each day of testing. Naïve mice were initially shaped to cross between zones of the chamber to terminate a foot shock (60 Hz, 0.5–1 mA, according to the minimally effective intensity for each mouse). With conditioned crossing behavior established, mice were then trained to associate sound (white noise, 5 s duration, 5 ms cosine ramps, 70 dB SPL) with foot shock initiated 5 s later. Crossing within the 5 s window was scored as a hit and the foot shock was avoided. Foot shock was initiated if the mouse failed to cross within the 5 s period (a miss) and was terminated upon crossing sides or 10 s, whichever occurred first. Once the hit rate stabilized at ≥ 70%, white noise was replaced with the narrow-band noise bursts and training continued until crossing behavior stabilized again. Psychometric functions were acquired by documenting the hit probability at different sound levels (−10 to 70 dB SPL in 10 dB steps) and pulse rates (60–300 Hz in 60 Hz steps). Stimuli were presented in a pseudo random fashion and repeated at least 15 times each. Inter-trial intervals were randomly drawn from a uniform distribution between 30 to 40 seconds. False positives were calculated as animal's

crossing probability during a 5 s window halfway through the inter-trial period. Typically, each animal performed 60 to 100 trials per day, 5 to 6 days per week.

For behavioral experiments involving detection of laser pulse trains, the implanted midbrain assembly was tethered to the laser with a patch cable. Mice were given 10 minutes to acclimate to tethering before conditioned crossing behavior was initially reestablished with broadband noise stimulus. Once the hit probability was comparable to that documented without tethering, the acoustic stimulus was replace by laser stimuli (1 ms laser pulses, 60 to 300 Hz in 60 Hz steps) without any additional behavioral shaping. Due to the variability of sensitivity introduced by injection volume and expression level of the opsins, the range of laser intensity tested was adjusted on a case-by-case basis for each animal to generate a range of subthreshold to suprathreshold behavioral responses. In all other respects, stimulus design and task organization were identical to the acoustic version of the task.

Histology

Animals were deeply anesthetized with ketamine and prepared for transcardial perfusion with 4% formalin solution. The brains were extracted and post-fixed in 4% formalin at room temperature for an additional 12 hours before transfer to 30% sucrose solution. Brain sections (60 μm thick) were counterstained with DAPI (Life Technologies). The location and size of the infection zone was inferred through visualization of the fluorescent label with a conventional fluorescence microscope (Zeiss).

Data analysis

Firing rate adaptation was quantified by calculating the ratio of the spike count to the first pulse divided by the average spike count to all remaining pulses within the 1 s period. To quantify the temporal fidelity of sound or laser evoked activities, a template-based classifier model was used. For any given recording site, half the trials of responses to all pulse rates were used to build peristimulus time histogram (PSTH) based templates; the other half of the trials were used as test cases. Test trials were compared with the

templates by calculating their cross correlation coefficients. The decoded pulse rate for a test trial was the pulse rate behind the most similar template (highest cross correlation coefficient). The decoding accuracy for all rates was calculated and averaged across recording sites. Pulse train detectability was quantified by dividing the PSTH into 100 ms bins and calculating the firing rates for each bin within the spontaneous and evoked periods on a single trial basis. For any given bin, its detectability was quantified as the rectified z-score of its spike count with respect to the baseline distribution. The difference between mean z-scores from the spontaneous and evoked periods for each trial provided the basis for calculating d'.

Statistical analyses

All statistical analyses were performed in Matlab (Mathworks). Repeated measures ANOVAs were used to compare neural or behavioral measurements over dependent variables such as pulse rate or sound intensity in the same group of animals. When comparing measurements across different groups of animals, mixed-designed ANOVAs were used, and the main effects were reported. Multiple comparisons were corrected with the Bonferroni method.

Results

Chronos expressing neurons can entrain a wide range of laser stimulation rates

To investigate the *in vivo* differences between ChR2 and the novel opsin Chronos, viruses expressing each opsin were introduced into murine ICc. Chronos and ChR2 expression in the auditory midbrain was confirmed by histological assessment of GFP and mCherry reporters respectively (Figure 2a). The ability of opsins to entrain light pulses of varying frequencies (200-300 Hz) with high fidelity was compared with the response of the ICc neurons to similar frequencies of NBN. We discovered that while ChR2 expressing neuronal multiunits were able to synchronize firing to laser stimulation up to pulse rates less than 80 Hz, Chronos expressing neurons demonstrated high fidelity entrainment to rates as high as 200 Hz. For even higher pulse rates, Chronos expressing neurons generated more sustained, albeit not fully synchronized, activity as compared with ChR2 (Figure 2b-c).

With repeated stimulation, neurons exhibit spike frequency adaptation whereby neurons become less excitable following the initial response. This is theorized to be caused by a variety of mechanisms including the slow recovery of sodium channels after the initial depolarization, as well as activity dependent activations of alternate ion channels that hyperpolarize the cell after the initial depolarization. As auditory neurons are known to operate with extreme temporal precision, we examined how spike frequency adaptation rates change in neurons activated by light-activating opsins as compared with NBN. The spike rate ratio was calculated between the first and the following stimulus pulses after each train of optogenetic or acoustic stimulation (Figure 2d). For all modes of activation (laser activation of opsins and NBN activation of intrinsic auditory pathways), rates of adaptation increased for increasing pulse rates (repeated-measures ANOVA, n=388, df=14, p=2.9x10^-6). ChR2 showed more significant adaptation than NBN (mixed-design ANOVA, n=117/160, df=1, p=2.9x10^-6) but at high pulse rates, Chronos expressing neurons demonstrated significantly less adaptation than even neurons activated by NBN (mixed-design ANOVA, n=111/160, df=1, p=9.0x10^-13).

In further analysis to characterize the fidelity of signal transmission, a subset of *in vivo* recordings were used to create PSTH-based classifier models for Chronos, ChR2 and NBN-induced neuronal activity (see *Data analysis* in *Methods*). The more distinct the patterns of spiking at each pulse rate, the more accurately the classifier model is able to predict the rates of new sets of laser stimuli from recordings of responding neural multi-units. Confusion matrices showing correct and incorrect classifications for laser stimulation of each opsin and for NBN are shown in Figure 3. Chronos-based classifier models were significantly more likely to correctly classify new stimulus rates compared with those constructed using ChR2 or NBN (mixed-design ANOVAs; Chronos vs. ChR2: n=117/160, df=1, p=9.0x10^-13; Chronos vs. NBN: n=111/160, df=1, p=6.0x10^-6). ChR2 and NBN models were not statistically significantly different (mixed design ANOVA; n=117/160, df=1, p=0.50).

These PSTH classifier models showed how the opsins allow midbrain neurons to encode different pulse rates, but do not characterize the salience of the neural information. To examine this, we counted the number of spikes in each 0.1s bin in the 1s interval before and after an episode of laser/acoustic stimulation (Figure 4a), and found the distributions of neural spikes during spontaneous and evoked times (4b). Spike counts from spontaneous and evoked periods were then converted into Z scores, which were taken as their absolute values because both positive and negative deviations may represent neural information (figure 4c). We then calculated the sensitivity index d' as the separation between the spike distributions for "hits" (firing during evoked periods) compared with "false positives" (spontaneous neural activity) (Figure 4d). We found that NBN and Chronos had significantly higher d' values compared with ChR2, which became increasingly obvious at higher stimulation rates (mixed-design ANOVAs; ChR2 vs. NBN: n = 117/160, df = 1, p = 1.5 × 10^−10; ChR2 vs. Chronos: n = 111/160, df =1, p=1.5 × 10^−12).

Thus, Chronos allows neurons to entrain high pulse rates with higher fidelity and greater discriminability than ChR2. These rates are compatible with the speeds commanded by neurons of the central auditory system. These findings inspired further

experiments to determine whether Chronos' advantage over older generation of opsins (ChR2) would support enhanced behavioral detection of photostimulation in the ICc.

Behavioral detection of ICc stimulation is similar for Chronos and ChR2 expressing animals at different pulse rates

We created the first murine behavioral model of an optogenetic auditory implant to investigate whether optogenetic tools can be used to simulate auditory percepts and determine if Chronos is superior to ChR2 in conveying an actionable auditory percept. Mice were injected with viral constructs expressing Chronos or ChR2 in the ICc (n=3 respectively). Similarly, 2 control mice were injected with saline. All animals were trained in an auditory avoidance task to report detection of acoustic or optogenetic stimulation of the ICc (Figure 5a). Mice learned to cross sides of a shuttle box after hearing acoustic stimuli at a range of pulse rates from 20 to 300Hz to avoid a foot shock. Psychometric functions were obtained for acoustic pulse rates and sound levels from -10 to 70 dB SPL. Subjects crossed with increasing probability for higher sound levels (Figure 5b). The slopes of the psychometric functions were estimated by a linear fit of the data, calculated to represent the salience of the acoustic stimuli and did not vary across injection types (mixed-design ANOVA; n=3/3/2, df=2, p=0.40). However, the psychometric slopes were affected by NBN pulse rate (Mixed-design ANOVA, Chronos vs. ChR2 vs. saline, n=3/3/2, df=4, p=1.5×10^{-3}; Figure 5d).

Murine subjects were then implanted with chronic optic fibers into the previously injected ICc. The sound detection task was repeated with photostimulation to the midbrain and Chronos and ChR2 animals immediate generalized the task across stimulation sources. Increasing laser amplitude resulted in improved crossing probability in all subjects (Figure 5c). However, the psychometric detection slopes of Chronos and ChR2 injected animals were not significantly different (one-way ANOVA: n=3/3, df=1, p=0.73; Figure 5e) and did not vary with pulse rate (one-way ANOVA: n=3/3, df=4, p=0.32). The rate of detection was at chance in saline-injected animals, suggesting that behavior in virus-infected animals could not be explained by thermal or visual cues during photostimulation.

In summary, behavioral studies of opsin expressing mice were not consistent with predictions that Chronos would be superior to ChR2 in encoding photostimulation in the auditory midbrain. Additional experiments by collaborators explored whether this could be explained by changes made to the transmitted signals at higher order regions of the auditory central nervous system. In the same mice that performed the behavioral task, multi-unit recordings were taking from the auditory cortex as animals were subjected to acoustic pulse trains or photostimulation of the ICc. Optogenetic activation of auditory neurons in the midbrain evoked brief, non-synchronized onset responses (occurring <50ms after time of photostimulation), followed by rapid suppression (Supplemental Figure A). In comparison, acoustic stimulation evoked significantly more robust responses than laser timulation of either ChR2 or Chronos-expressing neurons (one-way ANOVAs; NBN vs. ChR2: n=136/64, df=1, p<$1x10^{-196}$; NBN vs. Chronos: n=136/72, df=1, p<$1x10^{-196}$).

Discussion

Modern auditory prostheses are a result of decades of bioengineering and have improved the lives of deaf patients worldwide. For those who use cochlear implants and auditory brainstem implants, software and hardware improvements have dramatically changed the quality of the auditory output. However, the basic paradigm for electrically stimulating implants has not changed since Dr. William F. House first inserted a simple electrical wire into a patient's cochlea in 1972. The physical limitations of electrical implants inspired this thesis to explore new technologies in pursuit of a better hearing prosthesis. Optogenetics offers a method of stimulating neurons with light, bypassing the intrinsic limitations of electrical systems in hopes of reproducing the fine grained frequency resolution of the human auditory nervous system.

Inspired by the recent discovery of the ultra-fast opsin Chronos, we compared optical stimulation with Chronos and conventional ChR2 in the murine ICc. We found that Chronos supports a superior non-adapting code with high temporal fidelity at the speeds required to encode auditory information *in vivo*. The fidelity and salience of pulse trains encoded by Chronos was superior to those of ChR2 (Figures 2-4). However, these advantages were not apparent in behavioral experiments where Chronos and ChR2 expressing animals were trained to perform an auditory detection task (Figure 5). When signals produced by laser stimulation of the ICc were traced to the auditory cortex, Chronos and ChR2 encoded neural responses were non-synchronized and rapidly suppressed (Supplemental figure A).

The limitations of this current project are a reflection of time and resource constraints. First, the behavioral model consisted of only a detection task. The inability of our auditory detection task to discern the differences between Chronos and ChR2 could be further probed with a more complicated discrimination experiment. Animals could be trained to cross a shuttle box when exposed to a high acoustic pulse rate (e.g. 300 Hz) and ignore low pulse rates (e.g. 40 Hz). With photostimulation of the midbrain, Chronos and ChR2-expressing animals may show disparate abilities to perform this task. Presumably, given our *in vivo* neurophysiology results, Chronos-expressing mice would

be better able to generalize across stimulation modalities. In the event that ChR2 expressing animals are able to distinguish between the two pulse rates because the rapidly adapting onset responses at high pulse rates remain distinct from the more clearly encoded pulse train at lower frequencies, the discriminability of increasingly similar frequencies can be tested: animals could be serially trained to identify diminishing differences in high pulse rates (e.g. 300 Hz vs. 80 Hz, then 300 Hz vs. 120 Hz, etc), such that animals expressing Chronos may eventually demonstrate a competitive advantage because ChR2 expressing neurons encode high pulse rates with low fidelity.

However, these hypothetical experiments comparing Chronos and ChR2 in photostimulation discrimination tasks may prove unfruitful as we found that cortical responses to optogenetic stimulation of the auditory midbrain are poorly encoded and quickly suppressed. This is consistent with previous reports of weak cortical responses after electrical stimulation of single sites in the ICc, as compared with increased cortical activity when multiple isofrequency sites are stimulated simultaneously (53,54). This highlights a second limitation of this study in that the optical fiber size we used was relatively large compared to the size of the murine inferior colliculus. Stimulation through a single large laser beam may have triggered the activity of inhibitory interneurons (55,56). The cortex may therefore be more responsive to more complex afferent signals characterized by spatiotemporally differentiated patterns of information. More compact, denser arrays of optical electrodes would allow better localization of neuron stimulation that might then target specific areas of the auditory midbrain to mimic more natural patterns of neuron firing. Towards this end, research in material science has recently developed thin-film flexible LED arrays that can be miniaturized for such a biomedical application (57,58).

A third limitation to this study is found in the virals vectors we used, which were provided in the form of live virus so that we could not easily change the promoter sequences driving opsin expression. Chronos was driven by a Synapsin promoter that has been found to be active primarily in neurons (59), whereas ChR2 was preceded by a CAG promoter that drives high levels of gene expression in many mammalian cell-types (60,61). This introduced a confounding factor in our comparisons despite our histological

verification that both opsins appeared to be widely expressed in neurons of the ICc. Future experiments should explore a variety of promoters to target specific cell populations.

Future experiments

More sophisticated genetic and bioengineering approaches may make it possible to discern how spatially differentiated populations of neurons in the brainstem can be activated in a way that more closely mimics patterns of natural auditory information. This may be particularly important for central auditory prostheses given that the large drop in performance of ABIs as compared with cochlear implants may be attributable to the enormous complexity of the brain as compared with peripheral nerve fibers. While modern ABIs electrically stimulate vast areas of interconnected neurons and supporting cells, optogenetic tools might one day allow stimulation of targeted auditory networks. For example, viral constructs with cell-type specific promoters can be used to drive expression of opsins in excitatory neurons. One technical hurdle will be that some cell-specific promoters may not be driven with enough efficiency to allow consistent levels of opsin expression.

Alternatively, through bioengineering, a palette of opsins responsive to non-overlapping wavelengths of light can used to drive more complex patterns of stimulation. Multiple opsins can be introduced simultaneously, either via numerous viral constructs or a single construct expressing multiple channelrhodopsins that are stochastically expressed, such as in "Autobow" mice (62). This, combined with smaller and more dynamic optical arrays, can greatly improve the complexity of the transmitted code. With the use of multichannel micro LED arrays, new strategies for speech processing will need to be developed to code incoming sound into complex patterns of optical stimulation. These future experiments will simultaneously explore a more nuanced understanding of the natural patterns of neuronal firing that transmit the most accurate information to higher levels of the brain, as read out by behavioral and neurophysiological experiments.

After longitudinally studies in animal models, further technical and ethical issues regarding the safe and efficient usage of optogenetic technology in humans will need to

be explored. Notably, the use of live viruses in humans will be addressed not only in this field of research but across many that depend on the viral expression of novel genes as a component of therapy (63). To date, AAVs have proven safe for use in the human eye for the treatment of Leber's congenital amaurosis and is poised for application in a number of other clinical areas (64). Specific investigation into the species and strains of virus best suited for efficient infection and sustained gene expression in the auditory nervous system will be necessary, beginning with rigorous rodent and nonhuman primate studies.

Ultimately, the success of auditory implants over the past four decades serves to emphasize that the auditory prosthesis is actually a testament to the remarkable ability of the human brain to adapt. It is surprising that the small number of electrodes in modern cochlear implants can allow the majority of postlingually deafened patients to perceive speech and even some elements of music (29). In patients with ABIs, results have been more varied for different surgical candidates but have been particularly successful for younger patients, no doubt due to their superior neural plasticity as compared with older individuals. A 12 year follow-up of deaf children with ABIs found that 41% developed open set speech recognition (65). The improved, optogenetic auditory prosthesis of the future need not be perfect, but simply better able to simulate the complex neural circuits that govern auditory perception.

Conclusion

Optogenetic technology opens new doors to the design of a better auditory prosthesis. Here, we have demonstrated that the novel opsin Chronos can encode a wider range of temporal stimulation rates with better discriminability compared with standard ChR2. Specifically, Chronos-expressing neurons can entrain pulse rates comparable to the fast firing speeds of central auditory neurons. However, in our behavioral model of an optogenetic midbrain implant, Chronos does not enable better detection of an auditory stimulus triggered by laser stimulation. This project underscores the complexity of the central auditory neural circuits that prostheses aim to replicate and sets a foundation for future experiments towards an optogenetic auditory implant.

Summary

Auditory implants have improved the lives of thousands of patients worldwide who suffer from hearing loss. Modern devices like cochlear implants and auditory brainstem implants use electricity to stimulate neurons of the auditory system to generate sensations of sound. Electrical implants are limited in their ability to target small groups of neurons because crosstalk between neighboring electrodes blurs the resulting signal. Thus, light stimulation has been proposed as light can be more easily focused. To sensitize neurons to light, neurons are infected with viruses expressing membrane channel proteins that allow ions to move into and depolarize neurons in the presence of photons. This technology is called "optogenetics."

In this project, we created the first mouse model of an optogenetic auditory implant that stimulates auditory neurons of the midbrain. Using this model, we compared a new ultra-fast channel protein called Chronos with the well-known but slower protein channelrhodopsin 2 (ChR2). We found that Chronos allows auditory neurons to fire much faster than those expressing ChR2, at speeds comparable with those of midbrain neurons excited by sound. However, we found that this advantage was not apparent when we trained animals to perform a behavioral task in response sound. When their auditory neurons were stimulated with light, Chronos and ChR2 expressing mice performed the task equally well. This project represents an important step towards building the optogenetic auditory prosthesis of the future.

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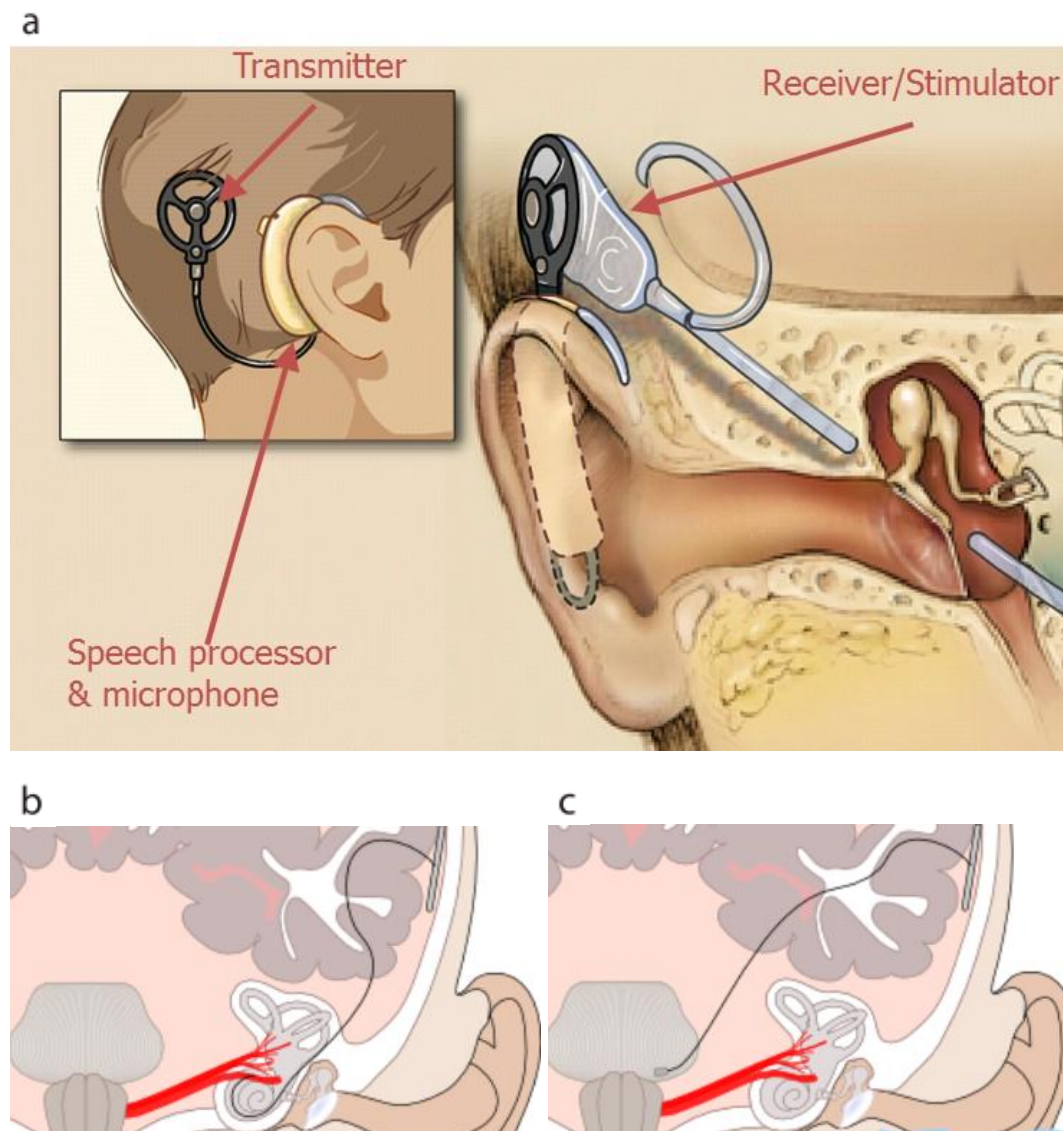
Figures

Figure 1. Functional components of auditory implants. (a) Sound from the external environment is collected in the microphone and transformed into an electrical code in the speech processor. This information is sent from an external transmitter to an internally implanted receiver/simulator. (b) In cochlear implants, the stimulator triggers an array of electrodes in the cochlea to activate spiral ganglion neurons. (c) In ABIs, the stimulator activates an array of electrodes that stimulate neurons of the auditory brainstem. Illustrations courtesy of the Medical Research Council – Cognition and Brain Sciences Unit (66) and NF2 Information and Services (67) websites.
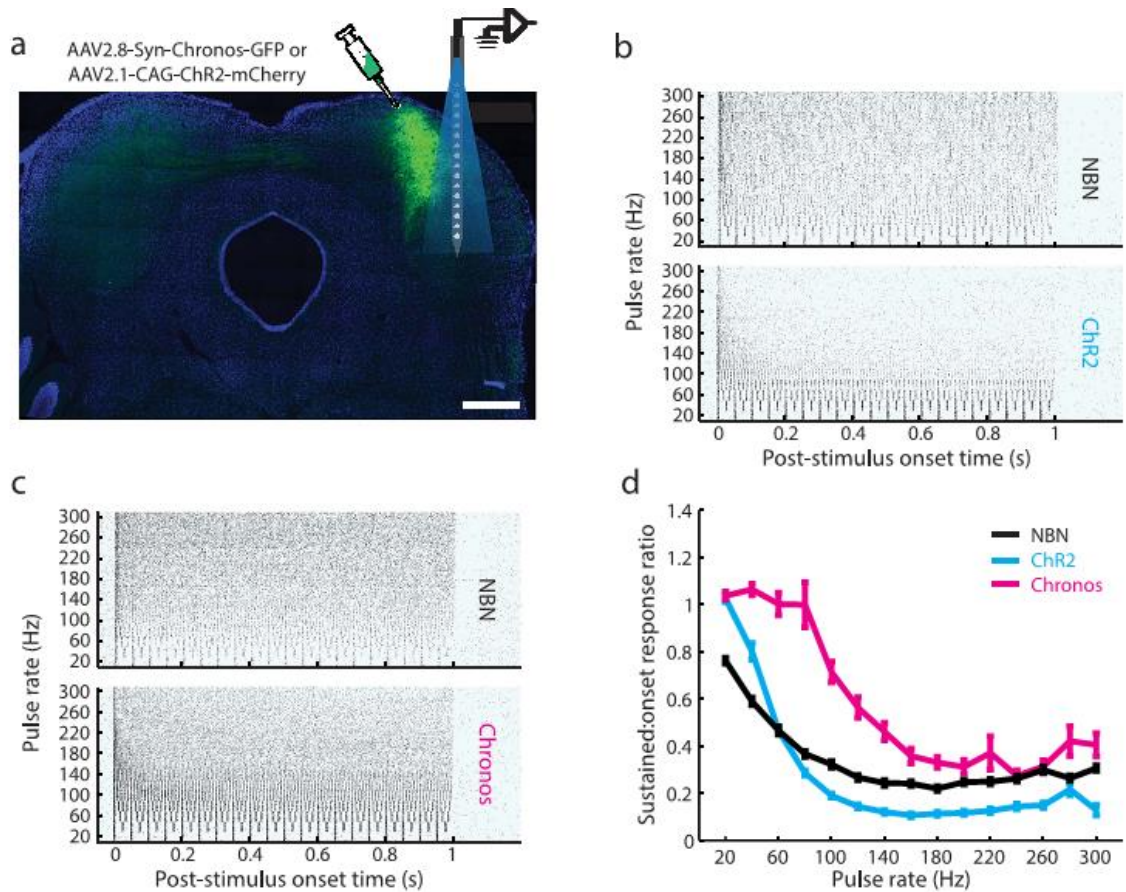
Figure 2. Chronos is better able to entrain high frequency pulse rates than ChR2. (a) Coronal sections of murine ICc were counterstained with DAPI to reveal robust Chronos-GFP expression with fainter fluorescence in the commissural axons that pass to the contralateral ICc. Optrode recording probes were inserted at the site of the prior injection. Scale bar = 0.5mm. (b-c) Rastergrams of ICc recordings in response to trains of laser stimulation or NBN. (d) Firing rate adaptation as represented by the ratio of spikes evoked by the first and the following stimulus pulses during each train of optogenetic or acoustic stimulation. Figure from Guo et al. 2015 (52).
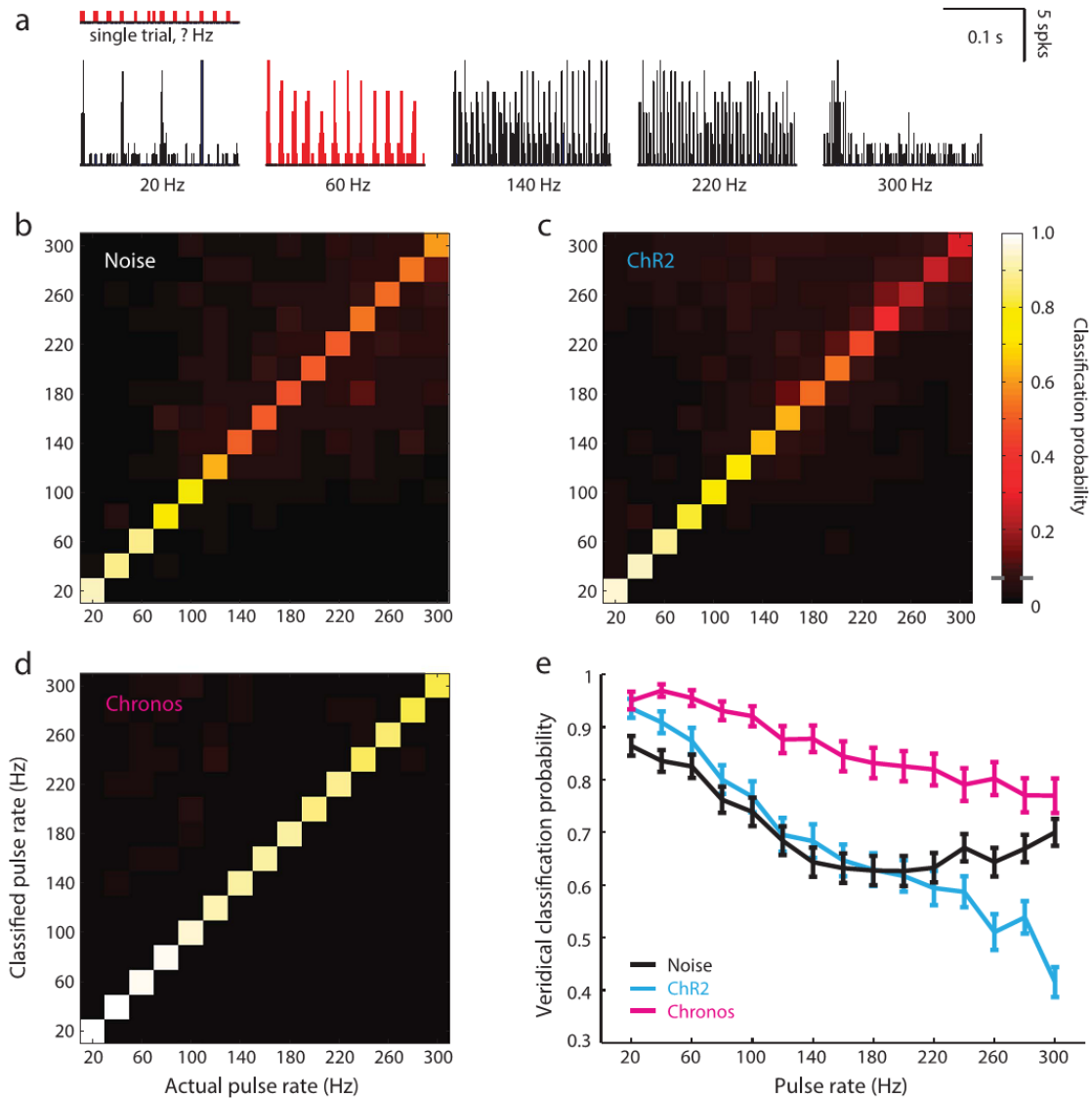
Figure 3. Chronos supports a superior neural code for stimulation rate, as quantified by PSTH-based classifier models. (a) Half the recordings (n=10) were used to develop templates of responses for a range of frequencies (bottom row) and the remaining trials were sorted to the frequency template that they most closely resembled (top row). (b-d) Confusion matrices showing correct and incorrect classifications indicate that Chronos more accurately classifies high pulse rates than ChR2 or NBN. (e) Mean probability of veridical classification decreases with increasing pulse rate for all stimulation modalities but Chronos is consistently superior to ChR2 at high frequencies. Error bars = SEM. Figure from Guo et al. 2015 (52).
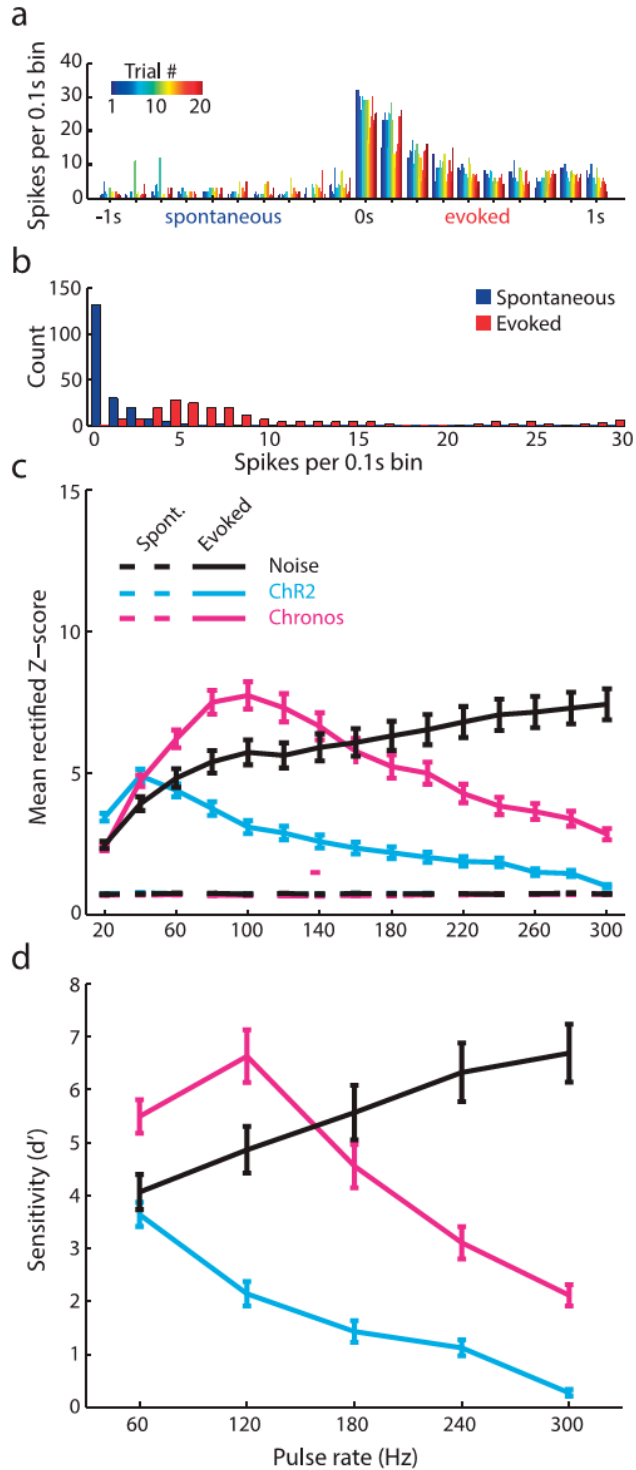
Figure 4. Chronos-mediated neural activity supports a more discriminable code in the murine midbrain. (a) Recorded activity from a 2s period surrounding the onset of photo or acoustic stimulation (at t=0s) was divided into 0.1s bins. (b) The distribution of

spike rates during the spontaneous (t=-1 to 0s) and evoked periods (t=0 to 1s) were derived. (c) Z-scores of neural activity from spontaneous and evoked periods were calculated and taken absolute values as any changes from the baseline firing rate could represent information encoding the incoming stimulation. (d) The difference between spontaneous and evoked z-scores was quantified as a d' metric, a measure of the discriminability of the evoked rate code. Figure from Guo et al. 2015 (52).
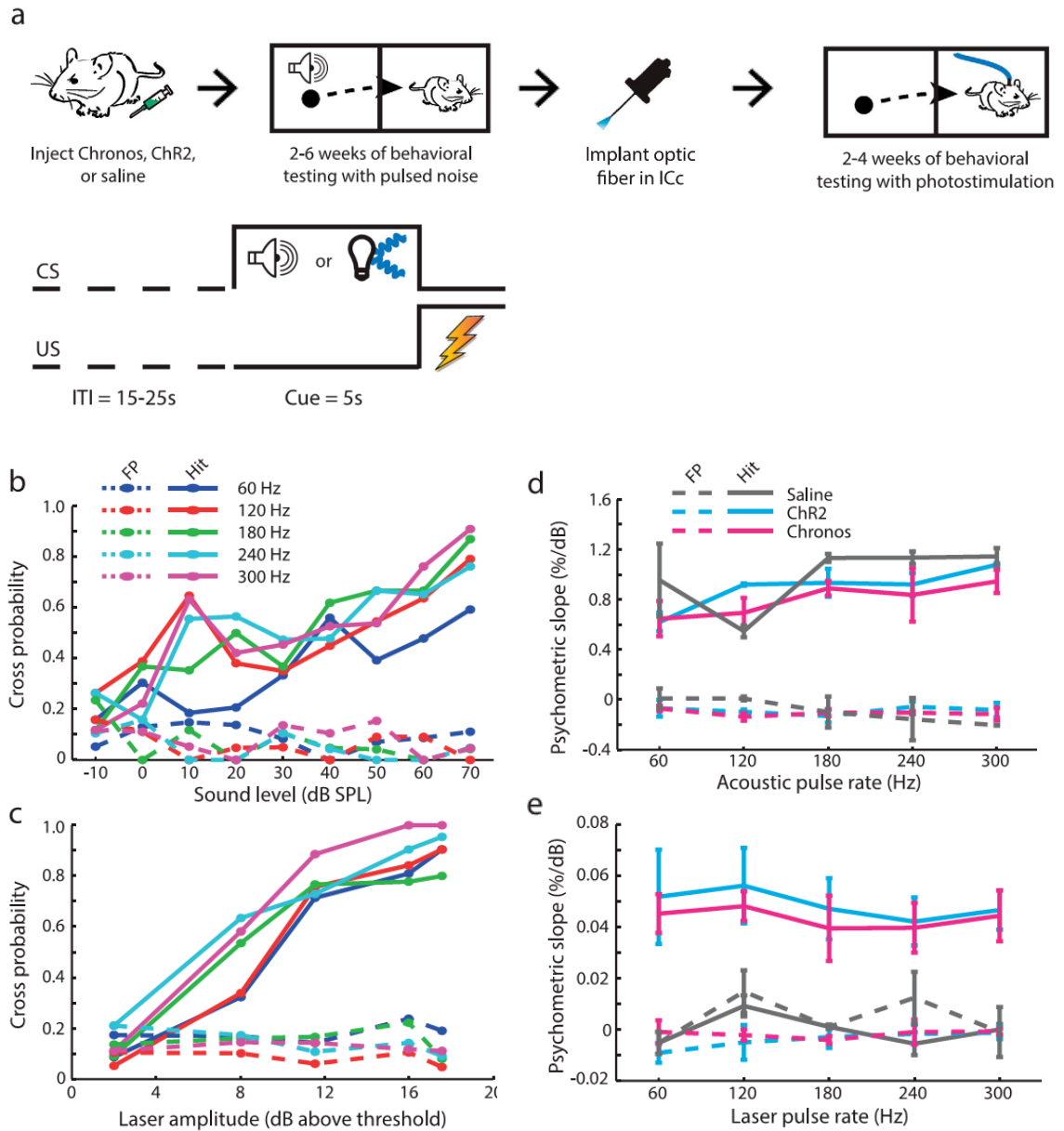
Figure 5. Behavioral detection of photostimulation is not superior in Chronos-expressing midbrains. (a) Animals with Chronos, ChR2 or saline injected into their ICc were trained to perform a behavioral detection task in response to pulsed noise. Animals cross the shuttle box to avoid a foot shock. After successful training, animals were implanted with chronic optical fibers at the site of prior injections and the detection task was repeated with photostimulation. Correct and false positive crossing probabilities are calculated as a function of sound level (b) and laser amplitude (c). The slope of the linear fit of psychometric curves is used as a proxy for pulse detection. Psychometric slopes for ChR2 and Chronos mice are not significantly different across changing rates of acoustic (d) or laser stimulation (e). Figure adapted from Guo et al. (52).

Supplemental Methods

Acute electrophysiology in the auditory cortex

ChR2$^+$ and Chronos$^+$ mice were anesthetized with ketamine and xylazine, and a craniotomy was made over the right auditory cortex. The exposed dura was covered with high viscosity silicon oil. Extracellular recordings of multiunit activity were made with tungsten electrodes (FHC Co.) positioned in the middle cortical layers. Acoustic stimuli were delivered to the contralateral ear via a calibrated in-ear acoustic system. Laser stimuli were delivered through the implanted optic fiber in the ipsilateral ICc. Acoustic and laser stimulus parameters were identical to the approach used for ICc recordings. Since animals at this stage had all completed behavioral training and assessment, the peak amplitude used for acoustic and laser stimulation was set to a suprathreshold level according to the corresponding behavioral from each mouse (60 dB SPL and 12 dB above the laser detection threshold, respectively).
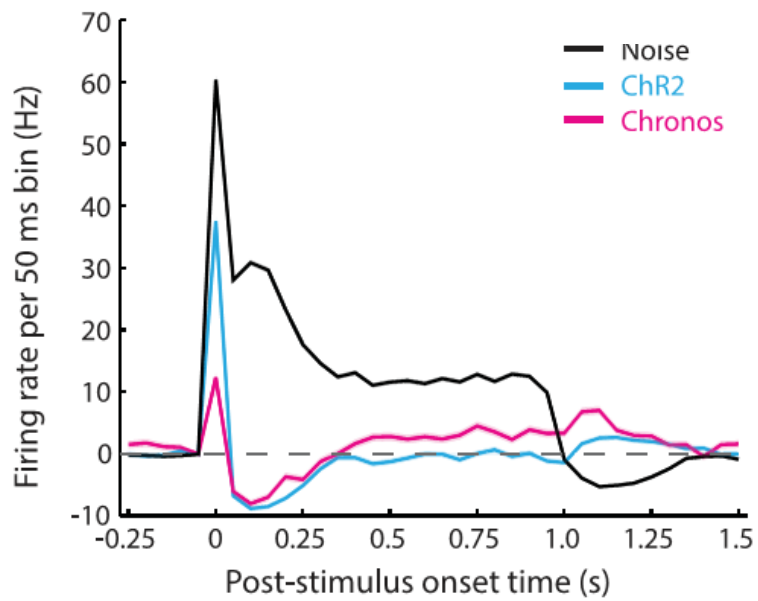
Supplemental Figure A. PSTHs across all pulse rates show that optical stimulation of opsin expressing neurons in the ICc results in brief and quickly suppressed responses at the level of the auditory cortex, as compared with more sustained responses when stimulated by noise. Figure adapted from Guo et al. 2015 (52).