Published in final edited form as:

Brain Res. 2013 May 20; 1511: 138–152. doi:10.1016/j.brainres.2012.10.030.

Assessment of the AAV- mediated expression of channelrhodopsin-2 and halorhodopsin in brainstem neurons mediating auditory signaling

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Abstract

The physiology and circuitry associated with dorsal cochlear nucleus neurons (DCN) have been well described. The ability to remotely manipulate neuronal activity in these neurons would represent a step forward in the ability to understand the specific function of DCN neurons in hearing. Although, optogenetics has been used to study the function of pathways in other systems for several years, in the auditory system only neurons in the auditory cortex have been studied using this technique. Adeno-associated viral vectors with either channelrhodopsin-2 fused with GFP (ChR2-GFP) or halorhodopsin fused with mCherry (HaloR-mCherry), capable of expressing light sensitive cation channels or chloride pumps, respectively, were delivered into the dorsal cochlear nucleus (DCN). One to 18 months later, expression of ChR2 and HaloR was observed throughout the DCN. Rhodopsin distribution within the DCN was determined to be within several cell types identified based on morphology and location within the DCN. Expression of ChR2-GFP and HaloR-mCherry was found at both the injection site as well as in regions receiving projections from the site. Wavelength appropriate optical stimulation *in vivo* resulted in neuronal activity that was significantly increased over pre-stimulation levels with no return to baseline levels during the time of the light exposure. We also examined the effects of optically driven neuronal activity on subsequent tone driven responses in the DCN. In the DCN 75 % of the 16 electrode sites showed decreased neuronal activity in response to a tone immediately following light stimulation while six percent were decreased following tone stimulation and 19% of the electrode sites showed no change. This is in contrast to tone driven neuronal activity prior to the light exposure in which the

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majority of electrode sites showed increased neuronal activity. Our results indicate that expression and activation of rhodopsin within neurons involved in auditory processing does not appear to have deleterious effects on hearing even 18 months following expression. In addition, virally targeted rhodopsins may be useful as tract tracers to delineate as well as modulate the activity of pathways and specific neurons. In the future rhodopsins can be targeted to specific subpopulations auditory neurons. Ultimately, photostimulation may provide a physiologically relevant method for modulating the function of auditory neurons and affecting hearing outcomes.

Keywords

Channelrhodopsin; halorhodopsin; dorsal cochlear nucleus; Chop2; HaloR; auditory; hearing; gene expression; transfection; ABR; auditory brainstem response

Introduction

Microbial rhodopsins have been cloned and when functionally expressed were found to form light-gated cation channels (ChR2) and chloride pumps (HaloR) (Boyden et al., 2005; Han and Boyden, 2007; Nagel et al., 2003; Zhang et al., 2007). Recently, several studies have been able to take advantage of these rhodopsin proteins. Once ChR2 and HaloR are inserted into the cell membrane a neuron can be depolarized or hyperpolarized remotely via specific wavelengths of light (Han and Boyden, 2007; Lima et al., 2009; Zhang et al., 2007). Even ectopic expression of one of these microbial rhodopsins, channelrhodopsin-2 (ChR2) or HaloR, was able to restore visual responses in mice with photoreceptor degeneration (Bi et al., 2006; Zhang et al., 2009).

With the proliferation of cochlear prosthetic devices that are implanted at various levels of the auditory system becoming a routine treatment for profound deafness in both young people and adults, developing the best possible device for implantation as well as providing the best possible platform for the reintroduction of hearing by understanding the effects of implantation and stimulation becomes critical. In the auditory system, restoration of hearing with cochlear prostheses is achieved through spread of electrical currents to neurons that are in direct contact with the implanted electrodes. The spatial sensitivity of this approach is limited by the spread of current. Recent approaches using lasers (Richter et al., 2008; Wenzel et al., 2009) to stimulate auditory neurons are able to activate specific regions of the cochlea thereby controlling the spread of activity providing discrete control of groups of neurons.

In the current study our goals were to determine whether constructs containing ChR2 and HaloR would be expressed across cell types in the dorsal cochlear nucleus (DCN), how long the expression would persist, whether rhodopsin expression was restricted to ascending and descending pathways related to the injection site and whether appropriate wavelengths of light could activate neurons *in vivo*.

In the future, transfection of neurons with ChR2 and HaloR will allow us to take advantage of a non-contact approach for neuronal stimulation and will provide us with the advantage of being able to target specific cell types with designer channelopsins expressed in specific cell

types and activated by different wavelengths of light ultimately resulting in more precise control of the auditory experience.

Results

Both ChR2 and HaloR can be expressed in DCN neurons with no deleterious effects on hearing

In animals (adult male Sprague Dawley rats) expressing either ChR2-GFP or HaloR-mCherry normal hearing thresholds were observed (20 kHz 29 – 31 dB) within one year of the injection (Table 1). In ChR2 animals, hearing thresholds were significantly elevated ($p = 0.05$) in animals 14 – 22 months of age (12 – 18 months following injection).

ChR2-GFP—Within five weeks of stereotaxic injection the expression of ChR2-GFP was observed in all layers of the DCN, with the heaviest fluorescence observed in DCN2 and DCN3 (Figure 1). Even 18 months after the injection, robust ChR2-GFP labeling was observed, identical to that found five weeks after injection. Many of the GFP expressing cells also labeled with the neuronal markers MAP2 and TUJ1 (Figure 2). Fluorescence for GFP was observed throughout the neurons, including somata, dendrites and axon terminals (Figures 1 and 2) with heavier labeling in cell membranes as well as in dendritic spines and axonal varicosities (See Figure 1F arrows). Labeling was not restricted to a specific type of neuron or neurotransmitter class. Labeling was observed throughout the neuropil across all layers of the DCN.

HaloR-mCherry—Just as found with ChR2-GFP, expression of HaloR-mCherry was observed in all layers of the DCN (Figure 3) with the strongest fluorescence observed in the fusiform (layer two) and the deep core (layer three) layers. Many different cell types within the DCN were capable of expressing the HaloR-mCherry fusion protein driven by the CMV promoter. Somata, dendrites and axons show a more diffuse labeling when compared to the expression of ChR2 (compare Figure 3 to Figure 1).

The rAAV2 incorporating ChR2-GFP and HaloR-mCherry constructs can trace neuronal pathways

We have determined that other regions of the auditory brainstem exhibit ChR2-GFP and HaloR-mCherry expression five weeks following a pressure injection of ChR2-GFP or HaloR-mCherry into the DCN. Both ChR2-GFP (Figure 4A-C) and HaloR-mCherry (Figure 4D-F) expression was observed in the contralateral IC, most robustly in the central nucleus. As expected, the expression observed in the IC was primarily localized to terminals (Figure 4C and 4F) presumably associated with somata located in the DCN. When the injection site included part of the ventral cochlear nucleus (VCN) (Figure 1A) labeling was observed in the ipsilateral superior olivary complex (SOC; Figure 5). The terminals were observed in the medial nucleus of the trapezoid body (MNTB) and the middle limb of the lateral superior olive (LSO). When the injection site contained little to no VCN, very little labeling was observed in the SOC (Figure 5C-D). The injection site often included a small region of the cerebellum, the interpositus nucleus (Int). The Int sends and receives projections from the inferior olive (IO). GFP labeled somata were observed in the Int (Figure 6A-D) while

terminals were found in the IO (Figure 6E-F) the downstream target of Int neurons. Different cell types could be identified depending upon the region of the Int observed. In the IntA (Figure 6C) cells with small somata ($\sim 10 \mu\text{m}$ diameter) and a single dendrite expressed ChR2-GFP, while cells in the IntDL (Figure 6D) expressing ChR2-GFP had larger somata ($\sim 20 \mu\text{m}$ diameter) with multiple dendrites.

Rhodopsin mediated neuronal activity in DCN neurons

Following exposure of the DCN and removal of the surrounding cerebellum, a probe with either five or 16 sites was lowered $500 \mu\text{m}$ below the surface (Figure 7A-C). In the case of the five channel probe, all of the sites were active (Figure 7C) with only one site each contained in the molecular (DCN-M) and fusiform (DCN-F) layers and at least two sites in the deep core layer (DCN-DC). Each site on the five channel probe had an area of $4000 \mu\text{m}^2$ separated by $120 \mu\text{m}$ (Figure 7A). For the 16 channel probe each site was $413 \mu\text{m}^2$ with $50 \mu\text{m}$ between each site (Figure 7B). The 16 channel probe contained only seven active sites. While ten sites were positioned within the DCN only six were active (Figure 7C). Of the six active sites within the DCN two were in the DCN-F and four were in the DCN-DC. No active sites were in the DCN-M. These five and 16 channel electrodes (Figure 7) were used for most of the experiments assessing optical activation of DCN neurons (Figure 8). In addition, 16 channel electrodes with the same configuration, but for which all of the recording sites were active were used in experiments where tone activation of DCN neurons was compared to optical activation of neurons (Figures 9 – 10).

ChR2—ChR2 is known to have peak wavelength sensitivity around 455 nm (Nagel et al., 2003). In control animals with no ChR2-GFP injection, regardless of whether the LED emitted light at wavelengths of 455 nm (Figure 8A) or 590 nm (Figure 8B), no changes in neuronal activity were observed in the DCN when comparing light on (10, 5 and 20 seconds X 3) vs light off conditions (30 seconds per trial). However, in animals with viral delivery of ChR2-GFP constructs to the DCN, exposure of neurons to 455 nm of light resulted in more neuronal activity corresponding to the time (10, 5, and 20 seconds) of the light exposure as compared to when the light was off (Figure 8C). We did not detect changes in multiunit activity when the same neurons in animals with viral delivery of ChR2-GFP constructs were exposed to 590 nm of light (Figure 8D). When comparing the light on condition with the light off condition during exposure to blue light (Figure 8E) vs amber light (Figure 8F), control animals sometimes showed small, but significant increases in standard deviation (SD; 0.168 ± 0.004 off 0.174 ± 0.007 on; $p = 0.035$) during stimulation with 455 nm light. However, the ChR2-GFP injected animals reliably resulted in robust significant increases in standard deviation (0.185 ± 0.002 off 0.389 ± 0.022 on; $p = 3.8 \times 10^{-9}$) when the blue light was “on” (Figure 8E) suggesting that repeated exposure to wavelengths of 455 nm of light resulted in corresponding increases in multiunit activity. During 590 nm light exposure (Figure 8F), neither controls (0.288 ± 0.005 off 0.389 ± 0.286 on; $p = 0.466$) nor ChR2-GFP injected animals (0.158 ± 0.002 off 0.163 ± 0.002 on; $p = 0.186$) demonstrated a significant difference in standard deviation when the amber light was “on” (Figure 8F). Regardless of whether ChR2-GFP transfected cells were activated for 5, 10 or 20 seconds, no diminution in signal was observed during a trial (Figure 8C).

ChR2 activation, but not expression alone affects tone driven activity in DCN neurons

Three weeks following viral delivery of ChR2 to neurons of the medial DCN the effects of light stimulation on both neuronal activation as a consequence of electrode position (medial vs lateral) and electrode channel location (dorsal vs ventral) as well as tonal activation were observed (Figures 9 and 10). When examining neuronal activity in a region closest to the injection of ChR2 (medial), each of the 16 electrode channels showed a response to the sound stimulation prior to light activation (Figure 9A). During exposure to a 455 nm wavelength light the majority of dorsal channels indicated that neuronal activity continued to increase as long as the light was on. However, the more ventral electrode channels showed that neurons were quickly activated at the onset of light exposure and maintained the maximum level of activation for the duration of the light exposure, ~ 1 minute (Figure 9B). Immediately following the light stimulation, a second noise exposure was performed and the majority of the 16 channels (75%) showed decreased responses when compared to pre- light stimulation levels 19% of the channels showed no change and only 6% of channels showed increased neuronal responses (Figure 9C). When the electrode was removed and placed in a more lateral region of the DCN, sound exposure again resulted in activity for all 16 channels (Figure 10A). Light stimulation in the more lateral location resulted in a pattern similar to that observed in the medial location with a distinction in the response of neurons located dorsally compared to those located ventrally. However, the response was much less robust (Figure 10B). Immediately following the light stimulus the animals were exposed to a broadband noise. Less than half (44%) of the channels in the lateral DCN showed increased responses to the sound stimulus, 44% of channels showed no change and 12 % of channels showed decreased responses (Figure 10C).

Discussion

Advantages and caveats of optogenetic tools for use in auditory brainstem neurons

Selection of an appropriate viral vector for delivery to neurons in DCN—

Although optogenetics has been used now for nearly a decade, to date, no published reports of expression and activation have been reported in auditory brainstem neurons. This may be due in part to unique properties exhibited by neurons in the central auditory pathway. For example, selection of a vector that allows delivery, transduction and function across cell types is important for proof of concept studies. We report that the adeno-associated virus 2 (AAV2) as a viral vector for delivery of rhodopsins to DCN neurons offers several advantages. Robust expression of ChR2-GFP and HaloRmCherry at the injection site depends on high copy numbers of the genes. The ability of AAV to result in high titers and the straightforward manner with which large numbers of neurons can be infected make the virus a good candidate for use. Although AAV has been used previously to deliver rhodopsins to neurons in the primary auditory cortex (Lima et al., 2009), eleven AAV serotypes have been reported. Serotypes for AAV differ broadly in transduction efficacies and tissue tropisms that may result in preferential targeting of specific cell types. The targeting specificity of a virus for a particular neuronal population can be determined by the ability of expressed features on the viral membrane to interact with receptors present on the neurons (Burger et al., 2004; Nathanson et al., 2009a) as well as by cell-type-specific promoters used to drive expression of the transgene (Brenner et al., 1994; Mayford et al.,

1996; Blomer et al., 1997; Jakobsson et al., 2003; Dittgen et al., 2004; Nathanson et al., 2009a). However, few cell-type-specific promoter fragments are small enough to be packaged with AAV2. In previous studies, these viral tropisms have lead to infection of only select neurons e.g., inhibitory neurons (Nathanson et al., 2009b). Based upon morphology, location and known characteristics of the neurons we examined, the viral construct used in the current study appeared to result in expression of ChR2-GFP and HaloRmCherry in both excitatory and inhibitory brainstem neurons. Serotype differences have also resulted in differential spread from the injection site. In rodents, diffusion of the AAV2 serotype has been compared to other serotypes when microliter volumes were injected into the CNS (Burger et al., 2004 and Paterna et al., 2004). Injection of AAV2 resulted in restricted expression patterns within the CNS when compared to other serotypes. In the current study, expression of ChR2-GFP and HaloR-mCherry was restricted to the injection site and was apparent throughout the extent of the infected cells. This could be useful when studying small cells, such as those in the molecular layer of the DCN, where stimulating and/or recording may be challenging. Optogenetics could provide a method by which functional information could be gleaned by exploiting the small spread of the virus via AAV2 infection in combination with light activation of these small neurons both at their origin in the DCN and their targets.

Cytotoxicity—Previously, high expression of transmembrane proteins such as ChR2-GFP and HaloR-mCherry has been reported to result in cytotoxicity due to endoplasmic reticulum (ER) stress (Kaufman and Zhang, 2006; reviewed in Schoenenberger et al., 2010). Often infected cells demonstrate aggregates of opsin proteins in the ER, particularly HaloR. These aggregates resulted in cells that were not responsive to optical stimulation and often cell death. These issues have been addressed by engineering transcripts containing ER exiting motifs. However, no indicators of cytotoxic stress were observed in DCN neurons expressing ChR2-GFP and HaloRmCherry even following delivery of high titers, robust expression and prolonged expression times.

Effects of expression on hearing—Using AAV2, we have genetically altered neurons in the DCN to express ChR2-GFP and HaloR-mCherry. We demonstrate that, neither ChR2-GFP nor HaloR-mCherry expression results in compromised hearing function in young adult animals. Animals that were older than one year showed a significant threshold shift one year following transfection when compared to pre-transfection hearing thresholds. The increase in hearing thresholds may be as a result of normal aging, but may be due, in part to expression of rhodopsins. Further detailed studies would be needed to test the interaction between age and rhodopsin expression. In the case of neurons expressing ChR2-GFP, tones were able to drive neuronal activity even following optical activation. These results suggest that neither the expression nor the activation of rhodopsins have deleterious effects on primary auditory function. However, future in depth studies exploring the effects of rhodopsin expression on the precise function of specific auditory neurons would be of great benefit.

Distribution of rhodopsin expression in DCN neurons—We have demonstrated that viral delivery of ChR2-GFP into the DCN results in expression within somata and

terminals within all layers of the DCN. We observed many DCN neurons with high levels of expression beginning three weeks after transfection (the earliest time point we examined) as well as after 18 months following introduction of the viral constructs. Expression of ChR2-GFP and HaloR-mCherry was identified across the layers of the DCN, likely within fusiform, giant, tuberculoventral and cartwheel neurons. Each of these neurons has previously been identified and associated with specific layers of the DCN. Our results are similar to those recently reported where ChR2 was virally targeted to neurons in the retina and auditory cortex (Ivanova and Pan, 2009; Lima et al., 2009). The results of our immunocytochemistry with MAP2 and TUJ1 support our hypothesis that the majority of the ChR2-labeled cells in the DCN following viral targeting are neurons, suggesting that DCN neurons contain the genetic machinery necessary to express and produce ChR2-GFP under control of the CMV promoter. In addition, we demonstrate results from the virally-targeted expression of light activated chloride pumps (HaloR) in DCN neurons. Like ChR2-GFP, expression of HaloR-mCherry was observed in neurons within each layer of the DCN. These studies suggest that both excitatory and inhibitory neurons in the DCN are capable of expressing ChR2-GFP and HaloR-mCherry. For both ChR2 and HaloR expression was observed even eighteen months following injections targeted to the DCN. While several studies have reported long term expression of ChR2 and HaloR (Han and Boyden, 2007; Bi et al., 2006; Ivanova and Pan, 2009) the current results indicate that cell tropisms do not impair long-term AAV2 mediated rhodopsin expression in DCN neurons.

Virus mediated transfection with rhodopsins can be used as a tract tracer—

Gene transfer of ChR2-GFP and HaloR-mCherry resulted in labeling of neuronal somata and terminals. This ability of ChR2-GFP and HaloR-mCherry to be expressed throughout the neuron allowed us to identify projections of DCN and cerebellar neurons. Unlike the herpes simplex virus (HSV) that has been used to mediate gene transfer of ChR2 in the auditory cortex (Lima et al., 2009) the AAV2 that was used in the current study does not cross synapses. Therefore, a neuron that produces ChR2-GFP had direct contact with the virus and may be identified in regions that have known direct connections with the injected area such as the DCN. However, as we demonstrated when the injection site included other regions (PVCN or cerebellum see Figure 1) labeling was observed in regions that received projections from the injection area. In the current study the tracer aspect of AAV2 appeared to be primarily anterograde in nature as has been noted previously in the brain (Chamberlin et al., 1998). These results suggest that viral delivery of ChR2 may provide a useful physiological tool for tract tracing and recording neuronal activity within specific auditory pathways.

Optical stimulation of DCN neurons—Timing—Using light with a wavelength compatible with ChR2 activation, responses were evoked and recorded from DCN neurons. However, the responses were dependent upon the light intensity with little depolarization observed with lower intensity light. The lack of responses observed when using amber light suggests that the observed neuronal activity was ChR2 specific and that factors such as heat from the LED or current changes from LED pulses do not contribute to the responses. The fact that ChR2 responses in DCN neurons increase immediately after the onset of light stimulation, is sustained for the duration of the light exposure and returns to background

levels immediately after light stimulation is stopped, suggests that ChR2 stimulation may allow precise, reliable photo-activation of DCN neurons.

Optical stimulation of DCN neurons—Location—Light stimulation of ChR2 transfected neurons in the DCN evoked different neuronal responses that were dependent upon the location of the neurons that were being stimulated within the DCN and the ability of the light to reach the neurons. Previously, illumination of ChR2 expressing neurons with 473 nm light at a power of 1–5 mW/mm² was sufficient to elicit action potentials (Boyden et al., 2005). Similar to studies in the auditory cortex (Lima et al., 2009), in the current study the light was positioned just above the surface of the brain. When the light power density was measured at the surface of the brain the intensity was ~ 39 mW which is more than twice the 17 mW reported in the auditory cortex (Lima et al., 2009) and that reported in studies of the retina (Ivanova and Pan, 2009). Based on previous studies of the transmission of light in brain tissue (Aravanis et al., 2007), the propagation of light in the current study may have allowed direct activation of neurons up to depths of 0.4 – 0.5 mm. This would comprise a column of neurons in a 0.7mm² area (the size of the spot of light on the brain surface) that included the molecular, fusiform and part of the deep layer of the DCN.

We also observed changes in the response of neurons to tone driven activity following ChR2 activation. The photo-activation dependent changes in tone driven neuronal responses were also dependent upon the recording site within the DCN, with recording sites lateral to the injection site exhibiting less robust responses. These data stress the importance of understanding placement and the spread of light used to activate ChR2 transfected neurons.

Optical stimulation of DCN neurons—Frequency—The auditory brainstem comprises neurons that are characterized as “fast-spiking” in nature. Several reports demonstrate that wild-type ChR2 is not precise above 40 Hz, resulting in spike doublets and failure to evoke spikes during sustained high-frequency light pulse trains (Berndt et al., 2011 and Freund 2003). We were able to demonstrate spiking activity ranging from 40–50 Hz. However, we did not demonstrate the precision of the spikes for specific auditory neurons. In the auditory system the reduced ChR2 capacity in spike frequency could be a limitation. In the future, variants of the opsins, ChETAs (ChR E123T/A) or Ca²⁺-permeable channelrhodopsin CatCh that address this issue with high frequency stimulation (Gunaydin et al., 2010; Kleinlogel et al., 2011) can be targeted to specific brainstem neurons.

Alternatively, variants of ChR2 and HaloR that demonstrate fast spiking may not be necessary. Although sustained spike frequency in the DCN generally remains below 100 Hz (Rhode and Greenberg, 1992), higher firing rates have been observed during transient responses. However, these transient responses are generally associated with complex spiking or spike bursts elicited by brief stimuli designed to evoke individual responses in isolation. Despite the limitation of frequency response, wild-type ChR2 could provide a sufficient initial stimulus, similar to electrical stimulation, to evoke these transient, high-frequency spike bursts.

There are several methods that can be used to target ChR2 and HaloR to specific neurons for optical control of neuronal activity (Kravitz et al., 2010; Lima et al., 2000). There are many

established Cre-mouse models. When these models are used in combination with floxed ChR2 or floxed HaloR the rhodopsins can be preferentially activated in neurons of interest. In the future, a similar approach could be used in the auditory system. Cell type-specific promoters could be used to target ChR2 and HaloR to specific neuronal cell types such as the recently reported vGluT3 labeled neurons in the CN (Fyk-Kolodziej et al., 2011) or dopaminergic neurons that have been previously identified in the inferior colliculus (Tong et al., 2005). However, this Cre approach currently is not appropriate for translation to the clinic. For this purpose, specific promoters combined with appropriate virus serotypes would be a more likely approach for translational research. In general, the strategy of photo-stimulation of auditory neurons offers the ability to avoid neuronal damage by using light stimulation instead of electrical current, more precisely target specific groups of neurons for repetitive excitation and/or inhibition of these neurons on a timescale that is physiologically relevant.

Methods

Ethics Statement

All procedures were approved by the Wayne State School of Medicine “Institutional Animal Care and Use Committee (IACUC)” and conform to the National Institutes of Health guidelines.

Animals

A total of 21 normal hearing adult male Sprague Dawley rats were used for these studies. The brains from four different groups were examined. Control animals (no transfection) were tested for responses to optical stimulation of DCN neurons with blue and amber light (n = 6). In addition, extracellular recordings were performed in animals injected with either ChR2-GFP (n = 6) or HaloR-mCherry (n = 3). Histological analysis was also performed in animals in which no extracellular recordings were performed, but injected with either ChR2-GFP or HaloR-mCherry (n = 6).

Auditory brainstem responses (ABRs)

To determine the hearing status of animals prior to surgery and to assess the effect of adenovirus injection into the DCN on hearing thresholds, we recorded auditory brain stem responses (ABRs). Just prior to ABR testing animals were anesthetized with xylazine (8 mg/kg) and ketamine (75 mg/kg) and placed in a sound proof booth (Hamilton-Kinder, Poway, CA). Three frequencies (4, 12, and 20 kHz) were tested and recorded using a small speaker placed directly into the ear canal (Beyer sound source and Pyramid PA 600x stereo power amplifier). The active electrode, reference needle and ground were inserted at the vertex (top of the head), below the recording ear, and the contralateral ear, respectively. The sound stimulus consisted of a 15-msec tone burst, with a rise–fall time of 1 msec. Waveforms were visualized and recorded using the Data Acquisition and Real-Time Analysis (Daqarta 4.0) software package (Interstellar Research, Ann Arbor, MI). The sound stimulus was started at 80 dB SPL and, in hearing group, was decreased in 10 or 15 dB steps until near threshold, then in 5 dB increments until threshold could be determined. For each

SPL, up to 1024 responses were averaged. In deafened animals the loudest stimulus tested was 100 dB.

Stereotaxic delivery of rhodopsins

Sprague Dawley rats were anesthetized with a mixture of xylazine (8 mg/kg) and ketamine (75 mg/kg) by intramuscular injection and body temperature was maintained with a water re-circulation heating pad. Using stereotaxic coordinates consistent with Paxinos and Watson (AP 11.6 mm relative to bregma, ML 3.2 mm from midline and DV 4.9 mm from pia) injections were made into the caudal DCN. Adeno-associated virus 2 (AAV2) with either a GFP (ChR2) or mCherry (Halo-rhodopsin) tag was injected bilaterally (1 μ l) using a Hamilton Syringe with 33-gauge needle ($\sim 1 \times 10^{10}$ or 6×10^{12} GP/ml for ChR2 and $\sim 1 - 9 \times 10^{11}$ GP/ml for HaloR). Following the injection the needle remained in place for five minutes before removal. The viral constructs used in this study have been previously described (Bi et al., 2006; Ivanova and Pan, 2009; Zhang et al., 2009).

Histology

Tissue from the brain was collected from 15 animals three weeks to 18 months after injection (ChR2 n = 11; HaloR n = 4). Animals were transcardially perfused with 9.25 % sucrose followed by 4% paraformaldehyde in 0.1 M PBS (pH 6.8). Brains were post-fixed in the same paraformaldehyde solution for 1 hr at room temperature and then placed in 30% sucrose for 24–48 hrs at 4°C. The fixed and cryo-protected tissues were cut with a cryostat into 30 – 40 μ m sections and stored on glass slides at –80°C. Sections from auditory (DCN, VCN, SOC and IC) as well as extra-auditory (cerebellum and inferior olive) regions were examined for ChR2-GFP or HaloRmCherry fluorescence.

Immunocytochemistry

In order to determine whether ChR2 and HaloR expression was neuronal, GFP and mCherry were examined for co-localization with the neuronal markers MAP2 and TUJ1. Sections through the DCN were incubated with a mixture of primary antibodies; mouse anti-MAP-2 (microtubule associated protein-2, cat. #MAB3418, Chemicon, Temecula, CA; 1:200) and mouse anti-TUJ1 (class III beta-tubulin, cat. #MAB1637, Chemicon; 1:200). After 24 hours at room temperature in a dark humid chamber, the sections were rinsed and the tissue incubated with a fluorophore conjugated secondary antibody (Cy3 donkey anti-mouse; 1:500 Jackson ImmunoResearch, West Grove, PA).

All images were visualized and captured with a Leica Microscope (DM 5000B) equipped with appropriate fluorescence filters, a Cool Snap EZ monochrome camera and Elements software (Photometrics Ann Arbor, MI). Figures were compiled using Adobe Photoshop software to combine, size and adjust the brightness and contrast of selected images.

Photostimulation of DCN neurons

Following xylazine (8 mg/kg) and ketamine (75 mg/kg) anesthesia, the occipital bone was exposed and removed just above the caudal DCN. A small portion of the cerebellum covering the surface of the DCN was carefully removed via suction until the DCN was

clearly visible. All surgical procedures were performed under sterile conditions and body temperature was monitored and maintained at normal temperatures with a heating pad.

Field potential responses were monitored with either a single shank five or a 16 channel electrode (NeuroNexus Technologies, Ann Arbor MI and a gift from Univ. of Michigan KHRI). Each channel was separated by 120 or 50 μm respectively. The probes were visually placed and guided into the DCN using a stereotaxic frame to lower the electrode 500 μm below the surface. A grounding electrode was placed just under the skin near the scapula.

For light activation of ChR2-GFP neurons, an LED was placed 2 cm above the DCN, which had been surgically exposed. A royal blue light-emitting diode (LED) with a spectrum ranging from 400–460 nm and a peak of 455 nm (LXHL-LR5C, Luxeon Brantford, Ontario, Canada) was used for activation of ChR2 (455 nm; ChR2 optimal wavelength) while an amber LED with a spectral range of 585–597 nm and a peak spectrum of 590 nm (LXHLL3C, Luxeon Brantford) was used for activation of HaloR. An intensity of 8.96×10^{16} photons/cm²/s at the surface of the DCN and a spot size of 0.7 mm² were the parameters used for optical stimulation using the Royal Blue LED (7.0 v). For the Amber LED (2.9 v) the parameters included an intensity of 1.01×10^{16} photons/cm²/s at the surface of the DCN and a spot size of 0.7 mm². Two conditions were tested in each group, flashing light stimulation and continuous light stimulation.

In the case of flashing light stimulation the light pulses were generated by a relay board (WINFORD RLY102) and controlled by an oscilloscope with a trigger (LG Precision Co. OS-5020G). Each LED emitted light at a rate of 5 cycles/s (1 cycle = light on 100 ms + light off 100 ms). Three trials lasting 5, 10, and 20 seconds were repeated 3 times each for each LED. Between each trial there was a 30 second recovery time without any light stimulation. For the continuous light stimulation the selected LED emitted a continuous pulse of light during the entire 5, 10, or 20 second trial. As in the flashing stimulation case, for continuous stimulation a 30 second recovery time without any light stimulation was implemented between each trial with a total of three trials.

In one case tones were presented to establish both the ability of the DCN to respond to a stimulus following surgical exposure as well as the effect of DCN activation by light on subsequent sound activation. The noise stimulus was a gaussian broadband noise from 0.10 kHz to 25 kHz. The noise was presented as 200 ms bursts (2/s) for 50 seconds before and after the light exposure. The noise was presented immediately after the light.

The evoked signals were amplified using a pre-amplifier and a MICRO 1401 amplifier with data recorded using Spike2 Software (v 4.20).

Acknowledgments

Contract grant sponsor: Ralph Wilson Foundation (to A.G.H), Capita Foundation (to A.G.H)

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Figure 1.

Channel rhodopsin (ChR2) can be expressed in DCN neurons. For transfection of DCN neurons a rAAV2-CAG-Chop2-GFP-WPRE expression cassette was used (A). Following a single injection of AAV2 containing the ChR2-GFP construct, labeling was observed throughout the DCN (B-C). Small cells were identified in DCN1 (D), while several cell types were labeled in DCN2 and DCN3 (D - G). Arrow in D indicates large cell oriented horizontally (enlarged in F) and arrow in E indicates cell with dendritic tuft (enlarged in G). Arrows in F point to spines on dendrites, perhaps indicating the location of synaptic inputs.

Scale bar in C also valid for B and scale bar in E is also valid for D. BGHpA - a bovine growth hormone polyadenylation sequence; CAG - a hybrid CMV enhancer/chicken β -actin promoter; Cb - cerebellum; DCN - dorsal cochlear nucleus; PVCN - posteroventral cochlear nucleus; icp - inferior cerebellar peduncle; sp5 - spinal trigeminal tract; DCN1 - molecular layer of the DCN; DCN2 - fusiform layer of the DCN; DCN3 - deep layer of DCN; ITR - inverted terminal repeat; WPRE - woodchuck post transcriptional regulatory element.

Figure 2.

Channel rhodopsin (ChR2) is expressed in neurons. When ChR2 labeling (**A**) is combined with immunocytochemistry for the neuronal markers MAP2 and TUJ1 (**B**), double labeling of neurons can be observed in the dorsal cochlear nucleus (**C**).

Figure 3.

Halo-rhodopsin (HaloR) can be expressed in DCN neurons. Following a single injection of AAV2 containing the HaloR-mCherry construct containing the rAAV-CMV-HaloRmCherry-WPRE expression cassette (A), labeling was observed throughout the DCN (B-C). Small cells were identified in DCN1 (D), while several cell types were labeled in DCN2 and DCN3 (D - G). Box in D indicates labeling in DCN2 (enlarged in E) and box in F indicates labeling in DCN3 (enlarged in G). Arrow in E points to a cell oriented horizontally in the DCN and arrow in G indicates a large cell in DCN3. Scale bar in C also

valid for B. BGHpA - bovine growth hormone polyadenylation sequence; Cb – cerebellum; CMV – cytomegalovirus promoter; DCN – dorsal cochlear nucleus; PVCN – posteroventral cochlear nucleus; icp – inferior cerebellar peduncle; DCN1 – molecular layer of the DCN; DCN2 – fusiform layer of the DCN; DCN3 – deep layer of DCN; ITR - inverted terminal repeat; WPRE - woodchuck post transcriptional regulatory element.

Figure 4. Channel rhodopsin (ChR2) and Halo-rhodopsin (HaloR) can be expressed throughout DCN neurons. Following an injection of AAV2 containing either the ChR2-GFP (green) or HaloR-mCherry (red) construct, labeling was observed in the inferior colliculus (**A-F**). For both ChR2 (**A-C**) and HaloR (**D-F**) labeling was primarily observed in terminals located within the CNIC (**B** and **E**) with sparse labeling in the DCIC (**C** and **F**).

Figure 5.

Channel rhodopsin (ChR2) and Halo-rhodopsin (HaloR) expression is faithful. Following an injection of AAV2 containing the ChR2-GFP (green) construct into the DCN that included labeling of PVCN (see figure 1B) labeling was observed in terminals throughout the superior olivary complex (**A-C**). When the injection of AAV2 containing the HaloR-mCherry (red) construct was confined entirely within the DCN, labeling for HaloR was not observed in the SOC (**D-F**). LSO – lateral superior olive; MNTB – medial nucleus of the trapezoid body; MSO – medial superior olive; LNTB – lateral nucleus of the trapezoid body; SPON – superior paraolivary nucleus; VNTB – Ventral nucleus of the trapezoid body; 7n – 7th Cranial Nerve (facial nerve); ILSO – lateral, LSO; mLSO – medial LSO; Scale bar in B also valid for A. Scale bar in E also valid for D.

Figure 6.

Expression of channel rhodopsin (ChR2-GFP) can be used to trace neuronal pathways.

Injection of the interpositus nucleus of the cerebellum (**A-B**) labels somata and dendrites in both the IntA (**C**) and the IntDL (**D**), both of which send projections to the IO (**E-F**). Box in **B** indicates region labeled in **A**. IntDM – dorsal medial interpositus nucleus; IntDL – dorsal lateral interpositus nucleus; IntA – anterior interpositus nucleus; IntDL – dorsal lateral interpositus nucleus; IO – inferior olive; py – pyramid

Figure 7.

Two different recording electrodes were used to assess optical activation of ChR2 expressing neurons. The five channel probe contained five 4000 μm² recording sites spaced 120 μm apart (A). While the 16 channel probe contained sixteen 413 μm² recording sites spaced 50 μm apart (B). For the five channel probe, active sites spanned the entire DCN (C). However, for the 16 channel probe active sites were only contained within the fusiform and deep core layers of the DCN (C). DCN-M – molecular layer of dorsal cochlear nucleus; DCN-F – fusiform layer of dorsal cochlear nucleus; DCN-M – deep core layer of dorsal cochlear nucleus

Figure 8.

Optical stimulation of DCN neurons transfected with ChR2. In animals that did not receive an injection of AAV2 ChR2-GFP (control), no neuronal activation was observed during exposure (horizontal lines) to blue (455 nm) light (**A**) or amber (590 nm) light (**B**). However, animals injected with AAV2 ChR2-GFP showed increased neuronal activity during trials when exposed to 455 nm light for 10, 5, or 20 seconds (**C**). However, no change in activity was observed when exposed to amber (590 nm) light (**D**). Standard deviation (SD) was compared when the blue (455 nm) light was on versus when the light was off in both control

and ChR2 injected animals (**E**) with the most robust increase in SD observed in the ChR2 injected animals when the blue light was on. Changes in SD were also compared when the amber (590 nm) light was on versus off in control and ChR2 injected animals (**F**). Horizontal lines in each panel denote times during which the light was on. Blue - exposure to 455 nm light; Amber – exposure to 590 nm light

Figure 9.

Tone driven activity in the medial DCN pre- and post- light stimulus. A 16 channel single shank Michigan Probe was used to record sound driven neuronal activity in a medial location (region closest to AAV2 ChR2-GFP injection site) of the CN (A). Sound stimulation was followed by a 455 nm light stimulus (B). When the light was on (left most arrow) six of the eight dorsal channels indicated gradual increases in neuronal activity with four channels showing a slow reduction in activity once the light was off (right arrow). The most ventral channels reflected a sharp increase in activity that was maintained during light stimulation and immediate decreases to pre-stimulus levels once the light was off (right arrow). Following the light stimulus the sound was played again (same as in A) and the majority of channels showed inhibition or no change in activity except for channel 9 which showed increased activity (C) compared to pre-light levels of neuronal activity (A). Channels are ordered from most dorsal (Ch 9) to most ventral (Ch 6). left arrow – initiation of light stimulus; right arrow – cessation of light stimulus

Figure 10.

Sound driven activity in the lateral DCN pre- and post- light stimulus. A 16 channel single shank Michigan Probe was used to record sound driven neuronal activity in a lateral location (region just lateral to AAV2 ChR2-GFP injection site) of the CN (**A**). Sound stimulation was followed by a 455 nm light stimulus (**B**). When the light was on (left most arrow) the majority of dorsal channels demonstrated a weak gradual increases in neuronal activity with all channels showing a sharp reduction in activity once the light was off (right arrow). Four of the most ventral channels demonstrated a very modest increase in activity that was maintained during light stimulation with an immediate decrease to pre-stimulus levels once the light was off (right arrow). Following the light stimulus the sound stimulus was played again and the majority of channels showed no change in activity (**C**) compared to pre-light levels of neuronal activity (**A**). Channels are ordered from most dorsal (Ch 9) to most ventral (Ch 6). left arrow – initiation of light stimulus; right arrow – cessation of light stimulus

Table 1
Summary of hearing thresholds following injections with Chr2 or HaloR constructs; Auditory brainstem responses

Time	Before and After Chr2 Injection			Before and After HaloR Injection		
	Pre	3 ^d	p value ^a	12 – 18 ^d	p value ^a	3 – 11 ^d p value ^a
Frequency						
kHz ^{b, c}	29±1.72	30±2.48	0.60	34±3.87*	0.02	33±2.08 0.49
2 kHz ^{b, c}	28±1.72	29±1.64	0.51	37±7.32*	0.01	31±2.08 0.67
0 kHz ^{b, c}	30±1.21	29±2.14	0.16	38±7.24*	0.03	31±1.89 0.63

^aSignificance p 0.05
^bHearing threshold, expressed in decibels (dB)
^cError (±) is expressed as STD
^dTime is expressed in months