Original Article

Histological and Physiological Investigation of Channelrhodopsin-2 and Halorhodopsin in the Dorsal Cochlear Nucleus

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Abstract   We have delivered viral vectors containing either Chop2 fused with GFP, Channelrhodopsin-2 (ChR2), or Halorhodopsin (HaloR) fused with mCherry (to form light gated cation channels or chloride pumps, respectively), into the dorsal cochlear nucleus (DCN). One to eighteen months later we examined the CN and inferior colliculus (IC) for evidence of virally transfected cells and processes. Production of ChR2 and HaloR was observed throughout the DCN. Rhodopsin localization within neurons was determined, with elongate, fusiform and giant cells identified based on morphology and location within the DCN. Production of ChR2 and HaloR was found at both the injection site as well as in regions projecting to and from the DCN. Light driven neuronal activity in the DCN was dependent upon the wavelength and intensity of the light, with only the appropriate wavelength resulting in activation and higher intensity light resulting in more neuronal activity. Transfecting cells via viral delivery of rhodopsins can be useful as a tract tracer and as a neuronal marker to delineate pathways. In the future rhodopsin delivery and activation may be developed as an alternative to electrical stimulation of neurons.

Key word   cochlear nucleus; channelrhodopsin-2; halorhodopsin; tracer; optical control

Introduction

Microbial channelopsins have been cloned and when functionally expressed were found to form light-gated cation channels (ChR2) and chloride pumps (HaloR). [1-5] 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. [6-7] Even ectopic expression of one of these channelopsins, channelrhodopsin-2 (ChR2), was able to restore visual responses in mice with photoreceptor degeneration. [8]

With the proliferation of cochlear prosthetic devices

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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 [9,10] 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 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 activated by different wavelengths of light ultimately resulting in more precise control of the auditory experience.

Methods

Stereotaxic delivery of rhododpsins

Normal hearing male Sprague Dawley rats (n=15) 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 a GFP (ChR2) or cytomegalovirus (CMV) with a mCherry (Halo-rhodopsin) tag was injected bilaterally (1 μl) using a Hamilton Syringe with 33-gauge needle. Following the injection the needle remained in place for five minutes before removal. The viral constructs used in this study have been previously described [8, 6, 11] and kindly provided by Dr. Z-H Pan (Wayne State University, Detroit, MI).

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℃. The fixed and cryo-protected tissues were cut with a cryostat into 30 ~ 40 μm sections and stored on glass slides at -80℃. Sections from auditory (DCN, VCN, SOC and IC) as well as extra-auditory (cerebellum and inferior olive) regions were examined for ChR2-GFP or HaloR-mCherry fluorescence.

Immunocytochemistry

In order to determine whether rhodopsin expression was neuronal, GFP 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

Extracellular recordings were obtained from rats injected with ChR2 (n=4) and from non-injected rats (n= 3). 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 a single shank five channel electrode (NeuroNexus Technologies, Ann Arbor MI and a gift from Univ. of Michigan KHRI). Each channel was separated by 100 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. A royal blue light-emitting diode (LED) with a spectrum ranging from 440-460 nm and a peak of 455 nm
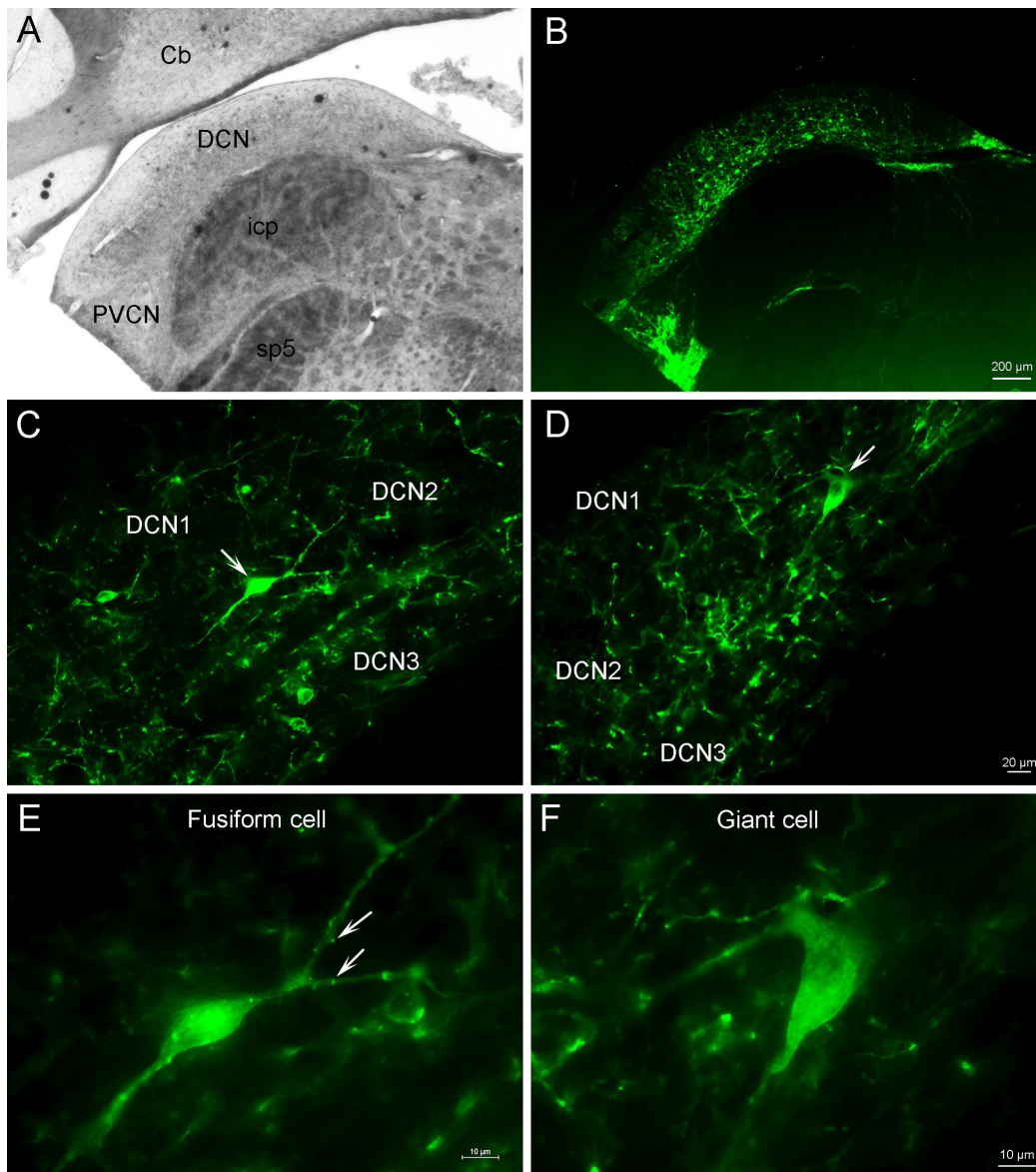
Figure 1   Channel rhodopsin (ChR2) can be produced in DCN neurons. Following a single injection of AAV2 containing the ChR2−GFP construct, labeling was observed throughout the DCN (**A−B**). Small cells were identified in DCN1 (**C**), while several cell types including fusiform and giant cells were labeled in DCN2 and DCN3 (**C − F**). Arrow in **C** indicates fusiform cell (enlarged in **E**) and arrow in **D** indicates giant cell (enlarged in **F**). Arrows in **E** point to spines on dendrites, perhaps indicating the location of synaptic inputs. Scale bar in **B** also valid for **A** and scale bar in **D** is also valid for **C**. 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

(LXHL−LR5C, Luxeon Brantford, Ontario, Canada) was used for activation of ChR2 (460 nm; ChR2 optimal wavelength) while an amber LED with a spectral range of 585−597 nm and a peak spectrum of 590 nm (LXHL−LL3C, Luxeon Brantford) was used for activation as a control stimulation. Both the royal blue LED (7.0 v, 8.96×10^{16} photons/cm²/s) and the amber LED

(2.9 v, 1.01×10^{16} photons/cm²/s) were placed 2 cm above the DCN for 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,
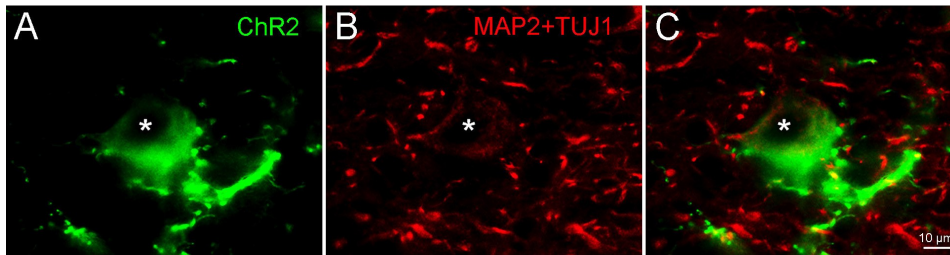
Figure 2 Channel rhodopsin (ChR2) is produced in neurons. When ChR2 labeling (**A**) is combined with immunocytochemistry for the neuronal markers MAP2 and TUJ1 (**B**), double labeling of neurons can be observed in the dorsal cochlear nucleus (**C**).
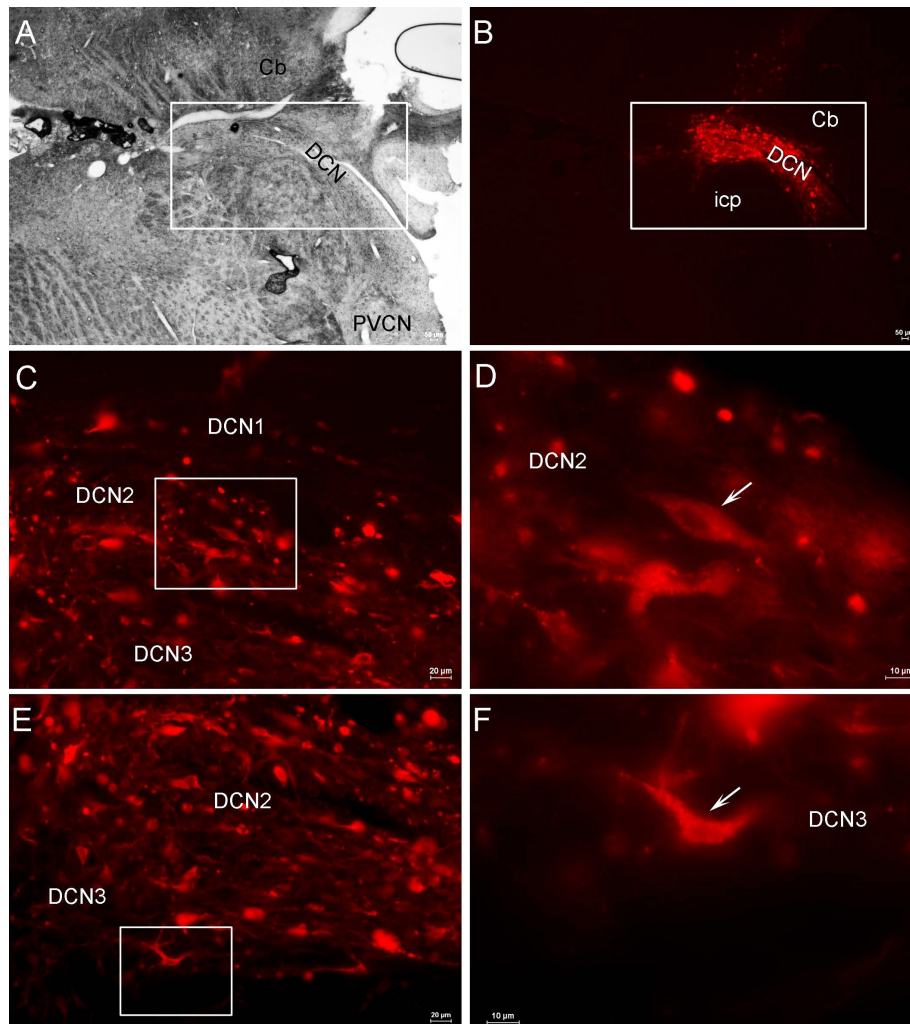

Figure 3 Halo−rhodopsin (HaloR) can be produced in DCN neurons. Following a single injection of CMV containing the HaloR−mCherry construct, labeling was observed throughout the DCN (**A–B**). Small cells were identified in DCN1 (**C**), while several cell types including fusiform and giant cells were labeled in DCN2 and DCN3 (**C – F**). Box in **C** indicates labeling in DCN2 (enlarged in **D**) and box in **E** indicates labeling in DCN3 (enlarged in **F**). Arrow in **D** points to a fusiform cell and arrow in **F** indicates a giant cell. Scale bar in **B** also valid for **A**. Cb−cerebellum; 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

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

All procedures were approved by the Wayne State School of Medicine "Laboratory Animal Care and Use

Committee" (PHS Animal Welfare Assurance number A3310−01) and conform to the National Institutes of Health guidelines.

Results

Both ChR2 and HaloR can be expressed and produced in DCN neurons

ChR2−GFP Within five weeks of stereotaxic injection the expression and production of ChR2−GFP was observed in all layers of the DCN, with the heaviest fluorescence observed in DCN2 and DCN3 (Figure 1). Many of the GFP producing cells were also labeled for the neuronal marker MAP2 and TUJ1 (Figure 2). Fluorescence for ChR2−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 on dendritic spines and axonal varicosities (See Figure 1E arrows). Labeling was not restricted to a specific type of neuron or neurotransmitter class. Cell types included fusiform, giant, elongate as well as cartwheel cells.

HaloR−mCherry Just as found with ChR2−GFP expression, production 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, including fusiform and giant cells were capable of producing the HaloR−mCherry fusion protein driven by the CMV promoter. Somata, dendrites and axons show a more diffuse labeling when compared to the production of ChR2 (compare Figure 3 to Figure 1).

The ChR2−GFP and HaloR−mCherry constructs can trace neuronal pathways

We have determined that other regions of the auditory brainstem exhibit ChR2 and HaloR 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) production was observed in the contralateral IC, most robustly in the central nucleus. As expected, the production observed in the IC was primarily localized to terminals (Figure 4C and 4F) presumably associated with somata located in the DCN. 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). Labeled ChR2−GFP somata were ob-
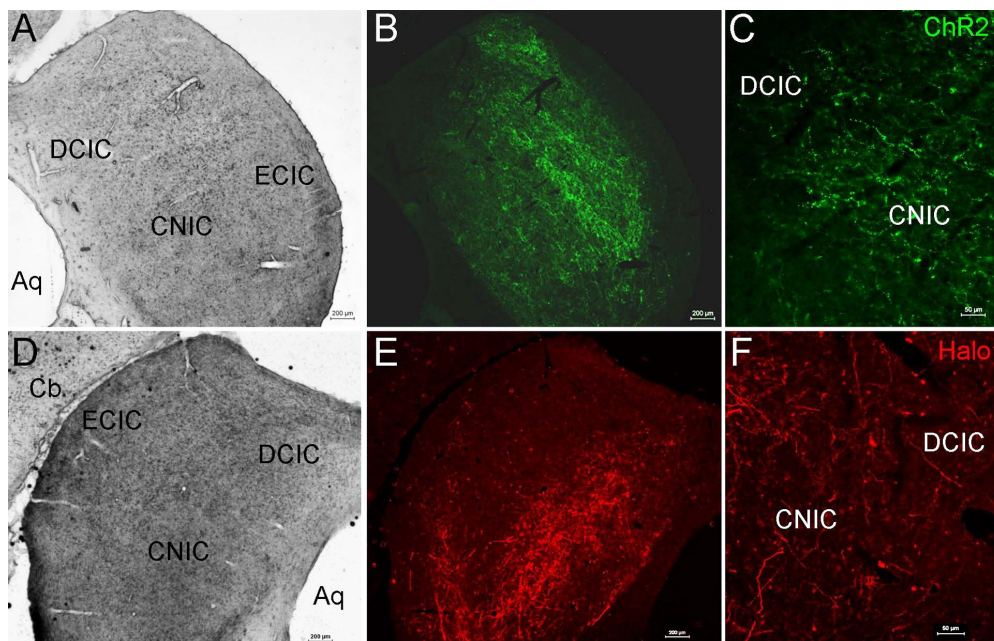

Figure 4   Channel rhodopsin (ChR2) and Halo−rhodopsin (HaloR) can be produced throughout DCN neurons. Following an injection of AAV2 containing the ChR2−GFP（green）or CMV containing the HaloR−mCherry（red）construct, labeling was observed in the inferior colliculus (**A−F**). For both ChR2 (**A−C**) and 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**).
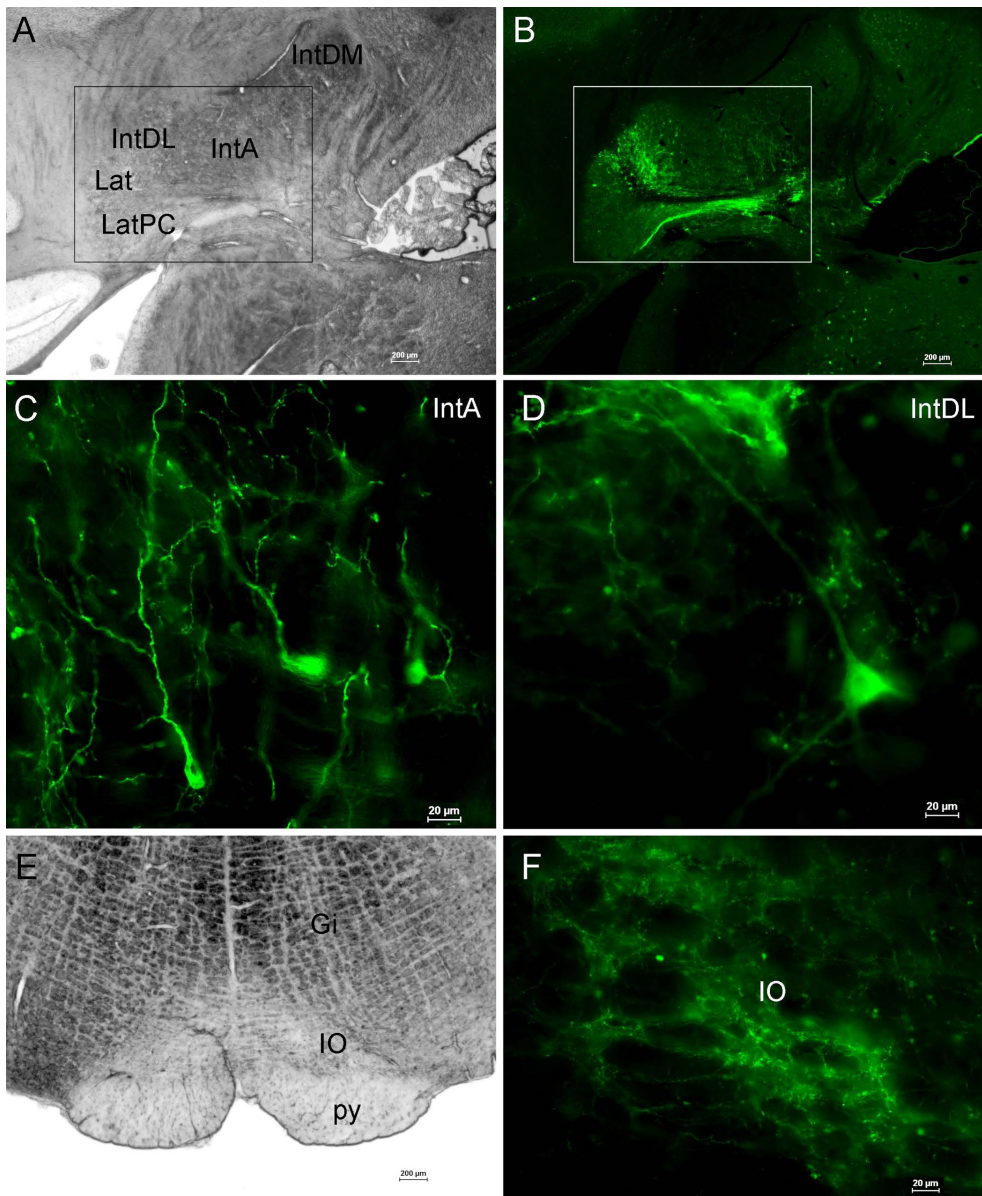
Figure 5   Production of channel rhodopsin (ChR2−GFP) can be used to trace neuronal pathways. Injection of the interpositus nucleus of the cerebellum (**A−B**) labels somata and dendrites in both the IntA (**C**) and the IntDL (**D**), both of which send projections to the IO (**E−F**). Box in **B** 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

served in the Int (Figure 5A−D) while terminals were found in the IO (Figure 5E−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 5C) cells with small somata (≤10 μm diameter) and a single dendrite expressed ChR2−GFP, while cells in the IntDL(Figure 5D) expressing ChR2−GFP had larger somata (≥20 μm diameter) with multiple dendrites. ***Photostimulation of ChR2 transfected DCN neurons***

is wavelength and intensity dependent

For light activation of ChR2−GFP neurons, an LED was placed 2 cm above the DCN, which had been surgically exposed. A light with a wavelength centered around 460 nm should open ion channels in neurons producing ChR2−GFP. To determine whether the effects of wavelength and intensity of light on neuronal activity we first exposed the ChR2 transfected DCN to three different intensities of blue light, (Figure 6A 8.60×10^{16}, 6B
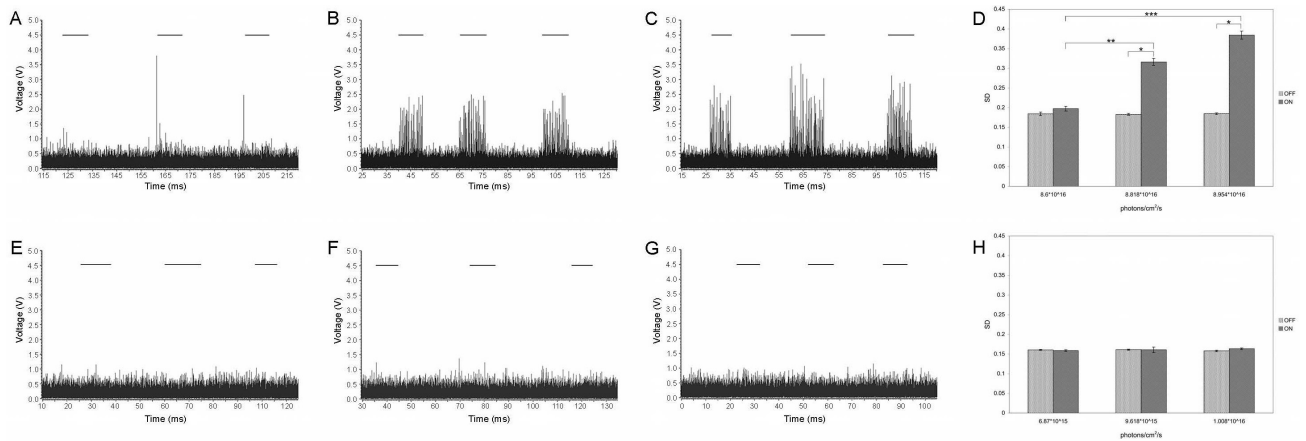
Figure 6 Optical activation of ChR2 producing neurons is intensity and wavelength specific. Using 455 nm light (A–D) neuronal activity in the DCN was compared at 8.60×10^{16} (**A**), 8.82×10^{16} (**B**), and 8.95×10^{16} photons/cm^2/s (C) during three trials in which the light was on for ~10 s (indicated by horizontal bars in **A−C**) and off for ~10s. The most robust activation was observed during the highest intensity of light stimulation (**C−D**). Exposure to 590 nm light (light on indicated by horizontal bars in (**E−G**) did not produce activation of ChR2 infected neurons at any of the intensities tested (**E−H**). The Y axis (**D** and **H**) indicates standard deviation while the X axis shows the effects of different intensities of 455 nm light (**D**) and 590 nm light (**H**). Asterisks indicate significant differences ($P \leq 0.05$).
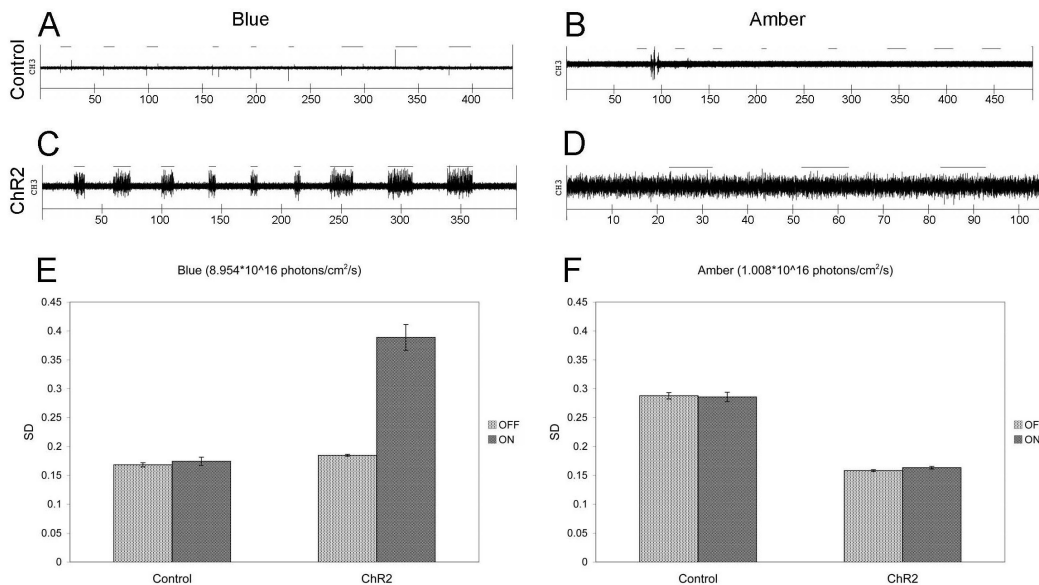

Figure 7 Light stimulation of DCN neurons transfected with ChR2. In animals that did not receive an injection of AAV2 ChR2–GFP (control), no neuronal activation was observed during exposure (horizontal lines) to blue (455 nm) light (**A**) or amber (590 nm) light (**B**). However, animals injected with AAV2 ChR2–GFP showed increased neuronal activity during trials when exposed to 455 nm light for 10, 5, or 20 seconds (**C**), but 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

8.82×10^{16}, 6C 8.95×10^{16} photons/cm^2/s) for three 10 second intervals. As the intensity of light increased from 8.60×10^{16} to 8.95×10^{16} photons/cm^2/s (Figure 6 A–D)

there was an increase in standard deviation (SD) observed between the light off vs the light on. However, the lowest intensity of blue light (8.60×10^{16} photons/cm^2/s)

resulted in no significant change in SD (Figure 6A and 6D). The two higher intensities resulted in significant increases in SD (150% p = 0.0001 and 200% p = 0.0138 respectively) when the light was on (Figure 6D) compared to light off. The DCN was then exposed to a different wavelength of light, amber (centered around 590 nm), also at three different intensities (Figure 6E 6.87 × 10^{15}, 6F 9.62 × 10^{15}, 6G 1.01 × 10^{16} photons/cm^2/s). The amber light resulted in no significant difference in the SD observed between the light on and the light off condition (Figure 6H).

Light activates DCN neurons producing either ChR2−GFP

In control animals with no ChR2−GFP injection, regardless of whether the LED emitted light at wavelengths of 455 nm (Figure 7A) or 590 nm (Figure 7B), no changes in neuronal activity were observed in the DCN when comparing light on (10, 5 and 20 seconds ×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 7C). 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 7D). When comparing the light on condition with the light off condition during exposure to blue light (Figure 7E) vs amber light (Figure 7F), control animals sometimes showed small, but significant increases (0.168±0.004 off 0.174 ± 0.007 on; *p* = 0.035) during stimulation with blue 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 × 10^{-9}) when the blue light was "on" (Figure 7E) suggesting that repeated exposure to wavelengths of 455 nm of light resulted in corresponding increases in multiunit activity. During 590 nm light exposure (Figure 7F), 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". Regardless of whether ChR2−GFP trans-

fected cells were activated for 5, 10 or 20 seconds, no diminution in signal was observed during a trial (Figure 7C).

Discussion

In the current study we have demonstrated that viral delivery of ChR2 or HaloR into the DCN results in expression within neurons and terminals within all layers of the DCN. Specifically, we identified ChR2 labeling in fusiform, elongated and giant cells. 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. [6, 7] 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. In addition, we demonstrate results from the viral−targeted expression of light activated chloride pumps (HaloR) in DCN neurons. Like ChR2−GFP, production of HaloR−mCherry was observed in neurons within each layer of the DCN.

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 produced 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[7] 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 connections with the injected area (DCN or cerebellum). In the current study the tracer aspect of AAV2 appeared to be primarily anterograde in nature as has been noted previously in the brain.[12] These results suggest that viral delivery of ChR2 and HaloR can be said to be a useful tool from the histological standpoint alone even excluding physiological views.

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 be able to be precise, reliable and performed on a time scale functionally relevant for CN neurons.

Currently implants designed restore hearing in the profoundly deaf comprise use electrical current and multiple electrodes designed to stimulate many neurons throughout the cochlea or a given brain region. The goal is to provide local control of discrete neuronal populations in order to generate signals that convey high or low frequency information depending on which neurons are stimulated. However, caveats of using electrical current to activate neurons in this manner include the inability to control the spread of activity across different groups of neurons decreasing the ability to clearly discriminate sounds. Recently, the use of pulsed infrared optical radiation has been used by others to activate spiral ganglion cells providing more discrete stimulation of these neurons allowing an increased number of independent sub-populations of neurons to be stimulated for parallel speech processing. [9] In the current study, transfecting neurons with ChR2 allowed us to take advantage of a similar non-contact approach.

In the future, cell type-specific promoters could be used to target these rhodopsins to specific neuronal cell types indentified such as the recently reported vGluT3 labeled neurons in the CN [13] or dopaminergic neurons that have been previously identified in the inferior colliculus. [14] This strategy offers the ability to avoid neuronal damage by using light stimulation instead of electrical current, more precisely target specific groups of neurons and repetitively excite and/or inhibit these neurons as necessary. Auditory neurons could be controled not by sound but by light with rhodopsins someday. That must be the great progress in the physiological, audiological and clinical study.

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