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# Voltage-gated $Ca_v1$ channels in disorders of vision and hearing

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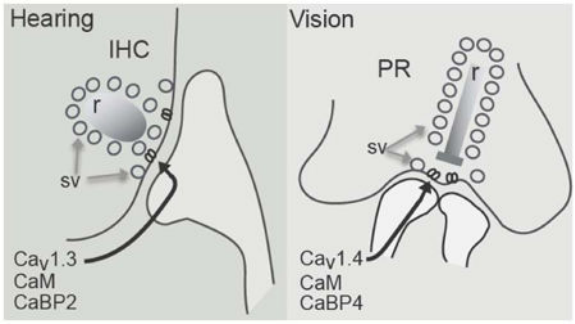
## Abstract

$Ca_v1$ channels mediate L-type $Ca^{2+}$ currents that trigger the exocytotic release of glutamate from the specialized "ribbon" synapse of retinal photoreceptors (PRs) and cochlear inner hair cells (IHCs). Genetic evidence from animal models and humans support a role for $Ca_v1.3$ and $Ca_v1.4$ as the primary $Ca_v$ channels in IHCs and PRs, respectively. Because of the unique features of transmission at ribbon synapses, $Ca_v1.3$ and $Ca_v1.4$ exhibit unusual properties that are well-suited for their physiological roles. These properties may be intrinsic to the channel subunit(s) and/or may be conferred by regulatory interactions with synaptic signaling molecules. This review will cover advances in our understanding of the function of $Ca_v1$ channels at sensory ribbon synapses, and how dysregulation of these channels leads to disorders of vision and hearing.

## Graphical abstract




Cav1.3 and Cav1.4 channel complexes of inner hair cells (IHC) and photoreceptor (PR) cells, respectively, share a number of biophysical properties and modulatory proteins at ribbon synapses (r = ribbon, sv = synaptic vesicles).

## Introduction

The perception of light and sound is initiated by photoreceptors (PRs) in the retina and inner hair cells (IHCs) in the cochlea, respectively. Both PRs and IHCs convert an environmental stimulus into an electrical signal that is communicated to second-order neurons and ultimately to the brain. An important structural feature of both PRs and IHCs is the synaptic

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ribbon. Associated with the presynaptic active zone, the ribbon tethers thousands of synaptic vesicles and allows for rapid and sustained release of glutamate in response to graded changes in membrane potential [1]. Voltage-gated $Ca^{2+}$ channels are positioned within nanometers of the ribbon [2], allowing for tight coupling of depolarization-dependent $Ca^{2+}$ influx and the molecular machinery involved in exocytosis. In contrast to the dominant role of $Ca_v2$ channels at most synapses in the central nervous system, $Ca_v1$ channels are required for exocytosis at ribbon synapses [3-5]. Visual and acoustic stimuli must be encoded continuously with high fidelity and sensitivity. This review will highlight the unique features and regulatory functions of $Ca_v1$ channels at the PR and IHC synapse, and their importance for faithful transmission of sensory stimuli.

## $Ca_v1.3$ channels and hearing

In the cochlea, sound-induced vibrations displace hair bundles rooted in the apical surface of IHCs. The subsequent modulation of mechanoelectrical transduction currents causes a graded receptor potential that regulates the opening of $Ca_v1$ channels clustered near the presynaptic ribbon. $Ca^{2+}$ influx through these channels triggers the exocytotic release of glutamate onto postsynaptic spiral ganglion neuron afferents, which transmit auditory information into the central nervous system. Even in the absence of sensory stimulation, IHC synapses are tonically active, and support spontaneous afferent firing rates that can exceed 100 Hz [6]. The intensity and timing of sound is encoded by an increase in steady-state afferent firing rates, and by the ability of IHCs to phase-lock transmitter release to frequencies up to 5 kHz, respectively.

Current evidence favors a crucial role for $Ca_v1.3$ channels as the major $Ca_v$ channel in IHCs. For example, in mice lacking $Ca_v1.3$ channels ($Ca_v1.3$ KO), IHCs are incapable of evoked exocytosis and exhibit whole-cell $Ca^{2+}$ currents that are ~90% of that in IHCs from wild-type mice [7, 8]. As a consequence, $Ca_v1.3$ KO mice are deaf, with no evidence of sound-evoked afferent activity in auditory brainstem responses (ABR) [7, 9]. Other components of the $Ca_v1.3$ complex in IHCs include the auxiliary $Ca_v\,\beta$ [10] and $\alpha_2\delta$ subunits.

Analyses of $Ca_v1.3$ channels in heterologous expression systems indicate a number of properties consistent with those of native $Ca_v$ channels and their physiological demands in IHCs. First, $Ca_v1.3$ activates rapidly compared to the related $Ca_v1.2$ channel abundant in the brain and heart [11, 12]. Whole-cell and single-channel recordings of $Ca^{2+}$ currents in IHCs indicate time constants for activation and latency to first opening in the submillisecond range [13-17]. Rapid activation kinetics of $Ca_v1.3$ channels are likely important for temporal aspects of sound coding, such as the rapid onset of sound and the ability to accurately trigger firing of the auditory nerve to reflect sound frequency (i.e., phase locking).

Second, $Ca_v1.3$ channels activate at relatively negative voltages [11, 12]. In single-channel recordings of immature mouse IHCs, channel openings were observed at voltages as negative as -70 mV [14]. Since the resting potential of these cells is ~ -60 mV [18], the negative activation threshold of Cav1.3 would be able to support tonic transmitter release at rest. Moreover, the stronger activation of these channels in response to sound-evoked

alterations in the receptor potential could encode increased sound intensity with greater rates of transmitter release.

Third, $Ca_v1.3$ channels in IHCs exhibit little $Ca^{2+}$- or voltage- dependent inactivation (CDI, VDI), in contrast to their behavior in heterologous expression systems and in the heart [11, 16, 17, 19-23]. CDI is a negative feedback regulation mediated by permeating $Ca^{2+}$ ions and relies on calmodulin (CaM) interacting with an IQ domain in the cytoplasmic C-terminal domain of the $Ca_v1.3$ $\alpha_1$ subunit. CDI is not seen with $Ba^{2+}$ as the charge carrier since $Ba^{2+}$ binds poorly to CaM, so $Ba^{2+}$ currents exhibit VDI, which is generally slower than CDI, (reviewed in [24]). The low levels of CDI in IHCs are thought to support the continuous encoding of sound information through sustained transmitter release. Possible factors contributing to the limited CDI of $Ca_v1.3$ channels in IHCs include the expression of $Ca_v1.3$ variants lacking a functional IQ domain [25]. In addition, the synaptic scaffolding protein, Rab3-interacting molecule 2 (RIM2), inhibits CDI and VDI of $Ca_v1.3$ channels in transfected tsA-201 cells. However, RIM2 is primarily expressed in the immature cochlea [26], such that other factors must contribute to the slow CDI and VDI in mature IHCs.

One possibility concerns CaBPs, a family of CaM-like $Ca^{2+}$ binding proteins that are highly expressed in the brain, retina, and inner ear [20, 27-31]. Like CaM, CaBPs have 4 EF-hand $Ca^{2+}$ binding domains, at least one of which is non-functional [27]. Antibodies against CaBP1, CaBP2, CaBP4, and CaBP5 label both immature and mature IHCs [20, 28]. In transfected HEK293T cells, CaBPs suppress CDI of $Ca_v1.3$ [20, 28, 32]. The mechanism may involve displacement of CaM by CaBPs from the $Ca_v1$ $\alpha_1$ IQ domain [33-35]. However, CaBPs bind to additional sites in $Ca_v1$ $\alpha_1$, and so may allosterically modulate CaM interactions with the channel [36-38]. CaBP2 may be an important physiological regulator of $Ca_v1.3$ in IHCs since a mutation in the CaBP2 gene causes moderate-to severe sensorineural hearing loss. The mutation causes premature truncation of CaBP2, such that the mutant CaBP2 lacks the C-terminal EF-hands. In transfected HEK293T cells, the mutant CaBP2 is less able to suppress CDI of $Ca_v1.3$ channels [39]. While additional research is required, increased CDI of $Ca_v1.3$ currents may be insufficient to support sustained neurotransmitter release from IHCs required for proper sound coding.

The requirement for $Ca_v1.3$ for hearing is clearly illustrated by the profound deafness seen in individuals with a mutation in *CACNA1D* [40]. This mutation results in a glycine insertion into the transmembrane helix IS6 in $Ca_v1.3$ variants containing exon 8B. In transfected tsA-201 cells, mutant channels are trafficked to the plasma membrane but do not conduct $Ca^{2+}$ currents. The mechanism may involve reduced ability of voltage-sensors to couple to pore opening, or some failure of conductance downstream of pore-opening. In addition to deafness, the affected individuals exhibit severe sinus bradycardia [40], consistent with the role of $Ca_v1.3$ in regulating sinoatrial pacemaking and with the cardiac phenotype of $Ca_v1.3$ KO mice [7, 19].

Dysregulation of $Ca_v1.3$ channels is implicated in additional forms of hearing impairment. For example, severe hearing loss can result from thyroid hormone (TH) deficiency, mutations in thyroid hormone receptor β, or iodine deficiency [41]. Studies of TH-deficient rodents indicate abnormal maturation of IHC ionic currents and physiology. In normal

mouse IHCs, $Ca_v1.3$ currents and BK $K^+$ currents normally undergo a developmental decline or increase, respectively, prior to the onset of hearing in mice (postnatal day (P)12) [42, 43]. However, $Ca_v1.3$ currents remain elevated, and BK currents are undetectable, in TH-deficient rats and mice [44, 45]. The abnormally elevated $Ca_v1.3$ current density may result from alterations in $Ca_v1.3$ trafficking or turnover, since the corresponding mRNA levels are not affected by TH deficiency [44]. Due to the maintained high levels of $Ca_v1.3$, spontaneous $Ca^{2+}$-dependent action potentials, which normally disappear around P13 [8, 46], are still evident in TH-deficient mice [44, 45]. IHCs from these mice also exhibit abnormalities in afferent and efferent innervation that suggest that IHCs have not transitioned at the molecular or morphological level to support tonic, graded changes in neurotransmitter release in response to sound stimulation [44]. TH is required for multiple aspects of auditory development, including maturation of the tectorial membrane and the generation of the endocochlear potential [41]. The extent to which the synaptic defects in IHC maturation contribute to deafness due to TH deficiency remains an open question.

A failure to regulate $Ca_v1.3$ channels also is found in a mouse model of Usher syndrome – the most common cause of combined deafness and blindness in humans [47]. “Deaf-circler” mice (*dfcr*) bear a mutation in the harmonin gene, which is a target of human mutations causing Usher syndrome [48]. Harmonin contains PDZ-domains, which are well-established motifs for protein-protein interactions [49]. Harmonin binds to a consensus PDZ binding site at the C-terminus of the $Ca_v1.3$ $\alpha_1$ subunit, and this interaction inhibits $Ca_v1.3$ current density in transfected HEK293T cells. The mechanism involves enhanced proteosomal degradation of $Ca_v1.3$, which can be reversed by the proteosomal inhibitor MG132. The *dfcr* mutation prevents harmonin binding to $Ca_v1.3$ and causes increased $Ca_v1.3$ current density in *dfcr* compared to control IHCs [50]. Immunolabeling for harmonin at IHC synapses increases after hearing onset (P12) [50]. These results support a model in which harmonin promotes developmental trimming of $Ca_v1.3$ channels, which may be important for IHC maturation. Harmonin also enhances voltage-dependent facilitation of $Ca_v1.3$ channels [51], which may be important for boosting $Ca_v1.3$ function during sound stimulation. The primary cause of deafness associated with harmonin mutations is likely not to involve $Ca_v1.3$ channels given that harmonin is also a key regulatory of mechanosensory channels in IHC hair bundles [52]. However, synaptic abnormalities due to altered regulation of $Ca_v1.3$ trafficking and function could exacerbate the mechanosensory defect.

## $Ca_v1.4$ channels and vision

In the retina, rod PRs mediate vision in dim light, while cone PRs mediate daytime vision and color perception. At the relatively depolarized membrane potential of PRs in darkness ($\sim$ -40 mV), the opening of $Ca_v1$ channels clustered near the presynaptic ribbon triggers the sustained release of glutamate, which inhibits postsynaptic ON bipolar cells. Light-dependent hyperpolarization suppresses $Ca_v1$-dependent glutamate release, which depolarizes ON bipolar cells, ultimately enhancing the activity of third-order retinal ganglion cells that transmit visual information into the brain. Cone PRs form synapses with multiple types of ON or OFF bipolar neurons, which are either depolarized or hyperpolarized, respectively, by light stimulation of cones.

The major $Ca_v1$ channel at PR synapses is $Ca_v1.4$, which localizes in rod and cone PR terminals in the outer plexiform layer of the retina [53-57]. In mice with genetic inactivation of the $Ca_v1.4$ $\alpha_1$ subunit ($Ca_v1.4$ KO), $Ca^{2+}$ entry into PRs in response to depolarization is substantially impaired [58]. In electroretinograms, which measure visual function, $Ca_v1.4$ KO mice show no evidence of synaptic transmission between PRs and bipolar cells [56, 58]. However, $Ca_v1.4$ KO mice also exhibit PR degeneration with age [59] and defects in PR synapse morphology [58, 60] due to a failure in synapse maturation [56, 59, 61]. In addition to $Ca_v1.4$ $\alpha_1$, the $Ca_v1.4$ complex at PR synapses likely includes auxiliary $Ca_v\beta_2$ and $\alpha_2\delta_4$ subunits. Mice with reduced expression of $Ca_v\beta_2$ and $\alpha_2\delta_4$ exhibit a similar visual and synaptic phenotype as in $Ca_v1.4$ KO mice [62, 63]. Immunochemical studies support the association of $Ca_v1.4$ $\alpha_1$ with $Ca_v\beta_2$ and $\alpha_2\delta_4$ in mouse retina [64].

$Ca_v1.4$ channels in heterologous expression systems exhibit properties similar to native $Ca_v1$ channels in PRs [65]. Compared to $Ca_v1.2$ channels, $Ca_v1.4$ channels activate at more negative voltages, which allows for tonic transmitter release at the dark membrane potential [66-68]. Although the IQ domain is conserved in $Ca_v1.4$ $\alpha_1$, a C-terminal modulatory domain (CTM) of this channel inhibits CDI. The mechanism involves an intramolecular interaction between the CTM and a proximal region of the $Ca_v1.4$ $\alpha_1$ C-terminal domain, which inhibits $Ca^{2+}$-free (apo) CaM interactions with the IQ domain [69-72]. As for $Ca_v1.3$ channels at the IHC synapse, limited CDI of $Ca_v1.4$ channels at PR synapses supports the continuous release of glutamate that is required for sensory encoding.

In contrast to the presence of multiple CaBPs in IHCs, CaBP4 is the only CaBP family member expressed in PRs. CaBP4 interacts with and colocalizes with $Ca_v1.4$ in the outer plexiform layer of the mouse retina [29, 56, 64]. While CaBP4 does not regulate CDI of full-length $Ca_v1.4$ channels, deletion of the ICDI allows CaBP4 suppression of $Ca_v1.4$ CDI [73]. CaBP4 binds to the IQ domain in $Ca_v1.4$ $\alpha_1$ and inhibits the intermolecular binding of the CTM with the proximal $Ca_v1.4$ $\alpha_1$ C-terminal site [73]. In transfected HEK293T cells, CaBP4 enhances voltage-dependent activation and reduces VDI of $Ca_v1.4$ channels [29, 73]. The net increase in $Ca_v1.4$ channel availability due to CaBP4 may be important for facilitating glutamate release from PRs in darkness. Consistent with this hypothesis, mice lacking CaBP4 (CaBP4 KO) exhibit visual and retinal phenotypes similar to those in $Ca_v1.4$ KO mice. In addition to significantly impaired light-modulated PR transmission, the morphology of PR synapses is abnormal [29, 56].

A requirement for $Ca_v1.4$ in human vision is supported by the multiple visual disorders associated with mutations in the gene encoding $Ca_v1.4$ $\alpha_1$, *CACNA1F*. The visual phenotypes associated with loss-of function of $Ca_v1.4$ are generally less severe in humans than in mouse models of the disease, which may be due to the normal or compensatory expression of $Ca_v1.3$ channels in human PRs. These disorders include congenital stationary night blindness type 2 (CSNB2), which causes heterogeneous symptoms that may include low visual acuity, myopia, and nystagmus [74-77]. Despite the name, night blindness is not always associated with CSNB2 phenotypes [78]. Other disorders associated with *CACNA1F* mutations include Åland island eye disease [79, 80] and cone-rod dystrophy (CORDX) [74, 81, 82]. Visual phenotypes of these disorders are similar to those in CSNB2, although there is early and more severe loss of cone function in CORDX compared to in CSNB2 [83]. An

autosomal recessive form of cone-rod dystrophy is caused by mutations in the *CACNA2D4* gene encoding $\alpha_2\delta_4$ [82], consistent with its importance as a $Ca_v1.4$ subunit [64].

The clinical variability associated with *CACNA1F* mutations may result from the functional impact of a particular mutation on $Ca_v1.4$ channels. In electrophysiological analyses in heterologous expression systems, CSNB2 mutant $Ca_v1.4$ channels may exhibit a loss-of function, gain-of function, and sometimes no observable difference compared to wild-type channels [68, 84]. For example, one CSNB2 mutation causes substitution of a proline for leucine860 (L860P), which is located in a conserved amphipathic helix in the loop connecting domains II and III. L860P reduces the number of function $Ca_v1.4$ channels at the cell surface by increasing the turnover of the protein [85]. While this would conceivably reduce $Ca^{2+}$ signals supporting PR glutamate release in darkness, it is also possible that PR synapse development would be impaired, given that PR synapses do not form in $Ca_v1.4$ KO mice [56, 59, 61]. More complex mechanisms may result from truncating mutations that delete the CTM from $Ca_v1.4$ (K1591X [86], R1827X [87]). These mutations cause a loss-of function by increasing CDI, but also a gain-of function through enhance voltage-dependent activation, a process which the CTM normally suppresses[71, 85]. A mouse model of a gain-of function CSNB2 mutation (I745T) has provided some insights into the underlying pathophysiological mechanisms. This mutation causes an unusually severe form of CSNB2, and a large (-30 mV) shift in the voltage-dependence of $Ca_v1.4$ activation [88]. I745T knock-in mice exhibit abnormal PR synapse morphology, reduced cone and rod transmission, and degeneration of PRs due to apoptosis [55, 59].

Two mutations in the gene encoding CaBP4 also contribute to visual phenotypes similar to those in patients with *CACNA1F* mutations. One mutation is a valine substitution for glutamate and a frameshift that elongates the protein (E267fs). The second is a truncating mutation (R216X) that deletes the C-terminal EF-hands of CaBP4 [89, 90], which is essentially identical in nature to the CaBP2 mutation that causes autosomal recessive hearing loss [32]. These mutations do not affect the ability of CaBP4 to bind to $Ca_v1.4$, but prevent effects of CaBP4 on voltage-dependent activation and inactivation [73]. Decreased $Ca_v1.4$ channel availability would be expected to inhibit levels of PR glutamate release, thus reducing the dynamic range of PR signaling in these patients.

## Summary

In IHCs and PRs, respectively, $Ca_v1.3$ and $Ca_v1.4$ share a number of biophysical properties that are important for their roles in mediating tonic neurotransmitter release at relatively negative membrane potentials, which is modulated by sensory stimuli. The growing number of visual and auditory disorders linked to altered $Ca_v1.3$ and $Ca_v1.4$ function emphasizes the need to better understand the roles and regulation of these channels in their native cell-types. Complicating matters is the fact that both $Ca_v1.3$ and $Ca_v1.4$ are expected to interact with a variety of proteins [91, 92]. Defining how genetic alterations in $Ca_v1.3$ and $Ca_v1.4$, and their interacting proteins, affect the localization and function of these channels in IHCs and PR remains an important challenge for understanding the pathophysiology of the associated channelopathies, as well as for the development of novel therapies.

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