Presynaptic voltage-gated calcium channels in the auditory brainstem

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ABSTRACT

Sound information encoding within the initial synapses in the auditory brainstem requires reliable and precise synaptic transmission in response to rapid and large fluctuations in action potential (AP) firing rates. The magnitude and location of Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels (CaV) in the presynaptic terminal are key determinants in triggering AP-mediated release. In the mammalian central nervous system (CNS), the CaV.2.1 subtype is the critical subtype for CNS function, since it is the most efficient CaV2 subtype in triggering AP-mediated synaptic vesicle (SV) release. Auditory brainstem synapses utilize CaV2.1 to sustain fast and repetitive SV release to encode sound information. Therefore, understanding the presynaptic mechanisms that control CaV2.1 localization, organization and biophysical properties are integral to understanding auditory processing. Here, we review our current knowledge about the control of presynaptic CaV2 abundance and organization in the auditory brainstem and impact on the regulation of auditory processing.

1. Introduction

Fundamental to hearing is the ability to accurately identify and interpret sound information over a wide range of time scales down to submillisecond (Pickles, 2013; Schnupp et al., 2011). An inability to do so results in problems with speech perception and sound localization in the aging population, central auditory processing disorder (CAPD) and auditory deficits in autism spectrum disorders, and leads to cognitive impairments (Atcherson et al., 2015; Fitzgibbons and Gordon-Salant, 2010). The magnitude and location of Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels (CaV) in the presynaptic terminal are key determinants in triggering AP-mediated release. In the mammalian central nervous system (CNS), the CaV.2.1 subtype is the critical subtype for CNS function, since it is the most efficient CaV2 subtype in triggering AP-mediated synaptic vesicle (SV) release. Auditory brainstem synapses utilize CaV2.1 to sustain fast and repetitive SV release to encode sound information. Therefore, understanding the presynaptic mechanisms that control CaV2.1 localization, organization and biophysical properties are integral to understanding auditory processing. Here, we review our current knowledge about the control of presynaptic CaV2 abundance and organization in the auditory brainstem and impact on the regulation of auditory processing.

In the majority of CNS synapses, CaV2.1 is the most effective at triggering AP-mediated release (Iwasaki et al., 2000; Takahashi and Momiyama, 1993; Wheeler et al., 1994), since it is most abundant and in closer proximity to SV release sites than CaV2.2 and CaV2.3 channels (Eggermann et al., 2012; Wu et al., 1998; Wu et al., 1999). During neuronal circuit maturation, synaptic transmission becomes increasingly CaV2.1-dependent due to a selective presynaptic reduction or loss of CaV2.2 and CaV2.3 in many synapses (Iwasaki et al., 2000; Iwasaki and Takahashi, 1998; Scholz and Miller, 1995). In addition, during development of neurons that signal with rapid and temporally precise APs in the mature circuit, SV release kinetics become faster as distances between CaV2.1 and SV release sites become shorter (Baur et al., 2015; Chen et al., 2015; Eggermann et al., 2012; Fedchyshyn and Wang, 2005; Matsui and Jähr, 2003; Nakamura et al., 2013; Stanley, 2016; Voinova et al., 2015).

While the hair cells in the cochlea are essential for sound detection, the auditory brainstem is composed of specific neurons in distinct nuclei that are responsible for the computations required for processing the temporal features of sound and the localization of sound sources (Pickles, 2013) (Fig. 2). Unlike hair cell synapses which contain specialized active zone (AZ) structures and utilize CaV1 channels to encode sensory information in an analog fashion (Pangrlic et al., 2018; Wichmann and Moser, 2015), auditory brainstem synapses have

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conventional AZ ultrastructures, use presynaptic Ca\textsubscript{2+} channels with few exceptions, and utilize AP-evoked synaptic transmission to encode auditory information (Grothe et al., 2010; Leao, 2019). Therefore, elucidating the mechanisms that control presynaptic Ca\textsubscript{2+} channel abundance and organization in auditory brainstem synapses is fundamental to understanding the key underpinnings of the initial stages of auditory information processing and how defects in these processes lead to auditory processing disorders. Here we will summarize our current knowledge of presynaptic Ca\textsubscript{2+} channel abundance and organization in auditory brainstem synapses and review the mechanisms implied to regulate presynaptic Ca\textsubscript{2+} subtype levels and organization. Finally, we highlight future directions regarding Ca\textsubscript{2+} channel research that will be of importance to understanding auditory signaling.

2. Voltage-gated Ca\textsuperscript{2+} channel complex composition

VGCCs are the Ca\textsuperscript{2+} entry sites in the presynaptic AZ that are essential for triggering SV release in response to APs (Nanou and Catterall, 2018). In the CNS, VGCCs are multi-subunit complexes consisting of an \(\alpha_1\), \(\beta\), and \(\alpha_2\delta\), and sometimes the \(\gamma\) subunit (Simms and Zamponi, 2014) (Fig. 3A). The \(\alpha_1\) subunit is the critical pore forming subunit that determines VGCC kinetics, voltage dependence, pharmacology, and conductance. In the mammalian CNS, the \(\alpha_1\) subunit is encoded by ten different genes which give rise to three different VGCC families, \(\alpha_1\) (L-type), \(\alpha_2\) (P/Q, N, R type), and \(\alpha_3\) (T-type). The \(\alpha_2\) family is composed of three members, \(\alpha_2\) (L-type), \(\alpha_2\) (P/Q), \(\alpha_2\) (N), and \(\alpha_2\) (T). These are the dominant channels in the mammalian CNS that mediate AP-evoked SV release. The pore-forming \(\alpha_2\) subunit contains multiple cytoplasmic interaction sites that bind to various AZ proteins (Fig. 3B). These interactions are implicated in regulating presynaptic Ca\textsubscript{2+} abundance and control of SV release kinetics within the presynaptic AZ (Catterall, 1999, 2011; Felix, 2005; Lubbert et al., 2019; Lubbert et al., 2017; Muller et al., 2016; Simms and Zamponi, 2014). Ca\textsubscript{2+} subtype abundance in the presynaptic membrane are different from those found in the neuron soma membrane (Doughty et al., 1998; Fisher and Bourque, 1995; Miki et al., 2013), therefore the mechanism(s) that control presynaptic and somatic Ca\textsubscript{2+} abundance must be distinct.

Three cytoplasmic regions in the \(\alpha_1\) subunit are implicated as critical for regulating presynaptic Ca\textsubscript{2+} subtype abundance and the position of Ca\textsubscript{2+} channels relative to SV release sites (defined as coupling) (Simms and Zamponi, 2014), a dominant parameter controlling SV release kinetics (Nehler, 2015). These three motifs are: 1) loop I-II region, which contains the primary Ca\textsubscript{2+} subunit binding site (AID) 2) the loop II-III region, which contains the synprint motif (the interaction site with the SNARE proteins) and 3) the C-terminal region, which contains many motifs that mediate binding to AZ proteins (Simms and Zamponi, 2014) and is frequently mutated in Ca\textsubscript{2+} channelopathies (Pietrobon, 2010) (Fig. 3B). Since these regions are not highly conserved between the different Ca\textsubscript{2+} isoforms (Zamponi, 2016; Zamponi et al., 2015) and are subject to alternative splicing (Lipscombe et al., 2013), they are speculated to regulate the differences between the abundance and organization of presynaptic Ca\textsubscript{2+} subtypes through protein-protein interactions (Catterall, 2011; Felix, 2005; Lubbert et al., 2019; Simms and Zamponi, 2014).

The \(\alpha_2\delta\), \(\alpha_2\delta\), \(\gamma\) subunits are auxiliary subunits that are encoded by distinct gene families (Nanou and Catterall, 2018). In mammals, four distinct genes, \(CACNB1\), \(CACNB2\), \(CACNB3\), \(CACNB4\), encode the \(\alpha_2\) subtypes, \(\alpha_2\), \(\alpha_2\), \(\alpha_2\), and \(\alpha_2\) subunits, are heavily spliced, which results in many different splice variants (Buraei and Yang, 2013; Rima et al., 2016). The \(\alpha_2\) subunits are localized intracellularly and differentially impact the voltage dependence of activation and inactivation (Buraei and Yang, 2013). Furthermore, they are critical for triggering Ca\textsubscript{2+} channels to the plasma membrane where they are involved in regulating surface level expression. Studies analyzing \(\alpha_2\) subunits affinity for the Ca\textsubscript{2+} subunit indicate that Ca\textsubscript{2+} has the highest affinity compared to the other isoforms with order of affinities from strongest to weakest being Ca\textsubscript{2+} > Ca\textsubscript{2+} > Ca\textsubscript{2+} > Ca\textsubscript{2+} (De Waard et al., 1995). Finally, splicing generates many different Ca\textsubscript{2+} isoforms which differentially modulates regulation of these pathways (Buraei and Yang, 2013; Rima et al., 2016).

The \(\alpha_2\delta\) subunits are encoded by four different genes, \(CACN2D1\), \(CACN2D2\), \(CACN2D3\), \(CACN2D4\) and are extensively spliced (Ablinger et al., 2020; Dolphin, 2013). They are extracellular membrane proteins, that are implicated in regulating Ca\textsubscript{2+} trafficking, surface expression and voltage dependent activation of Ca\textsubscript{2+} channels (Dolphin, 2013). They have important roles in synapse formation, structure and function that are independent of regulating Ca\textsubscript{2+} trafficking and membrane levels (Ablinger et al., 2020; Geisler et al., 2015). Eight different genes in the \(CACNG\) family, \(CACNG1-8\), encode the \(\gamma\) subunits (Chen et al., 2007). Although the \(\gamma\) subunit is expressed in the brain, evidence for a role in Ca\textsubscript{2+} trafficking in the CNS is lacking.

Ca\textsubscript{2+} subtypes are the dominant Ca\textsubscript{2+} subtype in the majority of presynaptic terminals and the most efficient in triggering AP-mediated release, while Ca\textsubscript{2+} is the least efficient (Dietrich et al., 2003; Simms and Zamponi, 2014; Takahashi and Momiyama, 1993; Wheeler et al., 1994; Wu et al., 1998). Ca\textsubscript{2+} subtypes are differentially modulated by G-protein coupled receptors, metabotropic receptors, and the ability to undergo Ca\textsubscript{2+} current facilitation, which impacts synaptic transmission.

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**Fig. 1.** Voltage-Gated Calcium channel number and position relative to SVs impacts AP-evoked release. A) An increase in VGCC number results in increased Ca\textsuperscript{2+} entry, SV release and the magnitude of excitatory postsynaptic current (EPSC). B) Ca\textsubscript{2+} proximity to SVs, defined as coupling, underpins the effectiveness of Ca\textsubscript{2+} subtypes in eliciting AP-evoked release and SV release kinetics.
and plasticity (Huang and Zamponi, 2017; Nanou and Catterall, 2018). Global knock out (KO) of Cav2.1 in mice has severe effects on animal viability which results in lethality by ~3 weeks (Jun et al., 1999). Global KO of Cav2.2 results in partial lethality and global KO of Cav2.3 in mice has no impact on viability (Saegusa et al., 2001; Saegusa et al., 2000). Finally, Cav2.1 is the dominant Cav2 isoform associated with Cav2 channelopathies that manifest in neurological disorders in the CNS (Carbone and Mori, 2020; Heyes et al., 2015; Pietrobon, 2010). Taken together these data, indicate the importance of Cav2.1 channels relative to Cav2.2 and Cav2.3 in the CNS.

3. Presynaptic Cav2 channels in the auditory brainstem

In the auditory brainstem, the Cav2 channel family are the key VGCCs for driving AP-evoked synaptic transmission. Currently, studies examining presynaptic Cav2 subtype composition have been carried only in a few synapses in the lower auditory brainstem: the endbulbs of Held-spherical bushy cell (SBC) synapse, the calyx of Held-medial nucleus of the trapezoid body (MNTB) synapse, the MNTB-lateral superior olive (LSO) synapse, the MNTB-medial superior olive (MSO) synapse, and the SBC-MSO synapse (Alamilla and Gillespie, 2013; Barnes-Davies et al., 2001; Inchauspe et al., 2007; Iwasaki and Takahashi, 2001; Lin et al., 2011; Oleskevich and Walmsley, 2002; Zhuang et al., 2020) (Fig. 2).

3.1. Endbulbs of Held

The endbulbs of Held which arise from the ascending branches of the auditory nerve fibers are large presynaptic terminals which contact the SBCs in the cochlear nucleus and are required to drive temporally precise AP signaling (Brawer and Morest, 1975; Wichmann and Moser, 2015). Direct presynaptic whole-cell patch clamp recordings of Cav2+ currents at prehearing endbulbs of Held revealed that Cav2.1 currents are the dominant Cav2 current (upwards of ~90%) (Lin et al., 2011). Four days after hearing onset, although the Cav2.2 channels can elicit AP-evoked synaptic transmission, Cav2.1 channel blocker ω-agatoxin IVA reduced AP-evoked release by ~90% (Oleskevich and Walmsley, 2002). Analysis of adult animals found that ω-agatoxin IVA almost completely blocked endbulb AP-evoked release (~95%) on average, however a small amount of sensitivity to the Cav2.2 subtype blocker, ω-conotoxin GVIA still persisted (Zhuang et al., 2020). Based on these results the endbulbs of Held still contain a small population of Cav2.2 channels relative to Cav2.1 channels in the mature stage. Non-stationary fluctuation analysis of presynaptic Cav2+ currents estimate that the entire endbulb of Held contains on average ~6400 Cav2 channels, of upwards to on average 36 Cav2.1 channels.

The role of sound-evoked activity in modulating Cav2 subtype levels at the endbulbs of Held has been explored. Deafness mutant mice which are congenitally deaf from birth exhibited no change in their Cav2 subtype contribution to AP-evoked release at the endbulb (Oleskevich and Walmsley, 2002). However, experiments that manipulated sound-evoked activity levels though noise rearing or occlusion of the ear canal after the onset of hearing found that noise rearing does not impact Cav2 channel abundance while ear canal occlusion does (Zhuang et al., 2020). In particular, ear canal occlusion resulted in a significant reduction of Cav2.1 currents with a concomitant increase in Cav2.2 currents. Therefore, this indicates the mechanisms that regulate presynaptic Cav2 channel abundance at the endbulb of Held are impacted by the manner of sound-evoked activity manipulation and the developmental stage it is performed.

3.2. The calyx of Held

The calyx of Held, a giant axosomatic glutamatergic presynaptic terminal in the medial nucleus of the trapezoid body neuron (MNTB) arises from the globular bushy cell axon (Held, 1893; Morest, 1968). The calyx of Held is the sole input that relays afferent AP spike patterns from the cochlear nucleus to the MNTB, which in turn provides rapid and precise glycinegic inhibition to key mono- and binaural cell groups (Joris and Trussell, 2018). Direct whole-cell presynaptic patch clamp recordings at the calyx of Held and analysis of AP-evoked synaptic transmission at the calyx/MNTB synapse demonstrated that before

![Fig. 2. The Auditory brainstem and the synapses where Cav subtypes have been identified. A) Schematic representation of the auditory brainstem with emphases on the auditory nuclei involved in initial stages of auditory information processing. The cochlea contains hair cells which relay sound information via the auditory nerve (AN) fibers where they make synapses in cochlear nucleus (CN). The neurons within the (CN), globular bushy cells (GBCs), spherical bushy cells (SBCs) make synaptic connections to many nuclei in the auditory brainstem. Dashed line indicating the nuclei which form the superior olivary complex (SOC). Within the SOC, the GBC axons make synaptic connection to the medial nucleus of the trapezoid body (MNTB) through a single presynaptic terminal called the calyx of Held. The MNTB further innervates the lateral superior olive (LSO), the paraventricular nucleus (SPON), the medial superior olive (MSO), the ventral NTB (VNTB) and lateral NTB (LNTB). Outside the SOC, the MNTB innervates the ventral, intermediate, and dorsal nucleus of the lateral leminiscus (VNLL, INLL, DNLL, respectively) and the CN. Synapses that are studied for type of VGCCs and release are highlighted in yellow. B) Model illustrative of the synapses where VGCC subtypes have been studied. VGCC subtypes indicated are in the posthearing animal.](image-url)
hearing onset, the calyx of Held is a mixed Ca$_{\text{v}2}$ subtype terminal, with Ca$_{\text{v}2.1}$ being the dominant subtype (Borst and Sakmann, 1998; Bort and Sawang, 1998; Iwashita et al., 2005; Iwashita and Takahashi, 1998; Wu et al., 1999). In contrast, after hearing onset there is a shift to Ca$_{\text{v}2.1}$ exclusivity at the calyx of Held (Doughty et al., 1998; Iwashita and Takahashi, 1998).

Since the globular bushy neuron soma contains Cav1, Cav2, and Cav3 channels and their levels are distinct of the developmental changes at the presynaptic terminal (Doughty et al., 1998), this indicates that change in presynaptic Cav2 abundance are regulated by local mechanisms(s) and not global mechanism(s) regulating Ca$_{\text{v}2}$ channel abundance. Analysis of transcription of Cav2 subtype levels at the pre- and post-hearing stage at the calyx of Held indicate no appreciable change in the different Cav2 $\alpha_1$ subunit mRNA levels (Korber et al., 2014). Furthermore, viral vector-mediated overexpression of the Cav2.1 $\alpha_1$ subunit before the onset of hearing at the wild-type mouse calyx of Held increased Cav2.1 currents and channel numbers and at the prehearing state there was a concomitant decrease in Cav2.2 current amplitude (Lubbert et al., 2019). However, overexpression of Cav2.2 $\alpha_1$ subunit before and after hearing did not impact Cav2.1 levels (Lubbert et al., 2019). Ablation of Cav2.1 results in an increase in presynaptic Cav2.2 currents but not Cav2.3 currents before and after the onset of hearing (Inchauspe et al., 2004; Ishihkawa et al., 2005; Lubbert et al., 2017). However, the total presynaptic Ca$^{2+}$ current in the Cav2.1 null calyx is reduced compared to wild-type levels (Inchauspe et al., 2004; Ishihkawa et al., 2005; Lubbert et al., 2017).

Estimates using non-stationary noise analysis at the prehearing mouse calyx of Held estimate a total of ~20,000 Cav2.2 channels (Lin et al., 2011). Given that the average mouse calyx has ~400 active zones, each calyx of Held active zone will contain ~50 Cav2.2 channels (Lin et al., 2011). This estimate is in line with Sodium-Dodecyl-Sulfate Freeze Fracture Immunolabeling (SDFS-FRIL) EM measurements, which found that putative AZs contain ~20–80 Cav2.1 channels at both the pre- and post-hearing calyx (Lubbert et al., 2019; Nakamura et al., 2015). Although the total presynaptic Cav2$^{2+}$ current remains similar at the pre- and post-hearing calyx of Held, presynaptic Cav2.1 channel numbers in the AZ increase during development (Lubbert et al., 2019; Nakamura et al., 2015). Thus, although unitary presynaptic Cav2.1 currents may increase during development, the dominant mechanism responsible for maintaining the total presynaptic Cav2$^{2+}$ currents is the increase in presynaptic Cav2.1 channel numbers with the concomitant loss of presynaptic Cav2.2 and Cav2.3 currents. Deaf mice (Cav1.1$^{-/-}$) do not have a difference in the presynaptic Cav2.1 current amplitudes at the calyx (Erazo-Fischer et al., 2007), suggesting that sound evoked activity is not responsible for setting presynaptic Cav2 levels. However, it is unknown if the manner of sound-evoked activity manipulation and the developmental stage impacts presynaptic Cav2.2 subtype levels at the calyx. Recent findings indicate that individual calyx of Held AZs are not saturated with Cav2.1 channels, as Cav2.1 $\alpha_1$ subunit overexpression at P1 or P14 resulted in an increased number of Cav2.1 channels at the AZ, resulting in increased synaptic strength (Lubbert et al., 2019). However, the mechanisms that regulate Cav2.2 subtype levels are unknown.

### 3.3. Lateral superior olive

Synapses in the lateral superior olive (LSO) are critical for detecting interaural intensity differences (Grothey et al., 2010; Yin et al., 2019). Studies from mice two-three days after hearing onset demonstrate that synaptic transmission at GABA/glutameric projections from the MNTB to the LSO are ~75–90% dependent on Cav2.1, while the remaining contribution is equally split between Cav1 and Cav2.2 channels (Giugovaz-Tropper et al., 2011). Developmental profiles indicate that similar to the endbulbs and the calyx of Held, total Cav2 currents are constant but there is a dramatic reduction in Cav1 and Cav2.2 currents during the progression to the onset of hearing (Alamilla and Gillespie, 2013; Giugovaz-Tropper et al., 2011). Glutamatergic transmission in the immature LSO also follows a similar developmental pattern of presynaptic Cav2.2 channel subtype contribution prior to these synapses switching to purely glycinergic transmission (Alamilla and Gillespie, 2013). This indicates that the presynaptic Cav2.2 channel composition is independent of the neurotransmitter utilized. Similar to the calyx, ablation of Cav2.1 in the MNTB/LSO synapse leads to Cav2.2 dominance (on average ~80% Cav2.2) with a slight increase in Cav1 currents (Giugovaz-Tropper et al., 2011).

### 3.4. Medial superior olive

The medial superior olive (MSO) is critical for encoding interaural timing differences (Grothey et al., 2010; Yin et al., 2019). Analysis of presynaptic Cav2 current contribution to AP-evoked release at the glycinergic MNTB-MSO synapse revealed that both Cav2.1 and Cav2.2 channels contribute to AP-evoked release (Barnes-Davies et al., 2001). However, Cav2.1 channels are the dominant subtype. Analysis of the contralateral glutamatergic spherical bushy cell-MSO synapse found that both Cav2.1 and Cav2.2 equally contributed to AP-evoked release at hearing onset (Barnes-Davies et al., 2001). However, if Cav2.2 channel contribution at this synapse is decreased in the mature auditory brainstem is unknown. Based on these synapses in auditory brainstem nuclei that control timing and intensity processing, the mechanisms that increase Cav2.1 channel abundance during development are likely shared.
4. Presynaptic Ca$_{2+}$2 channel organization in the auditory brainstem synapses

In presynaptic terminals a key determinant that regulates synaptic transmission and plasticity is the organization of VGCCs with respect to SVs (Nusser, 2018; Stanley, 2016; Wang and Augustine, 2014). Presynaptic terminals contain SVs either tightly or loosely coupled to Ca$_{2+}$2, which directly correlates to the SV release modes utilized by synapses (Baur et al., 2015; Eggermann et al., 2012; Fedchyshyn and Wang, 2005; Neher, 1998; Neher and Sakaba, 2008) (Fig. 4). Microdomain synapses have fast and slow SV release rates and broad AP waveforms, which produce both synchronous and asynchronous release in response to APs. Since the AP waveform is relatively broad, Ca$_{2+}$2 can diffuse over distances of 50–100 nm in response to an AP. These synapses utilize many loosely coupled VGCCs (~100 nm between the SVs and VGCC cluster) to trigger SV release and synaptic transmission is sensitive to EGTA, a slow binding Ca$_{2+}$2 chelator. Microdomain release is supported by all three Ca$_{2+}$2 subtypes and used by many synapses in neuronal circuits that require presynaptic plasticity to encode dynamic changes in response to repetitive stimulation (Hefft and Jonas, 2005; Ohana and Sakmann, 1998; Rozov et al., 2001; Vyleta and Jonas, 2014). Nanodomain synapses are Ca$_{2+}$2.1 exclusive and have very fast SV release rates and a very narrow AP waveform, producing only synchronous release which is EGTA insensitive. Since the AP waveform is narrow, Ca$_{2+}$2 diffusion is relatively constrained to under 50 nm in response to APs. Nanodomain release is utilized by synapses in neuronal circuits that require rapid and temporally precise APs to encode information (Baur et al., 2015; Bucurenciu et al., 2008; Chen et al., 2015; Fedchyshyn and Wang, 2005; Hefft and Jonas, 2005; Matsui and Jahr, 2003). However, despite being Ca$_{2+}$2.1 dominant, presynapses in immature neuronal circuits that encode temporal fidelity transition from microdomain to nanodomain release (Baur et al., 2015; Fedchyshyn and Wang, 2005). Therefore, these data suggest that the Ca$_{2+}$2.1 channel is not the sole instructive signal for nanodomain release, and it is possible that mechanisms that facilitate reorganization of AZ proteins within the AZ are key.

Our current knowledge about calcium channel organization and control of SV release kinetics in the auditory brainstem is largely based on the calyx of Held. Prior to the onset of hearing, AP-evoked synaptic transmission at the calyx is EGTA sensitive (Borst and Sakmann, 1996; Fedchyshyn and Wang, 2005). However, at the mature calyx, AP-evoked synaptic transmission is relatively EGTA insensitive and the Ca$_{2+}$2 cooperativity of release decreases during development (Fedchyshyn and Wang, 2005). Therefore, it is well-accepted that the developmental transition to nanodomain release at the calyx of Held is critical for temporal coding of auditory signals (Joris and Trussell, 2018). Based on analysis of the AP-evoked release sensitivity to EGTA, it has been hypothesized that SVs in the prehearing calyx are ~60 nm away from Ca$_{2+}$2 clusters (Borst and Sakmann, 1996; Meinrenken et al., 2002; Wadel et al., 2007) and ~20 nm from Ca$_{2+}$2 clusters in the mature calyx (Wang et al., 2008). However, these original studies were not based on morphological data that directly measured Ca$_{2+}$2 channel organization in the presynaptic AZ. Therefore, modeling simulations on Ca$_{2+}$2 channel arrangements found many potential solutions to how the Ca$_{2+}$2 channels could be arranged to replicate AP-evoked release (Keller et al., 2015; Meinrenken et al., 2002; Wang et al., 2009). A major breakthrough came from studies using SDS-FFRIL detection of Ca$_{2+}$2.1 channels on the calyx release face, which revealed that Ca$_{2+}$2.1 channels are organized as clusters (Nakamura et al., 2015). Although SDS-FFRIL is unable to report SV positions at the AZ (Fujimoto, 1995, 1997), by combining the experimentally derived channel organization with 3D reaction-diffusion modeling simulations of AP-evoked SV release it was determined that a perimeter of SVs around clusters of Ca$_{2+}$2.1 channels was able to reproduce SV release in response to APs (Nakamura et al., 2015). Unlike other models based on AZ morphology and simulated SV and Ca$_{2+}$2.1 organization (Keller et al., 2015), the perimeter model was able to reproduce the developmental changes in AP-evoked SV release kinetics and Ca$_{2+}$2 cooperativity of release. Subsequent studies using the perimeter model found that during early development SVs in the readily releasable pool (RRP), the pool of SVs that are released by APs, are located on average ~50–60 nm from Ca$_{2+}$2 channels, while in the mature calyx of Held the pool of SVs that can be release by APs are ~5–25 nm away from Ca$_{2+}$2.1 channels (Chen et al., 2015). Therefore, based on these studies the developmental shift in the tightening of SV coupling to Ca$_{2+}$2.1 channels is the dominant parameter that underpins the fast

![Fig. 4. Release modes utilized by synapses to code information. Microdomain synapses (VGCC-SV distance ~50–100 nm) have broad APs, utilize many loosely coupled VGCCs, and have slower SV release rates which result in synchronous and asynchronous release. Nanodomain synapses (VGCC-SV distance <25 nm) have narrow APs, use a few tightly coupled VGCCs, and have fast SV release rates which result in synchronous release.](image-url)
temporal coding by the calyx of Held.

Although mature calyx of Held utilizes SVs tightly coupled to Cav2.1 channels, there is heterogeneity in the morphology and synaptic transmission properties (Grande and Wang, 2011). Since morphologically simple calyces of Held have distinct release properties compared to morphologically complex calyces of Held, it has been proposed this morphological functional continuum at the calyx of Held/MNTB synapse potentially expands the coding capacity of sound information (Grande and Wang, 2011). What underpins this morphological-functional continuum? Individual AZs in the calyces of Held contain a nanodomain transition at the calyx of Held (Grande and Wang, 2011). Since morphologically functional continuum at the calyx of Held/MNTB synapse properties (Grande and Wang, 2011). Since morphologically-functional continuum is proposed to expand the morphological-functional continuum that is proposed to expand the coding capacity for a broad spectrum of sound information. It should be noted, the role of the morphological-functional continuum at the calyx of Held in encoding sound information remains to be tested.

Currently, the organization of the Cav2.2 and Cav2.3 channels in the presynaptic AZ of the prehearing calyx is unknown. Unfortunately, the current commercially available antibodies for these channels that worked in SDS-FRIR in hippocampal synapses do not work at the calyx (unpublished observations). However, analysis of EGTA sensitivity of Cav2.2 and Cav2.3 channel-mediated AP-evoked release indicates that Cav2.2 and Cav2.3 channels are located further away from SVs than Cav2.1 channels at the calyx of Held (Wu et al., 1998; Wu et al., 1999). Subsequent analysis of AP-evoked SV release from Cav2.1 KO animals found that release was more sensitive to EGTA, which supports the idea that Cav2.2 channels are located more distal to SVs (Inchauspe et al., 2007; Inchauspe et al., 2004). However, since total Cav2 currents are decreased in the Cav2.1 KO animal it is difficult to draw a definitive conclusion if this the change is solely due to changes in distance or changes in synapsin “coupling” currents. Previous studies have shown that EGTA sensitivity of AP evoked release in the presence or absence of conotoxin is similar (Wu et al., 1999). Therefore, it has been proposed that Cav2.1 and Cav2.2 channels are located at similar distances to SVs in the presynaptic AZ (Wu et al., 1999). Studies at the endbulbs of Held that modulated sound-evoked activity using noise rearing or ear canal occlusion found no impact on the Cav2.1+ cooperativity of release (Zhuang et al., 2020). Since the Cav2.2 contribution to AP-evoked release is increased with ear canal occlusion (Zhuang et al., 2020), this suggests that Cav2.2 channels in the endbulb of Held are located at similar distances from SVs as Cav2.1 channels. However, how other forms of deafness or modulation of sound-evoked activity impact Cav2+ cooperativity and SV coupling at other auditory brainstem synapses are unknown.

Analysis of SV release kinetics at both the endbulbs of Held (Lin et al., 2011) and the endbulbs of ventral nucleus of the lateral lemniscus (VNLL) demonstrated that they have similar SV release kinetics as the calyx of Held (Berger et al., 2014). Since these terminals are proposed to be scaled down versions of the calyx of Held, it is possible that they have similar Cav2 channel arrangements. However, the arrangement of presynaptic Cav2 channels in synapses that encode interaural intensity differences are unknown. Recent work based on modeling studies from granule cell presynaptic terminals in the cerebellum, which do not use temporal coding, suggest these smaller synapses use an alternate Cav2 channel arrangement than at the calyx of Held and stellate cell presynaptic terminals in the cerebellum (Rebola et al., 2019). Therefore, it is possible this alternate arrangement may be similar in intensity encoding synapses or other synapses in the auditory brainstem that are not required for temporal coding (Keller et al., 2015; Rebola et al., 2019).

5. Molecular mechanisms controlling presynaptic Cav2 abundance and organization

Although many proteins interact with the Cav2 channel complex (Muller et al., 2010), the molecular mechanisms that control presynaptic Cav2 subtype levels independent of somatic and dendritic levels in their native environment are unsolved. While studies using cell culture have provided insight into possible mechanisms controlling presynaptic Cav2 levels, they have yielded conflicting results, leaving uncertain whether these mechanisms are essential in vivo (Acuna et al., 2015; Atasoy et al., 2007; Butz et al., 1998; Cao et al., 2004; Cao and Tsiens, 2010; Davydova et al., 2014; Hibino et al., 2002; Ho et al., 2006; Kaeser et al., 2011; Maximov et al., 1999; Meyer et al., 2019; Spafford et al., 2003a; Spafford et al., 2003b; Szabo et al., 2006; Wong et al., 2013; Wong et al., 2014; Wong and Stanley, 2010). Studies at the calyx of Held have demonstrated that mechanisms regulating Cav2 coupling are independent of those regulating Cav2 abundance (Acuna et al., 2015; Lubbert et al., 2017). However, the mechanisms that establish Cav2.1 exclusivity or dominance and Cav2 organization in auditory brainstem presynaptic terminals are largely unknown. Due to the central role of the Cav2 α1 subunit in regulating synaptic transmission through a multitude of interactions, we primarily highlight the Cav2 α1 subunit motifs and protein interactions implied to regulate presynaptic Cav2 levels and abundance and their potential role in the auditory brainstem.

5.1. Cavβ subunits

The Cavβ subunits are critical for regulating Cav2 α1 subunit trafficking, surface level expression and gating (Bureai and Yang, 2013; Rima et al., 2016). They bind to the Cav2 α1 subunit with high affinity at the α-interaction domain (AID) of the I-II loop (De Waard et al., 1994; Pragnell et al., 1994). Although a secondary β interaction exists in the Cav2 α and Cav2 β (Walker et al., 1998), loss of that region had no impact on Cav2.1 levels at the prehearing calyx of Held (Lubbert et al., 2017). In the auditory brainstem, four Cavβ isoforms are variably expressed (Allen Mouse Brain Atlas) (Lein et al., 2007) and microarray analysis revealed that globular bushy cells express all four Cavβ isoforms (Korber et al., 2014). However, Cavβ isoform localization within the calyx of Held and other auditory brainstem presynaptic terminals is currently unknown. Although global Cavβ knock out mouse lines exists (Bureai and Yang, 2013), a complete characterization of Cavβ isoforms using these Cavβ KO mouse lines in the auditory brainstem has not been carried out. Cavβ4 and Cavβ2a are located within the cultured hippocampal neuron presynaptic terminals (Xie et al., 2007) and Cav2.1 channels have higher
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Fig. 5. Potential Model of Cav2 channel subtype abundance and regulation of SV release A) In this model the Cavβ4 subunit serves as the key scaffold molecule for binding both RIMS and CAST/ELKS proteins. The interactions of RIMS and CAST/ELKS with multiple synaptic proteins regulate Cav2 subtype levels, control presynaptic Ca\textsuperscript{2+} entry, and SV release kinetics.

5.2. αδ5 subunits

αδ5 subunits are auxiliary components of the Cavα1 channel complex which are critical for Cav2 channel trafficking, surface level expression, biophysical properties and synapse development (Dolphin, 2016, 2018). They are proposed to bind the extracellular L5 loops of the Cavα1 subunit between repeats I to III (Wu et al., 2015). In cultured hippocampal neurons, αδ5 overexpression led to increased Cavα2 channel abundance (Hoppa et al., 2012; Schneider et al., 2015) and increases in AZ size (Schneider et al., 2015). In addition, since αδ5 overexpression increased the efficacy of AP-evoked released despite a reduction in presynaptic Ca\textsuperscript{2+} influx, this implicates that αδ subunits are controlling Cav2 coupling to SVs through unknown interaction partners (Hoppa et al., 2012).

Among the four αδ isoforms, αδ3-1-3 are expressed in the auditory brainstem with overall expression levels of αδ3 > αδ2 > αδ1 (Cole et al., 2005). Gene array studies at globular bushy cells showed expression of αδ1 and αδ3 (Korber et al., 2014), although the cochlear nucleus appears to only express αδ2 and αδ3 (Allen Mouse Brain Atlas) (Lein et al., 2007). Regardless, the isoforms of αδ located in the presynaptic terminal are unknown. αδ1, αδ2, αδ3 global knock out animals exist (Fuller-Bicer et al., 2009; Ivanov et al., 2004; Neely et al., 2010). αδ2 and αδ3 global knock out animals have deficits in hearing due to defects in hair cell function (Fell et al., 2016; Pirone et al., 2014). However, use of these animals to characterize the role of αδ subunits in the auditory brainstem has been minimal. Global αδ3 knock out results in a reduction in Cavα2.1 channel abundance in the soma of spiral ganglion neurons, a reduction in the size of smaller auditory nerve fiber synapses in the cochlear nucleus, and dramatic impacts on auditory signaling (Pirone et al., 2014). Studies analyzing Cavα2 currents in cultured spiral ganglion neurons from 3 week old αδ3 null mice showed a severe reduction in somatic Cavα2.1 and Cavα2.3 currents whereas somatic Cavα2.2, Cavα1 or Cavα3 currents remained unaltered (Stephani et al., 2019). However, in P5 αδ3 null spiral ganglion neurons, somatic Cavα2.1 and Cavα2.2 currents were not reduced, but Cavα1 currents were reduced (Stephani et al., 2019). In contrast, the cortex of αδ3 null mice showed increased Cavα2.2 and Cavα2.3 channel abundance but with no change in Cavα2.1 channel abundance (Landmann et al., 2018). Regardless, neither of these studies compared Cavα2 abundance at both the presynapse and soma. Moreover, the phenotypes seen with global knock out mouse lines may be due to homeostatic compensation in the CNS. Therefore, whether αδ3 regulation of Cavα2 levels is due to global mechanisms or mechanisms specific to the presynaptic terminal remain to be tested.

5.3. Synprint domain

The synprint region, which binds to SNARE proteins, is located in the Cavα2 α1 subunit domain II-III linker and is implicated in Cav2 trafficking, plasma membrane abundance and voltage-dependent activation (Nanou and Catterall, 2018). This region is highly variable between different Cavα1 α1 subunits. In addition, the Cavα2.3 channel does not contain a syntaxin 1A binding site (Jurkat-Rott and Lehmann-Horn, 2004; Rajapaksha et al., 2008; Simms and Zamponi, 2014). Studies that swapped synprint regions between the Cavα1 and Cavα2 channels demonstrated that the Cavα2 synprint region was necessary for trafficking to the presynaptic terminal and AP-evoked release in cultured superior cervical ganglion (SCG) neurons (Mochida et al., 2003a; Mochida et al., 2003b). Furthermore, these studies demonstrated no difference in AP-evoked release with Cavα2.1 chimeras containing the Cavα2.2 synprint region. This suggests that in cultured SCG neurons, the synprint regions in Cavα2.1 and Cavα2.2 are not critical for differences in presynaptic localization between Cavα2.1 and Cavα2.2 channels (Mochida et al., 2003a; Mochida et al., 2003b). Further analysis of AP-evoked cholinergic release at SCG neurons revealed that the syntaxin 1A binding site in the synprint domain was critical for Cavα2.1 and Cavα2.2 channel abundance (Mochida et al., 2003a; Mochida et al., 2003b). In contrast, syntaxin 1A binding sites are dispensable to support AP-evoked release in hippocampal neurons (Szabo et al., 2006). Furthermore, MNTB-LSO synapses contain presynaptic Cavα1 channels at the same level as Cavα2.2 channels in Cavα2.1 KO mice (Giugovaz-Tropper et al., 2011). Thus, how the mechanisms on the importance of the synprint region regulation of Cavα2 levels and organization in CNS neurons is unclear.

The synprint domain is extensively spliced in the syntaxin 1A binding domain (Rajapaksha et al., 2008). Therefore, differences in syntaxin 1A binding affinity could change Cavα2.1 presynaptic abundance and coupling. Cavα2.1 α1 subunit isoforms lacking a large portion of the synprint region containing the syntaxin 1A binding sites have reduced plasma membrane incorporation in neuroendocrine cells (Kamp et al., 2005; Rajapaksha et al., 2008). However, whether these synprint Cavα2 splice variants are present in auditory brainstem neurons is currently unknown. In addition, the Cavα2.2 and Cavα2.3 α1 subunit mRNA contains an alternatively spliced exon 18a in the synprint region that is not found in Cavα2.1 (Lipscombe et al., 2013). Therefore, it is possible that exon18a splicing may control Cavα2 subtype trafficking and incorporation into the plasma membrane at the presynaptic terminal. The Cavα2 exon18a variant levels are upregulated and found in distinct neuron types that
5.4. Presynaptic active zone molecules

Many presynaptic active zone proteins are implicated to control Cav2 abundance and coupling through interactions in the Cav2.2 α1 subunit (Chen et al., 2018; Sudhof, 2012). RIM1/2 proteins are large presynaptic AZ molecules that can directly bind the C-terminal DDXC motif in the Cav2.2 α1 subunit (Kaeser et al., 2011). They are important for regulating presynaptic Cav2 channel density and abundance in both invertebrate and vertebrate synapses, as deletion of RIMs results in significant reductions in Cav2 current amplitudes (Han et al., 2011; Kaeser et al., 2011; Liu et al., 2011). Furthermore, deletion of RIM1/2 at the prehearing calyx of Held resulted in a slight slowing of SV release rates (Han et al., 2011). MINT1 proteins are part of the tripartite complex with CASK and Velis (Butz et al., 1998; Maximov et al., 1999). MINT1 proteins directly interact with the Cav2.2 α1 subunit through the same DDXC motif as RIM1/2, which was necessary and sufficient for trafficking of Cav2.2 to the presynaptic terminals in immature primary culture hippocampal neurons (Maximov and Bezprozvanny, 2002). CASK proteins potentially regulate Cav2.2 abundance as part of the tripartite complex with MINT1. RIM binding proteins (RBPs) bind to the Cav3 family via a conserved PQTPLTPRP motif and may regulate Cav3.2 coupling to increase neurotransmitter release in neuroendocrine cells (Hibino et al., 2002). Further, RBPs are proposed to specifically regulate Cav2.1 abundance through a direct interaction with Bassoon (Davydova et al., 2014).

Despite the importance of these molecules, the mechanisms of action of these proteins is ambiguous. RIMs, RBPs, and MINTs direct interaction with Cav2.1 and 2.2 α1 subunits is proposed to be critical for setting Cav2 levels and controlling coupling (Han et al., 2011; Hibino et al., 2002; Kaeser et al., 2011). However, deletion of these direct interaction motifs in the Cav2.1 C-terminus had no impact on presynaptic Cav2.1 levels or SV release kinetics at the prehearing calyx (Lubbert et al., 2017). In addition, a unique region in the Cav2.1 α1 subunit was identified that play a role in the regulation of SV release kinetics and the readily releasable pool size (Lubbert et al., 2017). Overexpression of Cav2.1 α1 splice variants lacking the RIM, RBP, or MINT1 binding sites in a Cav2.1 KO background was able to rescue the Cav2.1 contribution to AP-evoked release (Cao et al., 2004; Cao and Tsien, 2010). Furthermore, biochemical assays have failed to detect a direct interaction between the Cav2.1 α1 subunit and RIM, RBP, and MINT1 (Wong et al., 2013; Wong et al., 2014; Wong and Stanley, 2010). In addition, ablation of MINT1 or CASK proteins in mice has no effect on AP-evoked release (Atasoy et al., 2007; Ho et al., 2006). However, deletion of RBPs leads to slower SV release rates with no change in Cav2 abundance (Acuna et al., 2015), indicating that RBPs do not regulate presynaptic Cav2 levels but may play a role in SV coupling. Therefore, these direct interactions with either RIM1/2 (Kaeser et al., 2011), MINT1, CASK (Maximov and Bezprozvanny, 2002; Maximov et al., 1999) and RBP (Davydova et al., 2014; Hibino et al., 2002) proteins are not essential for controlling presynaptic Cav2.1 levels and SV release in microdomain release mode synapse. However, it is possible these interactions regulate SV release in nanodomain release mode synapse.

Bassoon is the only AZ protein proposed to specifically control presynaptic Cav2.1 channel abundance (Davydova et al., 2014). However, rescue experiments with Cav2.1 channels lacking the bassoon binding interaction domain at the calyx of Held demonstrated that Cav2.1 channel abundance were similar to wild-type Cav2.1 channel abundance (Lubbert et al., 2017). In addition, knockdown of bassoon or dual knockout of bassoon and piccolo at the post hearing calyx, which is Cav2.1 exclusive, has no effect on basal AP-evoked release (Partbhat et al., 2017). Furthermore, analysis of Cav2.1 and Cav2.2 mEOS-tagged protein levels in cultured hippocampal neurons appear to be equivalent at bassoon positive puncta (Schneider et al., 2015). Therefore based on these data, a role for bassoon in specifically controlling presynaptic Cav2.1 channel abundance is unlikely, although it is possible that bassoon may play a role in setting presynaptic Cav2.1 abundance in other synapses.

The CAST/ELKS proteins are an evolutionarily conserved core presynaptic AZ protein family found in invertebrate and vertebrate synapses (Deken et al., 2005; Ohtsuka et al., 2002; Wag et al., 2006; Wang et al., 2002) that are key regulators of presynaptic calcium channel abundance (Dong et al., 2018; Kittel et al., 2006; Radulovic et al., 2020). In the auditory brainstem, studies on CAST/ELKS function have been restricted to the calyx of Held (Dong et al., 2018; Radulovic et al., 2020). Deletion of both CAST/ELKS at the prehearing and posthearing calyx of Held resulted in a reduction in presynaptic Cav2.2 channel currents and Cav2.2 numbers. The mechanism by which CAST/ELKS regulates Cav2.1 levels and biophysical properties is unknown. CAST/ELKS directly bind with high affinity to the Cav2.1 subunit (Kiyonaka et al., 2012) and to the RIM PDZ domain through the DDXC motif in the C-terminus of CAST/ELKS (Lu et al., 2005). In addition, CAST/ELKS has a weaker direct interaction with the Cav2.1a1 subunit in the loop II-III domain (Kiyonaka et al., 2012). Although the data favors a model in which CAST/ELKS is a key regulator (Fig. 5), the relevance of these interactions in vivo remain to be tested.

6. Conclusions

In this review we highlighted our current knowledge on presynaptic voltage-gated calcium channel abundance and organization in auditory brainstem synapses and the potential regulatory mechanisms. These studies have shown that presynaptic Cav2.1 channels are the dominant subtype regulating synaptic transmission and that auditory brainstem synapses increase their reliance of presynaptic Cav2.1 during neuronal circuit maturation. Interestingly, it appears that Cav2 subtype levels can be impacted by the manner of sound-evoked activity manipulation and the developmental stage it is performed. Based on these observations, this raises a multitude of questions. What are the molecular mechanisms that regulate presynaptic Cav2 abundance and organization in the auditory brainstem? How does manipulation of auditory activity impact presynaptic Cav2 channel abundance and organization? Why does the type of sound-evoked activity manipulation and developmental time period differentially impact presynaptic Cav2 subtype levels? In addition, MNTB-LSO contain presynaptic Cav1 channels. Is their presynaptic presence unique to MNTB-LSO synapses or are they found in other presynaptic terminals in the auditory brainstem? What is the role that presynaptic Cav2.2, Cav2.3 and Cav1 channels play during development of the auditory brainstem? Do changes in presynaptic Cav2 channels and organization in the auditory brainstem synapses contribute to auditory processing disorders?

Currently studies on presynaptic channels have been limited to a few synapses in the auditory brainstem. Given that presynaptic VGCCs can have vastly different biophysical, pharmacological, and modulatory properties, it is critical to further investigate other presynaptic terminals in the auditory brainstem. The molecular diversity within presynaptic terminals enables a wide range of synaptic properties (Nusser, 2018). Therefore, there will likely be a multitude of presynaptic mechanisms such as trafficking, insertion, retention and stability that regulate presynaptic VGCC levels and organization in the AZ (Lubbert et al., 2019; Lubbert et al., 2017). New genetic models and molecular tools will be needed that will allow for cell specific and temporal specific manipulation of presynaptic VGCCs. Fundamental and important work remains to be done to elucidate our understanding on how presynaptic VGCCs regulate the localization of sound sources, processing of the temporal features of sound, and the contributions of defects in these processes to auditory processing disorders.
Declaration of competing interest
We have no competing interests to declare. I am signing on behalf of myself and Dr. Priyadarsini Veeraraghavan.

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