Introduction

A functional nervous system requires the formation and maintenance of synapses. Chemical neurotransmission requires a sensing of action potentials by presynaptic voltage-dependent calcium channels (VDCCs) and the resultant Ca$^{2+}$ influx into the presynaptic terminal that induces the fusion of synaptic vesicles to pre- synaptic membranes. 1,2 Chemical neurotransmitters released via exocytosis will then bind and open ligand-gated channels in the postsynaptic membrane to modify the excitability of the postsynaptic cell.3-5 Thus, synaptic ion channels must be concentrated in the pre- and postsynaptic membranes for effective synaptic transmission.

The formation and maintenance of synapses require both transynaptic organizing signals and the assembly of intracellular scaffolds. The list of molecules capable of inducing synapses (termed synapse organizers) continues to grow and has been reviewed in detail elsewhere.6-15 Interestingly, recent findings demonstrate that synaptic channels are also directly involved in the formation and maintenance of synapses, in addition to their functional role as ion-conducting channels required for synaptic transmission. The goal of this review is to explore how the non-conducting functions of ion-channel subunits contribute to the formation and maintenance of synapses in concert with extra-cellular synapse organizers and presynaptic active-zone-specific proteins (Fig. 1).

Presynaptic VDCCs and Cytosolic Proteins at the Synapse

Presynaptic localization of VDCCs requires cytosolic domains on the VDCCs (Synprint region, C-terminus domain).16-19 During the early stage of synapse formation, VDCCs and presynaptic active zone proteins are transported to the axon terminals by preassembled protein/vesicle packets.20,21 A unitary assembly of a nascent synapse has been modeled based on these preassembled transport vesicles.20-22 At the presynaptic terminal, VDCCs are strategically located in close proximity to the neurotransmitter release sites, which reduces the delay between Ca$^{2+}$ entry and synaptic vesicle fusion.23-29 The ultrastructural location of presynaptic VDCCs has been suggested as the paired double rows of 100-Å membrane macromolecules that are identified on the protoplasmic fracture face of freeze-fracture electron micrographs.30-32 Electron tomography analysis revealed large protein complexes at the presynaptic terminal, including the structure called “pegs” that aligned well with the paired double rows of membrane macromolecules.33-35 Thus, it is likely that presynaptic VDCCs form a complex with proteins required for active zone organization and synaptic vesicle fusion.

Few groups have taken a proteomics approach to identify the proteins associated with VDCCs.36-39 The extensive lists of proteins in these VDCC complexes coincide with the macromolecules composing active zone material visualized by electron tomography.33,34,40 VDCC subunits interact with some of these proteins directly, and with other proteins in these lists indirectly. Cytosolic interactions of VDCCs and synaptic proteins (SNAP-25, syntaxin, synaptotagmin) that modulate channel functions are

Recent findings demonstrate that synaptic channels are directly involved in the formation and maintenance of synapses by interacting with synapse organizers. The synaptic channels on the pre- and postsynaptic membranes possess non-conducting roles in addition to their functional roles as ion-conducting channels required for synaptic transmission. For example, presynaptic voltage-dependent calcium channels link the target-derived synapse organizer laminin β2 to cytomatrix of the active zone and function as scaffolding proteins to organize the presynaptic active zones. Furthermore, postsynaptic δ2-type glutamate receptors organize the synapses by forming transsynaptic protein complexes with presynaptic neurexins through synapse organizer cerebellin 1 precursor proteins. Interestingly, the synaptic clustering of AMPA receptors is regulated by neuronal activity-regulated pentraxins, while postsynaptic differentiation is induced by the interaction of postsynaptic calcium channels and thrombospondins. This review will focus on the non-conducting functions of ion-channels that contribute to the synapse formation in concert with synapse organizers and active-zone-specific proteins.

Key words: Bassoon, CAST, Cbln1, GABA_A, GluRδ2, Narp, neurexin, neuromuscular junction, rim, voltage-gated calcium channel
reviewed in Turner et al. in this issue of Channels and elsewhere.\textsuperscript{41} Furthermore, the organization of the presynaptic protein complex has been reviewed in detail elsewhere.\textsuperscript{42,43} Thus, the first section of this review will focus on the recently identified direct interactions of VDCC subunits and presynaptic active-zone-specific proteins. Active zones are areas of the presynaptic membrane with electron-dense material where synaptic vesicles fuse.\textsuperscript{44-46} The so-called cytomatrix of the active zone accumulates specifically at presynaptic active zones and includes Bassoon,\textsuperscript{57} Bruchpilot,\textsuperscript{48} CAST/Erc2,\textsuperscript{49} Munc13,\textsuperscript{50} Piccolo,\textsuperscript{51} Rim1,\textsuperscript{52} and SYD-2.\textsuperscript{53-56}

**VDCCα subunits and active zone proteins.** Structural interactions of VDCCα subunits during presynaptic differentiation have been demonstrated in central and peripheral synapses. The Ca_{2.2} (N-type, α1B,) and Ca_{1.2} (L-type, α1C) VDCCs, but not Ca_{1.3} (L-type, α1D), bind directly with the active zone protein Rim through interactions between the cytoplasmic loop connecting domain II–III of VDCC and the C2A or C2B domains of Rim.\textsuperscript{57} Rim also interacts directly with the VDCCβ subunit, which will be discussed below.\textsuperscript{58} Furthermore, the Ca_{2.2} and Ca_{2.1} (P/Q-type, α1A) VDCCα subunits and Rim1/2 bind directly through interactions between the C-terminus domain of the VDCCα subunit and the central PDZ domain of Rim.\textsuperscript{59} The Rim PDZ domain is required for maintaining presynaptic Ca\textsuperscript{2+} channel localization, and the Rim N-terminus primes synaptic vesicles.\textsuperscript{59} Similarly, Ca_{1.2} (L-type, α1C) VDCCs, but not Ca_{1.3} (L-type, α1D), bind directly with the active zone protein Piccolo through interactions between the cytoplasmic loop connecting domain II–III of VDCC and the C2A or C2B domains of Piccolo.\textsuperscript{60} These interactions were suggested to play roles in insulin granule exocytosis, which seems to serve a similar function as the Rim interaction mentioned above.

The interactions of the VDCC and active zone proteins are conserved in invertebrate synapses as well. At the Drosophila neuromuscular junction, Cacophony (the P/Q-type VDCCα subunit homologue) interacts directly with the active zone-specific protein Bruchpilot (Brp; a CAST/Erc family member).\textsuperscript{61} In Brp mutants, reduced levels of VDCCs (Cacophony) accumulated at active-zone-like structures at neuromuscular junctions, and specialized active zone structures called T-bars were missing. Thus, some VDCCs can initially accumulate at active zones without Brp, but the VDCC-Brp interaction is responsible for effectively clustering more VDCCs beneath the active zone density.\textsuperscript{61}
VDCCβ subunits and active zone proteins. Like the α subunits, VDCCβ subunits interact directly with active zone proteins. VDCCβ subunits bind directly with Rim1 through interactions between the center domain of the VDCCβ subunit (containing the Src homology 3 domain, α1-interacting domain and guanylate kinase domain) and the C-terminus domain of Rim1 (including the C2B domain). Protein complexes consisting of the P/Q-type VDCCα subunit, VDCCβ subunits and Rim1 can be co-sedimented from mouse brain homogenates, demonstrating the formation of triad in vivo. This interaction suppresses the voltage-dependent inactivation of neuronal VDCCs. Together with the enhanced synaptic vesicle docking in the vicinity of VDCCs that is caused by Rim1, this interaction potentiates neurotransmitter release at the presynaptic active zone.

VDCCβ subunits also interact with the active zone proteins Bassoon and CAST/Erc2. These active zone proteins are co-immunoprecipitated as triad of the P/Q-type VDCCα subunit, β1b or β4 subunits, and Bassoon or CAST/Erc2. This interaction is essential to structurally organize the presynaptic active zones at neuromuscular junctions. In the absence of functional Bassoon, whole-cell Ca2+ current and Ca2+ influx at the presynaptic microdomain of inner hair cells (primarily reflecting Ca2+ influx at active zones) are reduced. These findings suggest that the role of the VDCCβ subunit-Bassoon interaction may be similar to the role of the interaction between the VDCCβ subunit and Rim1.

These interactions between VDCC subunits and active-zone-specific proteins allow for anchoring of the active zone cytomatrix to the presynaptic membrane. Active zone proteins are also known to interact between themselves. Consistently, Bassoon, Piccolo and Rim are found in protein complexes containing VDCCs in vivo. Double-knockout mice for P/Q VDCCs and N-type VDCCs have synapses specifically lacking active zones. Taken together, these interactions suggest a formation of a macromolecular protein complex on the cytosolic side of the presynaptic VDCC, which is likely to be the active zones visualized as an electron dense projection in the ultrastructural analysis by electron tomography.

Presynaptic VDCCs and Extracellular Synapse Organizers

Two types of VDCCs, the P/Q- and N-types, are concentrated at many presynaptic terminals in the central and peripheral nervous system and play essential roles for synaptic transmission. A growing number of reports demonstrate that VDCC subunits are involved in organizing presynaptic differentiation jointly with synapse organizers. The second section of this review will focus on the extracellular interaction between presynaptic VDCC subunits and synaptic molecules that is necessary for synapse formation.

P/Q-type VDCCs and laminin β2. VDCC α subunits are known to have non-conducting functions for presynaptic differentiation at the neuromuscular junction. Presynaptic P/Q- and N-type VDCCs bind directly to laminin β2, which is a synapse organizer secreted by postsynaptic muscle cells. The P/Q-type VDCC utilizes its 11th extracellular loop domain (46 amino acids, excluding the lip domain) to interact with laminin β2 at its C-terminal 20 kDa domain, which includes a leucine-arginine-glutamine sequence. This interaction organizes the synaptic vesicle release sites or active zones, at motor nerve terminals. Furthermore, this interaction is linked to active-zone-specific proteins by VDCCβ subunits, suggesting a mechanism to link synapse organizers to the cytosolic presynaptic proteins (described in section I). Single-knockout mice for P/Q-type VDCCs, N-type VDCCs or laminin β2, or double-knockout mice for P/Q-type VDCCs and N-type VDCCs show reduced numbers of active zones. Laminin β2 induces presynaptic differentiation in cultured motor neurons even in the presence of P/Q- and N-type VDCC blockade by agatoxin-IVA and conotoxin-GIVA, providing evidence for the dispensability of the Ca2+ influx into nerve terminals for active zone formation. This extracellular interaction of presynaptic VDCCs and synaptogenic molecules organizes the presynaptic differentiation of neuromuscular junctions.

Similar to the neuromuscular junction, laminin β2 is concentrated in the synaptic cleft at the photoreceptor synapses and aid the differentiation of retinal neurons. Instead of P/Q-type VDCCs, the Ca1.4 (L-type, α1F) VDCC is preferentially expressed in the retina and is concentrated at photoreceptor synapses. Genetic deletions of these genes in mice causes a disassociation of the ribbons, an active zone structure, from presynaptic membranes at the photoreceptor synapses. This active zone phenotype suggests that the interaction of presynaptic VDCCs and laminin β2 plays an essential role in organizing active zones at the photoreceptor synapse, similar to the neuromuscular junction.

N-type VDCCs and α-neurexin. A functional link between N-type VDCCs and presynaptic neurexin has been suggested. Accumulation of VDCCs at brainstem synapses was impaired in the triple-knockout mice for synapse organizer α-neurexins. Interestingly, this phenotype is specific to N-type VDCCs and presynaptic neurexins awaits confirmation. The presynaptic proteins CASK and Mint/X11 can link between VDCCs and α-neurexins. However, the possibility of this interaction is decreased by the fact that knockout mice for CASK demonstrated normal active zones and functional presynaptic VDCCs. Similarly, cultured triple-knockout neurons for Mint1/2/3 showed normal synaptic ultrastructure and a defect of presynaptic function that seems to be attributable to the upregulated Munc18-1.

In a similar manner, a functional link between postsynaptic L-type VDCCs and the extracellular matrix glycoprotein tenascin-C has been suggested for hippocampal long-term potentiation, but the evidence for a direct physical interaction between VDCCs and tenascin-C awaits confirmation.

VDCC αδ subunit. The VDCC αδ subunits play roles in presynaptic differentiation at the Drosophila neuromuscular junction. Mutant embryos lacking the αδ-3 subunit have malformed synaptic boutons. This role of the αδ-3 subunit...
is independent of the ion-conducting function of the calcium channel complex, and is separate from its role of properly localizing VDCCαx subunits at the neuromuscular junctions. Any role of synapse organizer for these phenotypes is currently unknown.

**Postsynaptic Channels and Extracellular Synapse Organizers**

A primary function of postsynaptic ligand-gated ion channels at chemical synapses is to bind neurotransmitters and then open to modify the excitability of the postsynaptic cells. The cytosolic domains of these ligand-gated channels interact with postsynaptic scaffolding proteins to organize the synapse and modify the channel functions, which is detailed in other reviews. In addition to these interactions, the subunits of postsynaptic channels also show synaptogenic activities or interact with synapse organizers. The third section of this review will focus on extracellular interactions of postsynaptic channel subunits and synapse organizers.

**Glutamate receptor δ2 and cerebellin1.** Postsynaptic δ-type glutamate receptors (GluRδ2, GluD2) form transsynaptic protein complexes with presynaptic neuromitins through synapse organizer cerebellin1 precursor proteins (Cbln1). GluRδ2 is selectively expressed in Purkinje cells of the cerebellum and is exclusively localized at parallel fiber-Purkinje cell synapses. GluRδ2 forms heteromeric channels with AMPA or kainate receptors in vitro, but can exist as a homomeric receptor in vivo. Importantly, GluRδ2 demonstrates synaptogenic activity in vivo, as demonstrated by knockout mouse studies.

The N-terminal domain of GluRδ2 induces presynaptic differentiation in vitro and in vivo. Finally, a GluRδ2-null cerebellum shows impaired long-term depression (LTD) of parallel fiber-Purkinje cell synaptic transmission.

The N-terminal domain of GluRδ2 binds directly to Cbln1 and Chn1 also binds directly to neurexins IB/2B/3B and 1α, containing the S4 splice site. The Cbln1 knockout mouse shows ataxia and a severe reduction in the number of synapses between Purkinje cells and parallel fibers, which closely resembles the GluRδ2-null mouse. Strikingly, the synaptic defect in the Cbln1 knockout mouse can be rescued within a day by a single injection of recombinant Cbln1. This triad interaction of GluRδ2, Cbln1 and neurexin is essential for synapse formation between parallel fiber and Purkinje cells in cerebellum. This interaction can align postsynaptic channels to the synapse organizer located at the presynaptic terminal.

**Glutamate receptors and the neuronal pentraxin family, N-Cadherin, and EphB.** The synaptic clustering of AMPA receptors (GluR1-4 subunits, GluA1-4) is regulated by neuronal activity-regulated pentraxin (Narp). Narp is a member of the neuronal pentraxin family of calcium-dependent lectins, which includes neuronal pentraxin1 (NP1) and neuronal pentraxin receptor (NPR). Narp and NP1 are secreted proteins that form heteromeric complexes on the extracellular surface, and NPR is a transmembrane protein. Of the neuronal pentraxin family members, Narp is the only immediately early gene regulated by synaptic activity.

The N-terminal domain of the AMPA receptor GluR4 subunit interacts with the pentraxin domain of NP1. Axonal NP1 and NPR in presynaptic neurons are required to recruit GluR4 to synapses. The N-terminal domain of GluR4 is necessary and sufficient for its recruitment to the synapses. The Narp knockout mouse shows Narp’s requirement for activity-dependent changes in the strength of excitatory inputs onto parvalbumin-expressing interneurons of the hippocampus. Triple-knockout mice for neuronal pentraxins indicate that the pentraxins are necessary for early synaptogenic refinements in the retina and dorsal lateral geniculate nucleus.

AMPAR receptors are rapidly endocytosed in an mGluR1/5 dependent manner. This endocytosis of the GluR1 subunit requires NRP, which is cleaved by the MMP tumor necrosis factor-alpha converting enzyme (TACE) in an mGluR1/5-dependent fashion. The analysis using knockout mice for NRP and TACE blockers showed that NRP and TACE-activity are required for mGluR1/5-dependent LTD in hippocampal and cerebellar synapses.

Similarly, the N-terminal domain of AMPA receptor subunit GluR2 (GluA2) interacts directly with N-Cadherin and promotes formation and growth of dendritic spines in vitro and regulates hippocampal LTD. The extracellular domain of the NMDA receptor subunit NR1 (GluN1) interacts directly with tyrosine kinase EphB2 and regulates synapse development.

**GABA_α receptor and neurexin.** Postsynaptic GABA_α receptors interact directly with presynaptic neurexins. GABA_α receptors can be purified from brain homogenates using immobilized neurexin-2B in a neurulin-independent manner. A recombinant protein of the extracellular domain of the GABA_α1 receptor binds the extracellular domain of neurexin. Overexpression of neurexins in cultured neurons selectively suppresses GABAergic synaptic transmission without decreasing the number of GABAergic synapses, and this effect is independent of neurulin. This extracellular interaction suggests a potential mechanism to control the inhibitory synaptic transmissions in the brain. It is interesting to note that an increasing number of synapse organizers and synaptic proteins (Chn1, α-Dystroglycan, GABA_α receptor, LRRTMs, neuroligins) are found to interact directly with neurexins and contribute to the organization of synapses.

**VDCC αδ subunit and thrombospondin.** The auxiliary subunit of the VDCC, αδ subunit, is a receptor for the glial-derived synaptogenic molecule thrombospondin. These two proteins bind directly through the interaction between the von Willebrand factor type A domain of the αδ-1 subunit and the type 2 EGF-like repeats of thrombospondin1-5. This interaction promotes the formation of excitatory synapses with postsynaptically silent synapses lacking AMPA receptors in the mammalian central nervous system. This αδ-thrombospondin-mediated synapse formation does not require the ion-conducting function of L-, N- or P/Q-type VDCCs. Eroglu and colleagues concluded that αδ is necessary and sufficient postsynaptically.
for this synaptogenic activity based on their analysis of αβ-1
overexpression and knockdown in the postsynaptic cells. Thrombospondin induces ultrastructurally normal synapses that are presynaptically active, but the involvement of presynaptic αβ subunits or the identity of the presynaptic receptor for the thrombospondin-induced presynaptic differentiation awaits further study. Interestingly, thrombospondin has been shown to also interact in vitro with the synapse organizer neuroligin, which localizes at the postsynaptic membrane. These interactions may cooperate for postsynaptic differentiation.

Knockout mice for the αβ-2 subunit exhibit morphological abnormalities of Purkinje cell dendrites in the cerebellum. Mice lacking the αβ-4 subunit exhibit a significantly reduced outer plexiform layer, and their intraretinal circuitry and functions are perturbed. The phenotypes in these mutant mice suggest roles of the VDCC αβ δ subunit in synapse formation, but ultrastructural or immunohistochemical analyses of the synapse in these mutants mice awaits further study. Whether thrombospondin (or other synapse organizers) contributes to the phenotypes in these αβ δ mutants remains unclear.

**Are there More Extracellular Interactions of Ion Channels at Synapses?**

Potentially, additional interactions of synaptic ion channel-synapse organizer remain to be identified. This possibility is supported by several examples of extracellular interactions of ion channel subunits identified outside the synapse. For instance, G-protein-activated inward rectifier K (GIRK) channels interact directly with integrins. The arginine-glycine-aspartate (RGD) sequence located in the extracellular domain of GIRK channel binds to integrins. This interaction increases the plasma membrane localization of GIRK channels. Also, the voltage-dependent sodium channel α subunit interacts with the extracellular carbonic anhydrase domain of receptor protein tyrosine phosphatase β (RPTPβ). RPTPβ also interacts with the cytosolic domains of the voltage-dependent sodium channel α subunit and β1 subunit. These interactions modulate channel function by the phosphatase activity of RPTPβ. The β1/2 subunits of voltage-dependent sodium channels also interact directly with cell adhesion molecules (connexin-43, contactin, N-cadherin, NrCAM, neurofascin-155, -186, tenasin-C/R) on axons and glia cells. The diverse functional roles of these homophilic and heterophilic cell adhesions of voltage-dependent sodium channels are reviewed in detail elsewhere. Genetic interactions of the mechanotransduction channel MEC-4/10 and the extracellular anchor protein MEC-5 through the extracellular link protein MEC-9, or with collagen unc-105 have been suggested, but these await confirmation of direct physical interactions. These examples show that extracellular interactions of ion channel subunits can be quite diverse and extensive.

**Summary and Perspectives**

The studies summarized in this review clearly establish non-conducting roles for ion-channel subunits in the formation and maintenance of synapses, in addition to their important role as ion-permeable channels for chemical neurotransmission. Both presynaptic and postsynaptic channel subunits interact with extracellular synapse organizers. Such structural interactions are extended into the cytosolic region by using the ion-channel subunits as scaffolding proteins. These trans-synaptic protein interactions allow alignment of pre- and postsynaptic specialization to achieve effective neurotransmission.

Important questions still remain to elucidate the role of transsynaptic molecular mechanisms involving ion channel subunits for organizing synapses. First, the initial interaction or the essential interaction to precisely position these synaptic channels at the pre- and postsynaptic sites remains unknown. Recent findings described in this review suggest that the location of these synaptic channels can be modified either from cytosolic side or extracellular side. Second, when or how much is ion-conducting function of channels required for the formation/maturat/maintenance of synapses? The molecular mechanism responsible for the initial phase of synapse formation does not seem to require synaptic activity. Even in the absence of synaptic transmission, morphologically normal synapses can be formed with active zones in knockout mice for choline acetyltransferase or munc18-1, or double-knockout mice for munc13-1/2 or P/Q-, N-type VDCCs. However, activity becomes important later for synaptic elimination and P/Q-type VDCCs plays an essential role at the climbing fiber-Purkinje cell synapse. Third, the timing of the synapse transmission control/ modification by these transsynaptic interactions is unknown. The extracellular interactions reviewed here can modify both excitatory and inhibitory synapses, but the control of these actions remains unknown. These are just few examples of questions that await further investigation to elucidate synapse formation and maintenance.

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