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SIGNALING MECHANISMS IN SYNAPSE ASSEMBLY

Secreted factors as synaptic organizers

Erin M. Johnson-Venkatesh[1] and Hisashi Umemori[1,2]
[1]Molecular & Behavioral Neuroscience Institute, University of Michigan Medical School, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, USA
[2]Department of Biological Chemistry, University of Michigan Medical School, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, USA

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Abstract

A critical step in synaptic development is the differentiation of presynaptic and postsynaptic compartments. This complex process is regulated by a variety of secreted factors that serve as synaptic organizers. Specifically, fibroblast growth factors, Wnts, neurotrophic factors and various other intercellular signaling molecules are proposed to regulate presynaptic and/or postsynaptic differentiation. Many of these factors appear to function at both the neuromuscular junction and in the central nervous system, although the specific function of the molecules differs between the two. Here we review secreted molecules that organize the synaptic compartments and discuss how these molecules shape synaptic development, focusing on mammalian *in vivo* systems. Their critical role in shaping a functional neural circuit is underscored by their possible link to a wide range of neurological and psychiatric disorders both in animal models and by mutations identified in human patients.

Introduction

A functional neural circuit requires the precise apposition of presynaptic and postsynaptic specializations, forming a functional synapse. Synaptic development is a process that involves: (i) axon extension and targeting, (ii) initial contact between the axon and its target, (iii) presynaptic and postsynaptic differentiation, (iv) synaptic maturation, (v) synaptic pruning, and (vi) maintenance (Sanes & Lichtman, 1999; Benson *et al.*, 2001; Goda & Davis, 2003; Scheiffele, 2003; Waites *et al.*, 2005; Fox & Umemori, 2006). Here we focus on the synaptic differentiation and maturation steps, the steps that convert axons and their targets into functional synapses. As a developing axon contacts its target, the growth cone transforms into a presynaptic terminal containing functional neurotransmitter release machinery. This process involves: (i) accumulation of synaptic vesicles and vesicle-associated proteins, (ii) active zone formation, (iii) calcium channel clustering, and (iv) amassing of mitochondria in the nerve terminal (Fig. 1). In the target cell, postsynaptic specializations are induced. This process involves: (i) clustering of neurotransmitter receptors, (ii) accumulation of scaffolding proteins, such as post-synaptic density protein-95 (PSD-95) at glutamatergic synapses and gephyrin at GABAergic synapses, and (iii) morphological changes, such as spine formation.

The organization of presynaptic and postsynaptic compartments is accomplished by a variety of molecules called synaptic organizers (Gautam *et al.*, 1996; Umemori *et al.*, 2004). Pioneering work at the neuromuscular junction (NMJ) identified two secreted factors, agrin and laminin-β2, as critical for postsynaptic and presynaptic differentiation, respectively (Sanes & Lichtman, 2001). Recent studies suggest that multiple secreted factors cooperate for neuromuscular synapse formation (Fox *et al.*, 2007). In the brain, molecules proposed to be critical for the organization of synaptic compartments include secreted factors and cell adhesion molecules. The secreted factors that are shown to play a role in synaptic organization in the mammalian brain *in vivo* fall into one of five families: fibroblast growth factors (FGFs), Wnts, pentraxins, neurotrophic factors and thrombospondins. Cell adhesion molecules (CAMs) that organize synapses include: leucine-rich repeat transmembrane proteins (LRRTMs), neurexins and neuroligins, SynCAM, the netrin G ligand family of adhesion molecules (NGLs), and ephrins and their receptors (Ephs). These adhesion molecules are described in the chapter by Washbourne and have been reviewed previously (Gerrow & El-Husseini, 2006; Biederer & Stagi, 2008; Jin & Garner, 2008). In this chapter, we focus on secreted molecules (Fig. 1) and describe synaptic differentiation induced by secreted factors at the NMJ and in the central nervous system (CNS), with particular emphasis on glutamatergic synapses. As these studies were carried out in many different animal models and both *in vitro* and *in vivo* systems, we particularly emphasize those conducted *in vivo* in mammalian systems.

The neuromuscular junction

Due to its accessibility, simplicity and stereotyped development, many molecules involved in synaptic development were first characterized at the NMJ (Table 1). The study of synaptic development was pioneered by the discovery that agrin is a postsynaptic organizer at the NMJ. Since this discovery, many other molecules have been implicated in

Correspondence: Hisashi Umemori, [1]Molecular & Behavioral Neuroscience Institute and [2]Department of Biological Chemistry, as above.
E-mail: umemoh@umich.edu

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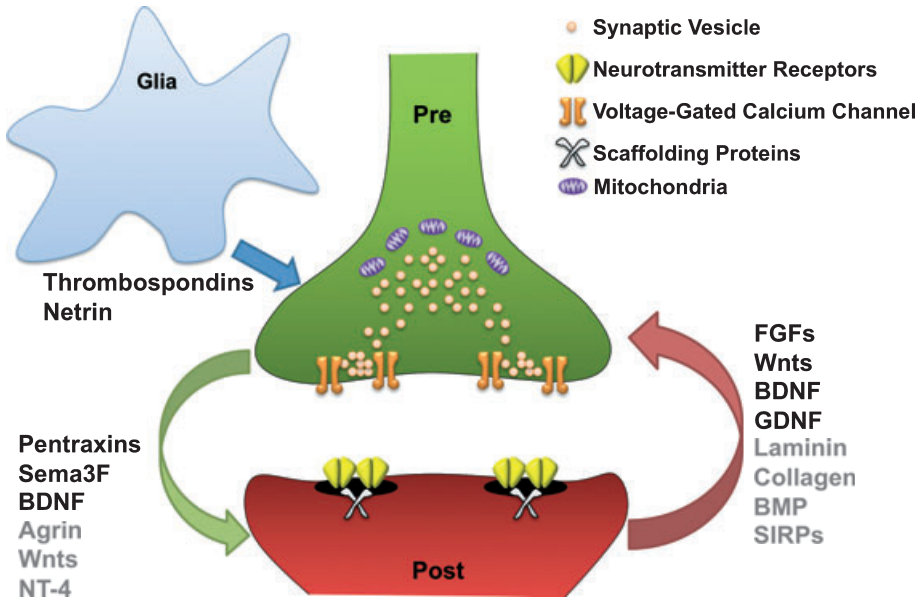

FIG. 1. Synaptic development involves the differentiation of both presynaptic and postsynaptic terminals by various secreted synaptic organizers. Differentiation of the presynaptic compartment includes the accumulation of synaptic vesicles, mitochondria and voltage-gated calcium channels, and the formation of active zones. Presynaptic organizers are released from the target cell and promote this differentiation; these factors include FGFs, Wnts, BDNF, GDNF (CNS and NMJ), laminin-β2, collagen IV, BMP and SIRPs (NMJ). Differentiation of the postsynaptic compartment involves the aggregation of neurotransmitter receptors and scaffolding proteins, and spine development. Postsynaptic organizers released from the presynaptic nerve terminal include pentraxins, Sema3F (CNS), BDNF (CNS and NMJ), agrin, Wnts and NT-4 (NMJ). Glia surrounding the newly forming synapse also contribute to CNS synaptic development by the release of thrombospondins and netrin.

NMJ development. However, agrin is still the only molecule that is shown to be critical for postsynaptic differentiation in mammals *in vivo*. For presynaptic differentiation, FGF7/10/22, laminin-β2 and collagen IV have been identified as target-derived presynaptic organizers at the mouse NMJ *in vivo*. In other systems, morphogens and signal regulatory proteins (SIRPs) have been shown to regulate development of the postsynaptic and presynaptic compartments of the NMJ. Neurotrophic factors appear to be involved in the modulation and maintenance of the NMJ.

Synaptic development of the mammalian neuromuscular junction in vivo

Agrin: the postsynaptic organizer

Prior to motor neuron approach, the developing muscle contains relatively sparse acetylcholine receptors (AChRs). Upon arrival of the motor neuron, the AChRs cluster at the motor neuron contact site. This clustering involves the mobilization of AChRs already in the membrane as well as the production of new AChRs. Early work at the NMJ delineated the agrin signaling pathway as critical for this postsynaptic receptor clustering (Sanes & Lichtman, 2001). Agrin was originally identified from the *Torpedo* electric organ as a basal lamina molecule able to cluster AChRs (McMahan *et al.*, 1992). This led to the 'agrin hypothesis', whereby agrin is released from the motor nerve terminal and then organizes the postsynaptic apparatus (McMahan, 1990). Agrin binds to its receptor, LRP4 (low-density lipoprotein receptor-related protein 4), which then activates MuSK (muscle-specific kinase) to cluster AChRs (Kim *et al.*, 2008; Zhang *et al.*, 2008). MuSK interacts with the cytoplasmic proteins Dok-7, Tid1 (tumorous imaginal disc) and the cytoplasmic adaptor protein rapsyn to induce AChR clustering (Okada *et al.*, 2006; Linnoila *et al.*, 2008; Song & Balice-Gordon, 2008). However, the exact signaling pathway that mediates AChR clustering remains largely unknown. Agrin-deficient mice have fewer and smaller AChR aggregates (Gautam *et al.*, 1996), indicating that agrin is critical for NMJ development.

Presynaptic organizers: distinct molecules for different developmental stages

The first well-characterized presynaptic organizer at the NMJ was laminin-β2 (also known as s-laminin), which is an extracellular matrix protein like agrin. Laminin-β2 is derived from muscle and concentrated in the synaptic basal lamina (Hunter *et al.*, 1989). In mice lacking laminin-β2, endplates form in the right location; however, active zones do not form properly and the frequency of mini-end-plate potentials is greatly diminished (Noakes *et al.*, 1995; Knight *et al.*, 2003). Voltage-gated calcium channels appear to serve as the receptors for laminin-β2 to mediate presynaptic development (Nishimune *et al.*, 2004).

Another well-characterized family of target-derived presynaptic molecules is the FGF family. FGFs are a family of intercellular signaling molecules with 22 members in mammals. They signal through four different receptors [FGF receptors (FGFRs)] that are all tyrosine kinase receptors (Ornitz & Itoh, 2001). FGF7/10/22 were identified as presynaptic organizers based upon their activity in a synaptic vesicle clustering assay in motor neurons (Umemori *et al.*, 2004; Fox *et al.*, 2007).

FGF7/10/22, along with laminin-β2 and collagen IV, are necessary for proper neuromuscular synapse development *in vivo* (Fox *et al.*, 2007). The expression pattern of these molecules and the phenotype of knockout animals suggest that they act sequentially to guide NMJ development and maintenance. FGF7/10/22, signaling through FGFR2b, is important for the induction of presynaptic terminals, followed by signaling from laminin-β2 for maturation and then collagen IV for maintenance of the NMJ (Fox *et al.*, 2007). These results delineate how multiple presynaptic organizers can permit separate control of distinct phases in the life of a neuromuscular synapse *in vivo*.

TABLE 1. Comparison of molecules implicated in synaptic development at the NMJ and/or in the CNS.

Class of molecule	Molecule	Role at the NMJ	Model	Role in the CNS	Model
FGF	FGF7	Presynaptic vesicle clustering Synapse repair	Receptor knockout mice FGFBP1 RNAi in mice	Presynaptic GABAergic vesicle clustering	Knockout mice and receptor knockout mice
	FGF22	Presynaptic vesicle clustering Synapse repair	Receptor knockout mice FGFBP1 RNAi in mice	Presynaptic glutamatergic vesicle clustering	Knockout mice and receptor knockout mice
	FGF2	Presynaptic vesicle clustering	Cultured spinal neurons	Presynaptic vesicle clustering	Cultured hippocampal neurons*
Laminin	Laminin-β2	Presynaptic maturation	Knockout mice	Not yet known	–
Collagen	Collagen IV	Presynaptic maintenance	Knockout mice	Not yet known	–
SIRP	SIRPα	Presynaptic vesicle clustering	Cultured motor neurons	Not yet known	–
Agrin	Agrin	Postsynaptic AChR clustering	Knockout mice	Presynaptic vesicle clustering Spine development	Knockout mice
BMP	BMP	Presynaptic development	*Drosophila*	Not yet known	–
Wnt	Wnt7	Not yet known	–	Presynaptic development Synaptic remodeling	Knockout mice and receptor knockout mice
	Wnt11r	Postsynaptic AChR prepatterning	Zebrafish	Not yet known	–
	Wnt3	Postsynaptic AChR clustering	Chick	Presynaptic development	Cultured hippocampal neurons
	Wingless	Presynaptic vesicle clustering Postsynaptic glutamate clustering	*Drosophila*	Not yet known	–
	Wnt5	Not yet known	–	Inhibition of presynaptic development	Cultured hippocampal neurons
	Wnt/*lin-44*	Not yet known	–	Inhibition of presynaptic development	*C. elegans*
Neurotrophic factors	BDNF	Maintenance of AChR clustering	*In vivo* mouse models	Presynaptic development Postsynaptic development	Knockout mice and receptor knockout mice
	GDNF	Presynaptic vesicle clustering	*Xenopus* nerve–muscle co-cultures	Presynaptic development Postsynaptic development	Heterozygous haplo-insufficient mice
Pentraxins	Narp	Not yet known	–	AMPA receptor clustering	Spinal neuron culture†
	NP1	Not yet known	–	AMPA receptor clustering	Cultured hippocampal neurons†
	NPR	Not yet known	–	AMPA receptor clustering	Cultured hippocampal neurons†
Semaphorin	Sema3F	Not yet known	–	Spine formation	Knockout mice and receptor knockout mice
Thrombospondin	Thrombospondin 1 and 2	Not yet known	–	Synapse formation	Knockout mice
Netrin	UNC-6	Not yet known	–	Presynaptic assembly Inhibition of presynaptic development	*C. elegans*

The function of synaptic organizers at the NMJ and/or in the CNS. The model systems used to uncover these functions are also listed. *Knockout mice have no reported phenotype. †Mice with all three of these molecules deleted have fewer glutamatergic synapses within the developing hippocampus.

FGF signaling is also involved in synaptic repair at the NMJ. Recent studies suggest that nerve injury induces the expression of FGF binding protein 1 (FGFBP1) in the muscle, which potentiates the effect of FGF7/10/22 for reinnervation (Williams *et al.*, 2009).

Other molecules proposed to be involved in neuromuscular junction development

Mammalian in vitro *systems*

Basic FGF (FGF2) was shown to be a candidate presynaptic organizer *in vitro* (Dai & Peng, 1995). Beads coated with FGF2 induce clusters of the presynaptic marker synaptotagmin in cultured spinal neuron neurites. However, an *in vivo* role for FGF2 in synaptic development has not yet been shown.

Like FGF7/10/22, the extracellular domain of SIRPs was identified for its activity in a synaptic vesicle-clustering assay in motor neurons (Umemori & Sanes, 2008). However, the importance of SIRPs *in vivo* has not yet been shown. SIRPα is a transmembrane immunoglobulin superfamily molecule expressed by muscle cells. The extracellular domain of SIRPα appears to be cleaved and released to induce presynaptic differentiation. Interestingly, SIRPα and FGF22 have different effects in cultured motor neurons: SIRPα-induced synaptic vesicle aggregates are significantly larger than those induced by FGF22, and SIRPα does not promote neurite branching like FGF22 does (Umemori & Sanes, 2008). In addition, SIRPα signals through the CD47 receptor and G-protein signaling followed by cAMP and MAP kinase, whereas FGF22 signals through tyrosine kinases with different downstream signaling targets. These presynaptic differences suggest that SIRPα and FGF22 work at distinct neuromuscular synapses and/or different stages of development.

Non-mammalian animal models

A variety of morphogens have been implicated in both presynaptic and postsynaptic NMJ development in non-mammalian models but none of these molecules has been validated in mammals. Despite this limitation, one strength of these studies is that many of the downstream signaling pathways have been elucidated.

Classically, morphogens, which include sonic hedgehog, Transforming growth factor beta/bone morphogenetic protein (TGFβ/BMP) and Wnts, have been studied for their role in tissue patterning; however, increasing evidence suggests that they also

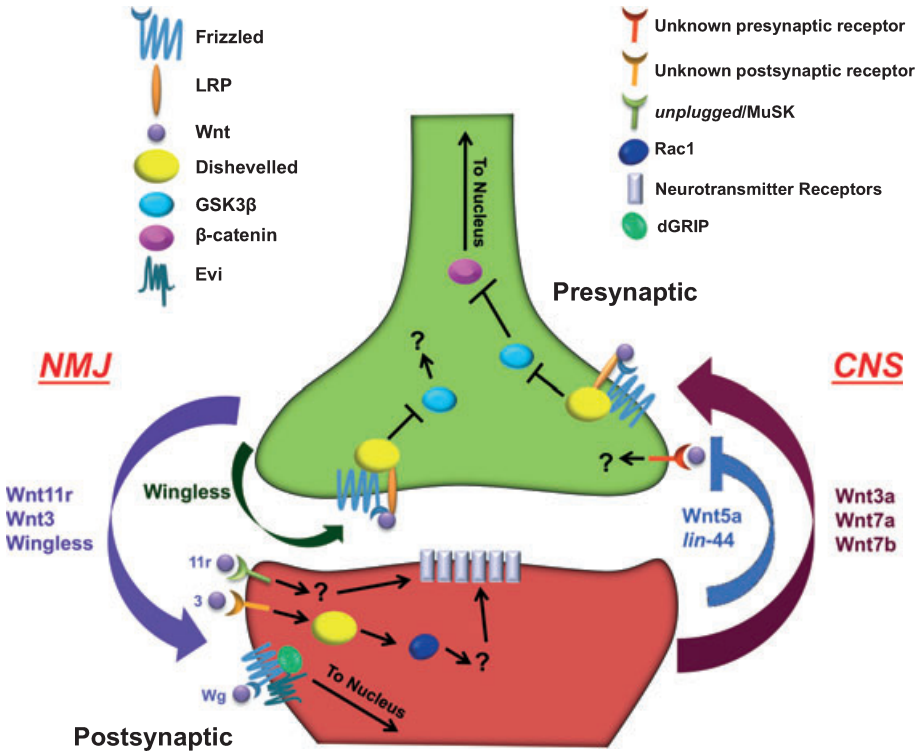

FIG. 2. Wnts organize presynaptic (CNS and NMJ) and postsynaptic (NMJ) specializations through multiple signaling pathways. Released from the presynaptic terminal, Wnt11r, Wnt3 and Wingless can all induce postsynaptic differentiation at the NMJ. Wnt11r may also be released from nearby somite cells (not depicted here). Wnt11r signals through the *unplugged*/MuSK receptor to induce clustering of AChRs. Wnt3 signals through an as yet unidentified receptor to activate Rac1, which induces clustering of AChRs. Wingless (Wg) is carried across the synaptic cleft by Evi, in the postsynaptic membrane it binds to DFrizzled-2 and, through association with dGRIP, is transported to the nucleus. Wg also organizes the presynaptic compartment through the beginning of the canonical pathway [Frizzled and LRP to Dvl to GSK3β]. Released from the postsynaptic target cell, Wnt3a, Wnt7a and Wnt7b are presynaptic organizers in the CNS that signal through the canonical Wnt signaling pathway (Frizzled and LRP to Dvl to GSK3β to β-catenin). Wnt5a and *lin-44*, on the other hand, inhibit presynaptic development through non-canonical signaling pathways that remain unknown.

play crucial roles in later stages of development (Sanchez-Camacho & Bovolenta, 2009). At the *Drosophila* NMJ, the expression of Wishful thinking (Wit), the homolog of BMP receptor II, is required in the motor neuron for proper development of the presynaptic compartment (Aberle *et al.*, 2002). Expression of a mutant form of *wit* results in smaller NMJs, decreased junction potentials and decreased expression of the cell adhesion molecule fasciculin II, the *Drosophila* neural cell adhesion molecule (NCAM) ortholog. This effect is mediated by the BMP homolog (Gbb) because *gbb* mutants have a similar phenotype to the *wit* mutants (McCabe *et al.*, 2003). In fact, *Gbb* expression is required in the postsynaptic muscle fiber for proper NMJ development.

Wnts have been shown to organize the postsynaptic compartment at the NMJ. Wnts signal through both canonical and non-canonical pathways (Fig. 2). In the canonical Wnt pathway, Wnt binds to the receptors LRP (low-density lipoprotein receptor-related protein) and Frizzled and associates them. This then activates the scaffolding protein Dishevelled (Dvl), which disassembles the glycogen synthase kinase 3β (GSK3β) destruction complex. Cytoplasmic β-catenin is then stabilized and transported to the nucleus where it activates Wnt-responsive genes. The non-canonical Wnt signaling pathways include a calcium pathway, planar cell-polarity pathway and Frizzled nuclear import pathway (Korkut & Budnik, 2009; MacDonald *et al.*, 2009).

At the zebrafish NMJ, AChR clustering occurs prior to, and even in the absence of, input from the motor neuron – a process known as prepatterning (Yang *et al.*, 2001; Flanagan-Steet *et al.*, 2005; Lin *et al.*, 2008). Wnt11r, which is expressed in the spinal cord and dorsolateral somites, organizes the postsynaptic structure in zebrafish *in vivo* (Jing *et al.*, 2009). Wnt11r regulates AChR prepatterning through a non-canonical signaling cascade via the *unplugged*/MuSK receptor. The AChR prepatterning then regulates motor neuron growth cone guidance.

At the chick NMJ, Wnt3, expressed by the motor neurons, can induce AChR clustering through a non-canonical signaling cascade that involves the activation of Rac1 (Henriquez *et al.*, 2008). These studies were conducted by implanting the chick wing with cells expressing Wnt3, prior to the stage when Wnt3 would typically be expressed, and then looking for AChR clustering. Under these conditions, AChR clusters form prematurely, and these clusters only stabilize in the presence of agrin. Thus, a potential way in which Wnts could promote postsynaptic differentiation is by interacting with other postsynaptic organizers, like agrin.

There is also evidence from *Drosophila* that Wnts may coordinate presynaptic and postsynaptic differentiation (Fig. 2). Wingless, the *Drosophila* Wnt, is secreted by the motor neuron. Presynaptically, at the ultrastructural level, synaptic boutons are smaller with fewer active zones and mitochondria in *wingless* mutants. Postsynaptically, synapses have an altered morphology and postsynaptic glutamate receptor clustering is decreased (Packard *et al.*, 2002). Packard and colleagues suggest that Wingless may thus serve as a coordinator for presynaptic and postsynaptic development. For presynaptic differentiation, Wingless signals through the local canonical Wnt pathway (Miech *et al.*, 2008). This local pathway involves LRP, Dvl and GSK3β, although β-catenin is not necessary. For the trans-synaptic

transmission of Wingless and postsynaptic Wingless signal transduction, the transmembrane protein, Evi, plays an important role (Korkut *et al.*, 2009). Presynaptic Evi-containing vesicular structures transport Wingless across the synaptic cleft, and postsynaptic Evi is required for the proper targeting of dGRIP, a PDZ protein required for the transport of the Wingless receptor DFrizzled-2 (Mathew *et al.*, 2005; Ataman *et al.*, 2006).

Neuromuscular junction modulators

Neurotrophic factors have been implicated in both presynaptic and postsynaptic development. However, their precise role in synaptic development is still unclear. The neurotrophins (NTs) are a family of neurotrophic factors that includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4. These factors signal through two types of receptors, the pan-NT receptor $p75^{NTR}$, which binds to all of the NTs with relatively equal affinity, and the Trk receptors, which include TrkA (specific for NGF), TrkB (specific for BDNF and NT-4) and TrkC (specific for NT-3). The NTs, particularly BDNF, modulate almost all aspects of synaptic development (Huang & Reichardt, 2001).

At the NMJ, TrkB and TrkC are expressed by the muscle, and BDNF, NT-4 and NT-3 are expressed in both the nerve and muscle (Ip *et al.*, 2001; Loeb *et al.*, 2002; Simon *et al.*, 2002). In cultured myotubes, application of BDNF and NT-4, but not NGF or NT-3, inhibits the postsynaptic differentiation induced by agrin (Wells *et al.*, 1999). TrkB receptors mediate this inhibition because direct activation of TrkB with TrkB antibodies mimics the effect of exogenous ligand application. BDNF, NT-4 and NT-3 are also important for the maintenance of AChR clustering and the NMJ (Gonzalez *et al.*, 1999; Belluardo *et al.*, 2001; Loeb *et al.*, 2002). If NT signaling is interrupted or NT expression suppressed, AChRs disperse, the NMJ fragments and muscles show greater fatigue. How these two sets of data relate to each other is unclear as the first set of experiments suggests that BDNF inhibits and the second that BDNF promotes maintenance of the NMJ. BDNF could inhibit or promote based upon expression levels or timing of its expression. Regardless, NTs may not be involved in synaptic differentiation *per se*, but rather act at later developmental stages as modulators.

Other neurotrophic factors have also been implicated in synaptic development at the NMJ. Glial cell line-derived neurotrophic factor (GDNF) is expressed in developing muscles (Henderson *et al.*, 1994). GDNF and its family member neurturin promote the differentiation of presynaptic terminals in *Xenopus* nerve–muscle co-cultures (Wang *et al.*, 2002). Exogenous application of GDNF or neurturin increases the number of clustered synaptic vesicles and the frequency of synaptic transmission. A role for GDNF in mammalian models or *in vivo* has not been delineated at this point. Thus, the exact role that neurotrophic factors play in neuromuscular synapse development remains a topic for future study.

The central nervous system

Among the factors shown to organize the mammalian NMJ *in vivo*, FGF7 and FGF22 are also important for presynaptic development within the CNS (Table 1). However, in the CNS, each FGF has a distinct function. A role for laminin-β2 and collagen IV has not been shown, and the role of agrin is less clear in the CNS. In addition to FGF7/22, Wnt7 is involved in presynaptic differentiation. Many members of the Wnt family also show presynaptic organizing activity *in vitro*, although, depending on the signaling pathway, organization is either promoted or inhibited. In the brain, pentraxins and semaphorins are implicated in differentiation of the postsynaptic compartment. In addition to these factors, neurotrophic factors appear to modulate the development of both the presynaptic and postsynaptic compartments. Several factors released from glia are also critical for synaptic development (Fig. 1). Thus, in the CNS, there appear to be more factors, which have specific functions, than at the NMJ. This may relate to the variety of synapses in the CNS.

Synaptic development of the mammalian central nervous system in vivo

Presynaptic organizers: multiple molecules for multiple synaptic types

In the mammalian brain, FGF7 and FGF22 are critical for synaptic development and serve as presynaptic organizers. FGF7, 10 and 22 were originally identified as target-derived presynaptic organizers in a screen from mouse forebrain extracts for the ability to cluster the synaptic vesicle-associated protein, synapsin, in cultured neurons (Umemori *et al.*, 2004). FGF22 is highly expressed by granule cells in the cerebellum. Its importance *in vivo* for the presynaptic differentiation of pontine axons (mossy fibers), which form synapses with cerebellar granule cells, was shown using a blocking reagent (soluble FGFR2b) and by disrupting FGFR2 expression postnatally by a conditional knockout approach. More recent work using FGF7 and FGF22 knockout mice finds distinct roles for FGF7 and FGF22 as presynaptic organizers within the brain (Terauchi *et al.*, 2010). FGF22 is important for the differentiation of glutamatergic synapses, whereas FGF7 is important for the differentiation of GABAergic synapses in the developing hippocampus. In FGF22 knockout mice, fewer vesicular glutamate transporter (VGluT)1 puncta are seen in area CA3 of the hippocampus and the frequency of miniature excitatory postsynaptic currents (mEPSCs) is decreased, whereas, in FGF7 knockout mice, fewer vesicular GABA transporter (VGAT) puncta are seen and the frequency of miniature inhibitory postsynaptic currents (mIPSCs) is decreased. Both the mEPSC and mIPSC amplitude remain unchanged. The change in frequency, but not amplitude, suggests that there is a presynaptic but not postsynaptic developmental defect. This study also highlights the importance of studying these molecules within an intact organism because, despite differential *in vivo* effects, exogenous application of FGF22 or FGF7 can both cluster glutamatergic and GABAergic synapses *in vitro* (albeit to different extents).

Wnt7a also regulates presynaptic development as a target-derived factor (Hall *et al.*, 2000; Ahmad-Annuar *et al.*, 2006). Wnt7a is expressed by cerebellar granule cells and, in Wnt7a knockout mice, synapsin I staining is transiently decreased and glomerular rosettes (a tripart synapse between granule cells, mossy fibers and Golgi cells) are less complex (Hall *et al.*, 2000). On the receptor side, Dvl1 is expressed in the mossy fibers, and Dvl1 knockout mice have a similar phenotype to Wnt7a knockout mice. Additional electrophysiological experiments in the Dvl1 knockout mice revealed a decreased mEPSC frequency (Ahmad-Annuar *et al.*, 2006). The canonical Wnt pathway appears to promote this presynaptic differentiation because the application of valproate or lithium, inhibitors of GSK3β, to cultured pontine explants mimics the differentiation of mossy fiber terminals by Wnt7a (Hall *et al.*, 2000, 2002). These results suggest that granule cells release Wnt7a, which then activates Dvl in the mossy fiber terminals and initiates a downstream signaling cascade by blocking GSK3β.

Wnt7 also appears to regulate synaptic remodeling as a result of plasticity in the adult hippocampus (Gogolla *et al.*, 2009). In fact, levels of Wnt7 are increased with environmental enrichment, resulting

in increased numbers of presynaptic active zones in large hippocampal mossy fiber terminals.

Postsynaptic organization

Within the mammalian CNS, FGFs and Wnts are well-characterized presynaptic organizers, but postsynaptic organizers have been less well studied.

Pentraxins are a diverse family of molecules that share a pentagonal structure (Goodman *et al.*, 1996). Three members of this family, Narp (neuronal activity-regulated pentraxin), NP1 (neuronal pentraxin 1) and NPR (neuronal pentraxin receptor), cluster AMPA receptors. Overexpression of Narp (also called NP2) in spinal neuron cultures increases clustering of GluA1-3 receptors but not gephyrin (O'Brien *et al.*, 1999, 2002). In non-neuronal cells transfected with GluA4, clusters of GluA4 are recruited at sites of contact between the transfected cells and the axons of co-cultured hippocampal neurons. VGAT NP1 and NPR mediate this GluA4 clustering (Sia *et al.*, 2007). RNAi knockdown of these pentraxins results in failure of GluA4 to cluster. The N-terminal domain of GluA4, with which NP1 and NPR interact, is critical for this clustering. Triple pentraxin knockout mice have fewer GluA4 clusters in the hippocampus (Sia *et al.*, 2007). Interestingly, study of the NP1/2 double knockout mice in the developing visual system suggests that the pentraxins are important for synapse maturation and refinement, but not the initial development of excitatory synapses (Bjartmar *et al.*, 2006).

Secreted semaphorins are also implicated in excitatory postsynaptic development in the brain. The secreted semaphorin, Sema3F, is shown to negatively regulate spine formation and distribution both *in vitro* and *in vivo* (Tran *et al.*, 2009).

Other molecules implicated in synaptic development

Mammalian in vitro *systems*

In cultured hippocampal neurons, exogenous application of FGF2 produces a higher density of synaptophysin and synapsin I puncta than control cultures. Furthermore, these puncta are associated with GluA1, suggesting that they are functional synapses (Li *et al.*, 2002). The importance of FGF2 *in vivo* appears to be limited as FGF2 knockout mice reportedly have normal synaptic development, although cell survival is decreased (Zhou *et al.*, 1998; Vaccarino *et al.*, 1999).

For presynaptic differentiation *in vitro*, Wnts can be divided into two groups: one that promotes and one that inhibits presynaptic differentiation (Fig. 2). These two groups use different signaling pathways: the canonical vs. the non-canonical pathways. Wnt7a, Wnt7b and Wnt3a promote presynaptic development in cultured hippocampal neurons (Davis *et al.*, 2008). Specifically, differentiation of presynaptic input was assayed for by staining for VGluT1. VGluT1 density is increased if Wnt3a, Wnt7a or Wnt7b is added to the culture media. Wnt3a, Wnt7a and Wnt7b preferentially activate the canonical pathway, as visualized by increased immunostaining for β-catenin in the nucleus.

Although the above studies represent Wnt signaling through the canonical pathway, the non-canonical pathway also plays a role in presynaptic differentiation. In contrast to Wnt3a, Wnt7a and Wnt7b, Wnt5a inhibits presynaptic development in cultured hippocampal neurons (Davis *et al.*, 2008). VGluT1 density is decreased if Wnt5a is added to the culture media. Wnt5a also decreases the levels of β-catenin in the nucleus, suggesting that Wnt5a is not signaling through the canonical pathway (Ishitani *et al.*, 2003; Topol *et al.*, 2003). Which non-canonical pathway is activated by Wnt5a remains to be investigated, and these results still need to be validated *in vivo*.

Non-mammalian animal models

Wnts have also been shown to be important for synaptic development in non-mammalian models, suggesting that this role is evolutionarily conserved. Wnt/*lin-44* in *Caenorhabditis elegans* also inhibits presynaptic specialization via its association with *lin-17*/Frizzled. *Dsh-1*/Dvl is required for this inhibition (Klassen & Shen, 2007). Mutations of β-catenin do not phenocopy Wnt/*lin-44* mutants, implying that the canonical pathway is not involved. However, mutations to Flamingo in the non-canonical planar cell-polarity pathway or CaM kinase (CaMK) in the calcium pathway also do not phenocopy Wnt/*lin-44* mutants, meaning that downstream signaling may be through a previously uncharacterized Wnt pathway.

Central nervous system synaptic modulators

Both agrin and neurotrophic factors are implicated in synaptic development but their actual role is still unclear.

Although traditionally studied at the NMJ, agrin can also promote synaptic development in cultured hippocampal neurons. Fewer synapses form, as measured by synapsin I clustering, if agrin expression is inhibited with antisense oligonucleotides or antibodies (Bose *et al.*, 2000). In the cerebral cortex, agrin localizes to a subset of excitatory presynaptic terminals (Ksiazek *et al.*, 2007). There are fewer spines and mEPSC frequency is decreased in mice lacking agrin in the brain (Ksiazek *et al.*, 2007), suggesting that, in contrast to the NMJ, agrin is involved in both presynaptic and postsynaptic development in the CNS. The precise role of agrin in the CNS remains to be elucidated.

Within the CNS, BDNF appears to be involved in many aspects of synaptic development. Presynaptically, exogenous application of BDNF to dissociated hippocampal cultures significantly increases the frequency of mEPSCs, the number of vesicles in the presynaptic terminal and synaptic density (Collin *et al.*, 2001). BDNF also enhances synaptic vesicle docking and increases mEPSC frequency in hippocampal slice cultures (Tyler & Pozzo-Miller, 2001). In retinotectal cultures, overexpression of the truncated TrkB receptor, which serves to sequester BDNF, results in decreased density of presynaptic specializations and fewer synaptic vesicles (Marshak *et al.*, 2007). Although BDNF also affects axonal branching, observations from *Xenopus* optic axons treated with BDNF suggest that its effects on synaptic clustering and branching are independent of one another because BDNF increases the synaptic cluster number per individual arbor (Alsina *et al.*, 2001). BDNF knockout mice have more pronounced synaptic fatigue and decreased short-term plasticity, both of which suggest excitatory presynaptic deficits (Pozzo-Miller *et al.*, 1999). At the ultrastructural level, fewer docked vesicles are seen at excitatory synapses in the BDNF knockout mice. GABAergic synapses are also affected as mice engineered to lack activity-dependent expression of BDNF have decreased VGAT immunoreactivity in the cerebral cortex (Hong *et al.*, 2008).

TrkB knockout mice, which do not survive until adulthood, have similar presynaptic deficits to BDNF knockout mice. Without TrkB, the hippocampus develops with decreased synaptic vesicle density and decreased expression of synaptic vesicle-associated proteins including: synaptotagmin I, synaptophysin, syntaxin 1 and synaptosomal associated protein-25 (SNAP-25) (Martinez *et al.*, 1998). In conditional TrkB knockout mice, decreased synaptic density is seen in the adult hippocampus with synapsin I-cre and humanGFAP-cre lines but not CaMKII-cre mice, suggesting that TrkB is necessary for synaptic

development (Luikart *et al.*, 2005). TrkB deletion after synapses have already formed (the CaMKII-cre mice) does not result in synaptic disassembly, suggesting that TrkB is not necessary for synaptic maintenance. A milder but similar phenotype to TrkB knockout mice is also observed in TrkC knockout mice, suggesting that NT-3 may also be involved in synaptic development (Martinez *et al.*, 1998).

In terms of postsynaptic development, in co-cultures with neurons from wild-type and TrkB knockout hippocampi, neurons lacking TrkB develop fewer postsynaptic specializations, visualized by antibodies against PSD-95, and express fewer AMPA receptors (Luikart *et al.*, 2005). Furthermore, exogenous application of BDNF to cultured hippocampal neurons results in increased clustering of both NMDA receptors and GABA receptors at synaptic sites (Elmariah *et al.*, 2004).

The importance of TrkB signaling for the *in vivo* development of GABAergic synapses has been demonstrated by a decreased number of symmetric synapses in the cerebellum of TrkB conditional knockout mice with Wnt1-cre (Rico *et al.*, 2002). Similarly, mice engineered to lack activity-dependent expression of BDNF have fewer inhibitory synapses, as measured by vesicular GABAergic transporter and GABA receptor co-localization, in cortical cultures (Hong *et al.*, 2008). Thus, BDNF can regulate both excitatory and inhibitory synaptic development.

Due to its importance for neuronal plasticity, there are many synaptic studies involving NTs, particularly BDNF. However, many of these studies solely focus on adult data. Thus, there are synaptic abnormalities *in vivo* without BDNF or its receptor, TrkB, but it is not clear at what stage of development BDNF is acting. An additional complexity with the BDNF data is that various studies demonstrate an exclusive role for BDNF in excitatory synaptic development, whereas others demonstrate an exclusive role for BDNF in inhibitory synaptic development. Whether these conflicting data represent regional differences within the CNS, different developmental stages or just different model systems remains an important topic of study.

GDNF, which is expressed in hippocampal neurons, also promotes both excitatory and inhibitory synaptic development (Ledda *et al.*, 2007). In cultured hippocampal neurons, exogenous application of GDNF increases the co-localization of presynaptic and postsynaptic markers. The GDNF-family receptor (GFRα1) is expressed in both the presynaptic and postsynaptic membrane, and, when beads coated with GFRα1 are placed on hippocampal cultures, they cluster both VGluT and VGAT in the presence of GDNF (Ledda *et al.*, 2007). Fewer presynaptic markers are seen in mice with GDNF haploinsufficiency, suggesting that GDNF also has an *in vivo* importance for synaptic differentiation (Ledda *et al.*, 2007).

Glial factors

Although most reports have focused on neuronally released factors, glia are also important for the development of synapses within the CNS. In fact, the presence of glia in cultures, or the application of glia-conditioned media, enhances the number of synapses formed in retinal ganglion cell cultures (Ullian *et al.*, 2001). Thrombospondin was identified as the glially-released factor that enhances synaptic density (Christopherson *et al.*, 2005). Fewer excitatory synapses are formed in the cerebral cortex of thrombospondin 1 and 2 double knockout mice (Christopherson *et al.*, 2005). The promotion of excitatory synaptic density by thrombospondin is mediated by the gabapentin receptor, $\alpha 2\delta$-1, on neurons (Eroglu *et al.*, 2009). In addition, thrombospondin 1 may signal through neuroligin 1 (Xu *et al.*, 2010). In hippocampal cultures, application of the extracellular domain of neuroligin 1 or knockdown of neuroligin 1 with shRNA blocks the thrombospondin 1-mediated increase in the rate of synapse formation. Whether thrombospondin acts presynaptically and/or postsynaptically and what stage of development is regulated by glia remain open questions.

Another glially derived molecule, UNC-6/netrin, is also shown in *C. elegans* to promote the assembly of presynaptic terminals at the AIY-RIA synapse through the receptor UNC-40 (Colon-Ramos *et al.*, 2007). Interestingly, in the motor neuron DA9, UNC-6/netrin binding to its other receptor, UNC-5, excludes presynaptic components from dendrites (Poon *et al.*, 2008), reiterating the theme that the same molecules can have differential effects depending upon the downstream signaling cascade that is activated. The importance of netrin in mammalian models has not yet been shown.

Conclusions

A crucial step in synaptic development is the differentiation of the presynaptic and postsynaptic compartments. To date, FGF, laminin-β2 and collagen IV are the best-characterized presynaptic organizers, and agrin is the best-characterized postsynaptic organizer at the mammalian NMJ. Within the CNS, FGF7/22 and Wnt7a are the best-characterized presynaptic organizers. Pentraxins and semaphorins have been implicated in postsynaptic development but more axon-derived postsynaptic organizers await discovery in the CNS. As for the vast array of other molecules discussed above, further study is required to understand how they shape synaptic development in the mammalian nervous system. Some molecules need to be validated in *in vivo* systems, and others examined more closely to understand at what point in development they are necessary. For example, the NTs may not establish synapses, but may be important for their maturation and modulation later in development and adulthood.

Whether the NMJ and CNS utilize the same or different synaptic organizers is an interesting question (see Table 1). In the case of FGFs, their role of recruiting synaptic vesicles is similar at the NMJ and in the CNS; however, in the CNS, FGF7 and FGF22 subserve different, specific functions. Agrin is critical for postsynaptic development at the NMJ, but only involved in a minor way in the CNS. These results are consistent with the idea that the diversity of CNS synapses requires a broader array of factors that may serve more specific roles than at the NMJ. However, integration of how factors function at the NMJ and in the CNS remains to be elucidated.

The reason for a wide diversity of signaling molecules (both secreted and cell adhesion molecules) may address the many layers of complexity inherent in synaptic development. One need for complexity derives from the variety of neurotransmitters used in the CNS. For example, how glutamatergic vs. GABAergic synapses develop is largely unknown. Although factors like BDNF can regulate the development of both, new evidence from FGF7 and FGF22 suggests that different factors within the same family differentially regulate glutamatergic vs. GABAergic development. This notion is further supported by recent studies characterizing the role of different neuroligin and neurexin isoforms in GABAergic and glutamatergic synaptic development (Graf *et al.*, 2004; Boucard *et al.*, 2005; Chih *et al.*, 2006). Further investigation into how each type of synapse differentiates is ongoing. Another possibility is that secreted factors may serve as the initial organizing factors, whereas adhesion molecules are then critical for the stabilization of the nascent synaptic specializations and further maintenance of synapses.

Another question that remains largely unanswered is how these molecules organize synapses. Downstream signaling of Wnts has been best characterized. Clearly, which signaling cascade is activated is important because different pathways lead to either promotion or inhibition of vesicle clustering. Thus, other synaptic organizers could

have differential effects depending upon which signaling pathway is activated. For example, not only can the NTs signal through one of two types of receptors, but signaling through Trk can also activate a diverse array of signaling cascades showing multiple effects (Huang & Reichardt, 2001). A second potential mechanism of action could be through interaction either directly or through divergence of downstream signaling cascades with the cell adhesion molecules. FGFRs appear to physically and functionally interact with the cell adhesion molecules L1, NCAM, N-cadherin (Williams *et al.*, 1994; Saffell *et al.*, 1997; Kiselyov *et al.*, 2003), EphA4 (Yokote *et al.*, 2005) and neuropilin (West *et al.*, 2005). Thus, crosstalk between secreted molecules and cell adhesion molecules could be one way to functionally differentiate synapses.

The importance of synaptic development is clear. Perturbation of synaptic development has been linked to a variety of neurological and psychiatric disorders such as epilepsy, schizophrenia, Rett syndrome, fragile X syndrome, bipolar disorder and autism (Betancur *et al.*, 2009; Caleo, 2009; Woo *et al.*, 2009). In particular, these diseases have been linked to the synaptic adhesion molecules. However, connections to the secreted organizers also exist. BDNF has been linked to all of the above disorders (Castrén *et al.*, 2002; Sun & Wu, 2006; Rybakowski, 2008). In fact, expression of the Val66Met polymorphism of BDNF has been linked to the severity of Rett syndrome and susceptibility to psychiatric disorders (Egan *et al.*, 2003; Chen *et al.*, 2006; Zeev *et al.*, 2009). FGFs have also been linked to epilepsy. FGF7 knockout mice have increased susceptibility to seizure, and FGF22 knockout mice have resistance to seizure (Terauchi *et al.*, 2010). Understanding how secreted organizers function and how perturbations of their action result in disease remains an important focus of current research.

Abbreviations

AChR, acetylcholine receptor; BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; CAMs, cell adhesion molecules; CaMK, CaM kinase; CNS, central nervous system; Dvl, Dishevelled; FGF, fibroblast growth factor; FGFBP1, fibroblast growth factor binding protein 1; FGFR, fibroblast growth factor receptor; GDNF, glial cell line-derived neurotrophic factor; GFR, GDNF-family receptor; GSK3β, glycogen synthase kinase 3β; LRP, low-density lipoprotein receptor-related protein; LRRTMs, Leucine-rich repeat transmembrane neuronal proteins; mEPSC, miniature excitatory postsynaptic current; MuSK, muscle-specific kinase; Narp, neuronal activity-regulated pentraxin; NCAM, neural cell adhesion molecule; NGF, nerve growth factor; NGL, netrin G ligand; NMJ, neuromuscular junction; NP, neuronal pentraxin; NPR, neuronal pentraxin receptor; NT, neurotrophin; PSD-95, postsynaptic density protein 95; SIRP, signal regulatory protein; SNAP-25, synaptosomal associated protein-25; TGFβ, transforming growth factor β; Tid1, tumorous imaginal disc 1; VGAT, vesicular GABA transporter; VGluT, vesicular glutamate transporter; wit, Wishful thinking.

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