Synapse Formation between Central Neurons Requires Postsynaptic Expression of the *MEN1* Tumor Suppressor Gene

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Synapse formation is a crucial step in the development of neuronal circuits and requires precise coordination of presynaptic and postsynaptic activities. However, molecular mechanisms that control the formation of functionally mature synaptic contacts, in particular between central neurons, remain poorly understood. To identify genes that are involved in the formation of central synapses, we made use of molluscan neurons that in culture form synaptic contacts between their somata (soma–soma synapses) in the absence of neurite outgrowth. Using single-cell mRNA differential display, we have identified a molluscan homolog of the multiple endocrine neoplasia type 1 (*MEN1*) tumor suppressor gene encoding the transcription factor menin as a gene that is upregulated during synapse formation. *In vitro* antisense knock-down of *MEN1* mRNA blocks the formation of mature synapses between different types of identified central neurons. Moreover, immunocytochemistry and cell-specific knock-down of *MEN1* mRNA show that postsynaptic but not presynaptic expression is required for synapses to form. Together, our data demonstrate that menin is a synaptogenic factor that is critically involved in a general postsynaptic mechanism of synapse formation between central neurons.

Key words: synaptogenesis; soma–soma synapse; gene expression; MEN1 *tumor suppressor gene; menin; transcription factor; antisense knock-down*

All functions of the nervous system critically depend on the formation of organized neuronal networks during development. A crucial step in this process is the formation of specific synapses between presynaptic and postsynaptic neurons. Molecular mechanisms that control synapse formation remain poorly understood, and most of our knowledge comes from studies on the neuromuscular junction (NMJ). These studies show that developing presynaptic and postsynaptic cells exchange signals that coordinate their mutual maturation, involving both the recruitment of preexisting proteins and the induction of new gene expression (Sanes and Lichtman, 1999). The same mechanisms play a role in the formation of central synapses, and recent investigations have started to shed light on the proteins and genes involved. For instance, Wnt factors (Hall et al., 2000) and neuroligin (Scheiffele et al., 2000) induce presynaptic differentiation at cerebellar granule cell synapses, whereas postsynaptic differentiation is controlled by presynaptically released neuregulin (Ozaki et al., 1997), and in *Caenorhabditis elegans*, the intracellular presynaptic protein regulator of presynaptic morphology-1 controls the formation of central synapses (Schaefer et al., 2000).

These recent findings have raised the question as to how these different molecules act together and which genes are upstream and downstream of them, thus defining the molecular pathways that lead to synapse formation (Chang and Balice-Gordon, 2000). Whereas downstream elements may encode previously established synaptic proteins that are directly involved in synaptic transmission and plasticity, upstream elements may include transcription factors that coordinate the proper temporal expression patterns of synaptogenesis-associated proteins. At the developing NMJ, ETS family (Schaeffer et al., 1998) and MyoD family (Rudnicki and Jaenisch, 1995) transcription factors and cAMP response element-binding protein (CREB)-binding protein (Marek et al., 2000) play a role, and at interneuronal synapses, the ETS transcription factor ER81 recently has been shown to control synapse formation (Arber et al., 2000). Here, we have identified the molluscan homolog of the transcription factor menin, the product of the multiple endocrine neoplasia type 1 (*MEN1*) tumor suppressor gene (Chandrasekharappa et al., 1997), as a critical mediator of synapse formation between central neurons. We show that postsynaptic expression of menin is necessary for the proper formation of various types of central synapses, both

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excitatory and inhibitory. This finding opens up the possibility of studying the underlying gene program that coordinates postsynaptic aspects of central synapse formation.

MATERIALS AND METHODS

Animals. Laboratory-raised stocks of *Lymnaea stagnalis* were maintained at room temperature and fed lettuce. Snails with a shell length of 18–20 mm (1–2 months old) were used for cell isolations; snails with a shell length of 20–25 mm (2–3 months old) were used to produce brain-conditioned medium (CM).

Cell culture. Animals were dissected under sterile conditions as described previously (Syed et al., 1990). Brains were washed with antibiotic saline (50 μg/ml gentamycin). To prepare CM, gentamycin-treated brains were incubated in defined medium (serum-free 50% L-15 medium; Life Technologies, Rockville, MD) as described previously (Wong et al., 1981). For cell isolation, gentamicin-treated brains were incubated in a 0.3% trypsin–collagenase–dispase solution and pinned down to the bottom of the dissection dish (Syed et al., 1990). Sigmacote (Sigma, St. Louis, MO)-treated, fire-polished glass pipettes were used to extract neurons, which were subsequently plated onto poly-L-lysine-pretreated coverslips in the presence of CM and incubated overnight. Soma–soma synapses were constructed as described previously (Feng et al., 1997).

Electrophysiology. Intracellular recordings were used to monitor synaptic activity (Syed and Winlow, 1991). Glass microelectrodes (1.5–2.0 μm internal diameter; World Precision Instruments, Sarasota, FL) were filled with a saturated solution of K_2SO_4 (resistance, 20–40 MΩ). Neurons were observed under an inverted microscope (Axiovert 135; Zeiss, Esslingen, Germany) and impaled using Narashige (Tokyo, Japan) micromanipulators (MM 202 and MM 204). Electrical signals were amplified using a NeuroData amplifier, displayed on a PM 3394 digital oscilloscope (Philips, Eindhoven, The Netherlands), and recorded on a TA 240S chart recorder (Gould, Cleveland, OH).

Differential display-PCR. Differential display PCRs (DD-PCRs) were performed on triplicates of soma–soma-paired neurons and unpaired control neurons as described previously (Van Minnen and van Kesteren, 1999). Differentially expressed cDNAs were isolated, cloned in pGEM-T or in pBluescript, sequenced, and compared on-line with GenBank nonredundant database entries using the BLASTN and BLASTX algorithms (Altschul et al., 1990).

Full-length cDNA cloning of L-MEN1. Sense and antisense primers were designed based on the sequence of the 300 bp *L-MEN1* DD-PCR product and used to PCR-screen *Lymnaea* brain-specific cDNA libraries in combination with vector-based primers. One clone was amplified that appeared to contain the complete ORF and sequenced on both strands from three independent amplifications. Because this cDNA contained only three nucleotides of 5′ untranslated region (UTR) before the predicted start codon and because no larger cDNAs were found in our libraries, we obtained an additional 425 bp of 5′ UTR by performing 5′ rapid amplification of cDNA ends on *Lymnaea* brain mRNA.

Anitisense knock-down experiments. Soma–soma synapses were prepared as described above. Initially, cells were paired in CM containing either 15 μM *L-MEN1* antisense oligonucleotide (5′-AAAGGCCGGCAACTT-3′) or 15 μM mismatch oligonucleotide (5′-AAAGCCCGCCATCTT-3′). The following day, cells were monitored for outgrowth by light microscopy; synaptic activity was monitored electrophysiologically as described above. For selective antisense knock-down experiments, cells were isolated and plated individually in hemolymph-coated dishes to prevent neuronal adhesion to the substrate (Syed et al., 1996). After overnight incubation in CM containing either antisense or mismatch oligonucleotides, cells were paired in poly-L-lysine-coated dishes. Soma–soma pairs were prepared between mismatch-treated presynaptic and postsynaptic cells (controls), between mismatch-treated presynaptic and antisense-treated postsynaptic cells (postsynaptic knock-downs), and vice versa (presynaptic knock-downs). Electrophysiological recordings were made 5–7 hr later.

Western blotting and immunocytochemistry. An antiserum was raised in mice against a synthetic peptide corresponding to amino acids 181–194 of L-menin (TAEVTWHGKGNED). This polyclonal antiserum was tested on a Western blot containing *Lymnaea* total brain extract to check for specificity and was then used to immunocytochemically stain soma–soma-paired *Lymnaea* neurons. Preimmune serum was used as a negative control. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.5% NP-40, incubated for 4 hr in primary antiserum diluted 1:500 in 1% Boehringer blocking reagent (BBR) (Boehringer Mannheim, Mannheim, Germany), and incubated for 1 hr in secondary antibody (rabbit anti-mouse coupled to horseradish peroxidase or alkaline phosphatase) diluted 1:2000 in 1% BBR. Between each step, cells were washed with PBS. Antibody binding was visualized using the appropriate enzyme substrate.

RESULTS

To identify genes involved in synapse formation, we made use of an *in vitro* preparation of identified presynaptic and postsynaptic central neurons of the mollusk *L. stagnalis*. These neurons can be individually isolated from the adult brain, and when juxtaposed in culture in a soma–soma configuration, they readily reform neuron-specific synaptic connections that are functionally indistinguishable from synapses in the intact brain (Feng et al., 1997). The principle advantage of soma–soma-paired cells is that they do not display neurite outgrowth (Feng et al., 2000), allowing the identification of genes that are specifically involved in synapse formation. We used a single-cell mRNA differential display technique (DD-PCR) to characterize changes in gene expression that occur during soma–soma pairing of two identified *Lymnaea* neurons [i.e., the right pedal dorsal 1 (RPeD1) and visceral dorsal 4 (VD4) neurons]. When plated in CM (Wong et al., 1981), the soma–soma-paired cells RPeD1 and VD4 form a characteristic bidirectional inhibitory synaptic contact within 16 hr (Feng et al., 1997). Cells plated individually (unpaired) in the same dish were used as controls. One of the genes that was found by DD-PCR to be expressed in soma–soma-paired cells but not in unpaired cells (Fig. 1*A*) is the *Lymnaea* homolog of the human *MEN1* gene (Chandrasekharappa et al., 1997), named here *L-MEN1* (Fig. 1*B*).

The *MEN1* gene was first identified as a tumor suppressor gene (Chandrasekharappa et al., 1997). *MEN1* orthologs were subsequently identified in rodents (Stewart et al., 1998; Maruyama et al., 1999), zebrafish (Khodaei et al., 1999), and *Drosophila* (Maruyama et al., 2000; Guru et al., 2001). The human *MEN1* gene product, a protein named menin, contains two basic nuclear localization signals that cause it to translocate to the nucleus (Guru et al., 1998). The *L-MEN1* gene product, here named L-menin, is a 759 amino acid protein, almost 150 amino acids larger than its vertebrate counterparts (Fig. 1*B*). L-menin and human menin share 49% sequence identity, most of which is located in the N-terminal part (amino acids 1–420) and in the most C-terminal part of the protein (amino acids 695–759; all amino acid positions refer to the L-menin sequence). Two nuclear localization signals are present at conserved positions in L-menin [i.e., RKGQRRR (amino acids 509–515) and RKRPRR (amino acids 751–756)], and there is one unique nuclear localization signal [i.e., RKRRYK (amino acids 318–323)] (Fig. 1*B*), suggesting that L-menin is also a nuclear protein. Moreover, nuclear localization of L-menin is predicted with 70% confidence using the PSORT algorithm (Nakai and Kanehisa, 1992).

To test whether a causal relationship exists between *MEN1* expression and synapse formation in *Lymnaea*, an antisense knock-down approach was used. We paired *Lymnaea* neurons in CM containing an antisense oligonucleotide against the translation initiation site of the *L-MEN1* mRNA. Control cells were paired in CM containing a 3 bp mismatch oligonucleotide. In addition to the inhibitory synaptic pair RPeD1–VD4 (Feng et al., 1997), two excitatory synaptic pairs [i.e., RPeD1–VD2 and VD4–left pedal dorsal 1 (LPeD1)] (Hamakawa et al., 1999; Woodin et al., 1999) were also tested. In the presence of the mismatch (control) oligonucleotide, 92% of the cell pairs tested formed normal synapses ($n = 13$) (Fig. 2*A*). However, in the presence of the antisense oligonucleotide, 93% of all pairs failed to develop

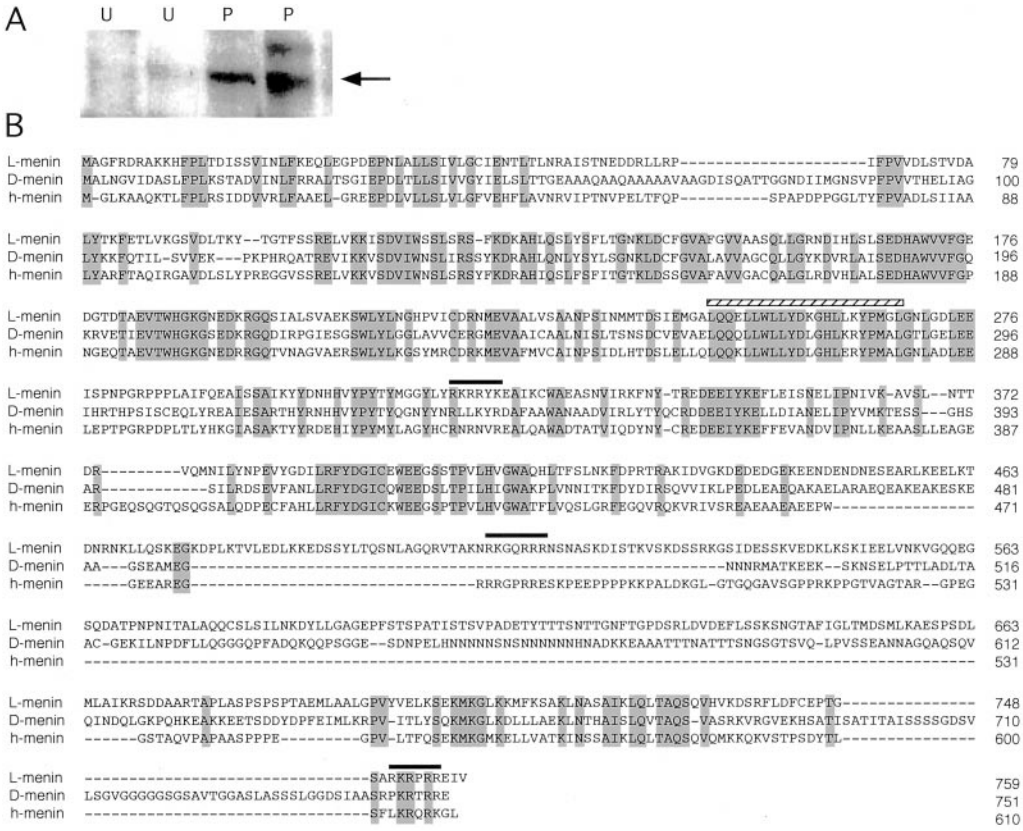

Figure 1. The *Lymnaea MEN1* gene is upregulated during synapse formation. *A*, Differential display gel showing the upregulation of a 300 bp PCR product (*arrow*) in paired cells (*P*) compared with unpaired cells (*U*). *B*, Amino acid sequence comparison of the *L-MEN1* gene product, L-menin, with *Drosophila* menin (*D-menin*; GenBank accession number AB040816) and human menin (*h-menin*; GenBank accession number NM000244). Identical amino acids in all three sequences are *shaded*. *Black bars* indicate nuclear localization sequences; the *hatched bar* indicates a conserved leucine zipper motif.

normal synapses ($n = 15$) (Fig. 2*A*). All cells were viable and showed normal morphology (Fig. 2*B,C*). Resting membrane potentials were normal compared with control cells (59.1 ± 6.5 mV vs 56.4 ± 8.8 mV for VD4, which is the most frequently used cell type in our experiments), and evoked spike amplitudes did not differ from controls (63.7 ± 6.4 mV vs 59.8 ± 3.3 mV for VD4). However, neither spontaneous nor induced action potentials generated postsynaptic potentials in 60% of the inhibitory pairs (Fig. 2*E*) and 50% of the excitatory pairs (Fig. 2*G*) in which L-menin expression was knocked down. In all other cases, synaptic transmission was significantly impaired. For instance, in two of four of the bidirectional inhibitory cell pairs (RPeD1–VD4), synaptic transmission was weak and in one direction only (data not shown), whereas in all excitatory pairs (RPeD1–VD2 and LPeD1–VD4) in which synaptic transmission was detectable, the amplitude of the EPSPs was significantly reduced [1.1 ± 0.5 mV ($n = 4$ in knock-down pairs) vs 7.1 ± 3.7 mV ($n = 7$ in control pairs); mean ± SD; $p = 0.012$] (Fig. 2*H–J*). Thus, there is a causal relationship between *L-MEN1* expression and the formation of functionally mature synaptic contacts, both inhibitory and excitatory.

Our next aim was to test whether L-menin is specifically involved in either presynaptic or postsynaptic mechanisms of synapse formation. To define the precise locus of L-menin expression, we first stained VD4–LPeD1 soma–soma pairs with an antibody that recognizes the L-menin protein (Fig. 3*A*). These data show that L-menin is selectively expressed in the postsynaptic cell (Fig. 3*B*), suggesting a postsynaptic function. The protein seems to be localized specifically in the perinuclear zone, suggesting nuclear translocation. Similar perinuclear staining has been observed for other transcription factors (Ratziu et al., 1998). The failure of our antibody to stain the nucleus itself could be explained by the fact that interaction with other nuclear proteins might mask the epitope. We subsequently prepared soma–soma pairs between presynaptic and postsynaptic cells that were incubated individually in either the antisense or the mismatch oligonucleotides and paired 24 hr later in CM. These experiments revealed that perturbation of *L-MEN1* expression in postsynaptic cells ($n = 5$) (Fig. 3*D*) but not in presynaptic cells ($n = 5$) (Fig. 3*E*) blocked synapse formation between paired neurons. Control pairs (presynaptic and postsynaptic cells incubated in mismatch oligonucleotides; $n = 5$) (Fig. 3*F*) always developed normal synaptic contacts. These data demonstrate that L-menin expression is required only in the postsynaptic cell to induce synapse formation.

DISCUSSION

Our data provide evidence that the transcription factor menin plays a crucial role in a postsynaptic mechanism of central synapse formation. In addition, our findings, together with its previously established role as tumor suppressor, suggest that menin may be part of a common regulatory mechanism for synaptic differentiation and cellular differentiation. Because the cellular actions of menin are for the most part unknown, one can only

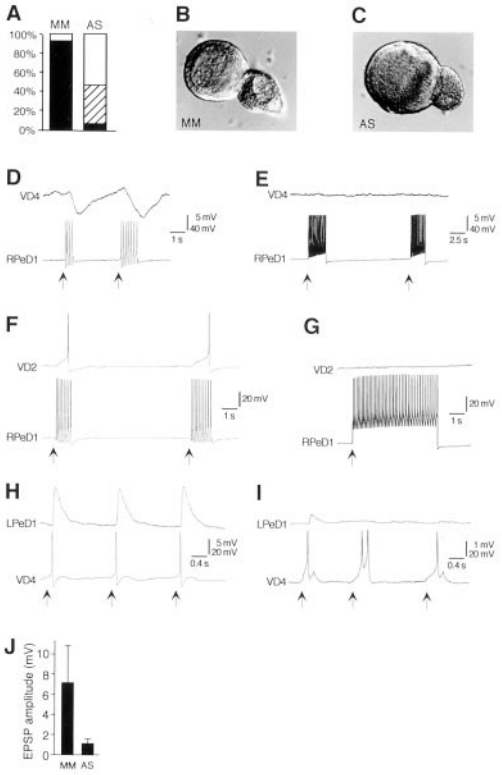

Figure 2. Antisense knock-down of L-menin prevents synapse formation between different types of *Lymnaea* neurons. *A*, Antisense knock-down of menin (*AS*) either prevented synapse formation (*open bar*) or significantly reduced synaptic efficacy (*hatched bar*) in 93% of the cell pairs compared with mismatch-incubated pairs (*MM*). A *black bar* represents the number of normal synapses. Cells incubated in antisense oligonucleotides (*B*) and cells incubated in mismatch oligonucleotides (*C*) had normal morphology and were comparable with control cells with respect to viability and membrane potential parameters. *D*, Characteristic inhibitory synapse from RPeD1 onto VD4 in cell pairs incubated in the mismatch oligonucleotide, showing compound IPSPs in VD4 after stimulation of RPeD1. *E*, In the knock-down pairs, stimulation of RPeD1 did not produce IPSPs in VD4. *F*, RPeD1–VD2 pairs incubated in mismatch oligonucleotides formed a characteristic excitatory chemical synapse from RPeD1 onto VD2. *G*, In the knock-down pairs, trains of action potentials in RPeD1 failed to induce EPSPs in VD2. *H*, VD4–LPeD1 pairs incubated in mismatch oligonucleotides formed a characteristic excitatory synapse, showing one-for-one EPSPs in LPeD1. EPSPs were consistently generated, as can be observed by comparing three consecutive EPSPs. *I*, In knock-down pairs, EPSPs could often be generated in LPeD1 with the first presynaptic action potential, but subsequent stimulations failed to produce postsynaptic responses. *J*, The average EPSP amplitude in VD4–LPeD1 knock-down pairs was significantly reduced compared with control pairs [1.1 ± 0.5 mV ($n = 4$) vs 7.1 ± 3.7 mV ($n = 7$); mean ± SD; $p = 0.012$]. *Arrows* indicate the onset of stimulation.

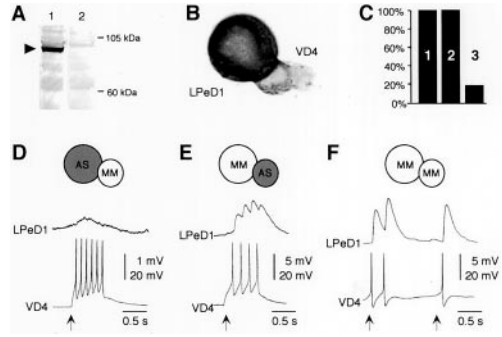

Figure 3. L-menin expression is required postsynaptically for synapse formation to occur. *A*, An antibody directed against L-menin recognizes a protein of the appropriate size (~85 kDa) on a Western blot of *Lymnaea* total brain extract (*lane 1*), which is not recognized by the preimmune serum (*lane 2*). *B*, Immunostaining of VD4–LPeD1 soma–soma pairs identified the postsynaptic cell (LPeD1) as the cell that expresses L-menin. *C*, When L-menin expression was knocked down selectively in either the presynaptic or the postsynaptic cell, synapses formed normally in control pairs (*bar 1*) and in presynaptic knock-down pairs (*bar 2*) but failed to form in 80% of the pairs when menin expression was knocked down in the postsynaptic cell only (*bar 3*). *D*, Absence of EPSPs in LPeD1 when L-menin expression is knocked down in LPeD1. *E*, Normal EPSPs in LPeD1 when L-menin expression is knocked down in VD4. *F*, Normal EPSPs in control pairs in which both cells were incubated in the presence of mismatch oligonucleotides. *Arrows* indicate the onset of stimulation.

speculate about the nature of such regulatory pathways. However, menin has been reported to interact with various other transcription factors, including the basic leucine zipper protein JunD (Agarwal et al., 1999) and the TGF-β-regulated protein Smad3 (Kaji et al., 2001). The interaction with Smad3 is of particular interest in this respect, because TGF-β not only acts as a tumor suppressor (Markowitz and Roberts, 1996) but has also been implicated in synaptic plasticity (Zhang et al., 1997). Thus, menin may be part of a common, TGF-β-induced signaling pathway for both cellular and synaptic differentiation.

Because we identified L-menin in a molecular screen that differentiates between outgrowing and synapse-forming neurons, one could question whether L-menin affects synapse formation per se or whether it does so indirectly by suppressing neurite outgrowth. Two observations strongly suggest that L-menin is directly involved. First, the upregulation of L-menin expression is only observed in postsynaptic neurons and not in presynaptic neurons, whereas in both cells neurite outgrowth is suppressed during synapse formation. Second, when *L-MEN1* expression was knocked down, we never observed an induction of neurite outgrowth from soma–soma-paired neurons (Fig. 2*C*). Thus, L-menin most likely controls synapse formation directly, whereas suppression of neurite outgrowth is controlled by a separate mechanism.

Although our knock-down experiments show that synapse formation is abolished in the absence of L-menin, this is not caused by a general effect on the viability and neuronal properties of the cells, because presynaptic neurons do not seem to be affected by *L-MEN1* knock-down with respect to membrane potential properties. Moreover, in many instances, postsynaptic potentials could be generated with the first presynaptic action potentials, but subsequent presynaptic spikes failed to produce a response in the postsynaptic cell (Fig. 2*I*). This demonstrates that cells are in principle capable of chemical transmission, but fail to develop functionally mature synaptic contacts in the absence of L-menin. The latter observation would suggest either a role in the maturation of the postsynaptic element itself or involvement in a retrograde feedback mechanism that induces presynaptic maturation. Interestingly, the latter alternative seems to hold true for another transcriptional regulator, CREB-binding protein, which is required postsynaptically to modulate the transmitter release properties of the presynaptic cell at the *Drosophila* NMJ (Marek et al., 2000). A candidate factor for the retrograde signal involved could be the transmembrane cell adhesion molecule neuroligin, because it was shown recently that postsynaptic expression of neuroligin is necessary and sufficient for presynaptic development at various central synapses (Scheiffele et al., 2000). It will be of interest to

resolve whether neuroligin expression is in any way under the control of menin during central synaptogenesis.

In addition to the aforementioned role in synapse formation, menin may also serve important functions in other developmental processes. For instance, menin expression and subcellular localization are tightly regulated during the cell cycle, suggesting a role in cell division and cell growth (Kaji et al., 1999). Recent studies have demonstrated that other molecules with early developmental functions can also be involved in the control of neurite outgrowth and synapse formation. For instance, the Notch transmembrane receptor, which is involved in lateral specification of cellular identity during early embryonic development, provides an important stop signal for outgrowing cortical neurons (Sestan et al., 1999). Similarly, Wnt factors, which are well studied with respect to their roles in determining cell fate and embryonic patterning, control presynaptic maturation at developing cerebellar mossy fiber synapses and may also be involved in synaptic plasticity in the mature brain (Hall et al., 2000). Thus, the cooptation of early developmental factors may represent a common mechanism in the control of synapse formation and synaptic plasticity. Most of these factors are able to alter gene expression directly or indirectly, and the identification of target genes will be of particular importance for further understanding the molecular mechanisms underlying synapse formation.

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