Chapter 5

Deletion of MEN1 does not affect formation of functional synapses in cultured hippocampal neurons

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Abstract

Synaptogenesis requires the expression and delivery of hundreds of proteins, but the transcriptional control of this process is poorly understood. Based on studies in invertebrates, the tumor suppressor gene MEN1 was suggested to be an essential transcription factor for synaptogenesis. Here, we investigated the role of menin in synaptogenesis in cultured hippocampal neurons, using MEN1-lox mice. Deletion of MEN1 with had no effect on the number of synapses, synaptic VAMP intensity or dendrite length in autaptic neurons. In addition, no differences were detected in spontaneous vesicle release, presynaptic strength or short-term presynaptic plasticity. Finally, the response to glutamate application was unaltered after removal of MEN1. Thus, menin does not seem to have an unique role in the formation of functional synapses in mouse hippocampal neurons.
Introduction
The formation of functional synapses is a crucial step in the development of a neuronal network. Initial contact between potential pre- and postsynaptic partners is established by cell adhesion molecules, and after a rigorous selection procedure, a fraction of these contacts mature into synapses [1]. The conversion of nascent contacts into fully operational synapses involves the sequential expression and delivery of hundreds of proteins to both pre- and postsynaptic terminals [2, 3]. The expression of genes necessary for synapse formation and maturation are likely to be controlled by transcription factors that are specifically activated during synaptogenesis (reviewed in [4, 5]). The specific transcription factors involved in this process, as well as their mechanism of action, is incompletely understood. In hippocampal neurons, the transcription factors MEF2A and MEF2D are critical to control the number of excitatory synapses, by controlling the expression of (among others) Arc and SynGAP [6]. Other transcription factors implicated in the formation of synapses in the hippocampus are NGN3 and CREB, which control the number and functionality of synapses, respectively [7-9]. At the neuromuscular junction, a role for ETS, MyoD and CREB was suggested [10-12].

Using cultured neurons derived from the mollusk Lymnaea stagnalis, we previously identified the transcription factor menin to be essential for synaptogenesis [13]. Menin is specifically upregulated during synapse formation in L. stagnalis. Conversely, postsynaptic knockdown of menin almost completely blocked the formation of functional excitatory and inhibitory synaptic contacts [13]. These findings make menin an excellent candidate for controlling transcription of genes critical for synaptogenesis.

Figure 5.1. Menin levels decline after expression of Cre recombinase in MEN1-lox neurons. A, top: Western blot stained for menin in control and various time points after Cre recombinase infection (DIV1). Bottom shows coomassie staining as input control. B. Quantification of menin protein levels with western blot. *P < 0.05, unpaired t-test. N = 3.
Encoded by the multiple endocrine neoplasia type 1 (MEN1) tumor suppressor gene [14], menin has a well described role in the formation of tumors in endocrine tissue [15]. MEN1 is well conserved across species, with 49% shared sequence identity between L. stagnalis and human [13] and 89% between mouse and human [16]. Menin is involved in the epigenetic control of gene transcription, through interactions with (among others) the histone methyltransferase MLL [17, 18], histone deacetylase mSin3a [19] and transcription factor JunD [18, 20, 21]. Deletion of MEN1 is early embryonically lethal and embryos display severe deficits in facial or cranial development [22]. MEN1 heterozygous mice are viable, but develop numerous tumors in endocrine tissues in early adulthood, and have increased lethality [22]. Besides expression in endocrine tissue, MEN1 is highly expressed throughout central nervous system during embryonic development, and in the hippocampus and cortex in adult mice [16]. Its function in neurons is unknown to date.

In the current study, we investigated the role of menin in synaptogenesis in mice. Using MEN1-lox mice [23], we found that deletion of MEN1 does not affect synapse formation, neuron morphology or synaptic transmission in cultured hippocampal neurons. Thus, in contrast to L. stagnalis, menin is not critical for the formation of functional synaptic contacts in mouse hippocampal neurons.

**Results**

*Deletion of MEN1 does not affect synapse formation in autaptic cultures*

To investigate if menin has a role in synapse formation in cultured hippocampal neurons, we made use of MEN1-lox mice, which allows for conditional deletion of MEN1 by expression of Cre-recombinase [23]. We first tested the time scale at which menin is lost after Cre infection. Mass cultures of MEN1-lox hippocampal neurons were infected at 1 day in vitro (DIV1) with lenti virus encoding for Cre, and were lysed several days later to measure menin protein levels (Figure 5.1A-B). We observed a gradual decrease in menin levels, reaching significance at DIV14 and 18 (P < 0.05, unpaired t-test, N = 3). Thus, MEN1 is efficiently deleted by Cre infection, and menin protein levels progressively decline over the course of two weeks.

To test if menin deletion affects synaptogenesis and neuron morphology, we made autaptic cultures of MEN1-lox hippocampal neurons, a culture system that is routinely used to study synaptic function and cellular morphology [24-26]. Cultures were infected with GFP or Cre-GFP at DIV1, fixed at DIV14 and analyzed for cell morphology with immunofluorescence [27] (Figure 5.2A). We did not observe a difference in the average number of VAMP puncta per neuron (P = 0.32, Kruskal Wallis ANOVA, N = 3; see Figure 5.2B), indicating that there was no change in the number of presynaptic terminals. Furthermore, the fluorescence intensity or surface area of VAMP puncta was unchanged, suggesting that there was no change in the number of synaptic vesicles per terminal (Figure 5.2B). We also did not find a difference in the total dendrite length or the number of VAMP puncta per µm dendrite. With Sholl analysis, a measure of dendritic arbor complexity, we found a small but significant reduction in dendrite branching at 30 µm from the soma (P < 0.05, Mann-Whitney test Figure 5.2C). Thus, deletion of MEN1 does not affect synaptogenesis, but may have an effect on dendrite branching.
Figure 5.2. Deletion of MEN1 does not affect synapse formation and cell morphology. A. Example images of 2-week old autaptic hippocampal neurons infected with GFP (left) or Cre-GFP (right). Scale bars represent 20 µm. B. Morphological properties of two week old neurons. Data derived from n = 32 (GFP), n = 61 (Cre), N = 3. C. Sholl analysis of two week old cells. Bin with largest difference (30 µm) is significant with P < 0.05 (Mann Whitney test). D. Example images of 3-week old autaptic neurons. E. Morphological properties of three week old neurons. Data derived from n= 24 (GFP), n = 27 (Cre), N = 1. F. Sholl analysis of three weeks old cells. Bin with largest difference (90 µm) is significant with P < 0.05 (Mann Whitney test). All data shown as average, error bars represent SEM. Black dots on bar graphs represent values of individual observations.
Since menin levels only slowly decline after infection with Cre (Figure 5.1), we hypothesized that the period to DIV14 is too short to observe an effect on synaptogenesis. We therefore repeated the analysis with older neurons, fixed at DIV21 (Figure 5.2D and E-F). Again, no differences were detected in the number, surface area, fluorescence intensity or density of VAMP puncta, as well as dendrite length (N = 2, Figure 5.2E). In contrast to experiments performed at DIV 14 (Figure 5.2D), Cre-infected neurons were indistinguishable in dendritic branching at short distance from the soma from the soma. Unexpectedly, neurons now appeared to have slightly more branching, at 100-150 µm (P < 0.05, Mann-Whitney test, Figure 5.2F). In conclusion, deletion of MEN1 does not affect synaptogenesis in cultured neurons, but might have a small effect on dendrite branching.

**Deletion of MEN1 does not affect synaptic transmission**

To test if Cre-infected neurons form functional synapses that support normal synaptic transmission, we performed whole-cell electrophysiological recordings on autaptic neurons (Figure 5.3). GFP and Cre-infected neurons did not differ in resting membrane potential (GFP: -50.5 ± 1.7 mV; Cre: -50.9 ± 1.9 mV) or series resistance (GFP: 9.15 ± 0.40 MΩ; Cre: 9.16 ± 0.37 MΩ), indicating there were no gross abnormalities in membrane composition or ion channel expression. Presynaptic strength was tested by induction of action potentials (AP) via the recording pipette. Excitatory postsynaptic currents (EPSCs) evoked by a single AP had comparable amplitudes in both GFP and Cre (N = 3, 10 pA200 ms)

![Figure 5.3. MEN1 deletion does not affect synaptic transmission in autaptic hippocampal neurons. A. Example traces of evoked EPSCs of GFP and Cre infected neurons. B. Average EPSC amplitude for GFP (n = 12) and Cre (n = 14), N = 2. C. Paired pulse ratio of EPSCs. D. Example traces of spontaneous release of GFP and Cre infected neurons. E. Average mEPSC frequency. F. Average mEPSC amplitude. GFP n = 10, Cre n = 9. N = 2. All data shown as average, error bars represent SEM. Black dots on bar graphs represent values of individual observations.](image-url)
Figure 5.3A-B). By applying two consecutive APs at varying intervals, we tested if MEN1 deletion affected the strength of presynaptic terminals. The ratio of the amplitude of both responses (the paired pulse ratio, PPR) is a reliable measure of presynaptic release probability [28]. Cre-infected neurons did not display a difference compared to GFP in PPR in every interval tested, suggesting that there is no change in presynaptic strength (Figure 5.3C). In addition, no difference was observed in spontaneous release, as measured by the frequency of miniature EPSCs (mEPSCs). The amplitude of mEPSCs (Figure 5.3C), as well as the kinetics of mEPSC events were also similar in both groups (data not shown), suggesting that also the strength of the postsynapse is unaffected. Thus, spontaneous and AP-evoked synaptic transmission are not affected by removal of MEN1.

**Postsynaptic response is not affected after deletion of MEN1**

Preliminary biochemical data suggests that menin might control the expression of postsynaptic glutamate receptors (Van Kesteren et.al., unpublished). To investigate the postsynaptic response of Cre-infected neurons in more detail, we measured the response to local application of glutamate using electrophysiology (Figure 5.4). The amplitude of the postsynaptic response to 100 µM glutamate was similar in both GFP and Cre (N = 3, Figure 5.4A-B). Furthermore, no difference was found in the total charge transferred (Figure 5.4C) or the half width of the response (Figure 5.4D). These results, together with the unaltered mEPSC amplitudes (Figure 5.3F), indicate that the amplitude and kinetics of the postsynaptic response to glutamate are unaltered in neurons lacking MEN1.
Discussion
Synapse formation involves the precise sequential expression and delivery of hundreds of proteins to both pre- and postsynaptic compartment. The processes that regulate the expression of these proteins are incompletely understood, and likely involve tight transcriptional control by multiple transcription factors [4, 5]. The tumor suppressor gene MEN1 was previously shown to be essential for synaptogenesis in the mollusk L. stagnalis [13]. In the current study, we investigated the role of menin in synaptogenesis in mice. Despite its clear role in mollusks, we failed to identify a function of menin in the formation of functional synaptic contacts in cultured hippocampal neurons from mice.

With immunocytochemical studies on cellular morphology, we did not detect an effect of MEN1 deletion on the number of synapses (Figure 5.2). Since there was no difference in VAMP intensity or surface area, we conclude that the number of synaptic vesicles per terminals is also unaltered. These terminals proved to be functionally indistinguishable from wild type, as we did not detect a difference in spontaneous release frequency, EPSC amplitude or PPR (Figure 5.3). Finally, the surface expression of glutamate receptors is unaltered after deletion of MEN1, since mEPSC amplitude and the response to glutamate application were unaffected (Figures 3 and 4). In conclusion, deletion of MEN1 does not affect functional synapse formation and surface expression of glutamate receptors in cultured mouse hippocampal neurons.

Surprisingly, we did observe small but significant differences in dendrite branching (Figure 5.2 D & F). Since there was no change in the total dendrite length per cell (Figure 5.2), this effect seems specific for dendrite branching, and is not caused by changes in dendrite outgrowth itself. Confusingly, the measured effects in DIV14 and DIV21 were opposite, with slightly decreased branching at DIV14, and increased branching at DIV21. Dendrite branching was not measured in L. stagnalis, as in this culture system direct soma-soma synapses are formed, without depending on neurite outgrowth [13, 29]. The differences measured in the current study were small, and it is questionable whether it will affect neuronal function.

How can the differences between the current study and the results obtained in L. stagnalis be explained? Due to the early embryonic lethality of the conventional MEN1 knockout mouse [22], we had to rely on MEN1-lox mice, with removal of MEN1 by expression of Cre recombinase. It is possible that removal of MEN1 at DIV1 is too late to decrease menin expression to a sufficiently low level. Although we did measure a 20% drop in menin protein levels 3 days after infection with Cre (DIV4), and menin levels continued to decline afterwards (Figure 5.1), this might not be sufficient to study the role of menin in synaptogenesis. We cannot formally rule out that the residual menin protein levels, especially in the first days after Cre infection, are sufficient to carry out menin’s function synaptogenesis. In line with this hypothesis, MEN1 heterozygous mice develop normally until adulthood, without any morphological or behavioral deficits [22]. Moreover, due to the lack of available menin antibodies suitable for immunocytochemistry, we could not confirm if MEN1 was efficiently deleted from every cell. We can therefore not exclude that some of the neurons analyzed in this study did still express or contain menin in sufficient amounts to support synapse formation and function. The >90 % reduction of total menin...
levels measured with biochemistry, however make the latter explanation less likely. A more likely explanation is that menin does not have a role in synaptogenesis in mouse hippocampal neurons, or that its function is redundant. Although its sequence is 49% conserved in humans [13], it could be that mutations in MEN1 over the course of evolution altered menin’s interactions with other proteins or genes. Menin has not been studied any further in mollusks, and it is thus unknown to which proteins menin binds in L. stagnalis and which genes it regulates. Neurite outgrowth and synapse formation are orchestrated by many transcription factors in parallel [4, 5, 30], and thus it is well possible that deletion of MEN1 can be compensated by other transcription regulators. The high expression levels in both embryonic and adult brain suggest that menin has a function in (the development of) neuronal cells [16]. This, however, does not seem to include a dominant role in synaptogenesis in mice, since we did not observe defects on dendrite outgrowth and the formation of functional synapses in cultured hippocampal neurons after the removal of MEN1.
References

23:6075-6085.