Activity-Dependent Regulation of MEF2 Transcription Factors Suppresses Excitatory Synapse Number

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In the mammalian nervous system, neuronal activity regulates the strength and number of synapses formed. The genetic program that coordinates this process is poorly understood. We show that myocyte enhancer factor 2 (MEF2) transcription factors suppressed excitatory synapse number in a neuronal activity- and calcineurin-dependent manner as hippocampal neurons formed synapses. In response to increased neuronal activity, calcium influx into neurons induced the activation of the calcium/calmodulin-regulated phosphatase calcineurin, which dephosphorylated and activated MEF2. When activated, MEF2 promoted the transcription of a set of genes, including *arc* and *synGAP*, that restrict synapse number. These findings define an activity-dependent transcriptional program that may control synapse number during development.

During nervous system development, synaptic activity influences the strength and number of synapses that form between neurons. Although much has been learned about the molecular mechanisms that influence local changes in synaptic strength, less is known about how neuronal activity regulates the number of synaptic connections a neuron will form and maintain.

Neuronal activity modulates synapse number both during brain development and in adult organisms. The number of synapses formed during development can be in excess of the number that is maintained into adulthood (*1*, *2*). These excess synapses are eliminated in a manner that depends on sensory experience and synaptic activity. Similarly, the induction of long-term potentiation (LTP) and long-term depression (LTD) in the mature hippocampus lead to the formation and deconstruction, respectively, of spine synapses (*3*, *4*). Because the activity-dependent remodeling of synapses is often coordinated throughout an entire neuron (*5*), a global activity-dependent process may operate to control synapse number.

Neurons can coordinate activity-dependent processes throughout the cell through the activation of new gene transcription (*6*). The influx of calcium into neurons in response to synaptic activity leads to the activation of a number of transcription factors. Members of the myocyte enhancer factor 2 family (MEF2A to MEF2D) of transcription factors are highly expressed in the brain and are tightly regulated by several distinct calcium signaling pathways (*7*, *8*). Here, we report that MEF2 transcription factors act as negative regulators of synapse number in hippocampal neurons.

In rat brain lysates, MEF2A and MEF2D proteins were detected during embryonic stages, but their expression increased during the first 3 weeks after birth, a time during which synapses form and are remodeled (*9*) (fig. S1). Because the developmental regulation of MEF2 expression was recapitulated in dissociated hippocampal cultures (fig. S1), we used RNA interference (RNAi) to investigate the function of MEF2 proteins in culture. Short hairpin RNAs (shRNAs) that target MEF2A and MEF2D caused the persistent depletion of these proteins in the nuclei of transfected hippocampal neurons over a period of at least 10 days (Fig. 1A and fig. S2). The membrane depolarization-induced expression of a luciferase reporter gene under the control of three consensus MEF2 response elements (MREs) was also reduced by the reduction of MEF2A and MEF2D (Fig. 1B). However, the membrane depolarization-induced expression of a cyclic adenosine monophosphate response element (CRE) reporter gene was unaffected by MEF2 RNAi (fig. S3).

To investigate whether MEF2 regulates the number of synaptic contacts a neuron receives, we transfected shRNAs into cells at a time when synapses are just beginning to form [day in vitro (DIV) 5] and analyzed the number of synapses that had formed 8 to 10 days later. We used a low-efficiency method of transfection so that we could examine synapses formed onto transfected neurons by untransfected neurons in the same culture. Cells were fixed and stained for the presynaptic marker synapsin-1, as well as the postsynaptic marker postsynaptic density protein 95 (PSD-95). These proteins were localized to discrete puncta that frequently overlapped, reflecting the clustering of these proteins at synaptic sites (Fig. 2A). We counted the number of sites where these puncta were colocalized

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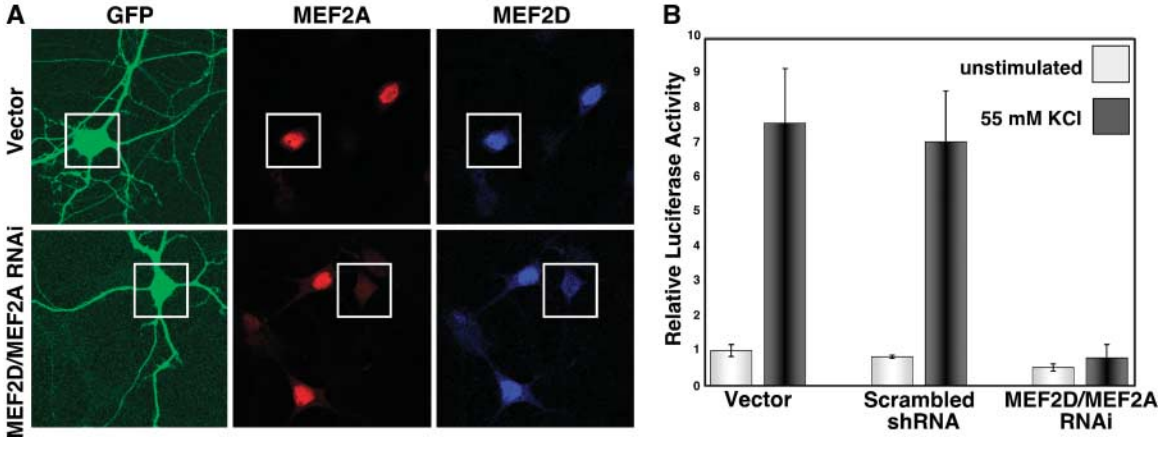

Fig. 1. Reduction of MEF2A and MEF2D attenuates MEF2-dependent transcription in hippocampal neurons. (**A**) Images of hippocampal neurons transfected with the indicated shRNA expression plasmids and stained with antibodies to MEF2A (red) and MEF2D (blue). Cells were transfected at embryonic day 18 (E18) + 5 DIV and fixed at E18 + 14 DIV. Transfected cells indicated by boxes. (**B**) Luciferase reporter assay with the use of 3xMRE-Luc cotransfected with the indicated shRNA expression plasmids. Two days after transfection, cells were pretreated with AP5 and tetrodotoxin (TTX) for 1 hour before depolarization with 55 mM KCl for 7 hours. Data are from two independent experiments, each of which was conducted in triplicate, and are means ± standard error of the mean (SEM) for unstimulated cells and +SEM for cells treated with KCl.

(henceforth referred to as cocluster density or synapse number) along the dendrites of transfected cells. Reduction of MEF2A and MEF2D resulted in a significant increase in the number of synapses formed onto neurons (Fig. 2B), suggesting that MEF2 proteins function to restrict the number of synapses received by a neuron. The effect of MEF2 reduction on synapse number is due to the loss of MEF2-dependent transcription, given that the expression of a constitutively active and RNAi-resistant form of MEF2 (MEF2-VP16) completely reversed the increase in synapse number caused by MEF2 RNAi (Fig. 2B). A DNA-binding deficient mutant (MEF2ΔDBD-VP16) did not alter synapse number, indicating that this effect is dependent on the ability of MEF2-VP16 to bind to DNA and activate transcription.

Because MEF2 proteins promote calcium-dependent neuronal survival in cerebellar granule neurons deprived of growth factors (*8*), we cultured our hippocampal neurons under conditions in which MEF2 is not required for survival. We also monitored cell viability by assessing the dendritic morphology and passive membrane properties of neurons that were transfected with control or MEF2-specific shRNAs and found no detectable differences (figs. S4 and S5).

To test whether the synapses formed on MEF2 shRNA-expressing cells were functional, we recorded miniature excitatory postsynaptic currents (mEPSCs) from neurons 8 to 10 days after transfection in a whole-cell voltage clamp configuration. The reduction of MEF2A and MEF2D caused a significant increase in mEPSC frequency, whereas the expression of a scrambled shRNA had no effect (Fig. 2C). We observed no effect of MEF2A and MEF2D RNAi on mEPSC amplitude or the mEPSC waveform, indicating that, apart from the effect on mEPSC frequency, the AMPA receptor–mediated postsynaptic responses to spontaneous glutamate release were unaffected in these cells (fig. S6). The MEF2 RNAi-dependent increase in mEPSC frequency was reversed by cotransfection with MEF2-VP16 but not MEF2ΔDBD-VP16, suggesting that increased MEF2-dependent transcription can reverse the effect of MEF2 RNAi on mEPSC frequency (Fig. 2C).

Presynaptic alterations might account in part for the increase in mEPSC frequency in MEF2 shRNA-expressing cells, but we also observed an increase in the number of excitatory synapses by immunocytochemistry, which suggests that an increase in synapse number likely contributes to this effect. The increase in mEPSC frequency could also be due in part to an increase in the size or strength of preexisting synapses so that mEPSCs below our threshold of detection become detectable in cells treated with MEF2 RNAi. However, no difference was detected in the distributions of mEPSC amplitudes between control and MEF2 shRNA-expressing cells, suggesting that this possibility is unlikely.

To test whether activation of MEF2 is sufficient to suppress excitatory synapse number after synapses have already formed, we used an inducible gene-activation system in which the ligand-binding domain of the estrogen receptor (ER) is fused to the C terminus of MEF2-VP16. The ER fragment is mutated so that it responds specifically to 4-hydroxytamoxifen (4OHT). In the absence of 4OHT, MEF2-VP16–ER remained sequestered in the cytoplasm of transfected hippocampal neurons, but it rapidly translocated to the nucleus and activated MEF2-dependent transcription upon addition of 4OHT to the culture media (fig. S7 and Fig. 3A).

We transfected MEF2-VP16–ER into hippocampal neurons and allowed synapses to develop in the absence of 4OHT. After synapses had formed (DIV 20 and 21), cells were fixed either before or after 12 or 24 hours of 4OHT treatment. We determined the density of synapsin-1–PSD-95 coclusters and also monitored the number of dendritic spines per unit length of dendrite. Dendritic spines are the primary sites of excitatory contacts in mature hippocampal neurons and provide an additional measure of the number of excitatory synapses (*10*). In neurons transfected with an empty vector or a MEF2ΔDBD-VP16–ER expression vector, the density of synapsin-1–PSD-95 coclusters and dendritic spines remained unchanged over a 12- or 24-hour time period when cells were treated with 4OHT (Fig. 3B and fig. S8). However, neurons expressing MEF2-VP16–ER displayed a significant decrease in both cocluster density and spine number after 12 or 24 hours of 4OHT treatment. These results indicate that the activation of MEF2-dependent transcription is sufficient to restrict synapse number.

MEF2 is activated by diverse extracellular stimuli, including growth factors, neurotrophins such as brain-derived neurotrophic factor (BDNF), and calcium influx resulting from neuronal activity (*7*). Because glutamate release at excitatory synapses is thought to play a critical role in synaptic remodeling, we tested whether the ability of MEF2 to suppress synapse number requires glutamate-stimulated calcium influx. We transfected a MRE reporter plasmid into hippocampal neurons at DIV 10. To block calcium influx resulting from spontaneous glutamatergic synaptic transmission, we exposed hippocampal cultures to the *N*-methyl-D-aspartate–type glutamate receptor (NMDAR) antagonist 2-amino-5-phosphonopentanoic acid (AP5) or the L-type voltage-gated calcium channel (VGCC) antagonist nimodipine and found that either treatment led to a decrease in expression of the MRE reporter gene (Fig. 4A). Conversely, application of glutamate caused an

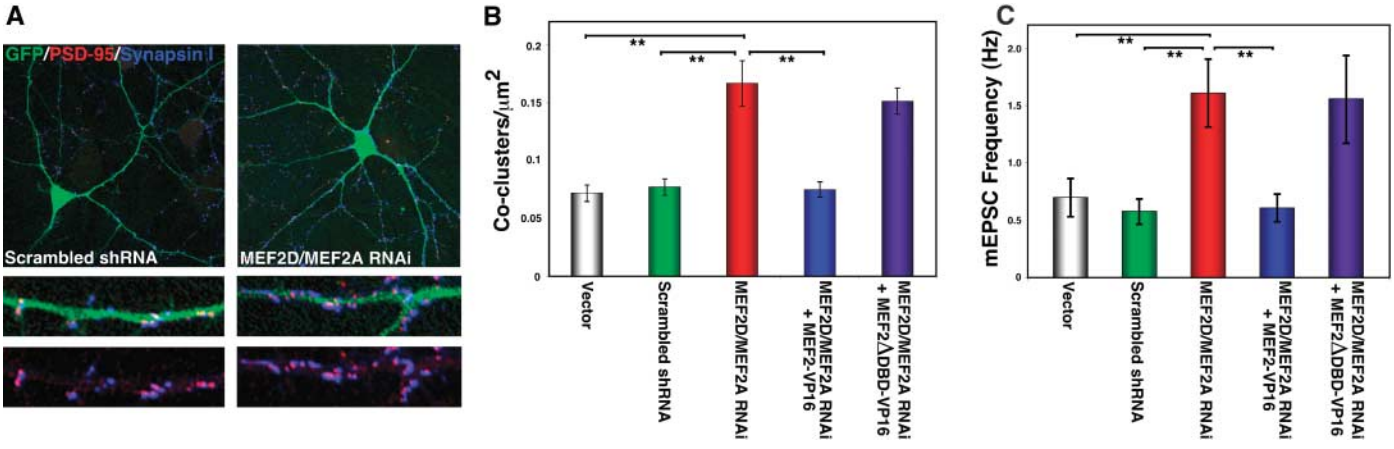

Fig. 2. Increased synapse number and mEPSC frequency after reduction of MEF2 in hippocampal neurons. (**A**) (Top) Images of hippocampal neurons transfected with the indicated plasmids and stained with antibodies to PSD-95 (red) and synapsin-1 (blue). (Bottom) Details of dendrites with and without cotransfected green fluorescent protein (GFP) displayed. (**B**) Average density of synapsin-1–PSD-95 coclusters along the dendrites of neurons transfected with the indicated plasmids. $P < 0.001$, analysis of variance (ANOVA); asterisks indicate statistical significance in pairwise comparison: $P < 0.001$, Bonferroni-Dunn post-hoc test. $n \geq 14$ cells per condition (137 total, including data shown in fig. S18) and data are means ± SEM. (**C**) Average mEPSC frequency for cells transfected with the indicated plasmids. $P < 0.01$, ANOVA; asterisks indicates statistical significance in pairwise comparison: $P < 0.005$, Bonferroni-Dunn post-hoc test. $n \geq 8$ cells per condition (55 total) and data are means ± SEM.

NMDAR-dependent increase in reporter gene expression, whereas membrane depolarization caused by increased extracellular concentration of potassium chloride (KCl) induced expression of the MRE reporter gene in a manner that required L-type VGCC-mediated calcium influx (Fig. 4, B and C). These data indicate that MEF2 transcriptional activity is induced by calcium influx through NMDARs and L-type VGCCs in hippocampal neurons.

To determine whether neuronal activity-dependent induction of MEF2 is required for the ability of MEF2 to suppress synapse number, we blocked synaptic activity by exposing cultured hippocampal neurons to the glutamate receptor antagonists AP5 and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and tested whether MEF2 functioned to restrict synapse number under these conditions. Blockade of synaptic activity does not alter the density of synapses formed onto control transfected hippocampal neurons (*11*). This likely reflects the evidence that activity is required for both the strengthening and weakening of synapses in these cultures. However, the reduction of MEF2A and MEF2D failed to cause an increase in the density of synapsin-1–PSD-95 coclusters in the presence of AP5 and CNQX (Fig. 4D). Thus, the ability of MEF2 to suppress synapse number requires the presence of glutamatergic synaptic activity, suggesting that the regulation of MEF2 by synaptic activity may be critical to its ability to suppress synapse number.

Because MEF2 phosphorylation is known to regulate its transcriptional activity (*7*), we tested whether phosphorylation of MEF2 is critical for calcium-dependent MEF2 activation in hippocampal neurons. In the absence of glutamatergic synaptic activity, we detected multiple species of MEF2A and MEF2D proteins in extracts from hippocampal neurons (Fig. 4E). The slower migrating forms of MEF2 appeared to be due to phosphorylation, because treatment of lysates from nondepolarized hippocampal cells with alkaline phosphatase led to the disappearance of these species. Membrane depolarization of hippocampal neurons also led to the disappearance of the slowly migrating forms of MEF2 in a manner that required calcium influx, suggesting that neuronal activity-induced calcium influx leads to the dephosphorylation of MEF2A and MEF2D. The calcium- and calmodulin-regulated phosphatase calcineurin (PP2B) has been implicated in the activity-dependent dephosphorylation of MEF2 (*12*). Using the specific inhibitors of calcineurin, cyclosporin A (CsA) and FK506, we observed that the membrane depolarization-induced dephosphorylation of MEF2A and MEF2D and MRE reporter gene expression were largely blocked by inhibiting calcineurin activity (Fig. 4, C and E). Moreover, MEF2D immunoprecipitated from nondepolarized neurons was dephosphorylated by recombinant calcineurin in vitro (fig. S9). These data suggest that calcineurin dephosphorylates MEF2 at specific sites that, when phosphorylated, inhibit the ability of MEF2 to activate transcription.

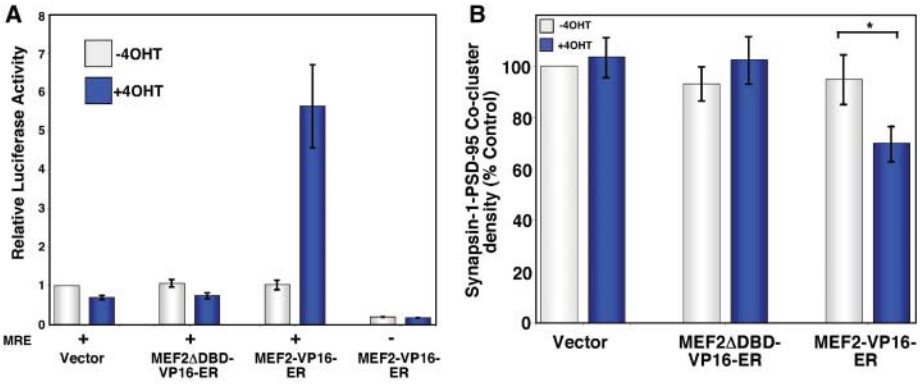

Fig. 3. Synapse loss after activation of MEF2-dependent transcription. (**A**) Luciferase reporter assay using 3xMRE-Luc or 3xMREmut-Luc cotransfected with the indicated expression plasmids. Two days after transfection, cells were stimulated with 4OHT for 6 hours. Data are from two independent experiments, each of which was conducted in triplicate, and are means ± SEM. (**B**) Average synapsin-1–PSD-95 cocluster density along the dendrites of neurons transfected and stimulated in the indicated manner for 24 hours. $P < 0.01$, ANOVA; asterisk indicates statistical significance in pairwise comparison: $P < 0.01$, Fisher's protected least significance difference (PLSD) test. $n \geq 21$ cells per condition (138 total); data are from three independent experiments and are means ± SEM.

To identify the sites of dephosphorylation, we used mass spectrometry and an analysis of MEF2 sequence conservation across species, which led us to focus on three serine residues in MEF2A and MEF2D—Ser^{221}, Ser^{255}, and Ser^{408} (on the basis of their positions in human MEF2A). Phosphorylation at Ser^{408} inhibits MEF2 activity in neurons (*13*). We generated three separate phosphospecific antibodies that recognize MEF2A when phosphorylated at Ser^{221}, Ser^{255}, or Ser^{408} (fig. S10). Membrane depolarization of hippocampal neurons resulted in a decrease in the phosphorylation of each of these three sites in a manner that was dependent on calcineurin activation (fig. S11). Application of BDNF, which activates MEF2 in neurons (*14*), did not detectably alter the phosphorylation of MEF2 at Ser^{221}, Ser^{255}, and Ser^{408} (fig. S12), suggesting that the dephosphorylation of these sites by calcineurin might confer a calcium-specific activation signal to promoters occupied by MEF2.

To test whether dephosphorylation of MEF2A at Ser^{221}, Ser^{255}, and Ser^{408} is important for neuronal activity-induced MEF2 transcription in neurons, we generated mutants of MEF2A in which an alanine replaced serine at all three serine residues, or Ser^{408} alone (fig. S13). We developed a protein replacement assay in which the expression of endogenous MEF2A and MEF2D in neurons was almost completely eliminated by RNAi, and MEF2 transcriptional activity was restored by expressing a MEF2A or MEF2D cDNA bearing silent mutations in the region targeted by the co-expressed shRNA, which renders the MEF2s resistant to RNAi (RiR) (Fig. 4F and fig. S14). MEF2 cDNAs lacking these silent mutations were still susceptible to RNAi and did not restore MEF2 activity. In this assay, both the triple mutant (Ser^{221}→Ala, Ser^{255}→Ala, and Ser^{408}→Ala) and the Ser^{408}→Ala mutant restored reporter gene expression to a greater extent than did wild-type MEF2A-RiR, indicating that phosphorylation of MEF2 at these serine residues inhibits MEF2 activity in neurons (Fig. 4F). In contrast, mutations of two highly conserved threonine residues that participate in stimulus-dependent MEF2 activation in other contexts did not alter the ability of MEF2A-RiR to restore calcium-dependent MEF2 activation (figs. S12 and S13) (*15*). Taken together, these data indicate that the calcium-dependent activation of MEF2 in neurons is distinct from these previously characterized mechanisms and requires the calcineurin-dependent dephosphorylation of MEF2.

We next tested whether, under culture conditions where calcineurin activity was inhibited so that MEF2 would not be dephosphorylated and activated, the reduction of MEF2A and MEF2D still caused an increase in synapse number. Chronic treatment of hippocampal neurons with CsA and FK506 led to a modest increase in synapse number (Fig. 4G). However, the reduction of MEF2A and MEF2D did not cause a significant increase in synapsin-1–PSD-95 cocluster number in the presence of calcineurin inhibitors (Fig. 4G). This suggests that the ability of MEF2 to suppress synapse number requires the neuronal activity-dependent activation of calcineurin and likely the dephosphorylation of MEF2.

To test whether MEF2 promotes the transcription of genes whose function is to negatively regulate the number of excitatory synapses that form onto neurons, we sought to identify activity-regulated genes whose expression is controlled by MEF2. We used lentiviral delivery

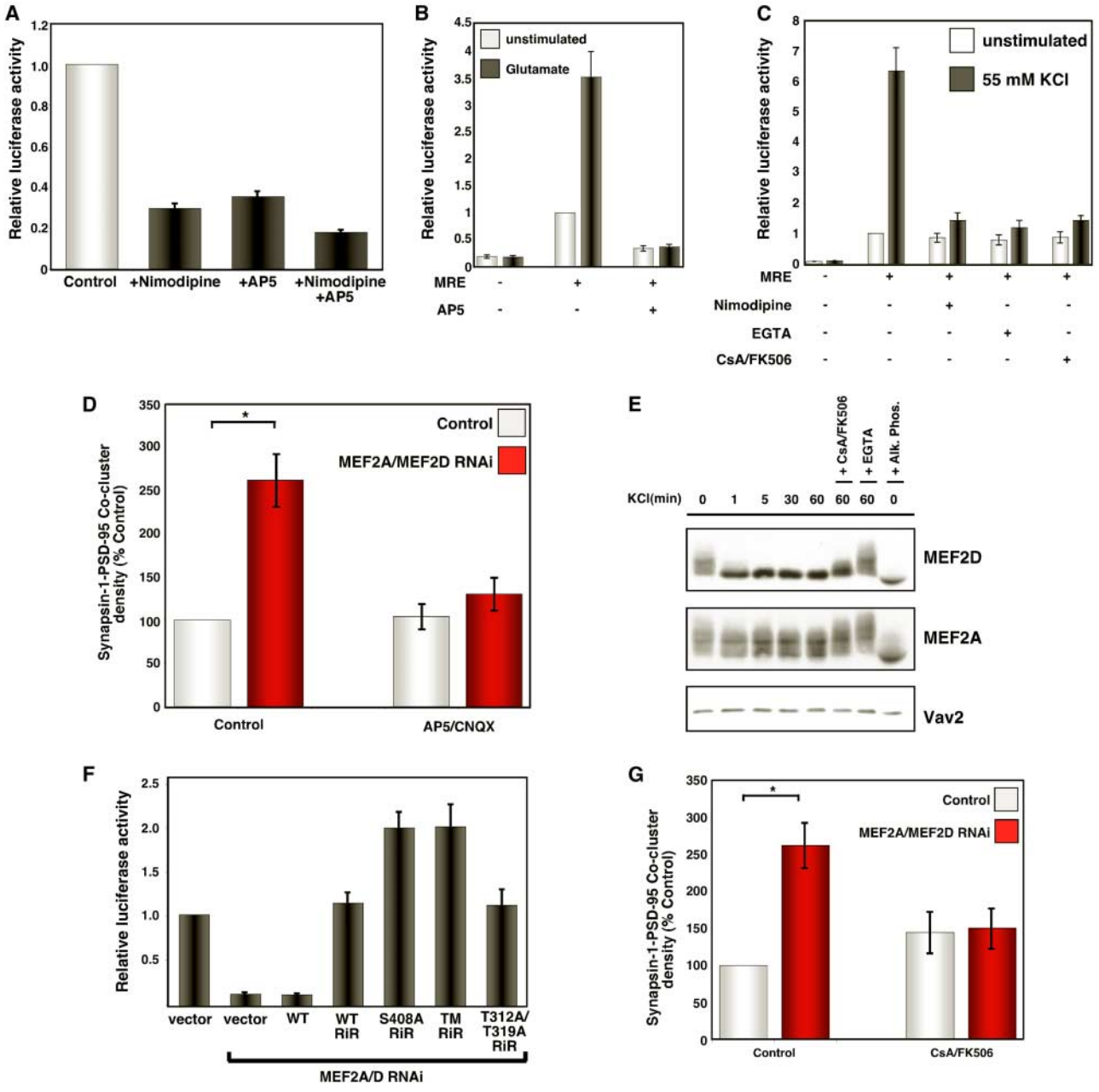

Fig. 4. Activity-dependent regulation of MEF2 by calcineurin. (**A**) Luciferase reporter assay with the use of 3xMRE-Luc transfected into E18 + 10 DIV hippocampal neurons. The indicated antagonists were added for 6 hours before lysates were collected. Data are means + SEM. (**B**) Luciferase reporter assay with the use of 3xMRE-Luc (MRE+) or 3xMREmut-Luc (MRE–). Transfected neurons were stimulated with glutamate (20 μM) for 4 hours at E18 + 10 DIV. Data are means ± SEM for unstimulated cells and means + SEM for glutamate. (**C**) Luciferase reporter assay using the same plasmids as in (B). Hippocampal neurons were transfected at E18 + 6 DIV and stimulated as in Fig. 1B. Data are means ± SEM for unstimulated cells and means + SEM for cells treated with KCl. (**D**) Density of synapsin-1–PSD-95 coclusters along the dendrites of neurons transfected with either a scrambled shRNA (control) or MEF2-specific shRNAs. $n \geq 12$ cells per condition (59 total) and data are means ± SEM. (**E**) Western blot analyses of MEF2 proteins extracted from hippocampal neurons at E18 + 8 DIV. Cells were pretreated with AP5 and TTX and stimulated with 55 mM KCl for the indicated times. (**F**) Luciferase reporter assay with the use of the same reporter plasmids as in (A), cotransfected with the indicated plasmids into hippocampal neurons at E18 + 4 to 5 DIV. Data are means + SEM. (**G**) Density of synapsin-1–PSD-95 coclusters onto neurons transfected with either scrambled shRNA (control) or MEF2-specific shRNAs. $n \geq 15$ cells per condition (63 total) and data are means ± SEM. For (D) and (G), $P < 0.001$, ANOVA; asterisks indicate statistical significance in pairwise comparison: $P < 0.001$, Bonferroni-Dunn post-hoc test. For (A) to (C) and (F), data are from two independent experiments, each of which was conducted in triplicate.

of shRNAs and Affymetrix microarrays to conduct a genome-wide screen for genes transcribed in response to membrane depolarization that are reduced in expression after reduction of MEF2. The reduction of MEF2A and MEF2D caused a decrease in the expression of genes belonging to several functional categories, including additional transcriptional regulators and signaling molecules such as kinases. We investigated two of these genes, the *activity-regulated cytoskeletal-associated protein* (*arc*) and *synaptic Ras guanosine triphosphatase activating protein* (*synGAP*), because the protein products of these genes negatively regulate synaptic development. Arc promotes the internalization of gluta-

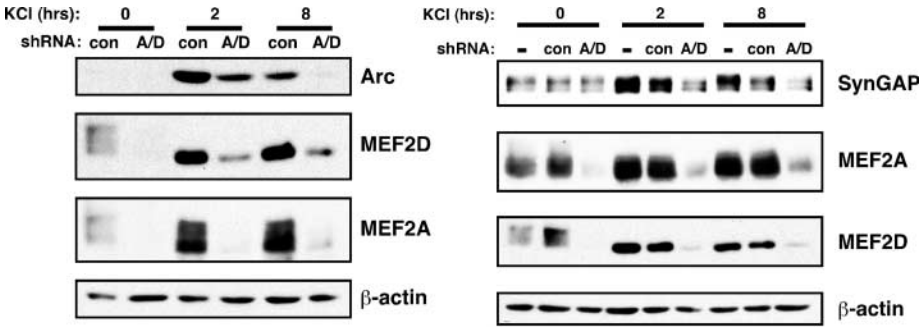

Fig. 5. Regulation of arc and synGAP by MEF2. Western blot analyses of arc (left) and synGAP expression (right) with the use of antibodies specific to the two proteins. Protein lysates were collected from neurons at E18 + 10 DIV. Neurons were transduced with lentiviruses at E18 + 3 DIV and depolarized with high KCl as in Fig. 1B before lysis. For synGAP expression analysis, neurons were lysed at E18 + 14 DIV. con, control; A/D, MEF2A and MEF2D RNAi.

mate receptors (*16*), whereas synGAP inhibits Ras–mitogen-activated protein kinase signaling in the postsynaptic compartment (*17*). We confirmed that MEF2 reduction leads to a decrease in the expression of *arc* and *synGAP* by real-time quantitative polymerase chain reaction and Western blotting (fig. S15 and Fig. 5). Moreover, we identified evolutionarily conserved regions at the genomic loci of both *arc* and *synGAP* and found that several of these DNA fragments were enriched in MEF2 chromatin immunoprecipitates (fig. S16). Thus, *arc* and *synGAP* may be direct transcription targets of MEF2.

Our results show that MEF2 transcription factors negatively regulate excitatory synapse number in an activity-dependent manner during synaptic development in vitro. They support a model in which the calcium- and calmodulin-regulated phosphatase calcineurin dephosphorylates and activates MEF2, which in turn promotes the remodeling of synapses by inducing a program of gene transcription that may involve *arc* and *synGAP*. Given that calcineurin is also required for the expression of certain forms of LTD (*18*), it is possible that MEF2 might also contribute to LTD. Whether MEF2 is important in vivo for such processes as synapse elimination, homeostatic control of synapse number, and/or LTD remains to be determined.

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Supporting Online Material

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Materials and Methods
SOM Text
Figs. S1 to S18

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A Calcium-Regulated MEF2 Sumoylation Switch Controls Postsynaptic Differentiation

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Postsynaptic differentiation of dendrites is an essential step in synapse formation. We report here a requirement for the transcription factor myocyte enhancer factor 2A (MEF2A) in the morphogenesis of postsynaptic granule neuron dendritic claws in the cerebellar cortex. A transcriptional repressor form of MEF2A that is sumoylated at lysine-403 promoted dendritic claw differentiation. Activity-dependent calcium signaling induced a calcineurin-mediated dephosphorylation of MEF2A at serine-408 and, thereby, promoted a switch from sumoylation to acetylation at lysine-403, which led to inhibition of dendritic claw differentiation. Our findings define a mechanism underlying postsynaptic differentiation that may modulate activity-dependent synapse development and plasticity in the brain.

The MEF2 family of transcription factors is highly expressed in the brain when neurons undergo dendritic maturation and synapse formation (*1*). MEF2A is especially abundant in granule neurons of the cerebellar cortex throughout the period of synaptogenesis (*1*) (fig. S1). In view of reported functions for transcription factors in distinct aspects of dendritic morphogenesis (*2–4*), we investigated a potential role of MEF2A in synaptic dendritic development in the cerebellar cortex.

During cerebellar development, granule neuron dendritic morphogenesis culminates in the differentiation of dendritic claws on which mossy fiber terminals and Golgi neuron axons form synapses (*5–8*). To visualize granule neurons undergoing postsynaptic differentiation, we transfected organotypic cerebellar slices prepared from postnatal day 9 (P9) rat pups with an expression plasmid encoding green fluorescent protein (GFP) (*9*). Transfected granule neurons in the internal granule layer had the typical small cell body with associated parallel axonal fibers and few dendrites (Fig. 1, A and B). Many dendrites harbored structures with the appearance of dendritic claws that were identified on the basis of classic descriptions as (i) located at the end of a dendrite, (ii) having cuplike or sicklelike appearance, and (iii) displaying undulating or serrated inner surfaces (*5–8*) (Fig. 1, C and D). Dendritic claws showed punctate expression of the postsynaptic protein PSD95 (Fig. 1D).

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