Argonaute (AGO) proteins are essential components of the microRNA (miRNA) pathway.AGO proteins are loaded with miRNAs to target mRNAs and thereby regulate mRNA stability and protein translation. As such, AGO proteins are important actors in controlling local protein synthesis, for instance, at dendritic spines and synapses. Although miRNA-mediated regulation of dendritic mRNAs has become a focus of intense interest over the past years, the mechanisms regulating neuronal AGO proteins remain largely unknown. Here, using rat hippocampal neurons, we report that dendritic Ago2 is down-regulated by the proteasome upon NMDA receptor activation. We found that Ser-387 in Ago2 is dephosphorylated upon NMDA treatment and that this dephosphorylation precedes Ago2 degradation. Expressing Ser-387 phosphorylation-deficient or phosphomimetic Ago2 in neurons, we observed that this phosphorylation site is involved in modulating dendritic spine morphology and postsynaptic density protein 95 (PSD-95) expression in spines. Collectively, our results point toward a signaling pathway linking NMDA receptor-dependent Ago2 dephosphorylation and turnover to postsynaptic structural changes. They support a model in which NMDA receptor-mediated dephosphorylation of Ago2 and Ago2 turnover contributes to the de-repression of mRNAs involved in spine growth and maturation.

Neuronal homeostasis requires a tight regulation of turnover for proteins involved in cellular signaling and structural adaptation (1, 2). More particularly, activity-dependent local protein synthesis has been shown to be necessary for several forms of synaptic plasticity (3–5). Consequently, many messenger RNAs (mRNAs) are transported to dendrites and then locally translated into proteins (6–9).

In recent years, microRNAs (miRNAs)3 have emerged as key factors in post-transcriptional regulation for a majority of mRNAs (10, 11). miRNAs are short noncoding RNAs (~20–24 nucleotides) that target their complementary sequences in specific mRNAs, thereby causing the inhibition of translation and/or a decrease in mRNA stability (12–15). The prominent role of miRNAs in neurons has been underlined by the discovery that miRNAs are significantly involved in regulating neuronal morphology and function (6, 16–19).

The regulation of mRNA translation by miRNAs occurs in the miRNA-induced silencing complex (miRISC), which enables miRNAs to target their mRNAs (12, 14, 20, 21). Several studies have shown that the association of miRISC with different scaffold or RNA-binding proteins underlies the translation regulation associated with synaptogenesis and synaptic plasticity (22–27).

At the core of miRISCs are Argonaute (AGO) proteins, which are loaded with miRNAs and enable miRNAs to bind their target mRNAs and induce subsequent translational repression (12, 14, 20, 21). Mammals express four to five different AGO proteins as follows: Ago1–4 and in mouse Ago5 (28, 29). Ago1 and Ago2 are the predominant forms of AGO proteins in the brain (30). Ago2 is the only mammalian AGO protein to possess endonuclease activity, which is induced by siRNAs and catalyzes cleavage and rapid degradation of targeted mRNAs (31, 32). Several different post-translational modifications of Ago2, like phosphorylation or ubiquitination, have been shown to alter Ago2 subcellular localization, protein stability, and its interaction with RNAs (20, 33). Despite the importance of Ago2 as a core component in miRNA-mediated control of dendritic mRNA translation, the regulation of Ago2 in response to neuronal activity remains largely unknown.

Here, we report that Ago2 is down-regulated in a proteasome-dependent manner upon NMDA-receptor (NMDA-R) activation in cultured hippocampal neurons. We find that prior to Ago2 degradation, NMDA treatment causes dephosphoryl-
Ago2 phosphorylation affects its turnover and spine density

Ago2 phosphorylation affects its turnover and spine density

Results
NMDA-R activity regulates proteasomal degradation of dendritic Ago2

To analyze the effect of synaptic activity on Ago2 protein localization and turnover in dendrites, we briefly incubated mature dissociated hippocampal neuronal cultures with NMDA. Immunocytochemistry analysis revealed a significantly lower amount of dendritic Ago2 in neurons treated with NMDA compared with untreated neurons (Fig. 1A). To exclude that NMDA-dependent neurotoxicity might account for our effects, we performed trypan blue staining 24 h after treatment with different NMDA concentrations for different durations. As shown in Fig. S1, the employed NMDA concentration did not induce neurotoxicity. Next, we asked whether a more general increase in synaptic activity would also cause a reduction in dendritic Ago2. We therefore treated neuronal cultures with the GABA$_A$ antagonist picrotoxin (PTX) to generally increase synaptic transmission in our neuronal cultures. Incubation with PTX induced a significant loss of Ago2 in dendrites (Fig. S2), which was blocked by co-incubation with the NMDA-R antagonist APV (AP5) thereby indicating again that NMDA-R stimulation is causing the loss of Ago2. Having observed that NMDA-R stimulation leads to a down-regulation of dendritic Ago2, we turned our attention toward the underlying mechanism. It has been shown in several non-neuronal cell types that Ago2 protein levels are regulated by proteasomal degradation (34–37). In addition, synaptic activity and NMDA-R activation, in particular, are known to up-regulate the proteasomal pathway in dendrites and spines (38–42). We therefore hypothesized that NMDA-R activation could trigger proteasomal degradation of Ago2 in neuronal dendrites. To test this hypothesis, we added the proteasome inhibitor lactacystin 1 h prior to NMDA application to neuronal cultures. As shown in Fig. 1B, treatment of neuronal cultures with lactacystin prevented NMDA-R–dependent Ago2 decrease in dendrites, indicating that Ago2 down-regulation involves the proteasomal pathway. We next repeated this experiment with an alternative mAb against Ago2 to validate our results. With this alternative antibody we obtained the identical result, i.e. a significant decrease of Ago2 upon NMDA-R stimulation, which is prevented by pretreatment of neuronal cultures with the proteasome inhibitor lactacystin (Fig. 1C).

NMDA-R activation triggers dephosphorylation of Ago2 at Ser-387

Hoping to understand the mechanism underlying the reduction of Ago2 upon NMDA-R stimulation, we investigated the dynamics of Ago2 phosphorylation upon NMDA stimulation. We observed in NMDA-treated neurons a decrease in dendritic Ago2 pSer-387 in parallel to a decrease in dendritic total Ago2 (Fig. 2B). However, the decrease in Ago2 pSer-387 was greater in proportion than the decrease in total Ago2, as indicated by the lower ratio of Ago2 pSer-387 to total Ago2 in dendrites stimulated with NMDA. This result corroborates our initial Western blotting results from Fig. 2A.

Our observation that NMDA-R activation leads to a specific reduction of Ser-387–phosphorylated Ago2 could be explained by two different scenarios: (a) either NMDA-R activation triggers the degradation of Ser-387–phosphorylated Ago2; or (b) NMDA-R activation leads to Ago2 Ser-387 dephosphorylation, and it is the dephosphorylated Ago2 that is preferentially subject to degradation. To test these two mutually exclusive hypotheses, we incubated neuronal cultures with the proteasome inhibitor lactacystin and analyzed whether we would still observe NMDA-dependent dephosphorylation of Ago2 pSer-387 in the absence of proteasomal degradation. To prevent the theoretical possibility that newly synthesized unphosphorylated Ago2 could affect the ratio of Ago2 pSer-387 to total Ago2, we added the protein synthesis inhibitor cycloheximide to neuronal cultures. Western blot analysis revealed that neurons treated with lactacystin still showed a significant reduction in Ago2 Ser-387 phosphorylation with NMDA-R activation, which was comparable with the reduction seen in neurons not incubated with lactacystin (Fig. 3, A–C). However, lactacystin did prevent decrease of total Ago2 protein amount upon NMDA-R activation (Fig. 3A), similarly to what we previously observed in dendrites by immunocytochemistry (Fig. 1, B and C). Furthermore, we tested an alternative proteasome inhibitor, MG132, and obtained the same result as with lactacystin, i.e. NMDA-induced reduction of Ago2 protein was blocked by MG132 (Fig. S4). These results indicate that...
Figure 1. NMDA-R activation leads to a rapid, proteasome-dependent decrease of Ago2 levels in hippocampal neurons. A, immunocytochemistry analysis of Ago2 protein (red) in dendrites of cultured hippocampal neurons (DIV19) counterstained with phalloidin-FITC (green) and treated with or without NMDA (40 μM NMDA, 15 min) prior to fixation. Left, representative images of primary dendrite segments. Right, whisker and box plot of Ago2 (AlexaFluor594) mean fluorescence intensity of 62.1-μm segments of proximal dendrites in different neurons (results normalized to control condition median; Control, n = 16; NMDA, n = 19; two-tailed Mann-Whitney U test, *, p < 0.05). B, immunocytochemistry analysis of Ago2 protein (red) with Wako clone 4D2 mAb (Ab#1) in dendrites of cultured hippocampal neurons (DIV19) counterstained with phalloidin-FITC (green), with or without lactacystin pretreatment (Lactacystin; 5 μM, 1 h) and with or without NMDA (40 μM NMDA, 15 min) treatment immediately prior to fixation. Shown is whisker and box plot of Ago2 (AlexaFluor594) fluorescence intensity in dendrites (results normalized to control condition median; vehicle, n = 24; vehicle + NMDA, n = 19; lactacystin, n = 22; lactacystin + NMDA, n = 18; Kruskal-Wallis test, p = 0.0002; Dunn’s multiple comparisons post-test, *, p < 0.05; **, p < 0.01). C, immunocytochemistry analysis of Ago2 protein (red) with a different antibody than in A, Abcam ab57113 mAb (Ab#2), in dendrites of cultured hippocampal neurons (DIV19) counterstained with phalloidin-FITC (green), with or without lactacystin pretreatment (Lactacystin, 5 μM, 1 h) and with or without NMDA (20 μM, 5 min) treatment immediately prior to fixation. Shown is whisker and box plot of Ago2 (AlexaFluor594) mean fluorescence intensity in dendrites (results normalized to control condition median; vehicle, n = 27; vehicle + NMDA, n = 27; lactacystin, n = 27; lactacystin + NMDA, n = 27; Kruskal-Wallis test, p = 0.0018; Dunn’s multiple comparisons post-test, *, p < 0.05; **, p < 0.01).
NMDA-R activation induces the dephosphorylation of Ago2 at Ser-387, which occurs independently from Ago2 degradation by the proteasome, suggesting that this dephosphorylation precedes NMDA-R–dependent Ago2 degradation by the proteasome.

**Ser-387 phosphorylation-deficient Ago2 is readily degraded upon NMDA-R activation**

Although our results so far point toward a link between Ago2 dephosphorylation at Ser-387 and Ago2 degradation upon NMDA-R stimulation, they do not provide direct evidence that the dephosphorylation of Ago2 is a necessary event for the targeted degradation of Ago2. To further elucidate the role of Ago2 Ser-387 phosphorylation in regulating Ago2 turnover, we cloned an eGFP-tagged Ago2 WT construct (Ago2WT) as well as an eGFP-tagged phosphoblock mutant of Ago2 with serine 387 substituted by alanine (Ago2S387A) and an eGFP-tagged phosphomimetic mutant of Ago2 with serine 387 substituted by aspartic acid (Ago2S387D). First, we transfected neuronal cultures with these constructs and analyzed their level of

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**Figure 2. NMDA receptor activation leads to a rapid decrease of Ser-387 phosphorylation in Ago2.**

A, Western blotting of hippocampal neurons (DIV23) treated with or without NMDA (20 μM, 5 min). Left, representative image of Ago2 pSer-387 versus total Ago2 Western blots (with corresponding β-actin loading control). Right, change in Ago2 pSer-387/total Ago2 ratio of paired samples for five independent experiments (each NMDA sample normalized to paired Control; one-tailed sign test, p < 0.05). B, Immunocytochemistry analysis of Ago2 pSer-387 protein (red) versus total Ago2 (green) in dendrites of cultured hippocampal neurons (DIV19) counterstained with phalloidin-FITC (green), with or without NMDA treatment (40 μM NMDA, 15 min) immediately prior to fixation. Shown is whisker and box plot for Ago2 pSer-387/total Ago2 relative ratio of mean fluorescence intensities in dendrites (results normalized to respective control condition median; Control, n = 24, NMDA, n = 19; two-tailed Mann-Whitney U test; **, p < 0.01).
expression. As shown in Fig. S5, expression of eGFP-tagged Ago2WT, Ago2S387A, and Ago2S387D caused a 2–3-fold increase in total Ago2 immunostaining compared with eGFP-expressing control neurons. Importantly, the overall expression level of Ago2WT, Ago2S387A, and Ago2S387D did not differ among the different constructs. We next transfected neurons with Ago2WT and tdTomato (a red fluorescent protein to visualize the complete neuronal structure and for standardization of eGFP-Ago2 fluorescence intensity) and tested whether ectopically expressed Ago2WT is regulated in a similar fashion like endogenous Ago2, i.e., it is degraded upon NMDA-R activation in a proteasome-dependent manner. Like before with endogenous Ago2, we observed that eGFP-Ago2-WT is decreased in dendrites by NMDA-R stimulation and that this down-regulation is prevented by proteasome inhibition (Fig. 3A).

To verify that Ser-387 dephosphorylation is an obligatory step for the proteasome-dependent down-regulation of Ago2...
Figure 4. Ago2-S387D phosphomimetic mutant blocks NMDA-induced Ago2 degradation. A, analysis of eGFP-Ago2WT in primary dendrites of DIV17 cultured hippocampal neurons, transfected with pEGFP-Ago2WT and pCI-tdTomato, treated with lactacystin (5 μM, 1 h) or vehicle (DMSO) and with or without NMDA treatment (40 μM NMDA, 15 min) prior to fixation. Shown is whisker and box plot of relative eGFP-Ago2/tdTomato ratio of mean fluorescence intensities in dendrites (results normalized to control condition median; vehicle, n = 14; vehicle + NMDA, n = 11; lactacystin, n = 14; lactacystin + NMDA, n = 15; Kruskal-Wallis test, p = 0.0278; Dunn’s multiple comparisons post-test, *, p < 0.05). B, analysis of eGFP-Ago2S387A and eGFP-Ago2S387D in dendrites of DIV15 cultured hippocampal neurons, transfected with pEGFP-Ago2S387A or pEGFP-Ago2S387D and pCI-tdTomato, with or without NMDA treatment prior to fixation (40 μM NMDA, 15 min). Shown is whisker and box plot of relative eGFP-Ago2/tdTomato ratio of mean fluorescence intensities in dendrites (results normalized to respective control condition median; S387A control, n = 17; S387A NMDA, n = 15; two-tailed Mann-Whitney U test, *, p < 0.05; S387D control, n = 19; S387D NMDA, n = 11; two-tailed Mann-Whitney U test, *, p < 0.05). C, analysis of eGFP-Ago2S387A in dendrites of DIV17 cultured hippocampal neurons, transfected with pEGFP-Ago2S387A and pCI-tdTomato, treated with lactacystin or vehicle (DMSO) and with or without NMDA treatment (40 μM NMDA, 15 min) prior to fixation. Shown is whisker and box plot of relative eGFP-Ago2/tdTomato ratio of mean fluorescence intensities in dendrites (results normalized to control condition median; vehicle, n = 17; vehicle + NMDA, n = 13; lactacystin, n = 11; lactacystin + NMDA, n = 11; Kruskal-Wallis test, p = 0.0110; Dunn’s multiple comparisons post-test, *, p < 0.05).
Ago2 phosphorylation affects its turnover and spine density

upon NMDA-R activation, we tested whether Ago2 with a phosphomimetic mutation at Ser-387 (Ago2S387D) is protected from NMDA-R–dependent degradation. Hippocampal neurons were transfected with expression vectors for either eGFP-Ago2S387A or eGFP-Ago2S387D and tdTomato. Although the phosphoblock form of Ago2 was down-regulated in dendrites, we observed that the phosphomimetic form was up-regulated upon NMDA treatment (Fig. 4B). Although this last result might suggest that the S387D mutant undergoes increased recruitment into stimulated dendrites, it more importantly shows that Ago2 Ser-387 dephosphorylation is a necessary prerequisite for Ago2 proteasomal degradation.

Our results in Figs. 3 and 4B suggest that NMDA-R activation induces a two-step process. First, NMDA-R activation causes Ago2 dephosphorylation at Ser-387. Second, NMDA-R activation causes subsequent Ago2 degradation by increasing proteasomal activity. To validate this two-step process, we tested whether Ago2 with a phosphoblock mutation at Ser-387 (S387A) is still subjected to increased proteasomal degradation after NMDA-R activation. eGFP-Ago2S387A and tdTomato were expressed together in hippocampal neurons and treated with NMDA with or without prior incubation with lactacystin. Again, we observed a reduction of Ago2S387A in dendrites upon NMDA-R activation, which is absent in lactacystin-treated neurons (Fig. 4C). Taken together, these findings show that dephosphorylation at Ago2 Ser-387 after NMDA-R activation permits the subsequent degradation of unphosphorylated Ago2 by the proteasome.

So far, we found that NMDA-R activation leads to the dephosphorylation and degradation of Ago2 in dendrites. However, in hippocampal neurons, NMDA-Rs are enriched at synapses on dendritic spines (49, 50). We therefore analyzed eGFP-Ago2 turnover in tertiary dendrites where spines are more abundant than in the primary dendrites analyzed so far. The ectopic expression of eGFP-Ago2WT together with tdTomato allowed us to compare the effects of NMDA application on Ago2 levels in spines versus dendrites. Similar to dendrites (Fig. 4A), we found that eGFP-Ago2WT levels are also decreased in spines upon NMDA-R activation (Fig. 5A and B). Interestingly, when comparing the turnover of eGFP-Ago2 in spines versus dendrites, we observed a greater decrease of Ago2 in spines compared with dendrites after NMDA-R stimulation (Fig. 5, C and D).

Ago2 Ser-387 dephosphorylation leads to increased spine density and PSD-95 enrichment

Finally, we turned our attention to the physiological consequences of Ago2 Ser-387 phosphorylation in dendrites. Reducing Ago2 phosphorylation at Ser-387 has been shown to decrease miRNA-induced repression of mRNAs (46). Furthermore, several research groups reported that miRNA-dependent translational regulation of local protein expression is associated with synaptogenesis and synaptic plasticity (22–27). Hence, we hypothesized that the NMDA-R–dependent dephosphorylation of Ago2 Ser-387 could be involved in regulating the release of mRNA repression and thereby allowing the local translation of mRNAs in dendrites and spines.

To test whether Ago2 Ser-387 phosphorylation affects spine growth and shape, we expressed Ago2WT, Ago2S387A, or Ago2S387D in cultured hippocampal neurons and quantified dendritic spines on tertiary dendrites. In Ago2WT-expressing neurons, NMDA-R activation was associated with an increase in spine density (Fig. 6A). Interestingly, expression of Ago2S387A by itself increased spine density in untreated transfected neurons, and NMDA treatment did not further increase spine density for Ago2S387A-expressing neurons, indicating that blocking Ago2 Ser-387 phosphorylation occludes the effects of NMDA-R stimulation on spine density. In untreated Ago2S387D-expressing neurons, spine density was similar to that of untreated Ago2WT-expressing neurons. However, in contrast to Ago2WT-expressing neurons, NMDA-R activation was not associated with an increase in spine density in neurons expressing Ago2S387D. These results indicate that mimicking Ser-387 phosphorylation of Ago2 blocks the effects of NMDA-R stimulation on spine density. Overall, our results that NMDA-R–dependent spine increase is mimicked by phospho-deficient Ago2 and blocked by phosphomimetic Ago2 support the involvement of Ago2 Ser-387 phosphorylation in modulating NMDA-R-dependent spine growth.

The finding that the expression of Ago2S387A in itself is associated with an increase in total spine density (Fig. 6A) prompted us to further analyze whether Ago2 Ser-387 phosphorylation affects spine maturation. Spines are classified based on their shape as mushroom-like, stubby, or thin spines, with mushroom-like spines being the most mature form (51). To verify whether Ago2 Ser-387 phosphorylation has an impact on the occurrence of the different spine types, i.e. mushroom, stubby, and thin spines, hippocampal neurons were transfected with either Ago2WT, Ago2S387A, or Ago2S387D (+ tdTomato). We found that mushroom spines are more numerous in neurons expressing Ago2S387A compared with Ago2WT or Ago2S387D, whereas no significant differences in thin and stubby spines could be observed (Fig. 6B). Given that mushroom spines are considered to be the most mature form of spines (51), our results suggest that Ago2 Ser-387 dephosphorylation contributes to spine maturation.

An important factor in dendritic spine maturation and excitatory synapse formation is the enrichment of PSD-95 in spines (52, 53). Importantly, Ago2 has been reported to be directly involved in the regulation of PSD-95 mRNA in the dendrites of hippocampal neurons (24). To analyze the possible role of Ago2 Ser-387 phosphorylation in regulating PSD-95 enrichment in spines, hippocampal neurons were transfected with either Ago2WT, Ago2S387A, or Ago2S387D (+ tdTomato), and PSD-95 expression was analyzed by immunocytochemistry. Although the increase in PSD-95 in neurons expressing Ago2S387A compared with Ago2WT was not statistically significant, we observed a significant increase in PSD-95 in neurons expressing Ago2S387A compared with neurons expressing phosphomimetic Ago2S387D (Fig. 6B, bottom).

Taken together, the effects of the phosphoblock mutation of Ago2S387A versus phosphomimetic Ago2S387D on dendritic spine density and morphology, as well as on PSD95 enrichment in spines, support the involvement of Ago2 Ser-387 dephosphorylation in spine growth and maturation.
Discussion

Mature miRNAs associate with AGO proteins to exert their regulatory effects on mRNA translation (12, 20, 21, 44). Several studies have shown that dendritic localization and interaction of Ago2 with components of the miRNA pathway are modulated by neuronal maturation and neuronal activity (22–24, 27, 54–57).

Here, we show that neuronal activity in the form of NMDA-R activation leads to Ago2 dephosphorylation at Ser-387 and proteasome-dependent degradation of Ago2 in dendrites of hippocampal neurons. Furthermore, neuronal expression of a phosphodeficient mutant of Ago2 that blocks phosphorylation of Ser-387 causes an increase in spine density. Overall, our observations support a model (Fig. 7) where NMDA-receptor activation through the regulation of Ago2 turnover contributes to the derepression of mRNAs involved in spine growth and maturation.

NMDA-R–dependent degradation of Ago2

Neuronal activity, especially NMDA-R activation, is known to up-regulate proteasomal activity and turnover of a variety of proteins in dendrites and spines (2–4, 40, 58). A widely accepted view, supported by several lines of evidence, proposes that synaptic activity leads in general to an acute increase in turnover of several mature miRNAs and derepression of their mRNA targets (19, 21, 59–61). Our result that NMDA-R activation causes proteasome-dependent Ago2 degradation therefore provides a mechanistic frame for the local synaptic dere-
expression of miRNA-targeted transcripts as well as miRNA turnover in neurons.

In addition, the activity- and proteasome-dependent turnover of Ago2, as described here, is similar to the degradation of MOV10 (an Ago2-interacting protein that is degraded after NMDA-R activation) and FMRP (a RNA-binding protein that also interacts with Ago and is degraded after mGluR activation) in cortical and hippocampal neurons, as reported previously (26, 62). Importantly, MOV10 and FMRP have both been found to regulate Ago2 association to mRNAs (62, 63). Taken together, these findings and our results suggest a common scheme where neuronal activity regulates the dendritic miRNA pathway through proteasomal degradation of its key effector proteins.

**NMDA-R-dependent dephosphorylation of Ago2**

Ago2 Ser-387 phosphorylation favors Ago2 localization to processing bodies (P-bodies), i.e. to cytoplasmic granules where
miRNA-targeted mRNAs are stored or degraded (46, 47). Therefore, our observation that Ago2 Ser-387 is dephosphorylated upon NMDA-R activation (Fig. 3) is in line with findings that neuronal activity, NMDA-R activation in particular, leads to acute disassembly of dendritic P-bodies and release of Ago2 (54, 55). Dephosphorylation of Ago2 Ser-387 has also been shown to reduce miRNA-induced repression of mRNAs (46). Derepression of miRNA-targeted mRNAs involves in general the dissociation of Ago2 from these mRNAs and may lead to the unloading of Ago2 from its miRNAs (20, 21). The pool of Ago2 that is unloaded of its mRNA and/or miRNA is then preferentially targeted for degradation (33, 64). In accordance with these results, we find that dephosphorylation of Ago2 at Ser-387 after NMDA-R activation is associated with increased Ago2 degradation. Two possible mechanisms could account for this effect as follows: either the dephosphorylation of Ser-387 is by itself sufficient to target Ago2 for degradation or the dissociation of Ago2 from RNA in consequence to Ser-387 dephosphorylation renders Ago2 vulnerable to degradation.

Finally, our findings add Ago2 Ser-387 phosphorylation in neurons to a growing number of post-translational modifications of Ago2 that regulate its turnover in non-neuronal cells (20, 33), like the phosphorylation of tyrosine 393 and the sumoylation of lysine 402 that have been shown to also regulate miRNA loading and stability of Ago2 (48, 65, 66). Given the larger molecular weight of phosphorylated Ago2 in Fig. 3, it seems plausible that additional modifications, like sumoylation of Ago2, occur in neurons. However, the role of these additional modifications in regulating Ago2 stability or function in neurons after NMDA-R activation remains unclear.

Modulation of dendritic spines by Ago2 dephosphorylation

Dendritic spines constitute postsynaptic compartments that are composed of an intricate network of scaffolding proteins. During development and synaptic plasticity, changes in spine number and shape are highly dependent on local dendritic protein translation (5, 67). The importance of the miRNA pathway in regulating local mRNA translation in dendrites is well established (6–8, 21), and many miRNAs have been found to impact dendritic morphology (16, 59, 60, 68–71). In accordance with the important role of the miRNA pathway in synaptic maturation and plasticity, we find that the expression of Ago2 with a phosphoblock or a phosphomimetic mutation at Ser-387 impacts dendritic spine density (Fig. 6A).

Apart from dendritic spine morphology, we find that neurons expressing Ago2S387A display a synaptic PSD95 enrichment compared with Ago2S387D-expressing cells. PSD-95 is a marker for synaptic maturation (72–74), playing an important role in concentrating glutamate receptors at synapses (52). The PSD-95 mRNA undergoes localized translation in dendrites (67) and was shown to be regulated by Ago2/miRNA-125a in conjunction with FMRP in dendrites (24).

Taken together, our results link NMDA-induced Ago2 Ser-387 dephosphorylation to increased spine density and maturation. Given that proteasomal degradation of MOV10 (an Ago2 interaction partner) is, like Ago2, regulated through NMDA-R activity (26), it appears that NMDA-R–dependent proteasomal degradation of components of the miRNA pathway constitutes an important element for deregression of dendritic mRNAs and local protein synthesis triggered by synaptic activity. Our
findings therefore contribute to an emerging picture where activity-dependent turnover of translational regulators is intimately linked to activity-dependent mRNA translation (26, 75–78). Ultimately, this relationship may offer an explanation as to how proteasome activation is linked to protein synthesis in the context of changes in structural plasticity (38) as well as in long-term memory formation (58, 79).

Materials and methods

DNA constructs

pEGF-P-Ago2WT was cloned by inserting human Ago2 from pcDNA3-myc-Ago2 (31) into the pEGFP-C2 vector (Clontech). pEGF-Ago2S387A and pEGF-Ago2S387D were generated by PCR site-directed mutagenesis of pEGFP-Ago2WT (tagging with GFP followed a similar approach as described previously (22, 80)).

For S387A, mutagenic primers, 5′-GCAATTTGATGCAGTTCGCAGTTCAACACAGATCCCATCCACGCAGTGG-3′ and 5′-CGGACGTATGGATGCTTGGAAAGCTGCACTTCGGATCAATTTGC-3′, were used. For S387D mutagenic primers, 5′-GCAATTTGATGCAGTTCGCAGTTCAACACAGATCCCATCCACGCAGTGG-3′ and 5′-CGGACGTATGGATGCTTGGAAAGCTGCACTTCGGATCAATTTGC-3′ were used.

Animals

The animal protocol (permit no. 17–139) followed the guidelines of the “Comité de Déontologie de l’Expérimentation sur les Animaux” of the Université de Montréal.

Primary neuronal cultures

Hippocampi from E18 to E19 Sprague-Dawley rats were digested in trypsin (0.25%) and mechanically dissociated. Cells were plated onto poly-d-lysine (PDL)–coated 6-well (35 mm) dishes at high density (2–3 × 10^5 cells/ml) for Western blotting experiments and onto PDL/laminin-coated glass coverslips (12-mm round Corning BioCoat Cellware) in 24-well plates at low density (7–8 × 10^4 cells/ml) for imaging experiments. Cultures were maintained in Neurobasal medium with 2% B27 supplement and 0.5 mM GlutaMAX supplement (Gibco), with initial 2% FBS (HyClone), at 37 °C and 5% CO₂. At 3 days in vitro (DIV3), for high-density cultures, and DIV4, for low-density cultures, half of the media was replaced with media without FBS and with cytosine-β-d-arabinofuranoside (Ara-C, 2.5 μM) to inhibit glial proliferation. Subsequently, a third to half of the media was replaced without FBS every 4–7 days, depending on culture density and experimental time points.

Drugs and treatments

For all treatments, neurons were incubated at 37 °C, 5% CO₂ in conditioned media with all drugs and other substances added with fresh maintenance media. In initial experiments examining the effects of NMDA-R activation on Ago2 turnover in dendrites (Fig. 1C) and on Ago2 Ser-387 phosphorylation (Fig. 2A), NMDA (Sigma) was applied at 20 μM (for 5 min). However, for ectopically expressed Ago2 (eGFP-Ago2WT), we found that a stronger and longer stimulation, i.e. 40 μM NMDA for 15 min, would lead to more robust/significant effects. Therefore, all other experiments requiring NMDA incubation were performed with 40 μM NMDA for 15 min. In most cases, neurons were fixed or lysed immediately following stimulation. However, for some experiments (Figs. 2A, 3, and 6A), neurons were washed with media and incubated for an additional 15 min to increase time-dependent effects of NMDA treatment. To inhibit proteasomal degradation, lactacystin (5 μM; Cayman Chemicals and AdipoGen) was applied an hour before NMDA-R stimulation. For the experiments in Fig. 3, we added the protein synthesis inhibitor cycloheximide (25 μg/ml, Acros Organics) to neuronal cultures to prevent the theoretical possibility that newly synthesized Ago2 could affect total Ago2 levels.

Fixation and immunocytochemistry/immunofluorescence

After treatment, neurons were immediately fixed with cold 4% paraformaldehyde (PFA) in PBS with 5 mM MgCl₂ for 10 min and additionally in 2% PFA in PBS for 10 min at room temperature.

For immunofluorescence labeling, cells were permeabilized and blocked in 0.2% Triton X-100 and 3% goat serum or 5% BSA in TBS for 20 min at room temperature. Neurons were then incubated with primary antibodies at 1:200 to 1:400 concentrations in TBS with 0.2% Tween 20 and 1.5% goat serum or 2.5% BSA for 1–2 h at room temperature or overnight at 4 °C. Secondary antibodies were applied at 1:100 to 1:300 dilution in TBS with 0.2% Tween.

Indirect immunofluorescent labeling was performed with the following primary antibodies: for (total) Ago2, mouse monoclonal Wako catalog no. 014-22023 (RRID: AB_1106837; Figs. 1, A and B, and 2B) or mouse monoclonal Abcam catalog no. ab57113 (RRID: AB_2230916; Fig. 1C); for Ago2 pSer-387, rabbit polyclonal EMC Biosciences catalog no. AP5291 (RRID: AB_2571732); for PSD-95, mouse monoclonal ThermoFisher Scientific catalog no. MA1-046 (RRID: AB_2092361). Secondary antibodies were conjugated with AlexaFluor 488, 546, 594, or 633 (ThermoFisher Scientific). Filamentous actin (F-actin) was stained with phalloidin conjugated to FITC (phalloidin-FITC; Sigma).

Transfections

Hippocampal neuronal cultures grown on coverslips (DIV14/15) were transfected with pCI-ttdTomato and pEGFP-Ago2 constructs using Lipofectamine 2000 (Invitrogen) and then transferred to conditioned media without Lipofectamine for 2–3 days (DIV16–19) before treatment and fixation.

Image acquisition and analysis

Image acquisition was performed with an Olympus Fluoview-1000 argon and diode laser-scanning confocal microscope equipped with a Plan-Apo ×60 oil objective (numerical aperture, 1.42). Confocal aperture and z-step size were adjusted automatically through the confocal microscopy platform Olympus Fluoviewer for optimal quantitative imaging of the fluorophores employed in each experiment. For experiments employing the same combination of fluorophores, confocal aperture and z-step size were the same. For each experimental

Ago2 phosphorylation affects its turnover and spine density
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set, acquisition settings (laser intensity and scan speed) and detector settings (photomultiplier levels, digital gain, and offset) were tested to optimize the quantitative aspect of the signal (at the single z-slice level), to avoid signal saturation or non-detection, oversampling or undersampling, and excessive background and noise. Importantly, all settings, manually or automatically adjusted, were kept constant within an experimental set.

Quantitative analysis of endogenous Ago2 immunolabeling was performed on averaged z-stacks without any image manipulation or enhancement. Quantification of eGFP-Ago2 constructs was performed on summed z-stacks to which background subtraction (automatic, rolling ball, ImageJ) was applied. For quantification of PSD-95 (Fig. 6B), thresholding prior to image quantification was used to select double-positive pixels tdTomato and PSD-95 to account for labeling of transfected neurons only.

For quantification of Ago2 in dendrites with immunofluorescence labeling or eGFP fusion constructs, the dendrite appearing to be the most prominent was identified, and a segmented line (i.e. continuous with bifurcation points) was drawn manually (ImageJ) for each neuron. To ensure unbiased analysis, the segmented line (for the line plot) was drawn with images only displaying the channel corresponding to the dendritic marker employed (phalloidin for immunolabeled neurons; tdTomato for transfected neurons), with the experimenter being blinded to (a) the channel corresponding to labeled or tagged Ago2 and (b) to the experimental condition (e.g. control versus NMDA treatment).

The line plot started at the base of the dendrite (border to the soma) and followed closely the center of the dendrite. Once the line was drawn in the channel corresponding to the dendritic marker, the experimenter added the channel corresponding to labeled or tagged Ago2 to the image to measure fluorescence intensity values in each channel using a line plot function (in Olympus Fluoviewer or ImageJ softwares). All line plots were cut off at a uniform length (300 or 400 pixels, depending on experiments), and the fluorescence intensity was averaged for the retained segments. For quantification of eGFP-Ago2 levels, mean eGFP fluorescence intensity was standardized by the corresponding mean tdTomato fluorescence intensity. The obtained values were then compiled and subjected to statistical analysis (followed by the experimenter not blinded to the experimental condition of the different groups).

For quantification of eGFP-Ago2 in dendritic spines (Fig. 5), images of secondary and tertiary dendrites were acquired. The eGFP/tdTomato ratio of mean fluorescence intensities was measured for each spine individually and matched to an equivalent region of interest (same shape and size) at the base of the spine in the dendrite.

For dendritic spine density and morphology analysis (Fig. 6), images of tertiary dendrites were acquired. Spines were counted in regions of constant length (19.2 μm) along the dendrite (using the tdTomato fluorescent signal to identify dendrites and spines). Spine counts are illustrated in Fig. 6, A and B, as the number of spines per 10 μm, i.e. counted number of spines per 10 μm. Spines were visually categorized in three groups: stubby, thin, or mushroom-like.

**Western blotting**

Immediately prior to lysis, cultures were washed once with ice-cold PBS with 5 mM MgCl₂ and 50 μM APV, and then with ice-cold plain PBS. Whole-cell extracts were obtained by scraping cells in denaturing lysis buffer (1% SDS TBS; 200 μl per 35-mm well) and boiling samples at 95 °C for 10 min. Lysates were centrifuged in a tabletop centrifuge for 15 min, and the supernatant was collected. For loading sample preparation, lysates were diluted with an equal volumes of 2× Laemmli buffer (10% β-mercaptoethanol) and boiled at 100 °C for 5 min. Samples were loaded onto 10% SDS-PAGE for electrophoresis and transferred to PVDF membranes. Membranes were blocked in 5% BSA in TBS 0.2% Tween (1 h at room temperature). To analyze Ago2 Ser-387 phosphorylation (Figs. 2A and 3), membranes were first incubated with Ago2 pSer-387 rabbit polyclonal antibody (EMC Biosciences catalog no. AP5291, RRID:AB_257132), diluted 1:1000 in blocking buffer, at 4 °C overnight, followed by incubation with peroxidase-conjugated anti-rabbit IgG (Rockland catalog no. 18-8816-33, RRID: AB_469529) and ECL (GE Healthcare). Afterward, membranes were stripped and probed for total Ago2. For total Ago2 quantification, membranes were incubated with Ago2 mouse mAb (Abcam catalog no. ab57113, RRID: AB_2230916), diluted 1:1000 in blocking buffer, at 4 °C overnight, followed by incubation with peroxidase-conjugated anti-mouse IgG (Thermo-Fisher Scientific catalog no. 31430, RRID:AB_228307) and ECL. β-Actin mouse mAb conjugated with peroxidase (Sigma catalog no. A2228, RRID: AB_476697) was used as loading control.

**Data representation and statistics**

Data sets for quantitative fluorescence analysis did not exhibit normal distribution. Therefore, to determine whether differences between datasets were statistically significant, the following nonparametric tests were employed: Mann-Whitney U test for experiments with two experimental conditions, and Kruskal-Wallis test (nonparametric ANOVA) with Dunn’s multiple comparisons post-test for experiments with more than two experimental conditions. The results for quantitative fluorescence analysis are represented nonparametrically with whisker and box plots (Figs. 1, 2B, 4, 5, and 6B). The rectangular box represents the range between the 1st quartile and the 3rd quartile. The median is represented as a horizontal line within the box. The whiskers represent the minimum, and the maximum and should not be confused with standard error of the mean or standard deviation. For visual simplicity and comparison purposes, all values for different treatments within an experiment were standardized to the median value of their respective control group.

For analysis of spine density (Fig. 6), the datasets exhibited normal distribution, allowing the use of standard one-way and two-way ANOVA followed by post-hoc Tukey test. For Western blot analysis, experiments were executed in a paired or matched design (i.e. independent sets of experiments with each set having its corresponding untreated control), and the obtained densitometric values of each sample were normalized.
by their respective control. Hence, the following nonparametric tests were used: a one-tailed sign test for paired samples subjected to two experimental conditions (Fig. 2A), and a Friedman test with Dunn’s multiple comparisons post-test for matched samples with four conditions (Fig. 3).

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