NEUROSCIENCE

BDNF-induced local translation of *GluA1* is regulated by HNRNP A2/B1

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The AMPA receptor subunit GluA1 is essential for induction of synaptic plasticity. While various regulatory mechanisms of AMPA receptor expression have been identified, the underlying mechanisms of GluA1 protein synthesis are not fully understood. In neurons, axonal and dendritic mRNAs have been reported to be translated in a cap-independent manner. However, molecular mechanisms of cap-independent translation of synaptic mRNAs remain largely unknown. Here, we show that *GluA1* mRNA contains an internal ribosome entry site (IRES) in the 5′UTR. We also demonstrate that heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 interacts with *GluA1* mRNA and mediates internal initiation of *GluA1*. Brain-derived neurotrophic factor (BDNF) stimulation increases IRES-mediated *GluA1* translation via up-regulation of HNRNP A2/B1. Moreover, BDNF-induced GluA1 expression and dendritic spine density were significantly decreased in neurons lacking hnRNP A2/B1. Together, our data demonstrate that IRES-mediated translation of *GluA1* mRNA is a previously unidentified feature of local expression of the AMPA receptor.

INTRODUCTION

All brain functions are mediated by the complex connectivity between synapses of individual neurons. In particular, learning and memory, the fundamental functions of the brain, are closely related to synaptic transmission, and the molecular mechanisms controlling these connections are exquisitely regulated. In particular, the AMPA receptor is an ionotropic glutamate receptor that facilitates fast excitatory neurotransmission in the brain. The number of AMPA receptors within synapses determines the strength of synaptic transmission, and abnormal expression is implicated in cognitive dysfunctions associated with neuropsychiatric and neurodegenerative diseases such as schizophrenia and Alzheimer's disease (*1*). The AMPA receptor consists of four subunits (GluA1 to GluA4), which assemble to form heterotetrameric structures. Among the four subunits, extensive studies have demonstrated that GluA1 has a pivotal role in long-lasting plasticity. Supporting this, long-term potentiation is impaired in the CA1 region of the adult hippocampus of GluA1 knockout mice (*2*). Moreover, GluA2/GluA3 double-knockout mice exhibit normal long-term depression, suggesting the presence of a GluA2/GluA3-independent long-term depression induction process (*3*). Together, these data indicate that GluA1 is a key factor that mediates long-term synaptic plasticity.

Accumulating evidence demonstrates that neuronal stimulation involving brain-derived neurotrophic factor (BDNF) regulates expression of the AMPA receptor subunit GluA1 in hippocampal neurons (*4*). BDNF activation affects the abundance of synaptic GluA1 through both trafficking of GluA1 and local translation of *GluA1* mRNA (*5*, *6*). Regulation of the AMPA receptor is of fundamental importance in tuning synaptic strength, not only for enhancing neuronal connections during brain development but also for storing information during learning and memory (*7*). However, little is known about the underlying molecular mechanisms of BDNF-induced local translation of *GluA1*. We hypothesized that this translational control of *GluA1* might be a previously unidentified mechanism of regulation for modulating the AMPA receptor reservoir pool and synaptic AMPA receptor levels.

Synthesis of several synaptic plasticity–related proteins is dependent upon posttranscriptional regulation, which includes the concerted actions of various RNA binding proteins including heterogeneous nuclear ribonucleoproteins (HNRNPs) (*8*). Moreover, several lines of evidence demonstrate that HNRNP dysfunction is associated with neurodegenerative diseases (*9*). HNRNPs are reported to bind to the 5′ untranslated region (UTR), 3′UTR, or coding region of various mRNAs to mediate mRNA transport, stability, or translation (*10*). One mechanism of translational regulation through interaction between hnRNPs and specific mRNAs is cap-independent translation. Ever since it was discovered in the viral genome (*11*), various studies have demonstrated that a subset of cellular mRNAs have internal ribosome entry site (IRES) activity in their 5′UTRs (*12*). In contrast to canonical cap-dependent translation, these mRNAs initiate translation by directly recruiting ribosomes in a cap-independent manner (*13*). Specifically, cellular IRES-mediated translation is associated with certain stress conditions (*14*), rhythmic oscillation of clock genes (*15*), and even dendritic mRNAs (*16*). Moreover, a recent study reported that IRES-mediated translation is important in normal mouse development (*17*). However, the molecular mechanisms and physiological consequences of IRES-mediated translational control of plasticity-related mRNAs are still poorly understood.

In this study, we report the first identification of IRES activity in *GluA1* mRNA, which is regulated by BDNF stimulation in hippocampal neurons. We also find that the RNA binding protein HNRNP A2/B1 interacts with the *GluA1* 5′UTR and acts as a trans-acting factor to enhance IRES-mediated translation of *GluA1*. Moreover, we use a novel technique, Puromycin–Proximity Ligation Assay (Puro-PLA) (*18*), to directly visualize de novo synthesis of GluA1 protein in situ. Furthermore, we show that inhibition of HNRNP A2/B1 decreases both GluA1 expression and dendritic spine density, suggesting that this HNRNP A2/B1–mediated IRES-dependent

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translation has a critical role in the expression of *GluA1* and in dendritic spine number.

RESULTS

BDNF induces global and local translation of *GluA1* mRNA

As previously reported, BDNF facilitates protein synthesis of plasticity-related dendritic mRNAs (*19*). To understand whether BDNF stimulation regulates GluA1 protein levels in dendrites, we used a recently described technique, Puro-PLA (*18*), to visualize newly synthesized GluA1 protein. In puromycin-labeled primary hippocampal neurons, newly translated GluA1 was detected in the somata and dendrites of neurons, in contrast to control neurons (no puromycin) or neurons treated with the translation inhibitor anisomycin. Moreover, preincubation with BDNF conferred increased Puro-PLA signals in both somata and dendrites (Fig. 1, A and B), suggesting that BDNF significantly up-regulates *GluA1* protein synthesis. *GluA1* mRNA levels, however, remained constant after BDNF treatment, unlike a previous report (Fig. 1C) (*4*). This may be due to different experimental conditions, including species of the neurons used and/or days of in vitro culture. These results suggest that BDNF induces translation of *GluA1* mRNA.

Because BDNF affects translation through the mammalian target of rapamycin (mTOR) signaling pathway (*20*), and mTOR activates cap-dependent translation through phosphorylation of 40*S* ribosomal protein S6 kinase 1 and eukaryotic initiation factor 4E-binding protein 1 (*21*), we hypothesized that inhibition of mTOR may block BDNF-induced GluA1 expression. To test this hypothesis, we stimulated primary hippocampal neurons with BDNF in the presence or absence of the mTOR inhibitor rapamycin. Rapamycin did not significantly inhibit the increase of GluA1 protein levels induced by BDNF stimulation. Moreover, neurons treated with rapamycin alone showed only marginal reduction in GluA1 expression (fig. S1). These results suggest that *GluA1* mRNA may be translated in a cap-independent manner, potentially activated by BDNF stimulation.

IRES activity in *GluA1* mRNA

To explore IRES activity in *GluA1* mRNA, we constructed a translation reporter that included the *GluA1* 5′UTR and firefly luciferase coding sequences that can be in vitro transcribed with either a G-capped or A-capped structure. In this system, the reporter mRNA harboring G-cap represents global translation of *GluA1* mRNA, while the A-cap harboring reporter mRNA describes only IRES-mediated translation efficiency because cap-binding proteins cannot bind to the A-capped mRNA (fig. S2A). We measured reporter activity, after in vitro transcription, followed by reporter mRNA transfection to human embryonic kidney (HEK)–293T cells for the transfection efficiency. We found that IRES-mediated translation comprises almost half of *GluA1* global translation (fig. S2B). Furthermore, we sought to validate IRES-mediated translation of *GluA1* through inhibition of general translation elongation or mTOR signaling by treatment with cycloheximide or RAD001, a rapamycin analog, respectively, in GluA1-expressing neuronal SHSY5Y cells. Compared with the vehicle control, cycloheximide treatment reduced GluA1 protein levels 9 hours after treatment, whereas RAD001 treatment did not alter GluA1 protein levels at the same time point (fig. S2C). These results suggest that GluA1 protein is likely synthesized by an alternative translation mechanism other than cap-dependent initiation, and this IRES-mediated translation may play an important role in *GluA1* protein synthesis.

To validate whether the *GluA1* 5′UTR directs IRES-mediated translation, we used a bicistronic reporter system. The 5′UTR of *GluA1* mRNA was inserted between the *Renilla* luciferase (Rluc) gene (translated in a cap-dependent manner) and the firefly luciferase (Fluc), which is translated only if the preceding sequences can recruit ribosomes. To exclude nonspecific effects of 5′UTR length, we inserted the reverse sequences of the *GluA1* 5′UTR into the bicistronic reporter (Fig. 2A). The ratio of Fluc to Rluc therefore reflects the IRES-mediated translation efficiency of the inserted intergenic sequences. We then introduced the reporters to widely used Neuro2a cells and found that insertion of the *GluA1* 5′UTR enhanced Fluc levels compared to the control reporter; these increased Fluc levels were reduced with the reporter containing the reverse sequences of the *GluA1* 5′UTR, suggesting that the *GluA1* 5′UTR can direct IRES-mediated translation (Fig. 2B). To ensure that IRES-mediated translation of the *GluA1* 5′UTR did not come from cryptic promoter activity or ribosome re-association, we constructed additional reporters without the cytomegalovirus (CMV) promoter or with the short hairpin structure upstream of Rluc (fig. S2, D and E). As expected, Rluc levels were negligible in the reporter lacking the CMV promoter. However, because Fluc activity was marginally detected (fig. S2, F and G), we performed Northern blot analysis using HEK-293A cells to confirm that a cryptic promoter does not exist in the *GluA1* 5′UTR (fig. S2H). Following introduction of the short hairpin reporter, Rluc activity was markedly decreased, but Fluc expression was maintained in Neuro2a cells (fig. S2, F and G). Together, these results demonstrated that *GluA1* mRNA induces an IRES-dependent translation mechanism mediated by its 5′UTR.

Because GluA1 expression and function is most abundant in the hippocampus, we asked whether the *GluA1* 5′UTR has IRES-mediated translation activity in mouse primary hippocampal neurons. To demonstrate this, we generated fluorescent bicistronic reporters, similar to the luciferase reporters described above. Rluc, expressed through cap-dependent initiation, was substituted with DsRed, and Fluc, translated via an IRES-dependent manner, was replaced with a modified form of green fluorescent protein ($_{myr}$dGFP) with myristoylation sequences for membrane anchoring and a destabilization motif to prevent signal saturation (*22*). The *GluA1* 5′UTR was then inserted in the intergenic region of these fluorescent genes to validate its IRES-mediated translation, and the 3′UTR was added downstream of $_{myr}$dGFP to confer dendritic targeting of the reporter mRNA (*23*). We introduced these fluorescent reporters to cultured hippocampal neurons and found that the GFP signal was clearly visible in dendrites and dendritic spines expressing the *GluA1* 5′UTR-inserted reporter compared with those expressing the control vector (Fig. 2, C and D). Together, these results suggest that the *GluA1* 5′UTR has IRES-mediated translation in both Neuro2a cell lines and primary neurons and may have critical roles in *GluA1* mRNA translation.

To further test whether the fluorescence signal of the bicistronic reporter is a product of translation, we used fluorescence recovery after photobleaching (FRAP) analysis in dendritic spines of reporter-transfected primary neurons. Both DsRed and GFP fluorescence in dendritic spines were significantly recovered after photobleaching (fig. S3A); however, recovery of these signals was reduced following treatment with the translation blocker anisomycin (fig. S3B). These

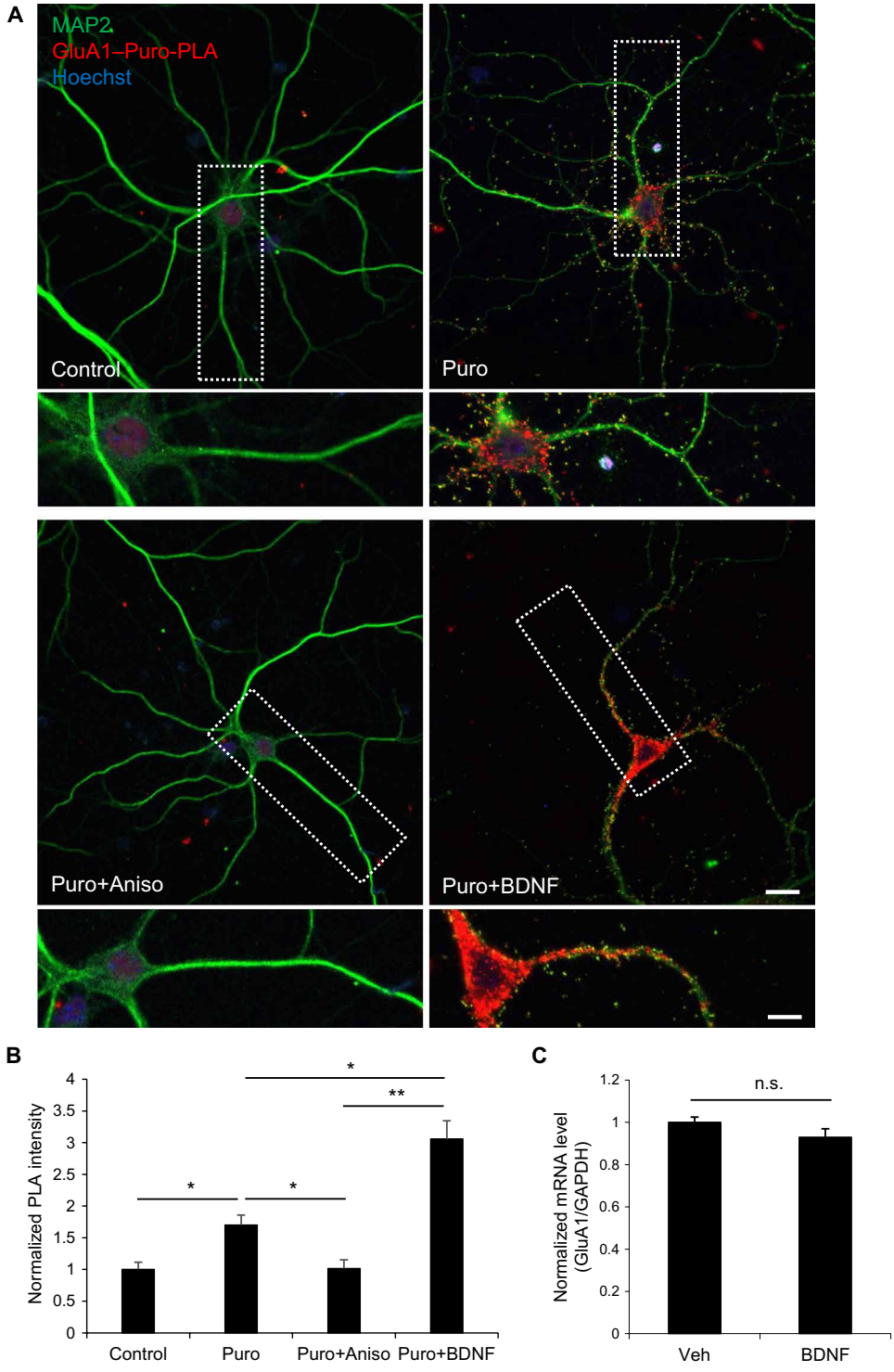

Fig. 1. BDNF-induced local translation of *GluA1*. (**A**) Representative hippocampal primary neuron images and close-ups of Puro-PLA signal for newly synthesized GluA1 without puromycin labeling (Control), with 15-min labeling of Puro alone (Puro), or with the protein synthesis inhibitor anisomycin (Puro+Aniso) or BDNF (Puro+BDNF). Scale bars, 25 μm (overview) and 15 μm (close-ups). (**B**) Summary graph of normalized PLA intensities of hippocampal primary neurons. The PLA intensity of control was set to 1. Data represent means ± SEM ($n = 8$, $^*P < 0.05$, $^{**}P < 0.01$). (**C**) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis for *GluA1* mRNA levels of hippocampal neurons after BDNF treatment. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA level was used for normalization ($n = 5$). n.s., not significant.

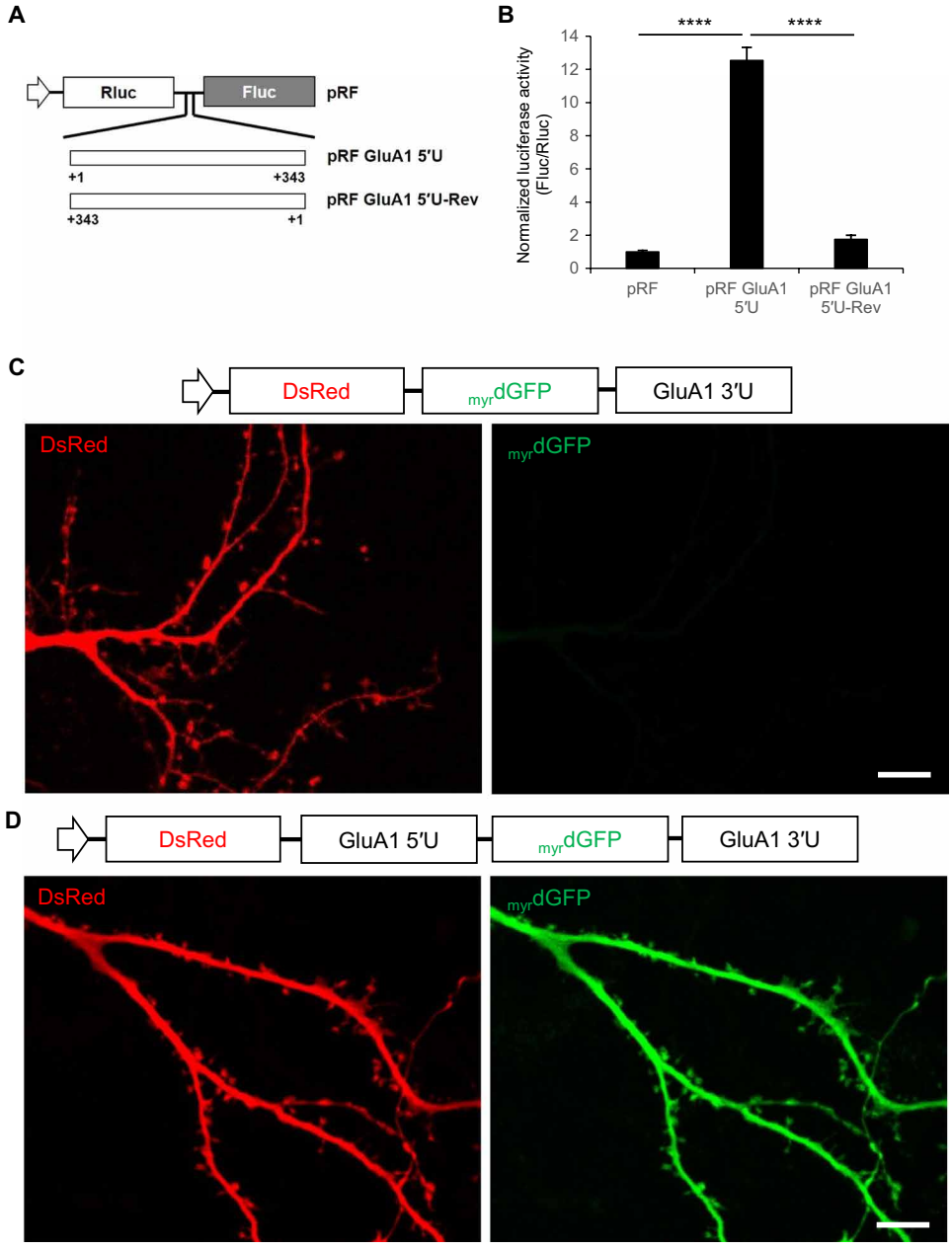

Fig. 2. Cap-independent translation of *GluA1* mRNA. (**A**) Schematic diagram of the bicistronic reporter plasmid containing the *GluA1* 5′UTR. The full length of *GluA1* 5′UTR or the reverse sequence of the *GluA1* 5′UTR was inserted between the Rluc and Fluc sequences of the pRF plasmid. (**B**) Neuro2a cells were transfected with bicistronic reporters, and the efficiency of cap-independent translation of each plasmid is shown. The ratio of the control pRF vector was set to 1. Data represent means ± SEM ($n = 5$, ****$P < 0.0001$). (**C** and **D**) Representative images of hippocampal neurons transfected with the control fluorescent bicistronic reporter (C) and reporter harboring the *GluA1* 5′UTR and 3′UTR (D). Scale bar, 20 μm.

results suggest that GFP signal recovery in dendritic spines is due to *GluA1* IRES–mediated translation.

Next, we asked whether BDNF activates IRES-mediated translation of *GluA1* mRNA. To explore this possibility, we introduced the DsRed and GFP fluorescent bicistronic reporters to primary neurons and treated with BDNF in the presence or absence of rapamycin. As expected, both DsRed and GFP fluorescence was enhanced with BDNF treatment. The DsRed signal, reporting cap-dependent translation, decreased with rapamycin treatment; GFP fluorescence, reporting IRES-mediated translation of *GluA1* mRNA, was not affected by rapamycin and was further increased with BDNF stimulation in the presence of rapamycin (Fig. 3, A and B). These results suggest that BDNF activates IRES-mediated translation of *GluA1* mRNA.

Identification of a cis-acting element in the *GluA1* 5′UTR

Most cellular IRES-mediated translation depends on RNA binding proteins that interact with specific regions in the secondary structures of mRNAs and directly recruit ribosomes (*12*). We performed in silico predictions to explore possible stem-loop structures in the

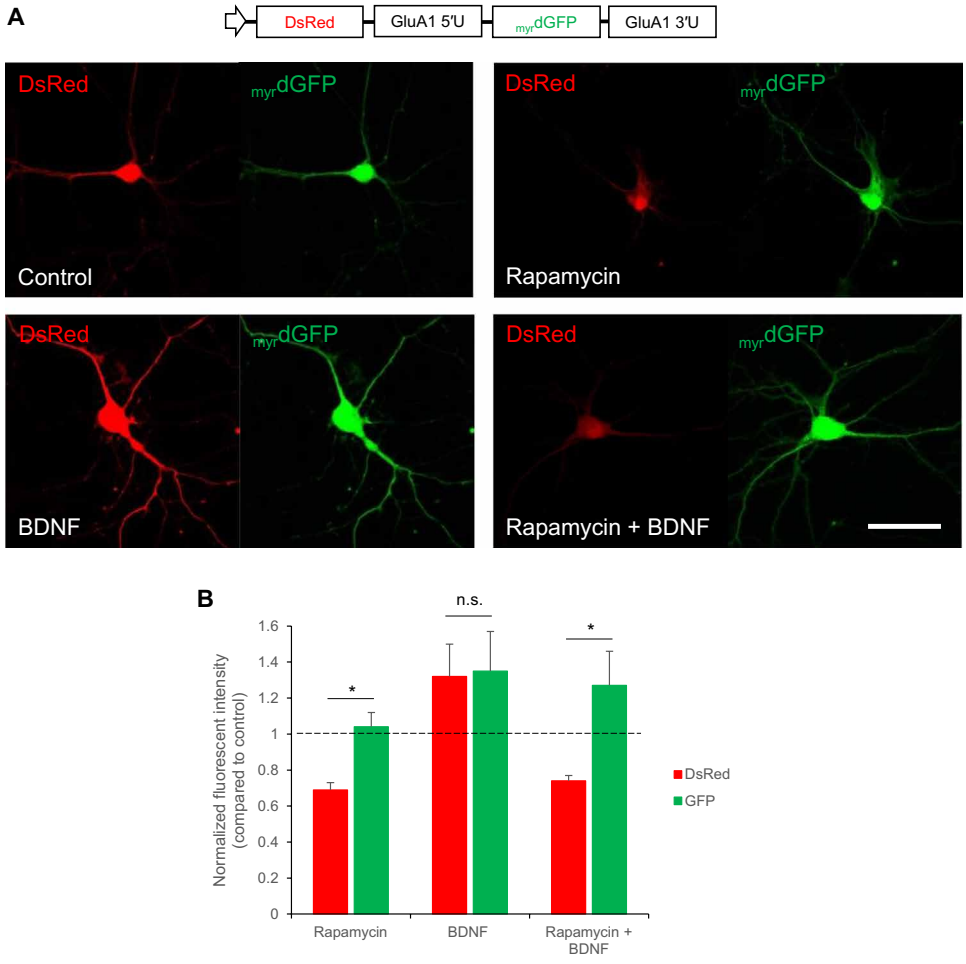

Fig. 3. BDNF activates IRES-mediated translation of *GluA1* mRNA. (**A**) Representative images of hippocampal neurons transfected with fluorescent bicistronic reporters after treatment with rapamycin, BDNF, or both. Scale bar, 50 μm. (**B**) Summary graph illustrating the fluorescence intensity of DsRed and GFP shown in (A). Intensities of samples were normalized with control samples. Data represent means ± SEM ($n = 7$ to 10 neurons per condition from three independent experiments, $^*P < 0.05$). n.s., not significant.

GluA1 5′UTR and found that these secondary structures gradually disappeared with serial deletions of the 5′UTR sequences. In addition, we predicted that certain RNA binding proteins may bind to specific secondary structures within the *GluA1* 5′UTR. To test this hypothesis, we generated serially deleted forms of the *GluA1* 5′UTR in bicistronic reporter constructs (Fig. 4A) and then introduced the reporters to Neuro2a cells. While there was a minor but significant reduction of IRES-mediated translation following introduction of the construct with 52 nucleotides deleted, introduction of the construct with 160 nucleotides truncated resulted in a marked decrease of IRES-mediated translation, similar to that induced by the control vector (Fig. 4B). Further, we constructed fluorescent serial deletion reporters and introduced them to primary hippocampal neurons, where they induced similar effects as those observed with the luciferase reporters described above. In dendrites and dendritic spines of DsRed-expressing neurons, no GFP signal was detected with the control vector (Fig. 4C), but GFP was visible in neurons expressing the reporter that included the full-length *GluA1* 5′UTR (Fig. 4D). However, this signal was markedly decreased when 160 nucleotides of the *GluA1* 5′UTR were deleted (Fig. 4E). These results suggest that the 160-nucleotide region at the 5′ end of the *GluA1* 5′UTR is critical for IRES-mediated translation.

hnRNP A2/B1 acts as a trans-acting factor of *GluA1* IRES–mediated translation

Several lines of evidence have demonstrated that numerous RNA binding proteins are localized at dendrites and dendritic spines of hippocampal neurons, and the levels of these proteins are regulated with synaptic stimulus (*24*). Moreover, it has been reported that expression of GluA1 is also increased in stimulation-dependent manner (*25*). Thus, we hypothesized that RNA binding proteins interacting with the *GluA1* 5′UTR may be a candidate trans-acting factor of *GluA1* IRES–mediated translation, resulting in increased GluA1 protein levels following neuronal stimulation. To validate binding factors of the *GluA1* 5′UTR, we conducted in vitro binding assays using a biotin-conjugated form of the *GluA1* 5′UTR with Neuro2a cell lysates. While the biotinylated *GluA1* 5′UTR alone could pull down several RNA binding proteins, this binding affinity was markedly reduced in combination with the nonbiotinylated competitor *GluA1* 5′UTR (Fig. 5A), suggesting that a specific interaction exists between the biotinylated *GluA1* 5′UTR and RNA binding proteins. Next, we extensively tested whether the RNA binding proteins that associated with the *GluA1* 5′UTR affected *GluA1* IRES–mediated translation. We found that knockdown of hnRNP A2/B1 significantly decreased IRES-mediated *GluA1* translation in

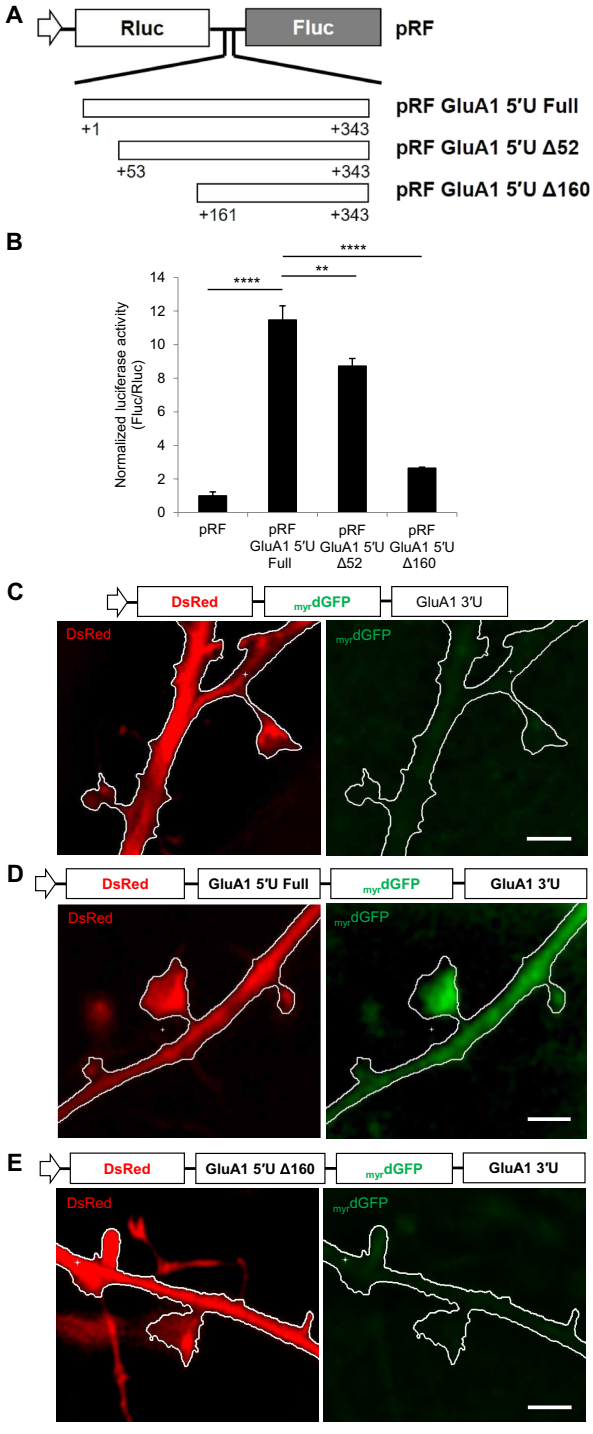

Fig. 4. Determination of the cis-acting element in IRES-mediated translation of *GluA1*. (**A**) Schematic diagram of the bicistronic reporter containing the serially deleted *GluA1* 5′UTR. (**B**) Neuro2a cells were transfected with serial deletion plasmids, and a luciferase assay was performed. The efficiency of cap-independent translation of each plasmid is shown. The ratio of the control pRF vector was set to 1. Data represent means ± SEM ($n = 5$, $^{**}P < 0.01$, $^{****}P < 0.0001$). (**C** to **E**) Representative images of hippocampal neurons transfected with control fluorescent bicistronic reporter (C), reporter containing the full-length *GluA1* 5′UTR (D), or reporter harboring a serially deleted *GluA1* 5′UTR (E). Images were visualized by super-resolution structured illumination microscopy. Dendrites and dendritic spines were outlined by white line. Scale bar, 1 μm.

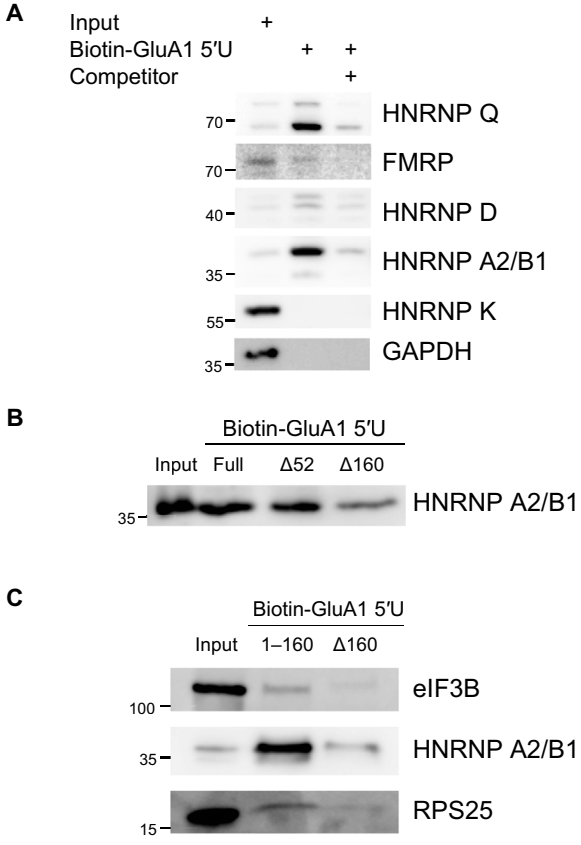

Fig. 5. HNRNP A2/B1 interacts with the *GluA1* 5′UTR. (**A**) Various RNA binding proteins that bound to the *GluA1* 5′UTR were determined using an in vitro binding assay. The biotin-conjugated *GluA1* 5′UTR was incubated with Neuro2a cytoplasmic cell extracts and subjected to immunoblotting using specific antibodies. A biotin-unconjugated *GluA1* 5′UTR was used as competitor. (**B**) Confirmation of the interaction between HNRNP A2/B1 and the *GluA1* 5′UTR. Biotin-conjugated full-length and serially deleted forms of the *GluA1* 5′UTR were incubated with the crude synaptosomal fraction from the mouse hippocampus and subjected to immunoblotting using hnRNP A2/B1 antibody. (**C**) Cis-acting region of *GluA1* 5′UTR preferentially interacts with eukaryotic initiation factor 3B, HNRNP A2/B1, and RPS25. Biotin-conjugated cis-acting region and truncated forms of *GluA1* 5′UTR were mixed with Neuro2a cytoplasmic cell lysates and followed by immunoblotting using specific antibodies.

Neuro2a cells (fig. S4, A and B) and levels of GluA1 protein without affecting *GluA1* mRNA levels in SHSY5Y cells (fig. S4, C and D), suggesting that HNRNP A2/B1 is one of strong candidate trans-acting factors in *GluA1* IRES–mediated translation. To confirm the specific relationship between HNRNP A2/B1 and *GluA1* mRNA, we tested whether hnRNP A2/B1 modulated expression of another glutamate receptor, NMDAR1. Our data showed that hnRNP A2/B1 knockdown did not affect NMDAR1 protein levels (fig. S4, C, E, and F). Moreover, we asked whether another hnRNP not associated with *GluA1* mRNA could regulate IRES-mediated translation of *GluA1*; our results demonstrated that hnRNP K knockdown did not affect IRES-mediated translation of *GluA1* mRNA in Neuro2a cells (fig. S4, G and H). These results suggest that hnRNP A2/B1 specifically regulates IRES-mediated translation of *GluA1* mRNA.

Notably, we also observed that the biotinylated serially deleted *GluA1* 5′UTR transcripts had reduced binding affinity with HNRNP

A2/B1 in mouse hippocampal crude synaptosomes (Fig. 5B). This result established a strong correlation with the data showing IRES-mediated translation of serially deleted *GluA1* 5′UTR luciferase reporters (Fig. 4B). To further confirm that the *GluA1* 5′UTR cis-acting region is sufficient to recruit HNRNP A2/B1, we performed in vitro binding assay with construct that has the cis-acting region of *GluA1* 5′UTR and 160 nucleotides truncated using Neuro2a cell lysates. The result shows that HNRNP A2/B1 preferentially binds to the cis-acting region of *GluA1* 5′UTR. This region also favorably interacts with eukaryotic initiation factor 3B and RPS25 (*26*), one of the 40*S* ribosomal proteins (Fig. 5C). Given that *GluA1* mRNA is localized in neuronal dendrites and dendritic spines (*23*, *27*, *28*), we used fluorescence in situ hybridization and immunocytochemistry to confirm the interaction and colocalization of *GluA1* mRNA and HNRNP A2/B1 in cultured hippocampal neurons. Although oligonucleotide probes for *GluA1* mRNA have been described (*28*), we constructed antisense and sense RNA probes that target cis-acting element in the *GluA1* 5′UTR; this allowed us to specifically verify the interaction between HNRNP A2/B1 and the *GluA1* 5′UTR. Our results illustrated that *GluA1* mRNA and HNRNP A2/B1 were colocalized at dendrites and dendritic spines of hippocampal neurons (fig. S5, A and B).

We speculated that if HNRNP A2/B1 were a bona fide trans-acting factor of BDNF-induced IRES-mediated *GluA1* translation, then HNRNP A2/B1 level would be increased with BDNF stimulation. To test this possibility, we evaluated protein levels of HNRNP A2/B1 and GluA1 in mouse hippocampal neurons after BDNF treatment and confirmed significant up-regulation of HNRNP A2/B1 and GluA1 by Western blot (fig. S6, A and B). We next validated the observed BDNF-induced up-regulation by immunocytochemistry following BDNF treatment in hippocampal neurons. Consistent with the Western blot data, the fluorescence intensities of HNRNP A2/B1 and GluA1 were increased under these conditions (fig. S6, C and D). These data suggest that HNRNP A2/B1 interacts with *GluA1* mRNA in neuronal dendrites and acts as a trans-acting factor of *GluA1* IRES–mediated translation.

HNRNP A2/B1 regulates BDNF-induced GluA1 expression and dendritic spine density

To confirm whether HNRNP A2/B1 modulates translation of *GluA1* mRNA in a BDNF stimulation–dependent manner, we transfected primary neurons with the short hairpin RNA (shRNA) targeting *Hnrnpa2b1* (sh_hnRNP A2/B1) (fig. S7, A and B) and performed Puro-PLA. As anticipated, control shRNA did not affect newly synthesized GluA1 (Fig. 6A), and BDNF increased the amount of newly translated GluA1 protein in somata and dendrites of untransfected neurons or those transfected with control shRNA (Fig. 6, C and E). However, we observed a significant decrease of newly synthesized GluA1 in HNRNP A2/B1–depleted neurons in a basal state (Fig. 6B). Moreover, newly translated GluA1 was increased in untransfected neurons upon BDNF stimulation, but not in neurons that were transfected with sh_HNRNP A2/B1 (Fig. 6, D and F), suggesting that BDNF-induced GluA1 de novo synthesis is regulated by HNRNP A2/B1.

Given that local translation of *GluA1* is regulated by HNRNP A2/B1, we asked whether HNRNP A2/B1 knockdown affects the total protein level of GluA1. To test this hypothesis, we transfected sh_HNRNP A2/B1 into hippocampal neurons. As previously confirmed, GluA1 protein levels were not changed in neurons transfected with a control shRNA under both vehicle- and BDNF-treated conditions (fig. S8, A, C, and E). GluA1 levels were reduced in HNRNP A2/B1–deficient neurons in a basal state (fig. S8B). In addition, BDNF stimulation up-regulated GluA1 levels in untransfected neurons, but not in HNRNP A2/B1–depleted neurons (fig. S8, D and F).

GluA1 is a subunit of the AMPA receptor, which is transported to synapses and eventually functions in postsynaptic sites. We showed that the knockdown of HNRNP A2/B1 affects local translation of *GluA1* and decreases GluA1 protein levels in an BDNF stimulation–dependent manner. In light of this evidence, we postulated that the cell surface expression of GluA1 would also be modulated by HNRNP A2/B1 in the presence of BDNF stimulation. To investigate this possibility, we directly assessed GluA1 surface expression following BDNF stimulation in hippocampal neurons. To detect surface GluA1, neurons were immunostained with antibodies that recognize extracellular epitopes of GluA1 in the absence of cell permeabilization. As previously described (*4*), BDNF stimulation efficiently increased surface GluA1 levels in control neurons (fig. S9, A, C, and E), but not in HNRNP A2/B1–depleted neurons (fig. S9, B, D, and F), suggesting that deficiency of HNRNP A2/B1 influences cell surface expression as well as total expression of GluA1 protein. Moreover, we further investigated whether HNRNP A2/B1 specifically regulates GluA1 surface expression by testing the surface level of GluA2. Our results showed that knockdown of HNRNP A2/B1 did not affect GluA2 surface expression (fig. S10, A and B), suggesting that HNRNP A2/B1 has a distinct role in regulating GluA1 expression.

Because mounting evidence has demonstrated that GluA1 surface expression is tightly correlated with the dendritic spines (*29*, *30*), we speculated that knockdown of HNRNP A2/B1 might affect dendritic spine density in hippocampal neurons. To test this possibility, we analyzed the density of dendritic spines after expressing sh_HNRNP A2/B1 in the neurons. In HNRNP A2/B1–reduced neurons, dendritic spine density was significantly decreased compared with control neurons (Fig. 6, G and H). These results suggest that HNRNP A2/B1–mediated translational control of GluA1 is crucial for normal dendritic spine density in hippocampal neurons.

DISCUSSION

Local protein synthesis in neuronal dendrites is regulated spatiotemporally in response to neuronal stimulation and provides a reservoir pool for the synaptic proteome. In the present study, we provide the first evidence suggesting that *GluA1* mRNA is translated via an IRES-dependent mechanism, at least, in part, regulated by RNA binding protein HNRNP A2/B1. Our study demonstrated that *GluA1* local protein synthesis in response to BDNF stimulation is mediated by HNRNP A2/B1 up-regulation. We used a recently described technique, Puro-PLA (*18*), to directly visualize newly synthesized GluA1 protein. Also, using bicistronic luciferase reporters, we found a substantial increase in *GluA1* IRES activity compared with control reporters. Consistently, our data showed clear expression of $_{myr}$dGFP in fluorescent bicistronic reporter experiments, confirming IRES-mediated translation of *GluA1* mRNA in hippocampal neurons; this effect was further validated through FRAP using the translation blocker anisomycin. The importance of HNRNP A2/B1 in modulating IRES-mediated translation of *GluA1* mRNA is corroborated by the data that total and surface expression of GluA1 were impaired in HNRNP A2/B1–deficient neurons after BDNF stimulation. Our data therefore suggest that BDNF stimulation–dependent local translation controlled

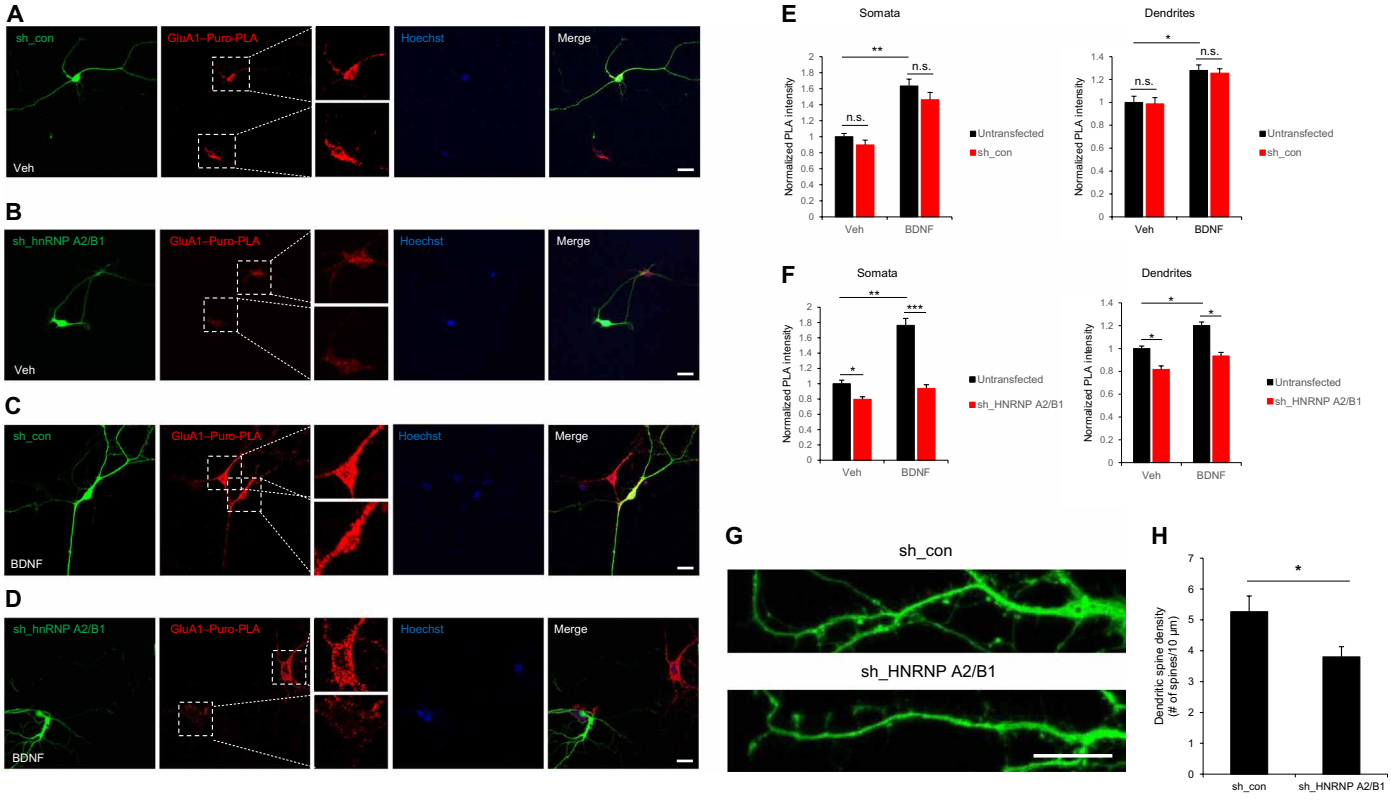

Fig. 6. HNRNP A2/B1 regulates BDNF-induced *GluA1* local translation. (**A** to **D**) Representative images of newly translated GluA1 after puromycin labeling in hippocampal neurons transfected with a control shRNA (A and C) or shRNA targeting *Hnrnpa2b1* (B and D). Neurons stimulated with BDNF are shown in (C) and (D). Insets of GluA1–Puro-PLA images are enlarged on the right. Scale bar, 20 μm. (**E** and **F**) Summary graphs showing PLA signal intensity in somata and dendrites of untransfected neurons or those expressing the control shRNA (E) or *Hnrnpa2b1* targeting shRNA (F). The fluorescence intensity of untransfected neurons without BDNF stimulation was set to 1. Data represent means ± SEM ($n = 11$ to 15 neurons per condition from three independent experiments, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$), n.s., not significant. (**G**) Representative images showing dendrites of control or HNRNP A2/B1–depleted neurons. Scale bar, 20 μm. (**H**) Summary graph of dendritic spine density obtained in neurons transfected with a control shRNA or *Hnrnpa2b1* targeting shRNA. Data represent means ± SEM ($n = 9$ to 15 neurons per condition from three independent experiments, $^*P < 0.05$).

by HNRNP A2/B1 is a previously unidentified regulatory mechanism for expression of the AMPA receptor subunit GluA1.

Previous reports implicate HNRNP A2/B1 in pre-mRNA splicing, mRNA trafficking, mRNA stability, and translational regulation (*31*). A recent study showed that HNRNP A2/B1 promotes IRES-mediated translation in lung cancer cell (*32*), suggesting an emerging role of HNRNP A2/B1 in translational activation. We therefore postulated that HNRNP A2/B1 interacts with translation initiation factors or ribosomal proteins to facilitate translation. Although its role in the central nervous system remains elusive, research suggests that HNRNP A2/B1 is implicated in several neurodegenerative diseases including fragile X–associated tremor/ataxia syndrome and amyotrophic lateral sclerosis (*33*, *34*). In this study, we suggest that HNRNP A2/B1 has a critical role in translational activation of the AMPA receptor subunit GluA1. In light of our findings, we propose a previously unknown role for HNRNP A2/B1 as a neuronal stimulation mediator in hippocampal neurons. Also, we found that the density of dendritic spine was reduced in HNRNP A2/B1–depleted neurons, but we could not exclude the possible mechanism of indirect action through alteration of global gene expression.

Consistent with previous studies (*24*), we showed that the expression of HNRNP A2/B1 was up-regulated by BDNF stimulation. Although it is not clear how BDNF enhances expression of HNRNP A2/B1 in hippocampal neurons, we can present several possible modes of action. First, BDNF may activate transcription of *hnrnpa2b1* mRNA. Interaction between BDNF and the receptor tyrosine kinase TrkB activates several intracellular signaling cascades including mitogen-activated protein kinase signaling and the phosphatidylinositol 3-kinase cascade (*35*). That is, BDNF-TrkB interaction may promote expression of HNRNP A2/B1 through activation of transcription factors. Second, BDNF stimulation may enhance accumulation of HNRNP A2/B1 in dendrites. BDNF regulates the motor protein KIF1A and KIF1A-mediated cargo transport (*36*), and HNRNP A2/B1 may be loaded in a cargo vesicle, which is transported by KIF1A. Third, it is possible that the *hnrnp2b1* transcript may be localized to dendrites, and its translation is augmented by BDNF stimulation.

GluA1 is a subunit of the AMPA receptor, which is trafficked to postsynaptic membranes and contributes to synaptic plasticity. Because we discovered that knockdown of HNRNP A2/B1 impairs surface expression of GluA1, it will be required to test whether the functionality of the AMPA receptor, including excitatory postsynaptic potential, is altered in HNRNP A2/B1–deficient neurons using electrophysiological experiments. Based on the known critical roles of AMPA receptor in synaptic plasticity, alteration of surface GluA1 levels may affect not only synaptic plasticity but also learning and

memory as a consequence. It will therefore be interesting to determine whether HNRNP A2/B1–mediated translational control of *GluA1* in vivo is also critical.

The AMPA receptor subunit GluA1 is essential for synaptic plasticity; thus, its expression should be tightly regulated in a spatiotemporal manner. Previous evidence showed that BDNF stimulation up-regulates expression of GluA1 (*4*); however, little is known about the underlying molecular mechanisms of translational regulation. Our study uncovers a previously unknown translational mechanism for *GluA1* in response to BDNF stimulation. Similar to other dendritic mRNAs (*16*), *GluA1* also uses IRES-mediated translation so that it is properly produced in the right context. There are currently different views about whether GluA1 subunit is trafficked from soma as a tetramer or locally synthesized. We suggest that this translational mechanism fits in both because we showed that BDNF treatment up-regulated HNRNP A2/B1, the critical factor for GluA1 translation, throughout the neuron. IRES-mediated translation is appropriate for a tightly regulated transcript because it requires specific trans-acting factors that can recruit translational machinery according to the environments of the cell to induce translational initiation. Thus, this example of IRES-mediated local translation of *GluA1* provides a previously unrevealed insight for translational control of dendritic mRNAs with spatiotemporal precision.

MATERIALS AND METHODS

DNA constructs

The mouse *GluA1* 5′UTR (NM_001113325.1) was amplified by polymerase chain reaction (PCR) from mouse complementary DNA (cDNA) using specific primers. To generate pRF-*GluA1* 5′UTR plasmids, amplified 5′UTRs were inserted into Sal 1/Sma 1 sites of the intercistronic region of a pRF bicistronic vector containing Rluc in the first cistron and Fluc in the second cistron (*15*). The PCR products were digested with Sma 1/Sal 1 and inserted into the pRF vector to create pRF-*GluA1* 5′UTR reverse constructs. Secondary structure in silico prediction of serially deleted sequences of the *GluA1* 5′UTR was performed by providing each sequence to RNA folding form applications in "The mfold Web Server" (http://mfold.rna.albany.edu). From many candidate structures, we acquired each structure with the lowest thermodynamic energy. To generate serial deletion constructs, 5′UTRs amplified using specific primers were inserted into Sal 1/Sma 1 sites of the pRF vector. The pHRF and ΔCMV vectors were constructed as previously described (*15*). For in vitro binding assay, the full-length or serially deleted *GluA1* 5′UTR was cloned into Eco R1/Xba 1 sites of pBluescript SK(+) (pSK). The *GluA1* 5′UTR was amplified and cloned into Sal 1/Bma H1 sites of pCY2-RF vectors, and the Rluc cistron was removed by digestion with Nhe 1/Xba 1 and then self-ligated to construct pCY2-*GluA1* 5′UTR-Fluc. For the DsRed-*GluA1* 5′UTR-$_{myr}$dGFP vector, DsRed-Express was cloned into Nhe 1/Hind 3 sites, the *GluA1* 5′UTR was cloned into Hind 3/Bam H1 sites, $_{myr}$dGFP was cloned into Bam H1/Not 1 sites, and the *GluA1* 3′UTR was cloned into Not 1 sites of pEGFP-N1 (Clontech). pcDNA3.1-5′$_{myr}$dGFP3′ was a gift from E. Schuman (Max Planck). Specific primer pair sequences are shown in table S1.

Cell culture

Neuro2a, SHSY5Y, HEK-293A, and HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (HyClone) containing 10% fetal bovine serum (FBS) (HyClone), penicillin (100 U/ml), and 100 μg of streptomycin (Welgene) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. For primary hippocampal neuron culture, we dissected hippocampi from mouse embryos (E17), dissociated them with trypsin (Sigma-Aldrich) and deoxyribonuclease I (Sigma-Aldrich) using a fire-polished Pasteur pipette, and plated them onto poly-L-lysine (Sigma-Aldrich)–coated dishes. Neuron cultures were maintained in Neurobasal medium (Gibco) supplemented with B27 (Gibco) and GlutaMAX-I (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C. Half of the media in each dish was exchanged with fresh culture media every 3 days.

Transfection

Plasmid or shRNA transfection for transient expression or knockdown was performed using the Neon Transfection system (Invitrogen) according to the manufacturer's recommendations. Cells were harvested 24 hours after transfection for subsequent experiments or otherwise stored at −70°C. Transient transfections of primary neuron cultures were performed using calcium phosphate or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Neurons were harvested for further experiments or fixed for immunocytochemistry.

Subcellular fractionation and synaptosome preparation

For cytoplasmic extracts from cell lines, cells were resuspended with buffer A [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride]. After 20-min incubation on ice, buffer B (2.5% NP-40 in buffer A) was added, and cells were resuspended and incubated for 10 min. Cells were centrifuged at 1000*g* at 4°C for 5 min, and supernatants were subjected to an in vitro binding assay. Mouse hippocampal synaptosome preparation was performed as previously described (*37*). Briefly, hippocampi were removed and dissected into ice-cold homogenization buffer (0.32 M sucrose, 4 mM Hepes-NaOH, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$; pH 7.3 with inhibitors of protease and phosphatase) and homogenized using a glass homogenizer. The homogenates were centrifuged at 1000*g* at 4°C for 15 min to remove the nuclear fraction. The supernatants were centrifuged at 12,000*g* for 15 min to yield the pelleted crude synaptosome.

In vitro transcription, in vitro binding assay, and immunoprecipitation

pSK vectors containing *GluA1* 5′UTR sequences were linearized with Xba 1 and in vitro transcribed in the presence of biotin–uridine 5′-triphosphate (UTP) using T7 RNA polymerase (Promega). The cytoplasmic fraction of Neuro2a cells or mouse hippocampal crude synaptosome lysates were incubated with or without in vitro transcribed biotin-labeled mRNA and subjected to incubation with streptavidin-agarose beads (Pierce). For competitor experiments, fivefold molar excess of non–biotin-UTP–labeled RNAs was added. Bead-bound proteins were pulled down and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting. For mRNA transfection, pCY2 vectors were linearized with Eco R1 and in vitro transcribed using SP6 RNA polymerase (Promega) with either the Ribo m^7G Cap Analog (Promega) or ApppG Cap Structure Analog (NEB). For RNA quality validation, all in vitro transcribed RNAs were subjected to agarose gel electrophoresis. For immunoprecipitation, cells were lysed with IP lysis buffer and lysates were incubated with an anti-Flag antibody. After overnight

incubation, protein G agarose beads (Thermo Fisher Scientific) were added to samples and further incubated for 4 hours. Washed beads were analyzed by SDS-PAGE, and specific antibodies were used for immunoblotting.

Luciferase assay

Cells were transfected with pRF vectors and harvested 24 hours after transfection. Cells were lysed with reporter lysis buffer, and *Renilla* and firefly luciferase activities were analyzed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. For monocistronic luciferase assay, cells were transfected with mRNAs and harvested 12 hours later. Half of the cells were used for the luciferase assay, and the rest were subjected to quantitative PCR (qPCR) for normalization of RNA transfection efficiency.

RNA interference

The shRNA sequences of *Hnrnpa2b1* were used as previously described (*38*). These oligonucleotides were annealed and inserted into Hpa 1/Xho 1 sites of the pLentiLox3.7 lentiviral vector. To knock down *HNRNPA2B1* in human neuroblastoma cell lines, shRNAs were used as previously described (*39*). Control shRNA and shRNA targeting *HNRNPA2B1* were a gift from A. Grimson (Cornell University). Small interfering RNA (siRNA) against endogenous *Hnrnpk* was purchased from Dharmacon (siGENOME SMARTpool HNRPK M-048992091).

Puro-PLA

The Puro-PLA procedure was performed as previously described (*18*). Briefly, after puromycylation with 3 μM puromycin for 15 min, neurons were fixed with 4% paraformaldehyde (PFA)–sucrose for 20 min at room temperature (RT). After fixation, cells were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 15 min, blocked in 10% FBS in PBS for 30 min, and incubated with primary antibody pairs diluted in blocking buffer for 1.5 hours at RT. PLA probes (Duolink In Situ PLA Probes, Sigma-Aldrich) were used according to the manufacturer's instructions. Ligation and amplification reactions were performed according to the manufacturer's recommendations (Duolink In Situ Detection Reagents Red, Sigma-Aldrich). After amplifications, cells were postfixed in 4% PFA-sucrose for 10 min at RT for better signal stability, processed further for immunocytochemistry with Hoechst staining for nuclei visualization, and mounted with fluorescent mounting medium (Dako).

Immunocytochemistry

After shRNA transfection, mouse hippocampal primary cultured neurons were fixed in 4% PFA in PBS for 20 min at RT. After PBS washing, neurons were permeabilized with 0.2% Triton X-100 for 10 min at RT, blocked with 10% FBS in PBS for 1 hour, and incubated with primary antibodies overnight at 4°C. For surface proteins, permeabilization step was omitted. Next, neurons were washed with PBS for 30 min, and Alexa Fluor–conjugated secondary antibodies were added for 1 hour at RT. After washing with PBS for 20 min, cells were stained with Hoechst for nuclei visualization for 10 min. Cells were then mounted with fluorescent mounting medium (Dako). Image acquisition was performed using a Nikon A1R$^+$ confocal microscope (Nikon) or a Leica TCS SP5 confocal microscope (Leica).

Fluorescence in situ hybridization

Digoxigenin (DIG) was used for DIG-labeled RNA probes. Fluorescence in situ hybridization using tyramide signal amplification was performed to detect *GluA1* mRNA. Mouse hippocampal neurons cultured on a chip were washed in 1× PBS and fixed in 4% formaldehyde for 10 min at RT. Cells were permeabilized with 0.1% Triton X-100 for 15 min at RT. After 1× PBS washing, cells were rehydrated for 5 min at RT, in 2× saline sodium citrate and 50% formamide, and hybridized for 2 hours at 37°C with the DIG-RNA probe denatured in 80°C for 5 min. Cells were washed for 1 hour in 0.1× saline sodium citrate, 50% formamide, and 0.1% NP-40 at 50°C. Cells were blocked with 10% FBS in TN buffer (0.1 M tris and 0.15 M NaCl; pH 7.5) and incubated with anti–hnRNP A2/B1 and anti–PSD-95, and a horseradish peroxidase (HRP)–conjugated anti-DIG antibody (Roche), at 4°C, overnight. After three washes for 10 min in TNT buffer (0.1 M tris, 0.15 M NaCl, and 0.05% Tween 20; pH 7.5), cells were incubated in Cy3-conjugated tyramide signal amplification plus working solution (PerkinElmer) for 5 min at RT. Cells were incubated with Alexa Fluor 488– or Alexa Fluor 647–conjugated secondary antibodies followed by three washes for 10 min each. Cells were then stained with Hoechst for nuclei visualization and mounted with fluorescent mounting medium (Dako).

Image analysis

For super-resolution image, we used super-resolution structured illumination microscopy (SIM; Nikon N-SIM). The raw images were reconstructed to three-dimensional SIM images using NIS-Elements software (Nikon). Images were acquired using 100× oil objective lens [numerical aperture (NA), 1.49] and equipped with an iXon DU-897 EMCCD camera (Andor Technology). Multicolor fluorescence was acquired using a diode laser (488 nm, 561 nm). For colocalization percentage analysis, acquired whole images were analyzed using Fiji (https://imagej.net/Fiji). For FRAP analysis, we used a Nikon A1R$^+$ inverted confocal microscope with 60× oil objective lens (NA, 1.49). A 3 μm × 3 μm photobleaching spot was chosen at a dendrite. Cells were maintained in a humidified, 5% CO_2 environmental chamber at 37°C (Live Cell Instrument), and images were acquired every 20 s for 10 min using NIS-Elements. Maximum intensity projection images were used, and each fluorescence intensities before bleaching were set to 100%. For dendritic spine density analysis, spines were defined on the basis of morphology from GFP volume filling. To be considered a spine, the compartment must be a clearly defined protrusion from the dendrite, extending at least 1 μm away from the dendritic shaft. Acquired images were analyzed using ImageJ [National Institutes of Health (NIH), https://imagej.nih.gov/ij/].

Western blot

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer, and protein samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Pall Corporation), and immunoblotted. Blocking was performed with 5% nonfat dry milk in tris-buffered saline and 0.1% Tween 20 (TBST) for 0.5 to 1 hour. Membranes were incubated with primary antibodies in blocking solution overnight at 4°C. After several washes with TBST, the membranes were incubated with secondary HRP-conjugated mouse (Thermo Fisher Scientific) or rabbit (Promega) secondary antibodies for 1 to 2 hours and visualized with SUPEX ECL reagent (Neuronex) and a LAS-4000 system (FUJIFILM), according to the manufacturer's instructions.

Acquired images were further analyzed with the Image Gauge program (FUJIFILM) or ImageJ (NIH, https://imagej.nih.gov/ij/).

RNA isolation and qPCR

Total RNA was extracted from cells using TRI reagent (Molecular Research Center) according to the manufacturer's instructions. Isolated RNAs were reverse-transcribed with the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's recommendations. RNA levels were detected by qPCR using the StepOnePlus Real-Time System (Applied Biosystems) with FastStart Universal SYBR Green Master (Roche) according to the manufacturer's instructions. Specific primer pairs were used to detect and quantify RNA levels. The sequences of the forward and reverse primers are as follows: *GluA1* (mouse), 5′-ACCACTACATCCTCGCCAAC-3′ and 5′-TCACTTGTCCTCCACTGCTG-3′; *Gapdh* (mouse), 5′-GCCATCAACGACCCCTTCATT-3′ and 5′-GCTCCTGGAAGATGGTGATGG-3′; *GLUA1* (human), 5′-TGTGACACCATGAAGGTGGG-3′ and 5′-AGCGCTTGTCTTGTCCTTGG-3′; *ACTB* (human), 5′-AGAGCTACGAGCTGCCTGAC-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′.

Antibodies and drug treatment

Antibodies used in this study are as follows: anti-GluA1, anti–hnRNP A2/B1, anti–PSD-95 (Abcam), anti–phospho-RPS6, anti–phospho-ERK (extracellular signal–regulated kinase), anti-FMRP (Cell Signaling), anti–N-term-GluA1, anti–N-term-GluA2, anti–hnRNP D (Millipore), anti-MAP2, anti–hnRNP A1, anti–14-3-3ζ (Santa Cruz), anti–hnRNP Q, anti-Flag (Sigma-Aldrich), anti-NMDAR1 (Synaptic Systems), and anti-actin (MPBIO). To inhibit translation, SHSY5Y cells were treated with 10 nM RAD001 or cycloheximide (100 μg/ml) and harvested at the indicated times. To block cap-dependent translation or induce synaptic stimulation, neurons were treated with 20 nM rapamycin (Sigma-Aldrich) or recombinant BDNF (100 ng/ml) (PeproTech), respectively.

Statistical analysis

All quantitative data are presented as means ± SEM. Comparisons between two groups were analyzed using two-tailed unpaired Student's *t* tests. For comparisons between more than two groups, a one-way analysis of variance was used with a Tukey's test. All microscopy experiments were repeated at least three times with different biological samples, and at least 10 somata and dendrites were analyzed per condition, per experiment. Signal intensities were quantified using ImageJ. A P value less than 0.05 was considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/47/eabd2163/DC1

View/request a protocol for this paper from *Bio-protocol*.

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BDNF-induced local translation of *GluA1* is regulated by HNRNP A2/B1

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