

Thrombopoietin receptor agonist eltrombopag alleviates cognitive impairment via NRG1/ErbB4 signaling

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Abstract

Background

Patients with minimal hepatic encephalopathy (MHE) show mild cognitive impairments. Thrombopoietin (TPO) has been shown to be neuroprotective. This study aimed to explore the therapeutic effect of Thrombopoietin receptor agonist eltrombopag (ELT) on MHE and the involvement of NRG1 signaling using primary rat neurons and a MHE rat model.

Methods

We explored the effects of ELT stimulation on NRG1/ErbB4 signaling and synapse formation in the primary rat neurons. Furthermore, we explored the cerebral TPO expression level and the effect of TPO replacement therapy in an MHE rat model.

Results

The results showed that ELT stimulation activated NRG1/ErbB4 signaling and enhanced synaptic protein expression in the primary rat neurons via sirtuin 1. An anti-NRG1 antibody, ErbB4 inhibitor, or knockdown of NRG1 or ErbB4 could significantly abolish ELT-induced upregulation of synaptic protein expression in the primary rat neurons. MHE rats had significantly decreased cerebral ELT expression compared with normal rats. ELT activated NRG1/ErbB4 signaling in MHE rat brains. Administration or overexpression of ELT or TPO promoted synapse formation and alleviated cognitive impairments in MHE rats.

Conclusions

These data suggest that ELT promotes synapse formation *in vitro* and *in vivo* via activating NRG1/ErbB4 signaling, serving as a promising therapeutic agent for MHE treatment.

Introduction

Minimal hepatic encephalopathy (MHE) is the earliest form of hepatic encephalopathy (HE), affecting up to 80% of patients with liver cirrhosis ¹. MHE is characterized by cognitive function impairments that negatively affect daily activities, quality of life, and overall survival of patients ². Currently, treatment for MHE has been focused on reducing serum ammonia levels; however, no treatment has been recommended as a clinical routine, apart from on a case-by-case basis ³. Therefore, it is necessary to develop a promising therapeutic agent for MHE treatment.

Thrombopoietin (TPO), a stimulator of megakaryocytic/platelet lineage, acts in the brain as a counterpart of erythropoietin (EPO), a hematopoietic growth factor with neuroprotective properties. TPO and its

receptor c-Mpl are expressed in the neurons of the human central nervous system (CNS) and murine neural cells. TPO is prominent in human cerebrospinal fluid 4. TPO was found to be neuroprotective in the CNS in hypoxic-ischemic neonatal rat brain models. TPO reduced brain damage and improved sensorimotor functions. In addition, TPO had a stimulating effect on neural cell proliferation and exerted an antiapoptotic effect 5 6. TPO improved neurological function and ameliorated brain edema after stroke 7. In the developing human CNS, the thrombopoietin gene is abundantly expressed. Considering that thrombopoietin contains a neurotrophic sequence, it may well play a role in neuronal cell biology 8. Thus, impaired TPO signaling is critical for the development of cognitive deterioration in the brain, suggesting that TPO replacement therapy might prevent cognitive disturbance in MHE. However, the expression pattern of cerebral TPO and the effect of ELT on cognitive function in MHE remain largely unknown.

Neuregulin1 (NRG1) is a transmembrane protein that belongs to the epithelial growth factor family. NRG-1 is highly expressed in the developing brain and in the adult nervous system, playing critical roles in regulating synaptic plasticity, neuroregeneration, and homeostasis of brain functions by interacting with tyrosine kinase receptors of the ErbB family, including erb-b2 receptor tyrosine kinase 4 (ErbB4) 9. Accumulating evidence have suggested that NRG1 prevents brain injury after stroke and protects neurons in Parkinson's disease and Alzheimer's disease 10–12. However, the relationship between TPO/MPL signaling and NRG1/ErbB signaling in the CNS remains unknown.

Eltrombopag (ELT) is an orally active thrombopoietin receptor agonist 13. In this study, we hypothesized that ELT might regulate synapse formation in MHE through the NRG1/ErbB4 pathway. To test our hypothesis, we explored the effects of ELT stimulation on NRG1/ErbB4 signaling and synapse formation in the primary rat neurons. We employed the loss-of-function assay to investigate whether NRG1/ErbB4 signaling mediates the regulation of ELT in synapse formation in rat neurons. Furthermore, we explored the cerebral TPO expression level and the effect of ELT replacement therapy in an MHE rat model. Our results suggest that ELT treatment promotes synapse formation and alleviates cognitive declines in MHE rats by activating NRG1/ErbB4 signaling.

Materials And Methods

Animals and MHE model

Sprague-Dawley rats (male, 220–250g) were purchased from the experimental animal center of the Chinese Academy of Sciences in Shanghai. This study was approved by Institutional Animal Care and Use Committee of Wenzhou Medical University (Wenzhou, Zhejiang, China). All animal experiments were conducted following the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and Institutional Animal Care and Use Committee of Wenzhou Medical University.

To induce liver cirrhosis, the rats (n = 50) were injected intraperitoneally with thioacetamid (TAA; 200 mg/kg in normal saline; Sigma-aldrich, St. Louis, MO, USA) twice per week for 8 weeks. The rats exhibited

delayed motor activity, lethargy, and subsequent coma were categorized into the HE group (n = 4) 14. The rats without HE symptoms (n = 46) were subjected to water-finding and Y-maze tests to confirm MHE.

Water-finding and Y-maze tests

Water-finding and Y-maze tests were performed before and after drug administration. For water-finding test, the rat was placed at the near-right corner of the apparatus and allowed to find and drink the water in the alcove within 3 min. The entry latency (the time take to enter into the alcove), contacting latency (the time between entering into the alcove and the first touching/sniffing/licking the water tube), and drinking latency (the time between entering into the alcove and drinking the water) were measured 15.

For Y-maze test, the rat was placed at the end of one arm in a three-arm apparatus to explore the maze freely for 8 min. The percentage of spontaneous alternation, defined as the arm choices/total choices ratio, was measured 16.

Before drug treatment, the TAA-treated rats with water-finding test score > the mean $\pm 1.96 \cdot SD$ or Y-maze test value > the mean $\pm 1.96 \cdot SD$ were categorized into the MHE group (n =4 4).

Animal treatment

For Thrombopoietin receptor agonist eltrombopag (ELT, Selleck Chemicals #S2229) administration, MHE rats were conducted oral administration (0,5,25mg/kg). For TPO or MPL overexpression, 2 μ L TPO- or MPL-overexpressing plasmids or control plasmids pCMV-Tag2A (0.5 μ g; Santa Cruz, CA, USA) was injected into the right lateral ventricles of MHE rats at a rate of 0.1 μ L/min. After water-finding and Y-maze tests, rats were anaesthetized with isoflurane and decapitated. The hippocampus and cerebral cortex were collected immediately and stored at -80 °C until use.

Cell culture and treatment

Primary rat hippocampal neurons (PHNs) were derived from the hippocampus and cerebral cortex, respectively, from 1-day-old Sprague-Dawley rat pups via trypsin and DNase digestion. PHNs were seeded in poly-L-lysine-precoated six-well plates at a density of 2×10^6 cells/well and cultured in Neurobasal® medium supplemented with 0.5 mM GlutaMAX™-I, B-27® 17. For dose-response analysis, PHNs and PC12 cells were treated with Thrombopoietin receptor agonist eltrombopag (ELT, Selleck Chemicals #S2229) (0, 6.25, 12.5, 25 μ M), Recombinant human Thrombopoietin protein (ab217448) (0, 50, 100, 200 ng/mL) or NRG1 (abcam, ab242321) (0, 1, 5, 20 ng/mL) for 24 h. For time-response analysis, the cells were treated with 12.5 μ M ELT or NRG1 for 0, 6, 12, 24, 48, or 72 h. To explore the effects of the antibody and inhibitors, the cells were pretreated with 10 μ g/mL anti-NRG1 mouse monoclonal antibody or mouse IgG, 50 μ mol/L ErbB4 inhibitor AG1478, or 10 μ mol/L sirtuin 1 (Sirt1) inhibitor sirtinol (Origene, Rockville, MD, USA) for 24 h, followed by ELT or NRG1 treatment for an additional 24 h. In the loss-of-function assay, cells were transfected with 0.25 μ g small interfering RNA (siRNA) against NRG1, siRNA against ErbB4, or scrambled siRNA (Santa Cruz, CA, USA), followed by ELT (12.5 μ M) treatment for an additional 24 h.

Semi-quantitative PCR and real-time quantitative PCR (qRT-PCR)

For semi-quantitative PCR, cDNA was synthesized from the total RNA using omniscript reverse transcriptase (Qiagen, Hilden, Germany), followed by PCR amplification using Taq DNA polymerase (Sigma-Aldrich).

For qRT-PCR, the cDNA was obtained using an iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) from the mRNA collected from tissue samples. RT-PCR was performed using an iQ™ SYBR®Green Supermix (Bio-Rad) on a CFX96 Touch system (Bio-Rad) according to the manufacturer's instruction. GAPDH was used as an internal reference. The primers (Invitrogen, Carlsbad, CA, USA) were as follows: NRG1, 5'AATGGACAGCAACACAAG3' (forward) and 5'TTAGCGATTACACTAGACAG3' (reverse); TPO, 5'GAAGAGCGACCCTCACATCAAG3' (forward) and 5'CTGCCAGTTCGTTTCAGTG3' (reverse); GAPDH, 5'TGTCATCAACGGGAAGCCCA3' (forward) and 5'TTGTTCATGGATGACCTTGGC3' (reverse).

Measurement of ammonia levels

A total of 300–800 mL 0.4 M HClO₄ containing 0.1 % (w/v) Na₂S₂O₅ was added to the liver/serum/hippocampus samples. The mixture was homogenized by sonication (Labsonic-U; Braun, Kronberg, Germany), followed by centrifugation at 200,009 *g* for 15 min at 4 °C. The aliquots of the supernatants were taken for the analysis of ammonia using high-performance liquid chromatography with electrochemical detection using modifications in the mobile phase.

Measurement of NRG1 secretion

The NRG1 levels in the culture medium of primary neurons were measured using high sensitivity sandwich enzyme-linked immunosorbent assay (ELISA) kits (ab213902, abcam) according to the manufacturer's instructions. The NRG1 levels were determined using a Multiskan MCC plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Western blot analysis and co-immunoprecipitation (Co-IP) assay

The total proteins was obtained from homogenized tissue samples or cells and determined using a Bradford quantification assay (Bio-Rad). The protein samples were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% (w/v) non-fat dry milk in phosphate-buffered saline (PBS), the membrane was incubated overnight at 4 °C with primary antibody against ErbB4, phosphorylated ErbB4 (pErbB4), ErbB2, pErbB2, NRG1, TPO, Sirt1, syntaxin, Homer, or β-actin (Abcam, Cambridge, UK), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Pierce, Thermo Fisher Scientific) for 1 h at room temperature. The blots were developed using ECL reagent (Amersham, Arlington Heights, IL, USA) and exposed to a Kodak Biomax film.

For Co-IP assay, the lysates of tissue samples or cells were incubated with above mentioned antibodies overnight at 4°C, followed by incubation with protein G-agarose beads (Millipore) for an additional 5h at 4

°C. The beads were washed with lysis buffer. The eluent was separated by SDS-PAGE and transferred to a PVDF membrane to probe proteins using primary and secondary antibodies mentioned above.

Functional labeling of presynaptic boutons with FM4-64

The primary neurons were incubated with 5 mg/mL FM4-64 (Invitrogen) and 50 mM KCl in Hanks' balanced salt solution for 1 min at 4°C. After reaction, free FM4-64 was removed by washing with Hanks' balanced salt solution. Images were acquired using Leica TCS SP8.

Immunofluorescence staining

Brain tissue sections or glass coverslips were fixed with 4 % paraformaldehyde for 30 min and then incubated with 0.1% Triton X-100 for 10 min at room temperature. After blocking with PBS containing 5% normal goat serum for 1 h at room temperature, the sections or coverslips were incubated with the primary antibody against ErbB4, NRG1, TPO, Sirt1, syntaxin, Homer, or MAP2 (Abcam) overnight at 4 °C, followed by incubation with Alexa Fluor 488 (green)- or Alexa Fluor 594 (red)-conjugated secondary antibody (Abcam) for 1 h.

For dendritic spine analysis, the primary neuron coverslips were incubated with the primary antibody against microtubule-associated protein 2B (MAP2B; 1:200; BD Transduction Laboratories, San Jose, CA, USA) or vesicular glutamate transporter 1 (vGlut1; 1:100; Neuromab, Davis, CA, USA) overnight at 4 °C, followed by incubation with Alexa Fluor 488 (green)- or Alexa Fluor 594 (red)-conjugated secondary antibody (Abcam) for 1 h. At least 10 primary neurons per coverslip were used for quantitative analysis.

Electrophysiological Analysis

Rats were anaesthetized with isoflurane and decapitated. The hippocampus was cut into 400-µm thick transverse slices using a vibratome. After an incubation in artificial cerebrospinal fluid (a-CSF) at room temperature for 60–90 min, the slices were placed in a recording chamber on the stage of an upright microscope (Olympus CX-31) and perfused with a-CSF (containing 1 mM MgCl₂) at a rate of 3 mL per min at 23–24°C. A 0.1 MΩ tungsten monopolar electrode was used to stimulate the Schaffer collaterals. The field excitatory postsynaptic potentials (fEPSPs) were recorded in CA1 stratum radiatum using a glass microelectrode filled with a-CSF with resistance of 3–4 MΩ. The field potential input-output curves were generated by measuring fEPSP slope responding to the stimulus intensity increased from 1 to 7 V, with an 0.5 V increment. The long-term potentiation (LTP) of fEPSPs was induced by 3 theta-burst stimulation (4 pulses at 100 Hz) with a 200-ms interval. The paired-pulse facilitation was examined by applying pairs of pulses, which were separated by 20–500 ms intervals. The magnitudes of LTP are expressed as the mean percentage of baseline fEPSP initial slope.

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analysis was performed using SPSS 18.0 (IBM, Armonk, NY, USA). Differences between groups were compared using one-way analysis of variance,

followed by Dunnett's post hoc multiple comparison test. The *P* values were adjusted using Bonferroni correction. A *P* value < 0.05 was considered statistically significant.

Results

ELT activates NRG1/ErbB4 signaling in rat neurons.

To investigate whether ELT protects the neurons through NRG1/ErbB4 signaling, we determined NRG1 and pErbB4 expression in primary rat neurons in response to ELT stimulation. Western blot analysis showed that compared with vehicle treatment, 12.5 μM ELT induced significant and maximum elevations in NRG1 and pErbB4 protein levels in both PHNs and PC12 cells (Fig. 1a and 1b). In addition, ELT treatment enhanced NRG1 protein expression and ErbB4 phosphorylation in both cell types in a time-dependent manner, peaking at 48 h after treatment (Fig. 1c and 1d). Immunofluorescence staining confirmed the upregulation of NRG1 expression and ErbB4 phosphorylation in PHNs in response to ELT (Fig. 1e). Consistent results were observed in NRG1 mRNA expression (Fig. 1f) and NRG1 secretion (Fig. 1g and h) in both cell types, except that the elevation of NRG1 mRNA level peaked at 180 min after ELT stimulation (Fig. 1f). Moreover, ELT treatment did not change the expression of NRG2, NRG3, or pErbB2 in PHNs (Supplementary Fig. S1 and S2), suggesting that ELT specifically activates NRG1/ErbB4 signaling at least in PHNs.

We further examined the effect of ELT on MPL/ErbB4 interaction. Co-IP revealed that 12.5 μM ELT significantly promoted MPL binding to ErbB4 in PHNs compared with vehicle treatment (Fig. 2). Taken together, these results indicated that ELT activates the interaction between MPL and ErbB4 in primary rat neurons.

ELT promotes synapse formation in rat neurons via NRG1/ErbB4 signaling.

Considering that both ELT and NRG1 are involved in synaptic plasticity and that ELT activates NRG1/ErbB4 signaling in primary rat neurons, we sought to investigate whether NRG1 mediates the regulation of ELT in synaptic plasticity. As shown in Fig. 3a and 3b, in PHNs, compared with vehicle treatment, ELT treatment remarkably enhanced the protein expressions of presynaptic marker syntaxin and postsynaptic marker Homer 18, whereas an anti-NRG1 antibody or ErbB4 inhibitor AG1478 effectively abolished ELT-induced syntaxin and Homer upregulation. Immunofluorescence staining confirmed that an anti-NRG1 antibody or AG1478 abolished ELT-induced syntaxin upregulation in PHNs (Fig. 3c). Furthermore, FM4-64 staining revealed that ELT treatment resulted in a significant increase in recycling synaptic vesicles in PHNs, whereas an anti-NRG1 antibody or AG1478 completely reversed the effect of ELT on recycling synaptic vesicles (Fig. 3d and 3e).

Then, we knocked down NRG1 or ErbB4 expression to further investigate the role of NRG1/ErbB4 signaling in the regulation of ELT in synapse formation. The siRNA-mediated NRG1 or ErbB4 knockdown efficiency was shown in Supplementary Fig. S3. We found that knockdown of NRG1 or ErbB4 significantly abolished ELT-induced upregulation of syntaxin and Homer protein expression in PHNs

(Fig. 3f and 3g). Then, we performed immunofluorescence staining for MAP2B and vGluT1 expression to examine the dendritic spine density. As shown in Fig. 3h and 3i, compared with negative control, knockdown of NRG1 or ErbB4 completely reversed ELT-induced increase in vGluT1-positive puncta in PHNs. Taken together, these data indicate that ELT promotes synapse formation in rat neurons via NRG1/ErbB4 signaling.

Sirt1 mediates TPO/NGR1-induced synapse formation in rat neurons.

It has been reported that Sirt1 is essential for cognitive function and synapse formation ¹⁹. Thus, we hypothesized that TPO/NGR1 might elicit synapse formation via Sirt1. Indeed, 12.5 μ M ELT markedly upregulated Sirt1 protein expression at 48 h after treatment in PHNs and PC12 cells (Fig. 4a–d). Immunofluorescence staining showed consistent results (Fig. 4e).

Similarly, 100 ng/mL TPO treatment also significantly enhanced Sirt1 protein expression at 48 h after treatment in PHNs (Fig. 4f–i). 5 ng/mL NRG1 treatment also significantly enhanced Sirt1 protein expression in PHNs (Fig. 4j–k). Moreover, ErbB4 inhibitor AG1478 effectively abolished ELT-induced upregulation of Sirt1/2 expression in PHNs, suggesting that Sirt1 is a downstream effector of TPO depended on NRG1/ErbB4 signaling in rat neurons (Fig. 4l–m). Furthermore, Sirt1 inhibitor sirtinol effectively abolished ELT-induced upregulation of syntaxin and Homer expression in PHNs (Fig. 4n–o). These results suggest that ELT/NGR1 promotes synapse formation via Sirt1 in rat neurons.

TPO is downregulated in MHE rat brains.

TPO contributes to synaptogenesis and neuroprotection ²⁰. We found that MHE rats had significantly reduced TPO protein levels in the hippocampus and cerebral cortex tissue samples compared with control rats (Fig. 5a and 5b). This finding was confirmed by semi-quantitative RT-PCR (Fig. 5c), qRT-PCR (Fig. 5d), and ELISA (Fig. 5e). Immunofluorescence staining showed significantly reduced MPL protein levels in the hippocampus and cerebral cortex tissue samples in MHE rats compared with control rats (Fig. 5f and 5g). Thus, we hypothesized that TPO treatment or overexpression might promote synapse formation in MHE rats.

ELT activates NRG1/ErbB4 signaling in MHE rat brains.

Next, we investigated whether ELT could activate NRG1/ErbB4 signaling to promote synapse formation in MHE rats. As shown in Fig. 6a–c, the administration of 1.2 μ g ELT significantly restored NRG1 and pErbB4 protein expression in the brain of MHE rats (Fig. 6a–g), compared with vehicle administration. Similarly, TPO overexpression (Fig. 6h and 6i) partially but significantly reversed MHE-induced losses of NRG1 and pErbB4 proteins in rat hippocampus (Fig. 6j–l) and cerebral cortex (Fig. 6m–o). Immunofluorescence staining confirmed the enhancive effect of TPO overexpression on pErbB4 expression in the hippocampus (Fig. 6p). In addition, 1.2 μ g ELT administration significantly restored hippocampal MPL expression in MHE rats (Fig. 6q and r), and TPO overexpression partially but

significantly reversed the loss of hippocampal MPL in MHE rats (Fig. 6s and t). These data collectively suggest that ELT activates NRG1/ErbB4 signaling in MHE rat brains.

ELT promote the expression of synaptic proteins in MHE rats.

Next, we assessed the effect of ELT on synapse formation in MHE rats. As shown in Fig. 7a–d, compared with vehicle treatment, administration of ELT resulted in significant restorations in Syntaxin and Homer protein levels in the brain tissue samples of MHE rats. Immunofluorescence staining confirmed the restoration of cerebral Homer expression in the MHE rat brain in response to ELT administration (Fig. 7e). Overexpression of MPL or TPO exhibited consistent results (Fig. 7f–k). Taken together, these results indicate that ELT promotes the expression of synaptic proteins in MHE rat brains.

ELT rescue cognitive and synaptic impairments in MHE rats.

Then, we examined the therapeutic effect of ELT on cognitive functions in MHE rats. The results of Y-maze test showed that administration of ELT or overexpression of TPO or MPL successfully reversed the decreases in the percentages of spontaneous alternation in MHE rats (Fig. 8a and 8b). The results of water-finding test demonstrated that administration of ELT or overexpression of TPO or MPL effectively reversed MHE-induced increases in the entry, contacting, and drinking latencies in rats (Fig. 8c and 8d).

Furthermore, administration of ELT or overexpression of TPO or MPL significantly countered MHE-induced decreases in the magnitudes of LTP (Fig. 8e and 8f) and MHE-induced increases in the serum and cerebral levels of ammonia in rats (Fig. 8g and 8h). These results suggest that ELT attenuates cognitive and synaptic impairments in MHE rats.

Discussion

In this study, we demonstrated that ELT promoted synapse formation via NRG1/ErbB4 signaling. In the primary rat neurons, ELT activated NRG1/ErbB4 signaling, which in turn promotes synapse formation via upregulating Sirt1 expression. In a MHE rat model that exhibited downregulation of cerebral ELT, ELT replacement therapy activated NRG1/ErbB4 signaling, promoted synapse formation, and alleviated cognitive impairment in rats. Our study provides ELT as a promising therapeutic agent to improve cognitive functions for patients with MHE.

Thrombopoietin (TPO), a 70-kDa glycoprotein, is the primary regulator of megakaryopoiesis and the primary hematopoietic growth factor responsible for platelet production ²¹. TPO shows significant homology with EPO (23%) at the amino-acid level and with neurotrophins (e.g., the highest homology with BDNF, 36%) in the N-terminal region ²². Prior to the isolation of TPO, its receptor was identified as the cellular proto-oncogene targeted by murine myeloproliferative leukemia virus and was therefore named c-Mpl. c-Mpl is predominantly expressed on megakaryocytes, mature platelets, and a subset of CD34⁺ hematopoietic stem cells ²³. The biological actions of TPO are initiated by specific binding to its receptors (i.e., c-Mpl) that are expressed on the surface of target cells. Signal transduction from activation

of the c-Mpl receptor following TPO binding has been demonstrated in many cell types. Importantly, both Tpo expression and cMpl expression have also been detected in nonhematopoietic tissues, including the brain 8^{24–26}. Thrombopoietin signals biological functions such as cell proliferation, survival, and maturation through several intracellular pathways (e.g., janus-activated kinase-signal transducer and activator of transcription (JAK-STAT), Ras/mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt)) 27. TPO and its receptor c-Mpl are expressed in the neurons of the human central nervous system (CNS) and murine neural cells. TPO is prominent in human cerebrospinal fluid 4. TPO was found to be neuroprotective in the CNS in hypoxic-ischemic neonatal rat brain models. TPO reduced brain damage and improved sensorimotor functions. In addition, TPO had a stimulating effect on neural cell proliferation and exerted an antiapoptotic effect 56. TPO improved neurological function and ameliorated brain edema after stroke 7. In the developing human CNS, the thrombopoietin gene is abundantly expressed. Considering that thrombopoietin contains a neurotrophic sequence, it may well play a role in neuronal cell biology⁸. Consistently, our results showed that MHE rats had significantly reduced cerebral ELT expression compared with normal rats, suggesting that loss of TPO or MPL is involved in the pathogenesis of MHE and that replacement therapy of ELT might be beneficial to MHE. Indeed, intracranial ELT administration or overexpression promoted synaptic protein expression, ameliorated cognitive and synaptic impairments, and reduced serum and cerebral levels of ammonia in MHE rats. Together, our evidence supports that ELT replacement therapy is a promising therapeutic approach in MHE treatment.

To identify the mechanism underlying the regulation of ELT in synapse formation, we determined the expression of NRG1/ErbB4 signaling. As a ligand of the ErbB receptor family, NRG1 possesses an epidermal growth factor domain that is required for binding and activating ErbB3 or ErbB4 9. NRG1 and ErbB kinases are widely expressed in the adult brain, and postsynaptic ErbB4 is particularly enriched at excitatory synapses 28. Activation of NRG1/ErbB4 signaling facilitates neural development, including neurotransmission, neuroplasticity, and pyramidal neuron synaptic plasticity 29. However, the expression pattern of NRG1/ErbB4 in MHE and the relationship between ELT and NRG1/ErbB4 signaling in MHE remain unexplored. In this study, we found that ELT activated NRG1/ErbB4 signaling and promoted synaptic protein expression in primary rat neurons and MHE rat brains. An anti-NRG1 antibody, ErbB4 inhibition, or knockdown of NRG1 or ErbB4 expression significantly reversed ELT-induced upregulation of synaptic protein expression, indicating that ELT promotes synapse formation via activating NRG1/ErbB4 signaling. In addition, like ELT, NRG1 and pErbB4 were downregulated in MHE rat brains, suggesting that restoration of NRG1/pErbB4 signaling might also benefits MHE. Indeed, our results showed that, like ELT replacement therapy, NRG1 administration or overexpression enhanced the expression of synaptic proteins, ameliorated cognitive and synaptic impairments, and reduced serum and cerebral levels of ammonia in MHE rats. Taken together, these findings suggest that decreased cerebral ELT may be associated with inactivated NRG1/pErbB4 signaling in MHE. Administration of ELT or overexpression of TPO or MPL may ameliorate cognitive impairment in MHE.

It has been reported that Sirt1 is essential for cognitive function and synapse formation. To identify the downstream effector of ELT/NGR1/ErbB4 signaling in MHE, we detected the expression of Sirt1 in the primary rat neurons in response to ELT or TPO treatment. We demonstrated that ELT or TPO treatment markedly upregulated Sirt1 protein expression in the primary rat neurons. Importantly, Sirt1 inhibitor effectively abolished ELT- or TPO -induced upregulation of synaptic protein expression in PHN, suggesting that ELT/TPO promotes synapse formation via Sirt1 in rat neurons. However, we did not investigate whether Sirt1 mediates ELT/TPO -induced synapse formation and cognitive enhancement *in vivo*, which will be addressed in future study.

Conclusion

In conclusion, our study demonstrated that ELT promotes synapse formation by activating NRG1/ErbB4/Sirt1 signaling in primary rat neurons. TPO expression is downregulated in MHE rat brains, along with inactivation of NRG1/ErbB4 signaling. Administration of ELT or overexpression of TPO or MPL promotes synapse formation and mitigates cognitive impairment by activating NRG1/ErbB4 signaling in MHE rats. These findings suggest that ELT are promising therapeutic agents for MHE treatment.

Abbreviations

a-CSF: artificial cerebrospinal fluid; cDNA: complementary DNA; CNS: central nervous system; Co-IP: co-immunoprecipitation; ECL: Electrochemiluminescence; ELISA: enzyme-linked immunosorbent assay; ELT: eltrombopag; EPO: erythropoietin; ErbB4: erb-b2 receptor tyrosine kinase 4; fEPSPs: field excitatory postsynaptic potentials; GAPDH: glyceraldehyde-phosphate dehydrogenase; HE: hepatic encephalopathy; JAK-STAT: janus-activated kinase-signal transducer and activator of transcription; LTP: long-term potentiation; MAP2: microtubule-associated protein 2; MAP2B: microtubule-associated protein 2B; MAPK: mitogen activated protein kinase; MHE: minimal hepatic encephalopathy; mRNA: Messenger RNA; NRG1: Neuregulin-1; PBS: phosphate buffered saline; PHNs: primary hippocampal neurons; PI3K: phosphoinositide 3-kinase; PVDF: polyvinylidene fluoride; qRT-PCR: quantitative real-time PCR; SDS-PAGE: sodium dodecyl sulfate -Polyacrylamide gel electrophoresis; siRNA: small interfering RNA; Sirt1: sirtuin 1; SPSS: Statistic Package for Social Science; TAA: thioacetamide; TPO: Thrombopoietin; vGlut1: vesicular glutamate transporter 1

Declarations

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Authors' contributions

Saidan Ding substantially contributed to the study conception and design, data interpretation, and manuscript revision. Xuebao Wang, Xiaoi Lu and Shuya Feng performed all the *in vitro* assays and data analysis. Leping Liu, Ruimin and Baihui Chen performed the *in vivo* experiments and data analysis. Jian Wang, He Yu and Qichuan Zhuge contributed to the manuscript preparation. All the authors contributed to the manuscript revision and read and approved the final article.

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Availability of data and materials

All data of the present study are available from the corresponding author upon reasonable requests.

Ethics approval and consent to participate

The study was approved by Institutional Animal Care and Use Committee of Wenzhou Medical University (Wenzhou, Zhejiang, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

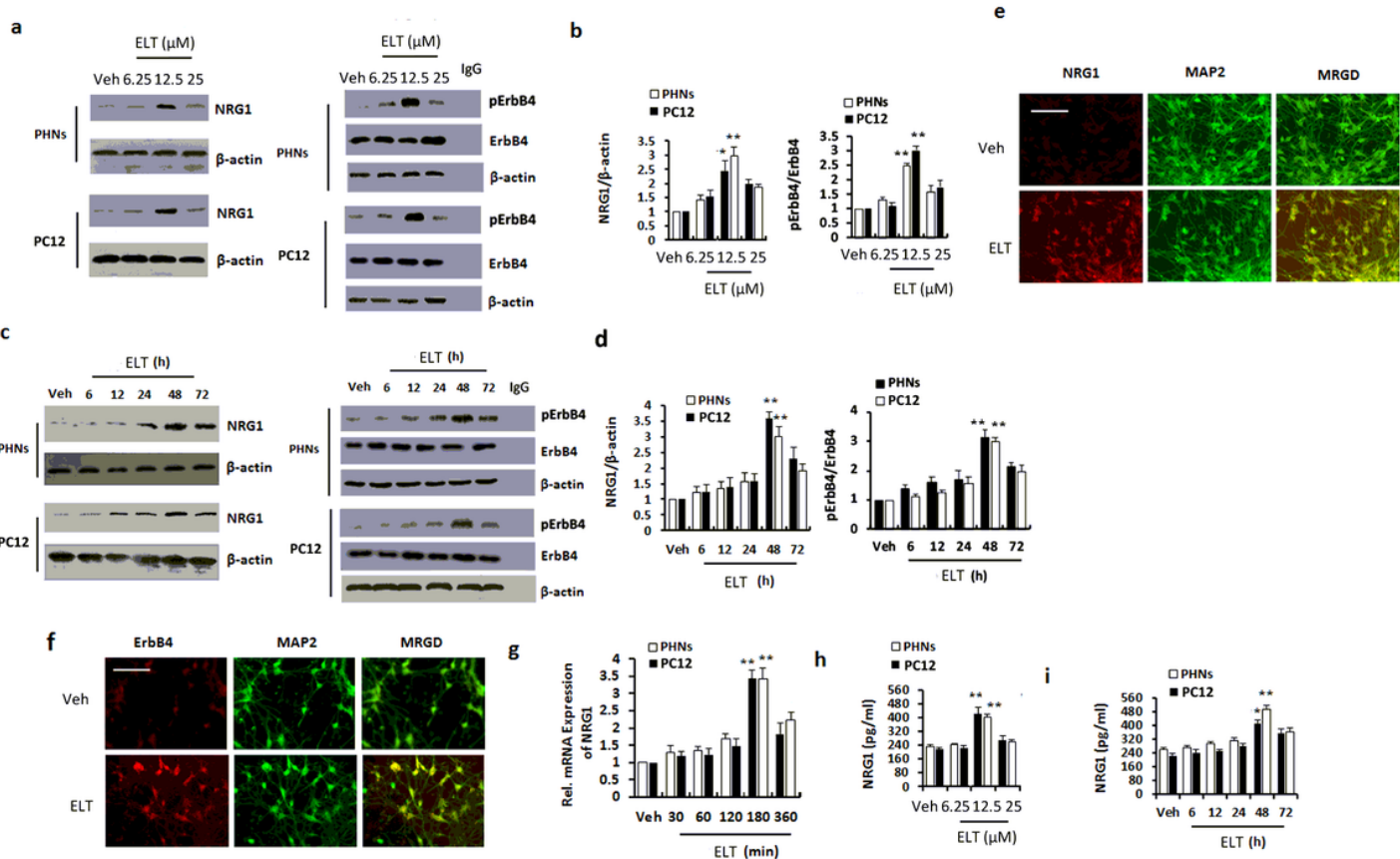


Figure 1

Insulin-like growth factor 1 (ELT) stimulation enhanced neuregulin 1 (NRG1) and erb-b2 receptor tyrosine kinase 4 (ErbB4) expression in primary rat neurons. (a) Primary rat hippocampal neurons (PHNs) or PC12 cells were treated with vehicle or ELT (0, 6.25, 12.5, 25 μ M) for 24 h. Western blot analysis was performed

to determine protein expression of NRG1, phosphorylated-ErbB4 (pErbB4), and total ErbB4. β -actin was used as an internal control. (b) Quantification of (a). (c) PHNs or PC12 cells were treated with 12.5 μ M ELT. Western blot analysis was performed at 0, 6, 12, 24, 48, and 72 h after treatment to determine protein expression of NRG1, pErbB4, and total ErbB4. β -actin was used as an internal control. (d) Quantification of (c). (e) Immunofluorescence staining was conducted to detect NRG1 (red), ErbB4 (red), and microtubule-associated protein 2 (MAP2; green) protein expression in PHNs. (f) PHNs or PC12 cells were treated as aforementioned. Quantitative realtime PCR (qRT-PCR) was performed to determine NRG1 mRNA expression. (g) NRG1 level was detected by ELISA in the medium of PHNs or PC12 cells at 1, 5 and 20 h after treatment with 12.5 μ M ELT. (h) ELISA was carried out to measure the NRG1 level in the medium of PHNs or PC12 cells at 0, 6, 12, 24, 48, and 72 h after treatment with 12.5 μ M ELT. Data are expressed as mean \pm standard deviation (SD). *P < 0.05, **P < 0.01 vs. vehicle-treated group. NS, non-significant. Scale bar = 25 μ m. MRGD, merged image.

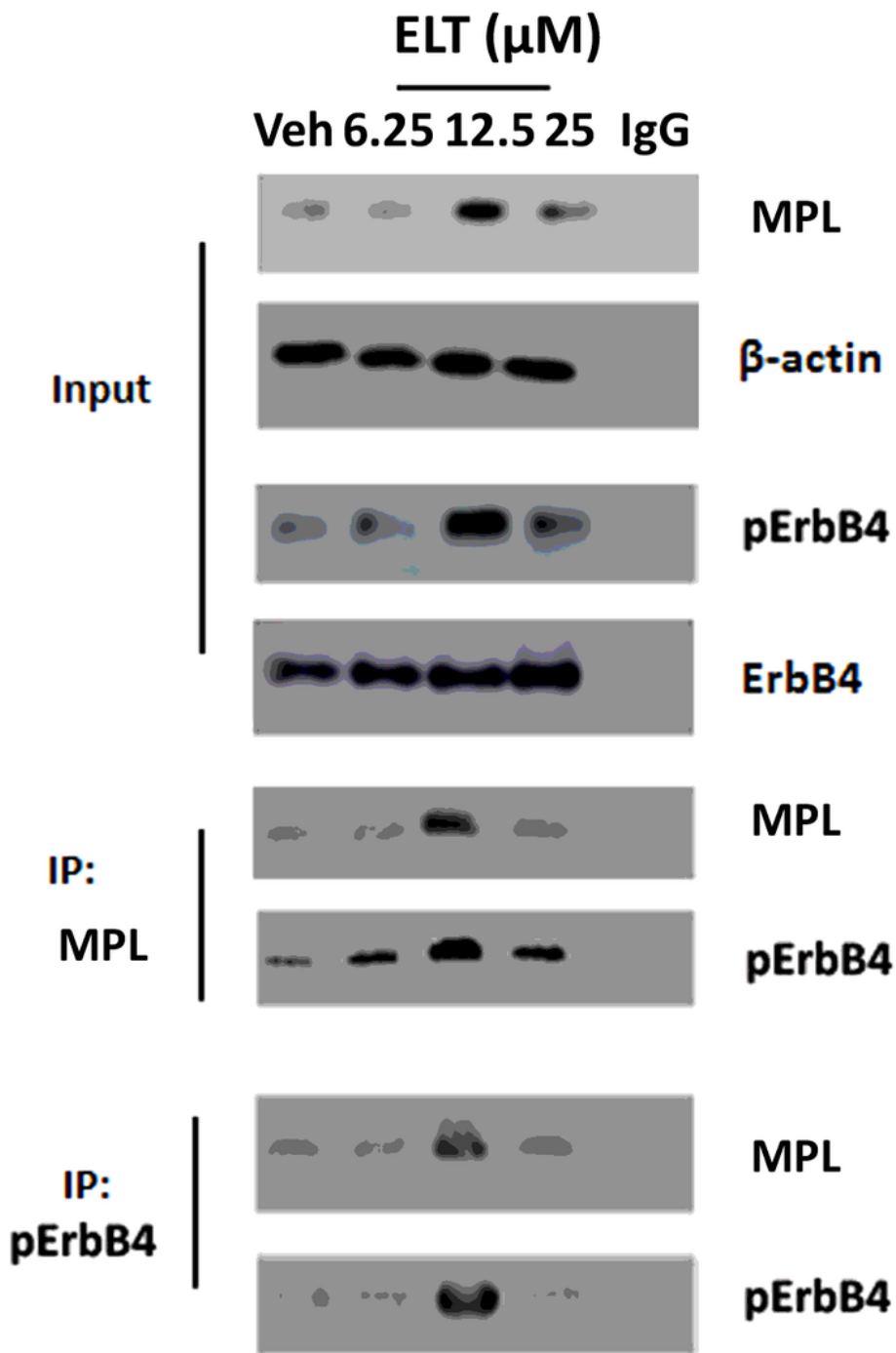


Figure 2

ELT promoted NRG1/pErbB4 interaction in primary rat neurons. PHNs were treated with vehicle or ELT (0, 6.25, 12.5, 25 μ M) for 24 h. Co-immunoprecipitation was conducted to examine the MPL/pErbB4 interaction. IP, immunoprecipitation.

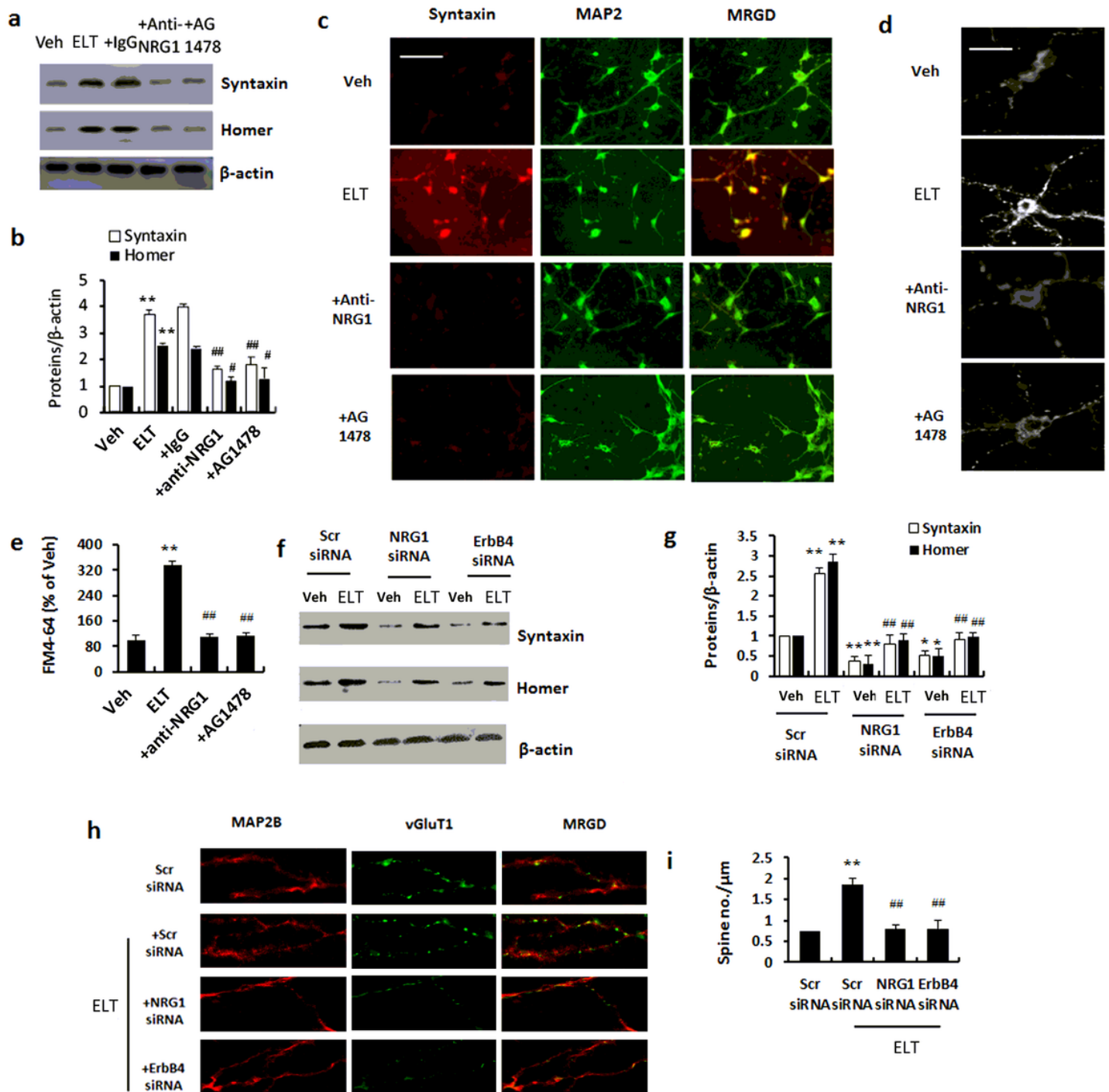


Figure 3

ELT promoted synapse formation in primary neurons by activating NRG1/ErbB4 signaling. PHNs were pretreated with 10 μ g/mL anti-NRG1 mouse monoclonal antibody, mouse IgG, or 50 μ mol/L ErbB4 inhibitor AG1478 for 24 h, followed by ELT (12.5 μ M) treatment for an additional 24 h. (a) Western blot analysis was performed to determine protein expression of syntaxin and Homer. β -actin was used as an internal control. (b) Quantification of (a). (c) Immunofluorescence staining was conducted to detect syntaxin (red) and MAP2 (green). Representative images are shown. (d) FM4-64 staining was performed to detect functional presynaptic terminals. Representative images are shown. (e) Quantification of (d). (f,

g) PHNs were transfected with scrambled siRNA or siRNA against NRG1 or ErbB4, followed by ELT (12.5 μ M) treatment for an additional 24 h. Western blot analysis was performed to determine protein expression of syntaxin and Homer. β -actin was used as an internal control. Immunofluorescence staining was conducted to detect MAP2 (red) and vGluT1 (green). Representative images are shown. (h, i) Quantification of (f) and (g). Data are expressed as mean \pm SD. * P < 0.05, ** P < 0.01 vs. vehicle/Scr siRNA-treated group; # P < 0.05, ## P < 0.01 vs. ELT/ELT+Scr siRNA-treated group. Scr, scrambled. Scale bar, 25 μ m. MRGD, merged.

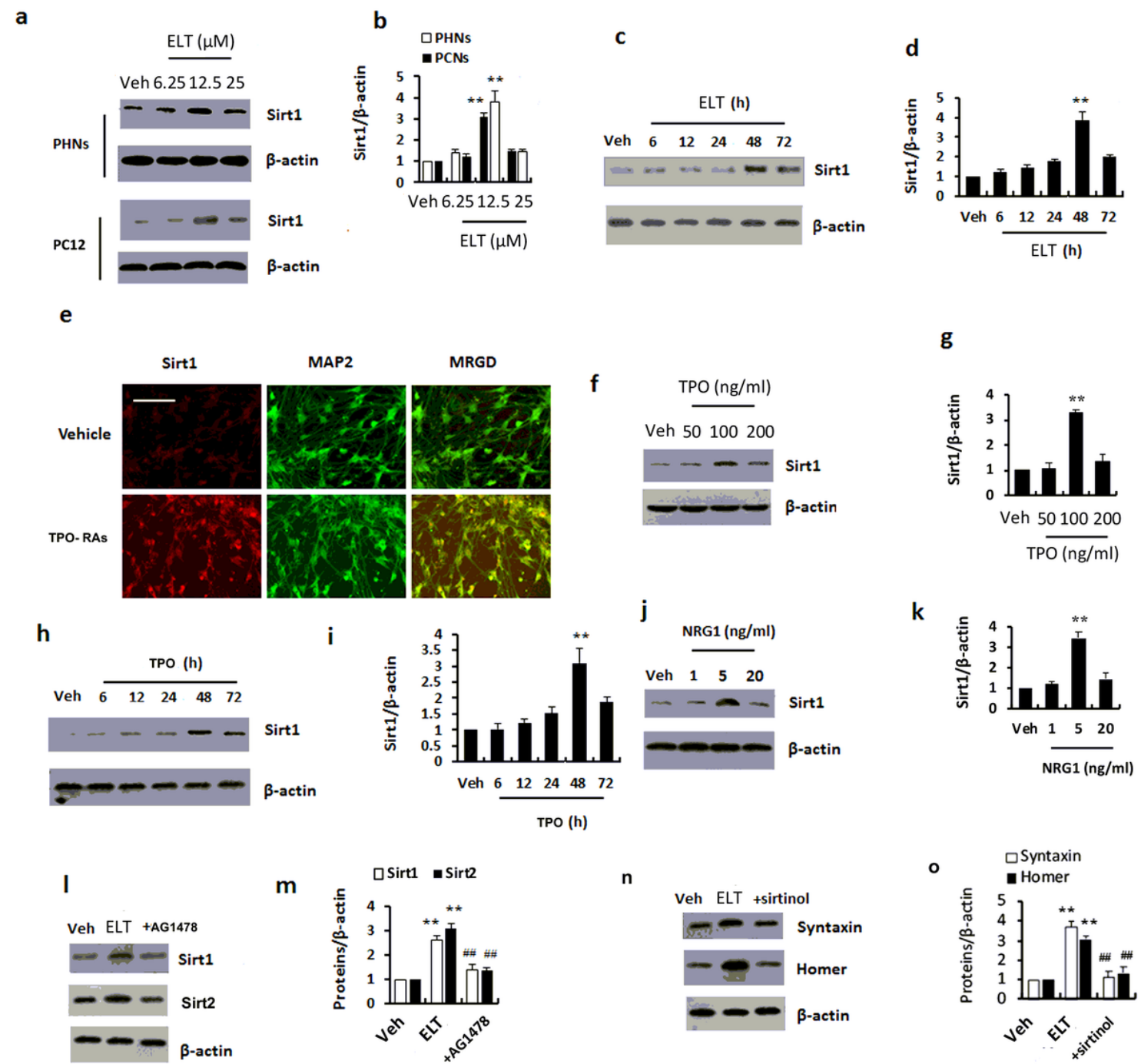


Figure 4

Sirtuin 1 (Sirt1) mediated NRG1-induced synapse formation in rat neurons. (a) PHNs or PC12 cells were treated with vehicle or ELT (0, 6.25, 12.5, 25 μ M) for 24 h. Western blot analysis was performed to determine protein expression of Sirt1. β -actin was used as an internal control. (b) Quantification of (a). (c) PHNs were treated with 12.5 μ M ELT. Western blot analysis was performed at 0, 6, 12, 24, 48, and 72 h after treatment to determine protein expression of Sirt1. β -actin was used as an internal control. (d) Quantification of (c). (e) Immunofluorescence staining was conducted to detect Sirt1 (red) and MAP2 (green). Representative images are shown. (f) PHNs or PC12 cells were treated with vehicle or TPO (50, 100, or 200 ng/mL) for 24 h. Western blot analysis was performed to determine protein expression of Sirt1. β -actin was used as an internal control. (g) Quantification of (f). (h) PHNs were treated with 100 ng/mL TPO. Western blot analysis was performed at 0, 6, 12, 24, 48, and 72 h after treatment to determine protein expression of Sirt1. β -actin was used as an internal control. (i) Quantification of (h). (j) PHNs or PC12 cells were treated with vehicle or NRG1 (1, 5, or 20 ng/mL) for 24 h. Western blot analysis was performed to determine protein expression of Sirt1. β -actin was used as an internal control. (k) Quantification of (j). (l) PHNs were pretreated with 10 μ mol/L AG1478 for 24 h, followed by treatment with 12.5 μ M ELT. Western blot analysis was performed to determine protein expression of Sirt1/2. β -actin was used as an internal control. (m) Quantification of (l). (n) PHNs were pretreated with 10 μ mol/L Sirt1 inhibitor sirtinol for 24 h, followed by treatment with 12.5 μ M ELT. Western blot analysis was performed to determine protein expression of syntaxin and Homer. β -actin was used as an internal control. (o) Quantification of (n). Data are expressed as mean \pm SD. * P < 0.05, ** P < 0.01 vs. vehicle-treated group; # P < 0.05, ## P < 0.01 vs. ELT-treated group. Scale bar, 25 μ m. MRGD, merged image.

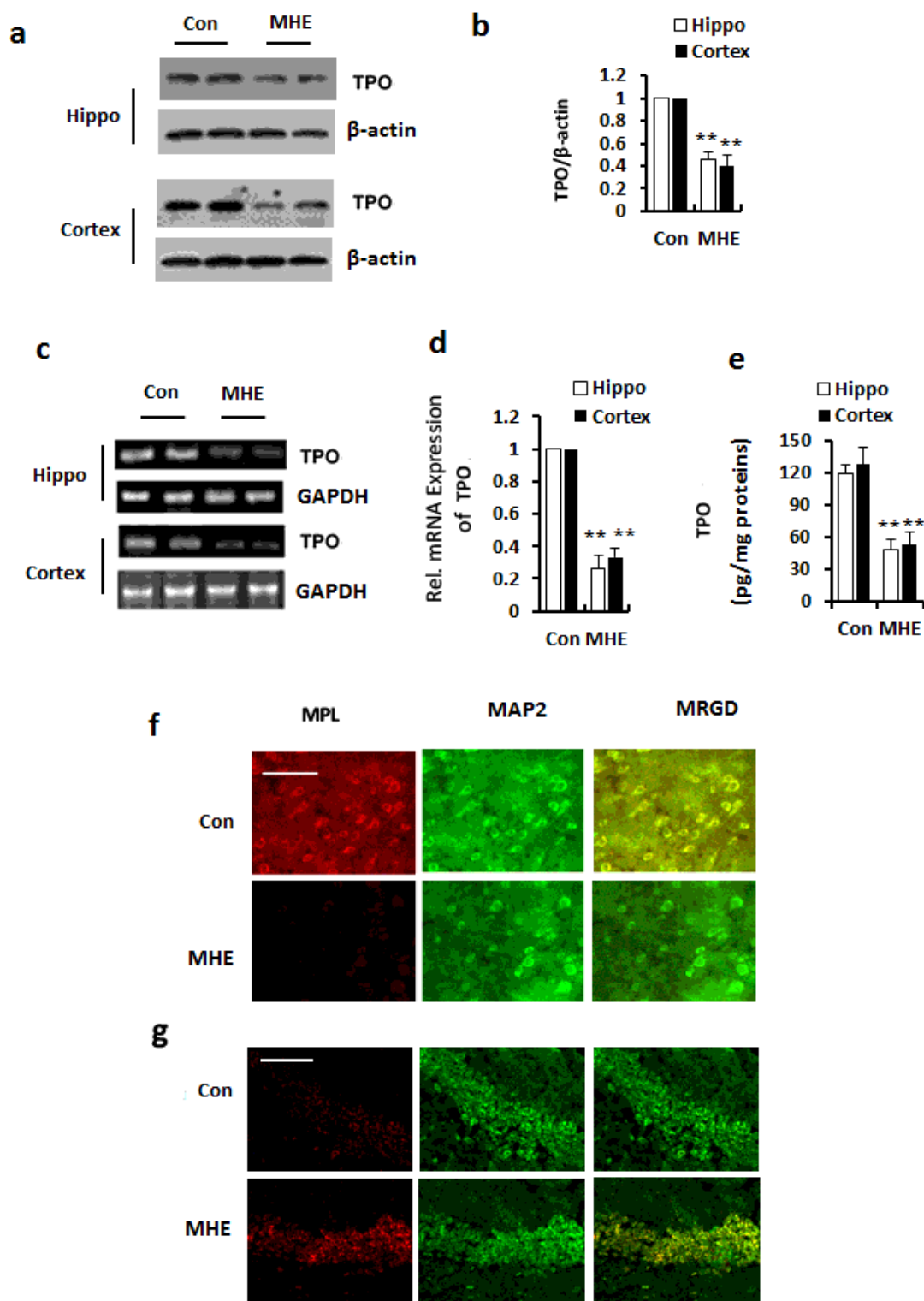


Figure 5

TPO expression was downregulated in MHE rat brains. Rats were sacrificed after treatment. The hippocampus and cerebral cortex were collected immediately and stored at -80 °C until use. (a, b) Western blot analysis was performed to determine protein expression of TPO in the hippocampus and cerebral cortex tissue samples. β -actin was used as an internal control. (b) Quantification of (a). (c, d) Semi-quantitative and quantitative real-time PCR were carried out to determine TPO mRNA expression in

the brain tissue samples. (e) ELISA was performed to measure the TPO levels in the brain tissue samples. (f, g) Immunofluorescence staining was conducted to detect MPL (red) and MAP2 (green) in free-floating cortical (f) and hippocampal (g) sections. Data are expressed as mean \pm SD. *P < 0.05, **P < 0.01 vs. control group. Scale bar, 25 μ m. MRGD, merged image.

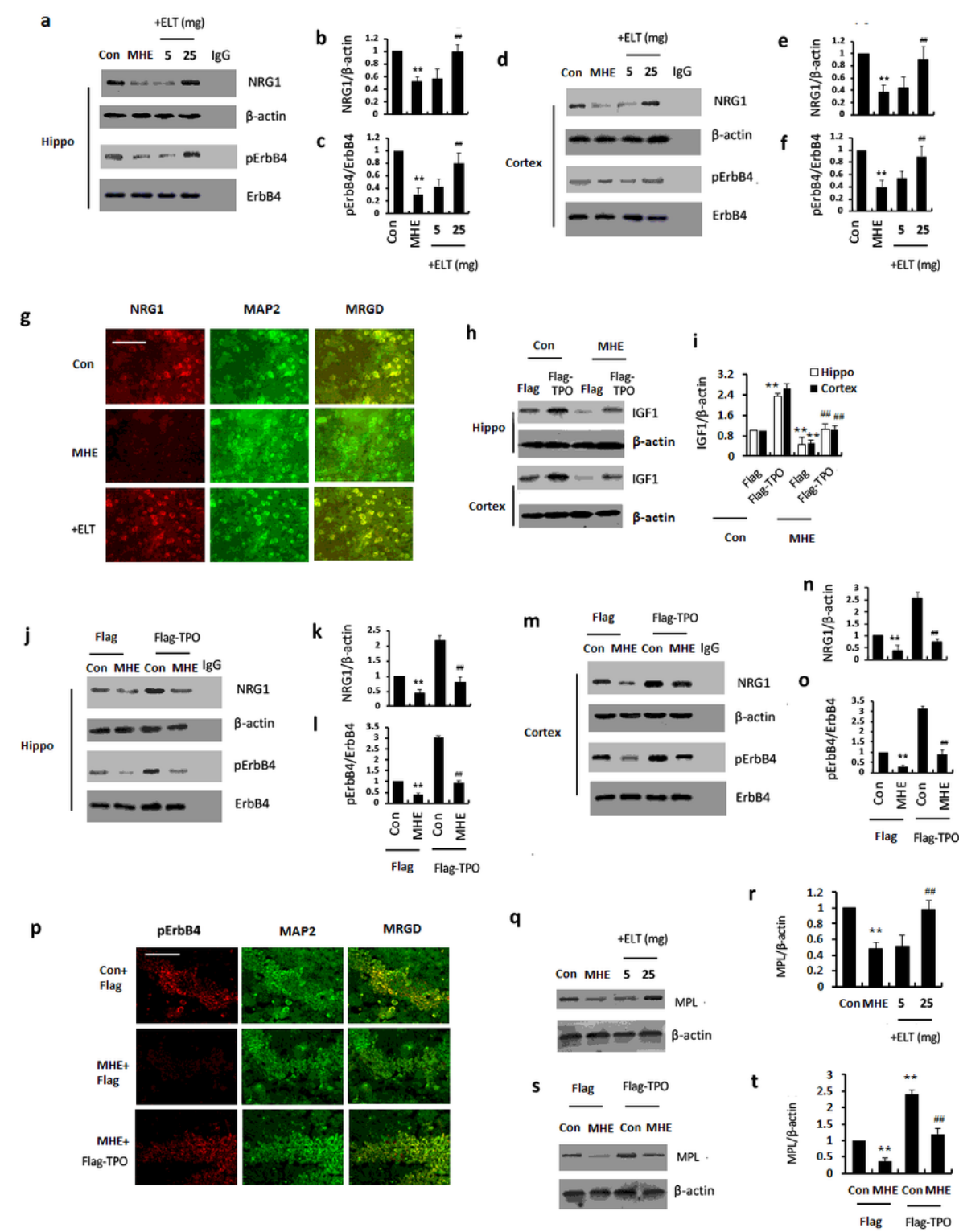


Figure 6

ELT activated NRG1/ErbB4 signaling in vivo. MHE rats were administered vehicle or ELT (5 or 25 mg) 3 times for a week via intracerebroventricular cannulation. (a–f) Western blot analysis was performed to determine protein expression of NRG1 and pErbB4 in the brain tissue samples. β -actin was used as an internal control. (g) Immunofluorescence staining was conducted to detect NRG1 (red) and MAP2 (green) in the brain tissue samples from MHE rats administered 25mg ELT. (h–o) MHE rats were administered ELT-overexpressing vectors (Flag-ELT) or empty vector (Flag) via intracerebroventricular cannulation. Western blot analysis was performed to determine protein expression of ELT (h, i), NRG1, and pErbB4 (j–o) in the brain tissue samples as indicated. β -actin was used as an internal control. (p) Immunofluorescence staining was conducted to detect pErbB4 (red) and MAP (green) in the brain tissue samples. (q–t) MHE rats were administered ELT (0.6 or 1.2 μ g; q, r) or Flag-ELT (s, t). Western blot analysis was performed to determine protein expression of MPL in the hippocampus tissue samples from rats. Data are expressed as mean \pm SD. * $P < 0.05$, ** $P < 0.01$ vs. control/control + Flag group; # $P < 0.05$, ## $P < 0.01$ vs. MHE/MHE + Flag group. Scale bar, 25 μ m. MRGD, merged image.

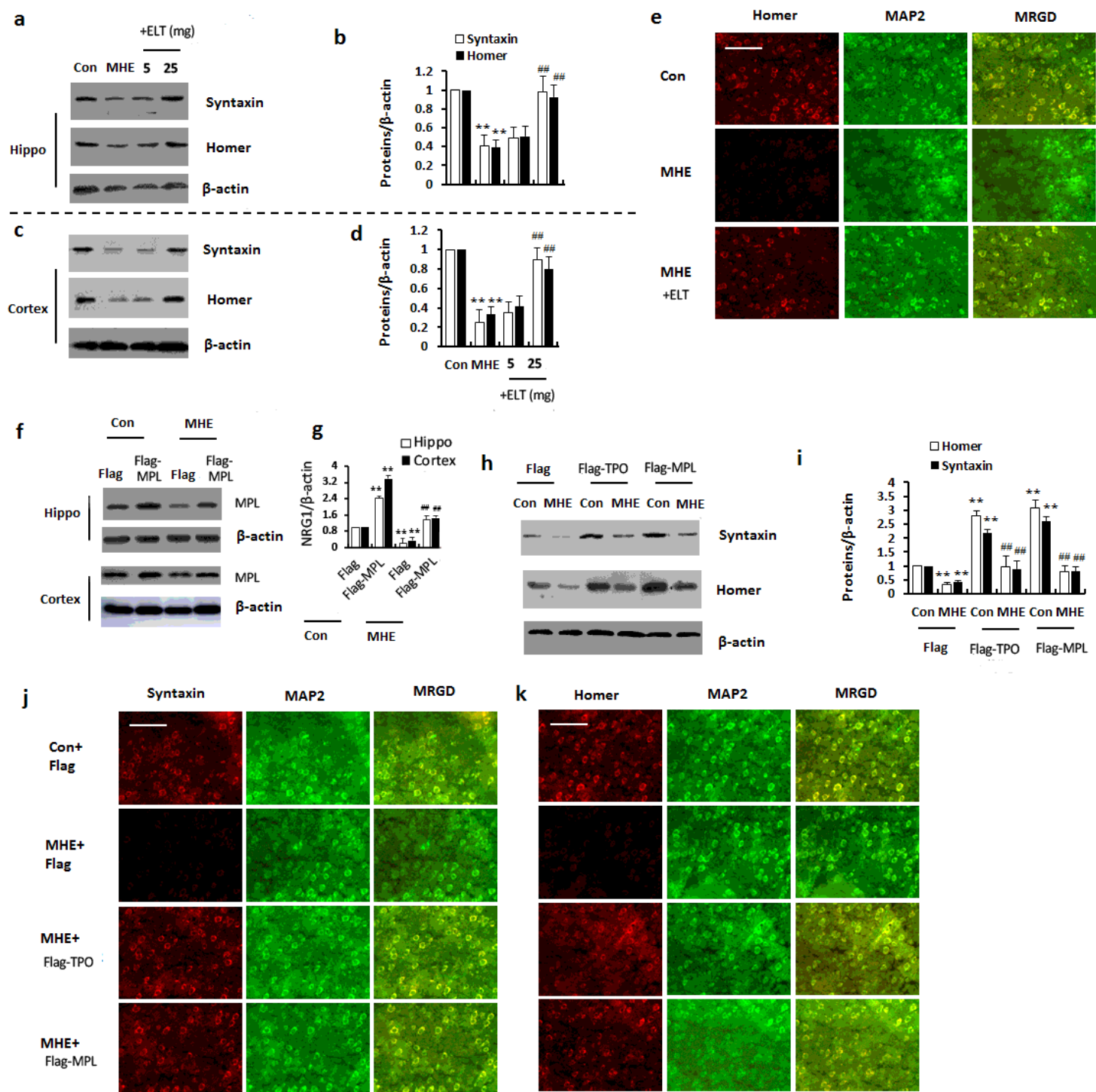


Figure 7

ELT or NRG1 promoted synapse formation in MHE rats. (a–d) MHE rats were administered vehicle, or ELT (5 or 25 mg). Western blot analysis was performed to determine protein expression of syntaxin and Homer in the brain tissue samples. β -actin was used as an internal control. (e) MHE rats were administered vehicle, ELT (25 mg). Immunofluorescence staining was conducted to detect Homer (red) and MAP2 (green) in the hippocampus tissue samples. (f, g) MHE rats were administered Flag-NGR1. Western blot analysis was performed to determine protein expression of NRG1. β -actin was used as an internal control. (h, i) MHE rats were administered Flag-TPO or Flag-MPL. Western blot analysis was

performed to determine protein expression of syntaxin and Homer in the brain tissue samples. β -actin was used as an internal control. (j, k) Immunofluorescence staining was conducted to detect syntaxin (red; j), Homer (red; k), and MAP2 (green) in the cortex tissue samples. Data are expressed as mean \pm SD. * $P < 0.05$, ** $P < 0.01$ vs. control/control + Flag group; # $P < 0.05$, ## $P < 0.01$ vs. MHE/MHE + Flag group. Scale bar, 25 μ m. MRGD, merged image.

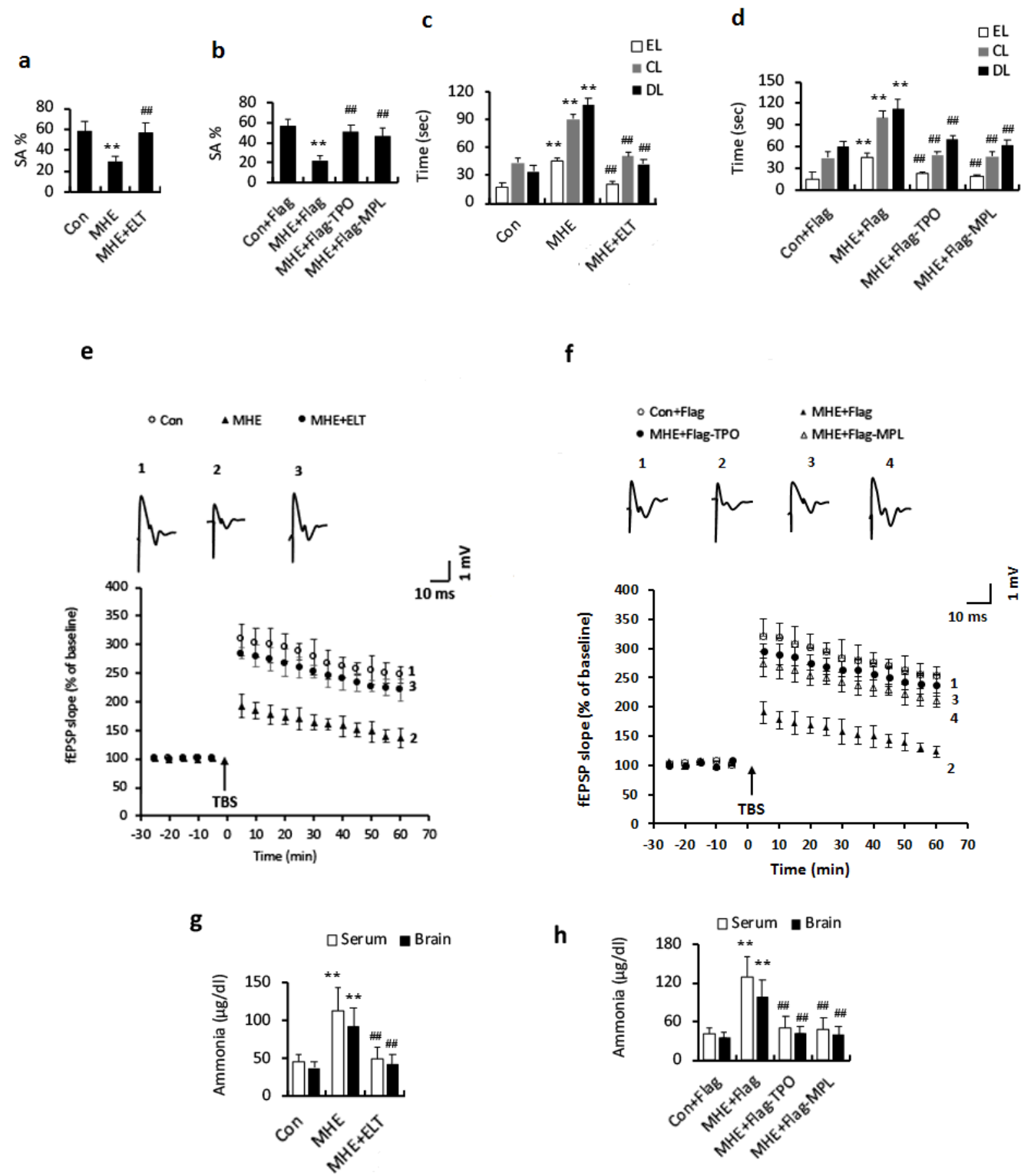


Figure 8

ELT or NRG1 alleviated cognitive impairment in MHE rats. MHE rats were administered ELT (25mg), Flag-TPO, or Flag-MPL. (a, b) Y-maze test was performed before and after drug administration. The percentages of spontaneous alternation (SA%) in rats were recorded. (c, d) Water-finding test was performed before and after drug administration. The entry latency (EL), contacting latency (CL), and drinking latency (DL) of rats were recorded. (e) Long-term potentiation (LTP) of field excitatory postsynaptic potentials (fEPSPs) was induced by 3 × theta-burst stimulation (TBS; 4 pulses at 100 Hz, repeated 3 times at 200-ms intervals). Representative fEPSPs are shown. 1, control rats; 2, MHE rats; 3, MHE rats administered 25mg ELT. (f) LTP of fEPSPs was induced by 3 × TBS. Representative fEPSPs are shown. 1, control rats treated with Flag; 2, MHE rats treated with Flag; 3, MHE rats treated with Flag-TPO; 4, MHE rats treated with Flag-MPL. (g, h) The serum and cerebral ammonia levels were measured. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01 vs. control/control + Flag group; #P < 0.05, ##P < 0.01 vs. MHE/MHE + Flag group.

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