

Neuritin Activates Insulin Receptor Pathway to Up-regulate Kv4.2-mediated Transient Outward K^+ Current in Rat Cerebellar Granule Neurons*

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Background: Neuritin is a new neurotrophic factor. The receptors for binding neuritin and its downstream signaling effectors, however, remain unclear.

Results: Neuritin increases Kv4.2-mediated I_A in rat cerebellar granule neurons by activating the insulin receptor pathway.

Conclusion: Neuritin may act as a ligand of the insulin receptor.

Significance: Our findings reveal an unanticipated role of the insulin receptor in previously unrecognized neuritin-mediated signaling.

Neuritin is a new neurotrophic factor discovered in a screen to identify genes involved in activity-dependent synaptic plasticity. Neuritin also plays multiple roles in the process of neural development and synaptic plasticity. The receptors for binding neuritin and its downstream signaling effectors, however, remain unclear. Here, we report that neuritin specifically increases the densities of transient outward K^+ currents (I_A) in rat cerebellar granule neurons (CGNs) in a time- and concentration-dependent manner. Neuritin-induced amplification of I_A is mediated by increased mRNA and protein expression of Kv4.2, the main α -subunit of I_A . Exposure of CGNs to neuritin markedly induces phosphorylation of ERK (pERK), Akt (pAkt), and mammalian target of rapamycin (pmTOR). Neuritin-induced I_A and increased expression of Kv4.2 are attenuated by ERK, Akt, or mTOR inhibitors. Unexpectedly, pharmacological blockade of insulin receptor, but not the insulin-like growth factor 1 receptor, abrogates the effect of neuritin on I_A amplification and Kv4.2 induction. Indeed, neuritin activates downstream signaling effectors of the insulin receptor in CGNs and HeLa. Our data reveal, for the first time, an unanticipated role of the insulin receptor in previously unrecognized neuritin-mediated signaling.

Neuritin, also known as CPG15, was discovered in a screen to identify novel genes involved in activity-dependent synaptic plasticity in the neocortex (1, 2). Neuritin was subsequently identified as an important neurotrophin expressed in the developing nervous system (3). Indeed, neuritin plays multiple roles in the process of neural development, synaptic plasticity, and

synaptic maturation (4, 5). For example, previous studies showed correlation of neuritin expression and its effect on neurite outgrowth and axonal function (6, 7). The function of neuritin in the voltage-gated ion channel in neurotropic responses, however, remains elusive.

Previous studies reported that exposure of primary embryonic hippocampal or cortical neurons to purified recombinant neuritin promotes neurite outgrowth and arborization (1, 2). Neuritin has also been shown to promote synaptic maturation (8). How neuritin mediates neuronal differentiation and maturation, however, is not known. Recent studies suggested that neuritin acts as a ligand to promote dendritic growth through intercellular signaling (2). Identification of neuritin receptor and its downstream signaling pathways, therefore, will be important for further investigation of neuritin in neuronal functions.

Cerebellar granule neurons (CGNs)² are small glutamatergic cells, and their differentiation gives rise to the numerous neuronal types in mammalian brain. In view of their well defined developmental pathway, CGNs are invaluable models for studying neuronal maturation differentiation and synaptic plasticity (9). Previous studies indicated that growth and differentiation factors can either stimulate or inhibit CGN development and maturation by regulating multiple signaling pathways (10, 11).

One of the characteristics during CGNs development and maturation is regulation of the expression of K^+ channels (12, 13). Primary culture of CGNs displays several voltage-activated outward K^+ currents (14). We previously reported that enhancement of delayed-rectifier outward (I_K) and fast transient outward (I_A) potassium currents was associated with CGN apoptosis (15, 16). In addition, I_K also plays a role in promoting CGN migration and maturation (11, 17). These studies,

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² The abbreviations used are: CGN, cerebellar granule neuron; DAB, 3,3'-diaminobenzidine tetrahydrochloride; DIC, days in culture; HNMPA, hydroxy-2-naphthalenylmethyl phosphonic acid; IR, insulin receptor; mTOR, mammalian target of rapamycin.

together, indicate that modulation of expression of different potassium channels may alter the status and function of CGNs.

The goal of this report was to examine whether neuritin modulates K^+ channels for its role in synaptic activity and neurodevelopment. To test this hypothesis, we examined the effect of neuritin on K^+ channels in rat CGNs. We find that neuritin increases I_A amplitudes by regulating the expression of Kv4.2 potassium channel subunits. In addition, we show that neuritin activates insulin receptor signaling pathway. Our study, for the first time, reveals neuritin-mediated signaling and its role in modulating K^+ channel expression for neuronal function.

EXPERIMENTAL PROCEDURES

Cell Culture—All experimental procedures were carried out in accordance with European guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC). Cells were derived from cerebellum of 7-day-old Sprague-Dawley rat pups as described previously (18). Isolated cells were plated onto 35-mm Petri dishes coated with poly-L-lysine (1 μ g/ml) at a density of 10^6 cells/ml. Cultured cells were incubated at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, insulin (5 μ g/ml), KCl (25 mM), and 1% antibiotic-antimycotic solution. After culture for 24 h, cytosine β -D-arabinofuranoside (5 μ M) was added to the culture medium to inhibit the proliferation of nonneuronal cells. Cells were used for experiments after 7–8 days in culture unless otherwise indicated. HeLa cells were maintained as a monolayer in high glucose DMEM, supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution.

Patch Clamp Recordings—Whole cell currents of granule neurons were recorded using conventional patch clamp technique. All currents were recorded using an multiclamp 200B amplifier (Axon Instruments, Foster City, CA) operated in voltage clamp mode. Data acquisition and analysis were performed with pClamp 8.01 software (Axon Instruments) and/or Origin 8 analysis software (Microcal Software, Northampton, MA). Prior to I_A recording, the culture medium was replaced with a bath solution containing 125 mM NaCl, 2.5 mM KCl, 10 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1 μ M tetrodotoxin, 20 mM triethanolamine, 10 mM glucose. Soft-glass recording pipettes were filled with an internal solution containing 135 mM potassium gluconate, 10 mM KCl, 10 mM HEPES (pH 7.3), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM EGTA, 1 mM ATP, and 0.1 mM GTP. The pipette resistance was 4–6 megohms after filling with internal solution. All recordings were performed at room temperature. The cultured granule cells selected for electrophysiological recording exhibited common morphological characteristics of healthy cells, such as fusiform soma with two main neurites of similar size. In addition, the mean capacitance of recorded cells for control group (7.17 ± 0.23 picofarads) and for neuritin treatment group (7.36 ± 0.11 picofarads) showed no significant difference ($p = 0.338$), indicating that comparable granule cells were used in the experiments.

In steady-state activation experiments, membrane potential was held at -100 mV, and I_A was evoked by a 200-ms depolarizing pulse from a first pulse potential of -60 mV to $+60$ mV in 10-mV steps at 10-s intervals. Data were analyzed using the

equation $G_K = I_K/(V_m - V_{rev})$, where G_K is the membrane K^+ conductance, V_m is the membrane potential, and V_{rev} is the reversal potential for K^+ . To study steady-state inactivation of I_A , currents were elicited using 1-s conditioning pre-pulses from -110 mV to 0 mV before a 200-ms test pulse of $+50$ mV. After normalizing each current amplitude to the maximal current, amplitude obtained from the -110 mV pre-pulse was used as a function of the conditioning pre-pulse potential and fitted with the function $I_A/I_A - \max = 1/(1 + \exp((V_{m1/2} - V_m)/k))$. Hence, we obtained an inactivation curve of I_A and calculated the V_{50} (the voltage at which the current amplitude was half-inactivated).

Data Analysis—Results were analyzed using one-way analysis of variance (ANOVA) for comparisons between multiple groups and by Student's t test for comparison of two samples. Data were presented as means \pm S.E. with n as the number of neurons for electrophysiological recordings, number of imaging experiments, or as the number of replicates for molecular biological experiments. For electrophysiological experiments, data were collected from at least four different batches of neurons prepared at different dates, thereby minimizing bias resulting from culture conditions. A p value < 0.05 was considered statistically significant.

Western Blot Analysis—Cells were lysed in HEPES-Nonidet P-40 lysis buffer (20 mM HEPES, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 2 mM EDTA, 100 μ M Na_3VO_4 , 50 mM NaF (pH 7.5), and 1% proteinase inhibitor mixture) on ice for 30 min. After centrifugation, the supernatant was mixed with 2 \times sodium dodecyl sulfate loading buffer and boiled for 5 min. The proteins were separated on a 10% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Millipore). The membrane was blocked with 10% nonfat milk and incubated at 4 °C overnight with mouse monoclonal antibody against Kv4.2, Kv4.3, Kv1.1 (1:2000, catalogue numbers 75-016, 75-017, and 75-007; University of California, Davis), or rabbit polyclonal antibody against the phosphorylated insulin receptor β subunit (1:1000, catalogue number 3024; Cell Signaling Technology), or rabbit monoclonal antibody against phosphorylated ERK1/2 or total ERK1/2 (1:1000, catalogue numbers 4370 and 4695; Cell Signaling Technology), or rabbit monoclonal antibody against phosphorylated Akt (1:2000, catalogue number 4060; Cell Signaling Technology), or rabbit polyclonal antibody against phosphorylated mTOR (1:1000, catalogue number 2971; Cell Signaling Technology), or rabbit polyclonal antibody against phosphorylated IRS-1 (1:500, catalogue number 3070; Cell Signaling Technology), or mouse monoclonal antibody against GAPDH (1:10,000, catalogue number KC-5G4; KangChen Bio-Tech, China). After extensively washing in TBST, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:10,000, catalogue numbers KC-MM-035 and KC-RB-035; KangChen Bio-Tech) for 2 h at room temperature. Chemiluminescent signals were generated using a SuperSignal West Pico trial kit (Pierce) and detected by exposure to x-ray film or using ChemiDoc XRS System (Bio-Rad Laboratories). Quantity One software (version 4.6.2, Bio-Rad Laboratories) was used for background subtraction and for quantification of immunoblotting data.

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Quantitative RT-PCR—To measure Kv4.2 mRNA levels, quantitative real-time PCR analysis was performed with the following sequences: forward primer, 5'-TGTCAGGAAGT-CATAGAGGCAGCGTG-3' and reverse primer, 5'-GGG-GTGGTTACTGGAGGTGTTGGAAT-3'. To control sampling errors, quantitative real-time PCR for the housekeeping gene cyclophilin D with the following sequences: forward primer, 5'-GGCTCTTCAAATGGACCCTTC-3' and reverse primer, 5'-GGCTCTTCAAATGGACCCTTC-3' was routinely performed on each sample and used as control. The reaction solution consisted of 2.0 μ l of diluted RT-PCR product, a 0.2 μ M concentration of each paired primer, and power SYBR Green PCR master mix (Toyobo, Osaka, Japan). The annealing temperature was set at 58 °C for Kv4.2 and 61 °C for cyclophilin D, and amplification cycles were set at 38 cycles. The absolute mRNA levels in each sample were calculated according to a standard curve set up using serial dilutions of known amounts of specific templates against corresponding cycle threshold (C_t) values. The normalized ratio of the target gene over cyclophilin D in each sample was presented. The specificity of the primers was verified by both gel electrophoresis and sequencing of the PCR products.

Immunoprecipitation—For the detection of Tyr-phosphorylated insulin receptor, CGNs incubated with or without neuritin were lysed in HEPES-Nonidet P-40 lysis buffer as described above. After centrifugation, cell lysate was mixed with rabbit polyclonal antibody against the phosphorylated insulin receptor β subunit (1:100, catalogue number 3024; Cell Signaling Technology) at 4 °C. After overnight incubation, protein A/G (1:25; Santa Cruz Biotechnology) agarose beads were added and incubated for additional 1 h at 4 °C. After centrifugation and multiple washing, immunoprecipitated complex were mixed with 2 \times sodium dodecyl sulfate loading buffer and boiled for 5 min. Samples were then examined by Western blot analysis using mouse monoclonal antibody against phosphorylated insulin receptor β subunit (1:1000, catalogue number sc-81500; Santa Cruz Biotechnology). To measure the level of phosphorylation of IGF-1 receptor, rabbit polyclonal antibody against IGF-1 receptor β subunit (1:100, catalogue number 3027; Cell Signaling Technology) was used for immunoprecipitation and the primary antibody for Western blotting was rabbit polyclonal antibody against phosphorylated IGF-1 receptor β subunit (1:1000, catalogue number 6113; Cell Signaling Technology).

Immunocytochemistry and 3,3-N-Diaminobenzidine Tetrahydrochloride (DAB) Staining—Cultured cells were fixed in fresh 4% formaldehyde in 0.1 M phosphate buffer for 15 min. Fixed cells were washed twice in ice-cold PBS and permeabilized with 0.25% Triton X-100 for 10 min. Cells were then washed three times in PBS for 5 min each and blocked in 1% BSA for 30 min. The labeling experiments were performed by incubating cells with antibody against phosphorylated insulin receptor β -subunit or phosphorylated IGF-1 receptor (1:100, catalogue numbers 3024 and 3027; Cell Signaling Technology) at 4 °C overnight. After vigorously being washed in PBS, the cells were incubated with the corresponding FITC- or Texas Red-conjugated donkey anti-rabbit IgG (1:200, catalogue numbers 111-095-003 and 111-295-003; Jackson Immunoresearch

Laboratories, West Grove PA) for 1 h at room temperature. Fluorescent-labeled cells were visualized with a Leica SP2 confocal laser scanning microscope (Leica, Mannheim, Germany) using a 40 \times objective lens.

DAB staining was detected according to the manufacturer's instructions. Briefly, after incubation with the primary antibody, cells were incubated with the horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000) (KangChen Bio-Tech, China) for 1 h at room temperature and then overnight with DAB reagent. Labeled cells were washed twice in PBS and examined.

Chemicals—Recombinant human neuritin was purchased from Pepro Tech (Rocky Hill, NJ). Hydroxy-2-naphthalenylmethyl phosphonic acid (HNMPA) was obtained from Santa Cruz Biotechnology. Triethanolamine, tetrodotoxin, insulin, U0126, rapamycin, PQ-401, and poly-L-lysine were purchased from Sigma. Fetal calf serum, DMEM, and antibiotic-antimycotic solution were purchased from Invitrogen. shRNA was purchased from GeneChem (Shanghai, China). Expression of shRNA was coupled with GFP to identify positively transfected cells.

RESULTS

Neuritin Increased I_A Amplitude of CGNs by Enhancing the Expression of Kv4.2 α -Subunit—To determine the potential role of neuritin on CGNs, we asked whether neuritin affected I_A . I_A was evoked by 200-ms depolarization to +40 mV from a holding potential of -100 mV in the presence of 20 mM triethanolamine, which suppresses I_K and permits better resolution of I_A . Incubation of CGNs with neuritin significantly enhanced I_A densities in a concentration-dependent manner. The data obtained from 99 neurons showed that incubation of CGNs with 15, 150, or 300 ng/ml neuritin for 24 h increased the current densities by 13.0% ($n = 27$), 45.5% ($n = 41$), and 44.4% ($n = 31$), respectively (Fig. 1A). To address whether neuritin-induced I_A densities were time-dependent, neurons were incubated with neuritin (150 ng/ml) for different times, and I_A densities were determined. After 12-, 24-, and 36-h incubation, neuritin was found to augment I_A densities by 22.9% ($n = 21$), 45.5% ($n = 41$), and 46.2% ($n = 20$), respectively (Fig. 1B). We also found that the effect of neuritin on the induction of I_A is dependent on the days that CGNs have been in culture (Fig. 1C). Neuritin, however, did not affect granule neuron I_A amplitude when applied acutely to bath solution by the perfusion system (data not shown). Together, these data indicate that incubating granule neurons from 3 days in culture (DIC) with 150 ng/ml neuritin for 24 h produced the most significant increase in I_A densities.

The effects of neuritin on the activation and inactivation properties of I_A were also explored using corresponding experimental protocols (see "Experimental Procedures"). I_A recorded using an activation protocol from neurons maintained in control medium or in medium containing 150 ng/ml neuritin were presented in Fig. 2A. The I_A activation curve was obtained by plotting normalized conductance as a function of the command potential (Fig. 2B). As shown in Fig. 2B, incubation with 150 ng/ml neuritin for 24 h did not affect the activation curve. The current was half-activated at -2.91 ± 1.98 mV ($n = 17$) and

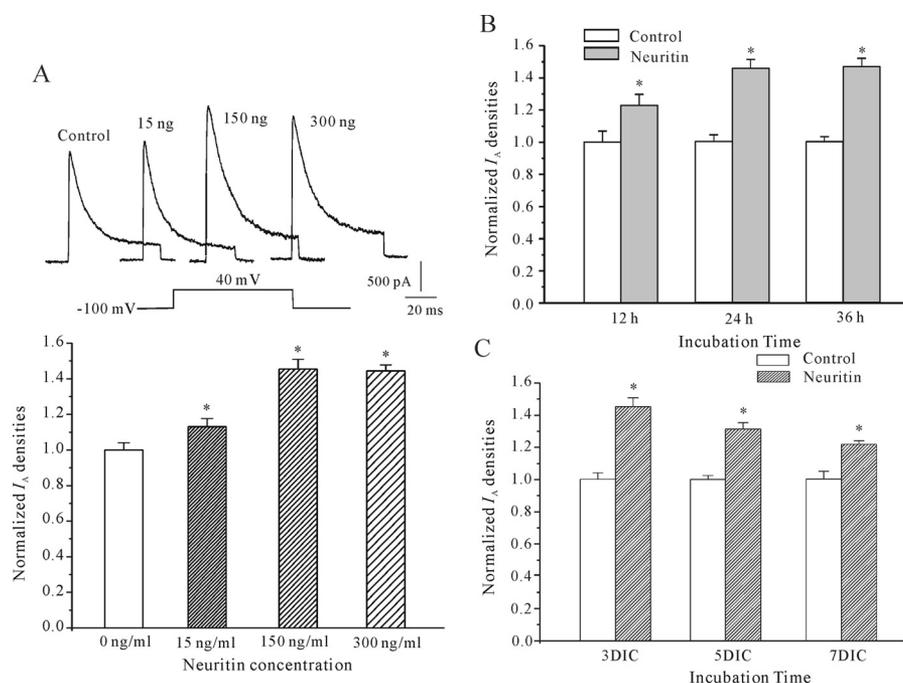


FIGURE 1. Neuritin enhanced I_A amplitudes in a concentration- and time-dependent manner in CGNs. *A*, I_A obtained from neurons maintained in control medium or medium with different concentration of neuritin. I_A was elicited by depolarizing pulses to +40 mV from a holding potential of -100 mV in the presence of 20 mM triethanolamine. *Upper panel* shows representative recording sample; *lower panel* shows statistical analysis. *B*, I_A densities in CGNs maintained for different incubation times in control medium or in medium containing 150 ng/ml neuritin. *C*, I_A densities in CGNs of different DIC maintained for control medium or in medium containing 150 ng/ml neuritin. Data are shown as means \pm S.E. (error bars). *, $p < 0.05$ compared with the corresponding control by unpaired *t* test.

-6.14 ± 2.27 mV ($n = 23$) for the control and neuritin-treated groups, respectively ($p > 0.05$). These data suggest that neuritin treatment does not alter the steady-state activation of the voltage dependence of the I_A .

Similarly, the inactivation curve of I_A was not significantly shifted after incubation with 150 ng/ml neuritin for 24 h (Fig. 2, *C* and *D*). The half-maximal inactivation voltage for the control and neuritin-treated groups was -74.43 ± 0.92 mV ($n = 25$) and -71.47 ± 0.93 mV ($n = 19$), respectively ($p > 0.05$). These data indicate that neuritin does not affect I_A inactivation kinetics. Together, electrophysiological recordings demonstrate that the neuritin-mediated enhancement of I_A amplitudes is not associated with the modification of I_A activation or inactivation properties.

Next, we hypothesized that neuritin-induced I_A densities might be due to up-regulation of potassium channel expression levels. Key α -subunits account for the I_A in CGNs include Kv4.2, Kv4.3, and Kv1.1 (37). Antibodies to Kv4.2, Kv4.3, and Kv1.1 α -subunit were used to measure their protein expression levels after incubation of CGNs with neuritin. Western blots indicated that protein levels of Kv4.2 were significantly enhanced following incubation of CGNs with neuritin for 24 h and from 3 to 7 DIC. There was, however, no significant change in the protein expression levels of Kv4.3 and Kv1.1 at 3–7 DIC (Fig. 3*A*). Kinetics of Kv4.2 induction was in parallel to the increase in I_A amplitudes upon neuritin treatment (Fig. 3*B*). Furthermore, specific primers to amplify Kv4.2 were used to measure mRNA expression levels by quantitative PCR after incubation with and without neuritin. Quantitative PCR revealed that the levels of Kv4.2 α -subunit mRNA were significantly increased (Fig. 3*C*). Taken together, these data indicate

that neuritin increases the I_A densities by up-regulation of the expression of Kv4.2.

Neuritin-induced I_A Densities and Increased Expression of Kv4.2 α -Subunit Are Sensitive to ERK or mTOR Inhibitors— Previous studies highlighted a critical role of extracellular signal-regulated kinase (ERK) cascades after synaptic activation (19, 20). We asked whether the ERK signaling pathway was activated by neuritin by measuring the levels of phosphorylation of ERK (pERK) (21, 22). Western blots showed that phosphorylation of both ERK1 (44 kDa) and ERK2 (42 kDa) was significantly increased by neuritin. After 15 min, 30 min, and 24 h treatment of neuritin, the levels of pERK relative to untreated control were 275, 377, and 27%, respectively (Fig. 4*A*). The effect of ERK activation by neuritin was ascertained by using MEK inhibitor U0126. As shown in Fig. 4*B*, incubation with U0126 completely abolished the effects of neuritin on the induction of I_A densities. In the presence of U0126, I_A densities induced by neuritin were reduced from 45.5% to 21.3% ($n = 41$, $p < 0.05$). Notably, administration of U0126 also blocked neuritin-induced Kv4.2 α -subunit protein levels (Fig. 4, *C* and *D*) and mRNA expression (Fig. 4*E*). The expression levels of the Kv4.2 α -subunit and mRNA level were decreased to $19.5 \pm 0.1\%$ ($n = 4$) and $0.08 \pm 0.03\%$ ($n = 4$), respectively. These data indicate that the ERK signaling pathway is required for the up-regulation of I_A densities and increased Kv4.2 expression by neuritin.

In addition to the ERK signaling, we also determined whether the mTOR pathway, which was reported recently to affect protein synthesis and relevance to synaptic plasticity (23), is required for the up-regulation of I_A densities and Kv4.2 α -subunit expression. After neuritin treatment, the phosphorylation

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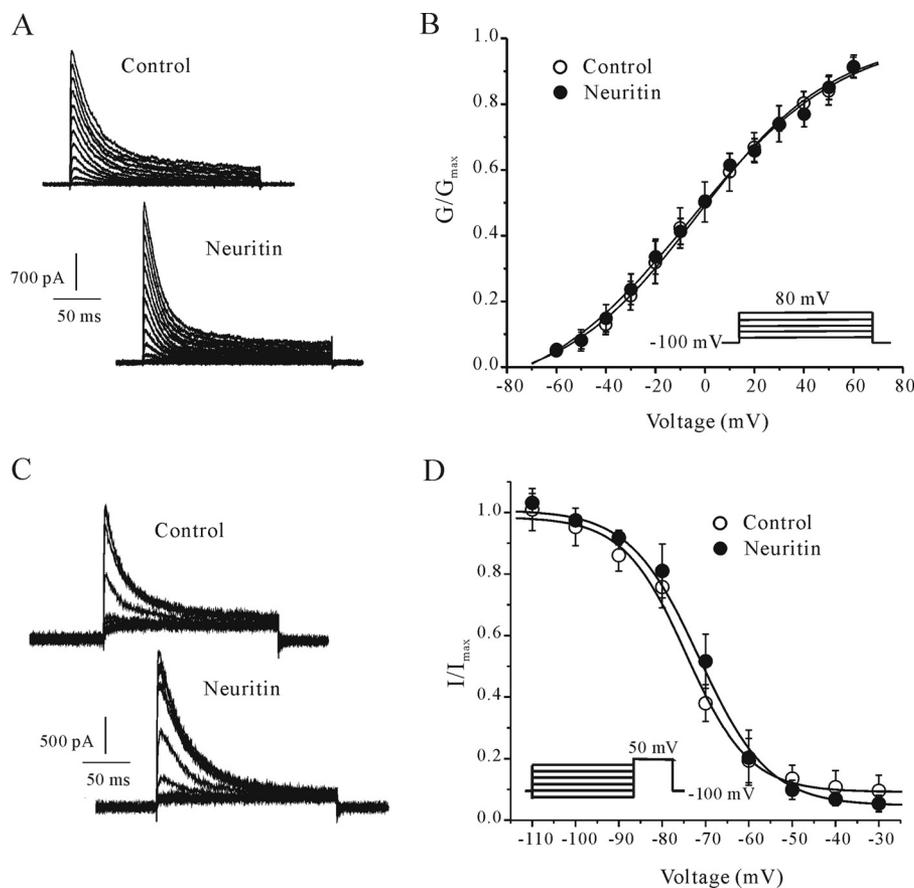


FIGURE 2. Neuritin did not alter the steady-state activation and inactivation properties of CGN I_A channels. *A*, I_A recorded using an activation protocol (see "Experimental Procedures") from neurons maintained in control medium or medium containing 150 ng/ml neuritin. *B*, steady-state activation curves of I_A obtained by plotting the normalized conductance as a function of command potential obtained from control and neuritin-treated groups. Data points were fitted using the Boltzmann function. Data points are shown as means \pm S.E. (error bars; $n = 18$ for the control group and $n = 22$ for the neuritin-treated group). *C*, steady-state inactivation curves for I_A obtained from control and neuritin-treated CGNs using inactivation protocols (see "Experimental Procedures"). Data points were fitted using the Boltzmann function. Data points are shown as means \pm S.E. ($n = 21$ for the control group and $n = 23$ for the neuritin-treated group).

level of mTOR (pmTOR) was significantly increased to 15.1% ($n = 5$) and 18.0% ($n = 5$) at 18 and 24 h, respectively (Fig. 5A). Activation of mTOR by neuritin was confirmed with the pharmacological inhibitor rapamycin. Fig. 5B showed that blockade of mTOR activity by rapamycin prevented neuritin-mediated increase of I_A densities and increased Kv4.2 protein expression. In the presence of rapamycin, I_A densities increased by neuritin were reduced from 45.5% to -5.3% ($n = 21$, $p < 0.05$). Similarly, the expression levels of the Kv4.2 α -subunit protein (Fig. 5, C and D) and mRNA level (Fig. 5E) were decreased to $-12.4 \pm 0.13\%$ ($n = 5$) and $16.6 \pm 0.04\%$ ($n = 5$), respectively. These data indicate that the mTOR pathway is also required for the up-regulation of I_A densities and increased Kv4.2 α -subunit expression by neuritin.

We also determined phosphorylation of Akt (pAkt), an upstream kinase of the mTOR pathway (23). Western blots indicated that phosphorylation level of mTOR and Akt in granule cells were increased significantly by treatment with neuritin (Fig. 5A). Quantitative analysis showed that the level of pAkt began to rise after neuritin treatment of 1 h and reached optimum activation by 6 h. The relative increased levels of Akt phosphorylation were 12.4% ($n = 4$) and 25.4% ($n = 4$), respectively. These data indicate that neuritin activates mTOR via the Akt kinase.

The role of Akt on neuritin-mediated induction of I_A densities and Kv4.2 expression was further ascertained by using pharmacological inhibitor LY294002. Fig. 5B showed that blockade of Akt activity by LY294002 inhibited neuritin-induced I_A densities and up-regulation of Kv4.2 protein expression. In the presence of LY294002, I_A densities increased by neuritin were reduced from 45.5% to 5.6% ($n = 17$, $p < 0.05$). Similarly, the induction of Kv4.2 α -subunit protein (Fig. 5, C and D) was decreased to $3.1 \pm 0.04\%$. These data indicate that Akt and mTOR pathways are required for the up-regulation of I_A densities and induction of Kv4.2 α -subunit expression by neuritin.

Neuritin Increased I_A and Kv4.2 Expression by Activation of Insulin Receptor—Previous studies indicated that the ERK and mTOR pathways were targets of growth factor activation such as NGF and insulin receptor (IR). Because some studies reported that neuritin is downstream in NGF-mediated neurite outgrowth (24), we hypothesized that insulin could mimic the induction effect of neuritin. Indeed, administration of insulin (50 or 150 ng/ml) induced I_A densities and increased Kv4.2 protein expression. In the presence of insulin, the levels of I_A densities and Kv4.2 protein level were increased by $39.3 \pm 0.02\%$ ($n = 16$) and $47.3 \pm 0.14\%$ ($n = 5$), respectively (Fig. 6, A, C, and D). These data indicate a role of

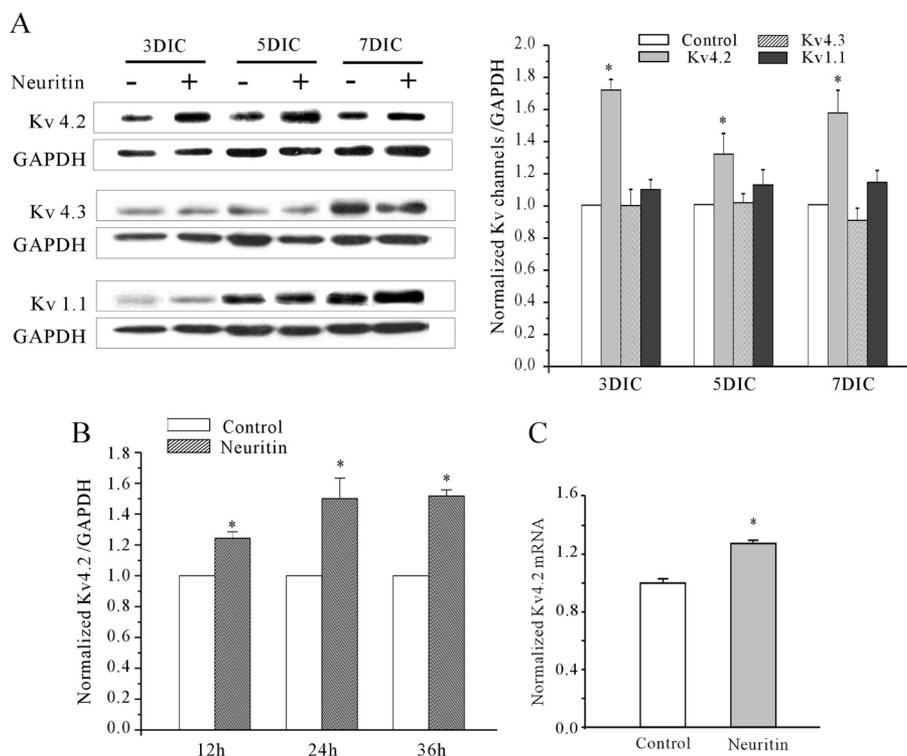


FIGURE 3. Neuritin increased the expression levels of Kv4.2 α -subunit rather than Kv4.3 or Kv1.1 in CGNs. *A*, Western blot showing effect of 150 ng/ml neuritin on different Kv channels expression levels in CGNs with different DIC. *Left*, representative Western blots. *Right*, statistical analysis. *B*, Western blot results of Kv4.2 α -subunit protein expression levels in CGNs, showing the effect of incubating with 150 ng/ml neuritin for 12–36 h. Chemiluminescent signals were detected using the ChemiDoc XRS System. *, $p < 0.05$ compared with corresponding control by unpaired t test. *Error bars*, S.E. *C*, Kv4.2 mRNA expression levels in control and neuritin-treated groups as measured by quantitative RT-PCR. Data were obtained from five independent experiments. *, $p < 0.05$ compared with corresponding control by unpaired t test.

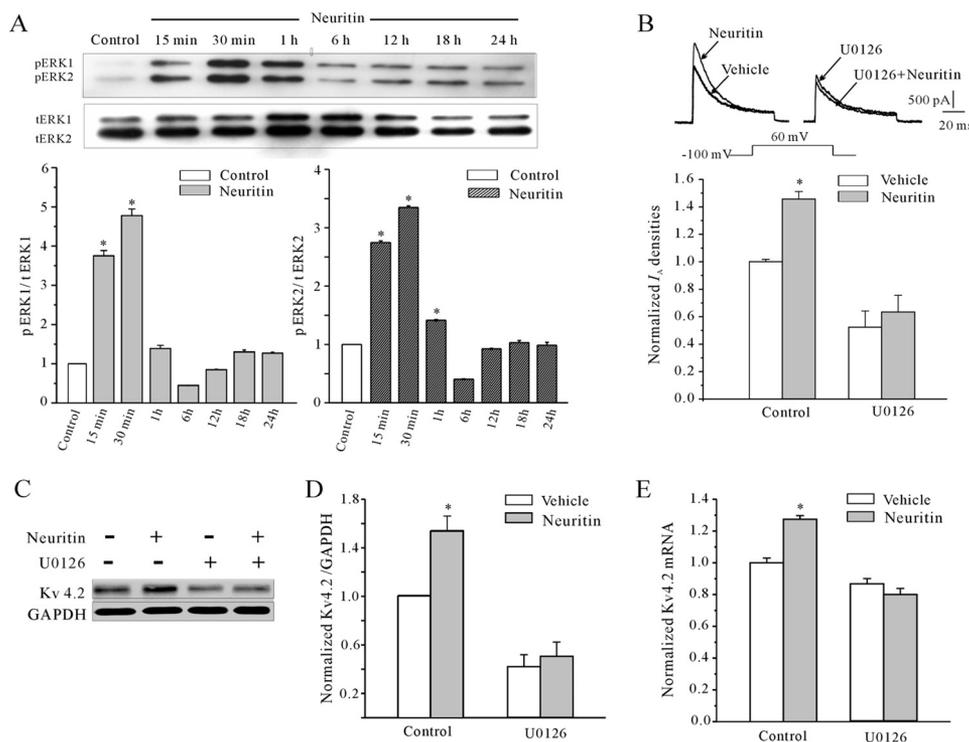


FIGURE 4. Neuritin-induced I_A densities and the increased expression of Kv4.2 α -subunit were dependent the ERK signaling pathway. *A*, Western blot showing the levels of phosphorylated ERK1 (pERK1) and phosphorylated ERK2 (pERK2) in CGNs incubated with 150 ng/ml neuritin for 15 min to 24 h. Chemiluminescent signals were detected by exposure to x-ray film. *B*, effect of MEK inhibitor, U0126, on neuritin-induced up-regulation of I_A densities. *, $p < 0.05$ compared with vehicle control (medium without neuritin) by unpaired t test. *Error bars*, S.E. *C* and *D*, effect of U0126 on neuritin-induced up-regulation of Kv4.2 protein level. *E*, effect of U0126 on neuritin-induced up-regulation of Kv4.2 mRNA expression measured by quantitative RT-PCR experiments. Data obtained from five independent experiments are shown as means \pm S.E. *, $p < 0.05$ compared with corresponding control by unpaired t test.

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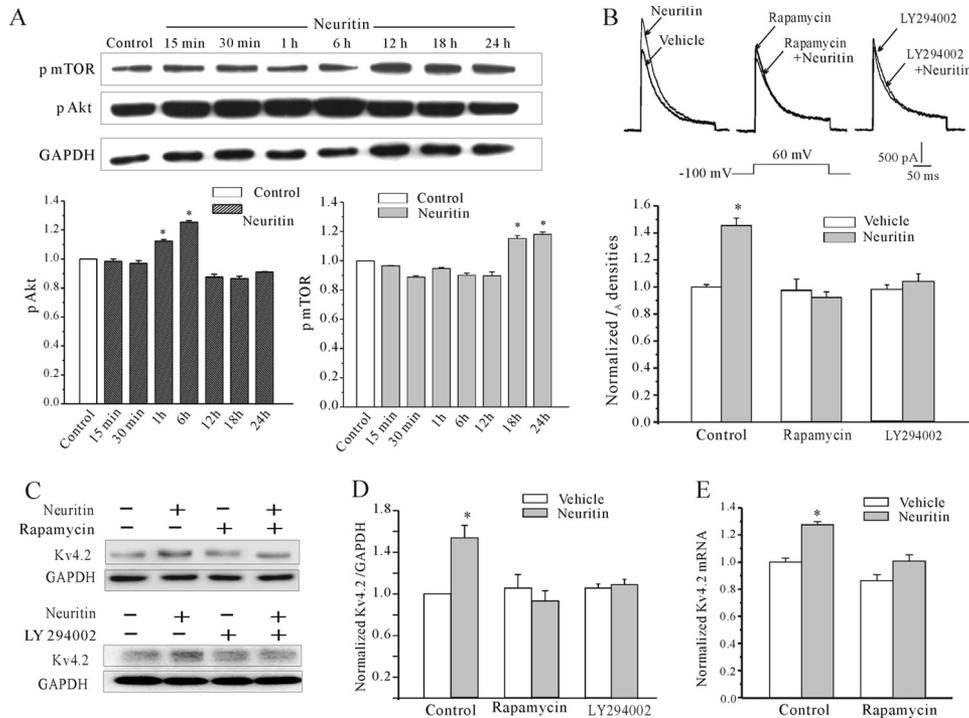


FIGURE 5. Neuritin-induced I_A densities and the increased expression of $Kv4.2$ α -subunit were dependent on the Akt/mTOR signaling pathway. *A*, Western blot showing the levels of activated Akt (pAkt) and activated mTOR (p-mTOR) in CGNs incubated with 150 ng/ml neuritin for 15 min to 24 h. Chemiluminescent signals were detected with the ChemiDoc XRS System. *B*, effect of mTOR inhibitor, rapamycin, and Akt inhibitor LY294002 on neuritin-induced up-regulation of I_A densities. *C* and *D*, effect of rapamycin and LY294002 on neuritin-induced up-regulation of $Kv4.2$ protein level. *E*, effect of rapamycin on neuritin-induced up-regulation of $Kv4.2$ mRNA expression measured by quantitative RT-PCR experiments. Data are shown as means \pm S.E. (error bars). *, $p < 0.05$ compared with corresponding control by unpaired *t* test.

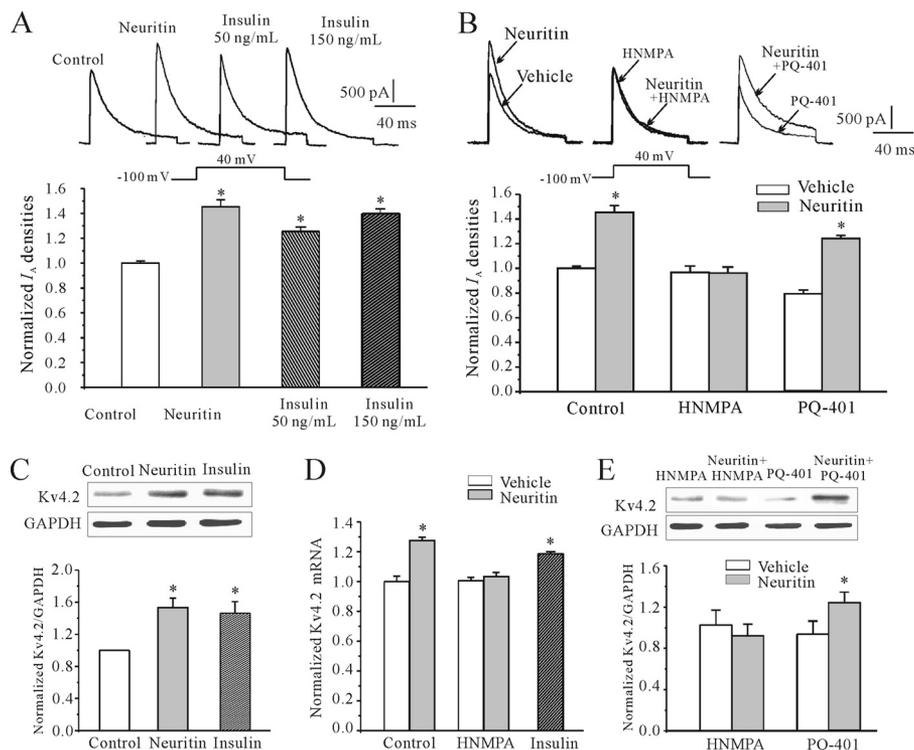


FIGURE 6. Insulin receptor was needed for neuritin-induced I_A densities and increased $Kv4.2$ α -subunit expression in CGNs. *A*, insulin mimicking the effect of neuritin and inducing I_A densities in CGNs. *B*, effects of insulin receptor blocker HNMPA and IGF-1 receptor blocker PQ-401 on the neuritin-induced increase of I_A densities. *C*, insulin mimicking the effect of neuritin and inducing $Kv4.2$ protein level in CGNs. *D*, effects of insulin and HNMPA on $Kv4.2$ mRNA expression measured by quantitative RT-PCR experiments. *E*, effects of HNMPA and PQ-401 on the neuritin-induced increase of $Kv4.2$ protein level of CGNs. Data were obtained from five independent experiments. *, $p < 0.05$ compared with vehicle (medium without neuritin) control by unpaired *t* test.

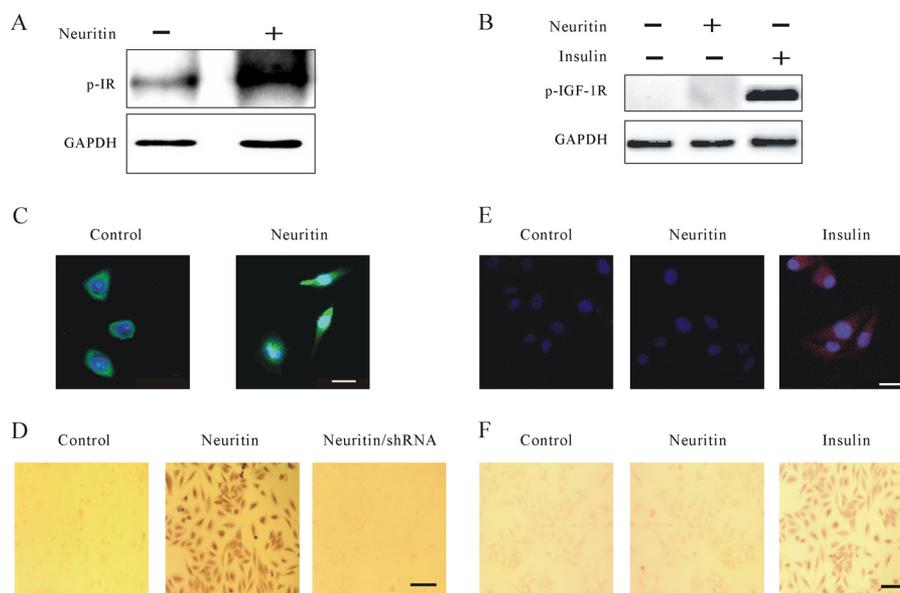


FIGURE 7. Effect of neuritin on IR or IGF-1 receptor activation in CGNs and HeLa cells. *A* and *B*, immunoprecipitation assays showing that neuritin induced phosphorylation of IR, but not IGF-1 receptor in CGN lysate. Effect of insulin on phosphorylation of IGF-1 receptor is also shown. *C*, immunofluorescence to detect phosphorylated IR in HeLa cells. Effect of neuritin is also shown. Representative immunofluorescence images from four independent experiments are shown. *Scale bar*, 30 μm . *D*, immunohistochemical studies with DAB staining detecting the amount of phosphorylated IR in HeLa cells after neuritin stimulation. Effect of shRNA to reduce IR expression is also shown. *Scale bar*, 120 μm . Similar data were obtained from four independent experiments. *E*, immunofluorescence to detect phosphorylated IGF-1 receptor in HeLa cells. Effect of neuritin or insulin is also shown. Representative immunofluorescence image from four independent experiments are shown. *Scale bar*, 30 μm . *F*, immunohistochemical studies with DAB staining to detect phosphorylated IGF-1 receptor in HeLa cells. Effect of neuritin or insulin is also shown. Similar data were obtained from four independent experiments.

insulin receptor pathway in the induction of I_A densities and Kv4.2 protein level.

Next, we asked whether blockage of the insulin pathway would affect the induction of I_A densities and increased Kv4.2 protein level by neuritin. We used HNMPA, which inhibits insulin receptor Tyr kinase, to address this question. Blockade of IR activity by HNMPA reduced neuritin-mediated induction of both I_A densities and Kv4.2 protein level (Fig. 6, *B* and *C*). In the presence of HNMPA, I_A densities increased by neuritin were significantly reduced from $45.5 \pm 0.05\%$ to $-0.01 \pm 0.05\%$ ($n = 23$, $p < 0.05$). Similarly, induction of the Kv4.2 α -subunit and mRNA expression were decreased to $-10.3 \pm 0.14\%$ and $0.27 \pm 0.02\%$, respectively (Fig. 6, *A* and *B*). These data indicate that neuritin may channel through the IR pathway for the induction of I_A densities and Kv4.2 protein level.

We also tested whether the IGF-1 receptor pathway was involved in neuritin-mediated effect using PQ-401 as antagonist of the IGF-1 receptor pathway. Unlike the effect of HNMPA, PQ-401 did not affect neuritin-induced up-regulation of I_A densities nor increased expression of Kv4.2 (Fig. 6, *B* and *E*). These data indicate that the IR, but not the IGF-1 receptor, may mediate the neuritin-induced effect on the increase of I_A densities and expression of Kv4.2 α -subunit.

Given that neuritin is an orphan ligand and its signaling receptor is not known, we hypothesized that neuritin might elicit IR activation. To test this hypothesis, we examined whether neuritin could stimulate Tyr phosphorylation of the IR by immunoprecipitation and immunocytochemistry. Immunoprecipitation assays showed increased amounts of phosphorylated IR, but not IGF-1 receptor, after neuritin treatment (Fig. 7, *A* and *B*). Immunofluorescence assays and immunocytochemistry further indicated that neuritin induced Tyr phosphoryla-

tion of the IR in HeLa cells (Fig. 7, *C* and *D*). Activation of the IR by neuritin, however, was reduced after transfection of shRNA to reduce IR expression in HeLa cells (Fig. 7*D*). As expected, neuritin did not induce Tyr phosphorylation of the IGF-1 receptor in HeLa cells (Fig. 7, *E* and *F*), supporting that neuritin activates the IR.

To further ascertain the effect of neuritin on IR activation, we determined the phosphorylation of IRS-1 and ERK, downstream effectors of IR signaling. Western blots showed that neuritin increased phosphorylation of IRS-1. Pretreatment of HNMPA, but not PQ-401, reduced the neuritin-induced Tyr phosphorylation of IRS-1 in HeLa cells (Fig. 8*A*). Similarly, neuritin increased the phosphorylation of both ERK1 and ERK2 after a 15-min stimulation. Pretreatment of HNMPA, however, reduced the neuritin-induced activation of ERK in HeLa cells (Fig. 8*B*). Taken together, these data indicate that the insulin receptor is involved in previously unrecognized neuritin-mediated signaling, which increases I_A densities and Kv4.2 protein level in native cerebellar granule cells (Fig. 9).

DISCUSSION

Neuritin is an important neurotrophin and plays multiple roles in the process of neural development, synaptic plasticity, and synaptic maturation (1, 2, 4, 5). The receptor for binding neuritin and its downstream effector signal pathway, however, are poorly investigated. Here, we show, for the first time, that neuritin could activate IR and ERK/mTOR signaling pathways to increase I_A amplitude and the expression of Kv4.2 in CGNs (Fig. 9).

CGNs contain two major voltage-dependent outward K^+ currents: a delayed rectifier potassium current (I_K) and a fast transient potassium current (I_A). Neuritin treatment for 24 h

Neuritin Up-regulates Kv4.2 and I_A via Insulin Receptor

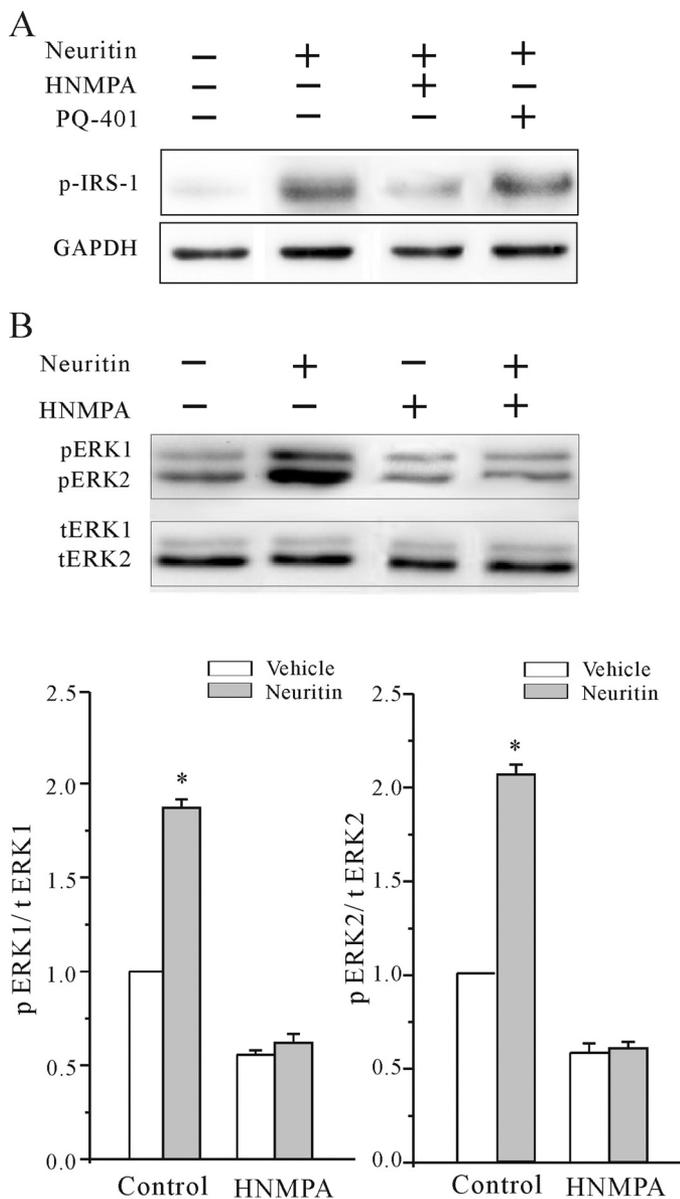


FIGURE 8. Effect of neuritin on IR or IGF-1 receptor activation in HeLa cells. A, Western blotting showed neuritin-induced phosphorylation of IRS-1 in HeLa cells. Effect of HNMPA and PQ-401 is also shown. B, Western blotting showed that neuritin increased phosphorylation of both ERK1 and ERK2 in HeLa cells. Effect of insulin receptor blocker HNMPA is also shown. *, $p < 0.05$ compared with vehicle control (medium without neuritin) by unpaired t test. Data were obtained from three independent experiments.

failed to modulate I_K in CGNs (data not shown). We thus examined the effect of neuritin on I_A in this study. I_A has been described in neurons from many regions of the central nervous system (25–27). Short term modulation of I_A densities could arise from a rapid mechanism due to changes in voltage-gating properties or intracellular trafficking of the channel proteins (28–31). Alternatively, a long term mechanism by up-regulation of channel mRNA and protein expression could be involved (32). We observed that neuritin did not affect I_A amplitude when it was applied acutely. Neuritin, however, significantly increased I_A densities without modification of I_A activation and inactivation properties, indicating that a long term mechanism of action by neuritin is involved.

Recent studies have suggested that the Kv4 *shal* family forms the major components of the I_A in the central nervous system (33, 34). Expression of the Kv4 family was found in CGNs, pyramidal neurons of the hippocampus and cortical neurons (32, 35, 36). We have shown previously that Kv4.2, Kv4.3, and Kv1.1 are the main α -subunits expressed in rat CGNs (37). Upon neuritin treatment, only Kv4.2 α -subunit mRNA and protein levels were significantly increased. More importantly, the kinetics of Kv4.2 induction was in parallel to the increase in I_A upon neuritin treatment. These data indicate that neuritin specifically increases the expression of Kv4.2.

Previous studies using cultured CGNs showed that subcellular redistribution of Kv4.2 from the soma to the dendrites and synapses is induced by synapse formation. Regulation of Kv4.2 can be further potentiated by synaptic activity (38). Indeed, expression of Kv4.2 in CGNs was increased with the duration of the microexplant culture period (12), in parallel to our findings that a concomitant increment of I_A and Kv4.2 from 3 to 5 DIC. Notably, after neuritin treatment, the levels of I_A and Kv4.2 from 3 DIC CGNs were similar to that of 5 DIC CGNs. Similar effect of neuritin on I_A and Kv4.2 expression was also found in cortical neurons (data not shown). These observations suggest that the effect of neuritin on Kv4.2 expression may be developmentally regulated and associated with neuronal maturation and synaptic plasticity as previous reported (38). The possible roles of neuritin on neuronal maturation via regulating Kv4.2 expression warrant further investigations.

Many studies have shown that ERK is important for regulation of neuronal function, including regulation of synaptic plasticity and long term memory formation (39, 40). One potential mechanism of this regulation by ERK is indirect via long term modulation of gene transcription and channel protein expression. For example, a previous study indicated that ERK activation could dually regulate Kv4.2 at the transcriptional as well as posttranslational level (41). Another possible mechanism is that ERK could directly phosphorylate subunits of ion channels to alter intrinsic channel properties to regulate membrane potential. The phosphorylation of Kv4 channel subunits by ERK was characterized by the changing of gating properties of Kv4.2 (29, 42) upon acute regulation of I_A by growth factors (43, 44). Our data show that neuritin does not alter the gating properties of I_A . Neuritin, however, modulates I_A by increasing Kv4.2 expression. Importantly, blocking activation of the ERK pathway eliminated the neuritin-induced effect on Kv4.2 expression. These data indicate that neuritin channels through the ERK pathway to control Kv4.2 gene transcription.

In addition to the ERK pathway, mTOR signaling is also involved in neuronal development. The mTOR pathway is also essential for several forms of synaptic plasticity for learning and memory (45, 46). Similar to the ERK pathway, mTOR kinase activity is modulated in response to various stimuli, such as neurotrophic factors and mitogenic hormones, to achieve transcriptional and translational control (23, 47–49). In this study, we show that mTOR-specific inhibitor rapamycin blocks neuritin-mediated induction of Kv4.2 mRNA and protein as well as subsequent increase in I_A . Thus, neuritin also channels through the mTOR pathway to control Kv4.2 gene transcription.

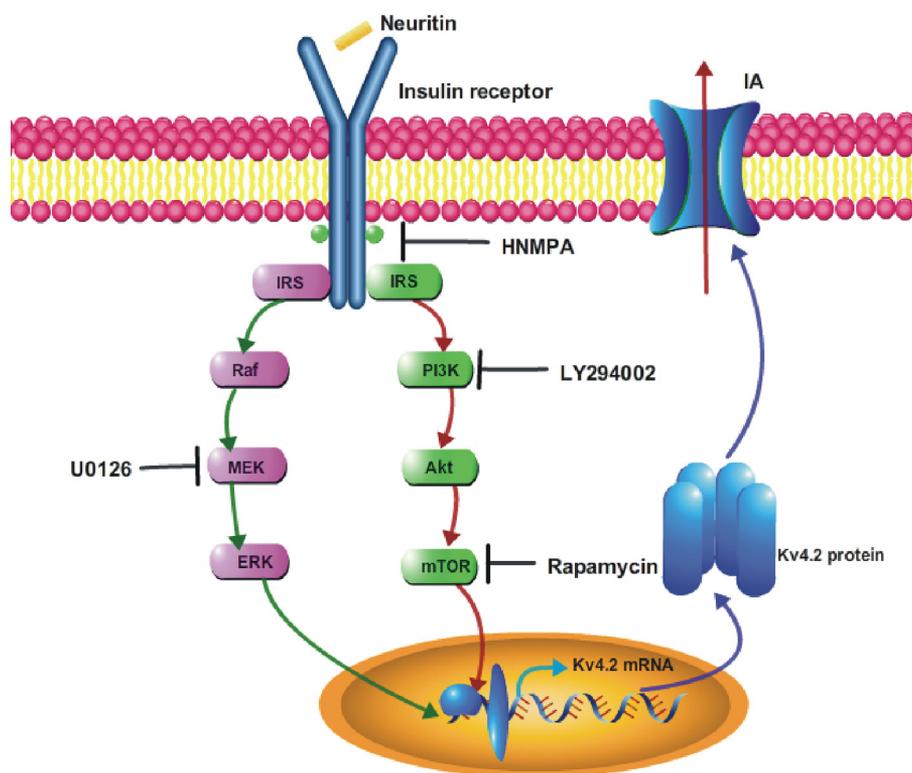


FIGURE 9. Schematic illustration depicting the mechanisms of neuritin in modulating expression of Kv4.2 and the subsequent increase in I_A amplitude.

Recent studies demonstrated that mTOR and ERK pathways cooperate with one another to affect many cellular functions. For example, cooperation between the ERK and mTOR pathways stimulates protein translation and induces tumor progression in a glioblastomas mouse model (50). In hippocampal neurons, it appears that cooperation of ERK and mTOR pathways regulates *de novo* protein synthesis, which is a prerequisite for long term potentiation (51). In this study, we did not explore the cross-talk between the mTOR and ERK signaling pathway in neuritin-induced Kv4.2 gene transcription. However, we believe that neither the ERK nor the mTOR pathway alone is sufficient to increase Kv4.2 gene transcription because administration of U0126 or rapamycin completely eliminated the effect of neuritin. Possibly, ERK and mTOR may assert a transcriptional cooperation on Kv4.2 induction, similar to the optimal activation operated by nuclear factor of activated T-cells (NFAT) transcription factor (52). Alternatively, ERK and mTOR could regulate at the transcriptional and translational level, respectively. For example, ERK-induced Kv4.2 transcripts may encode 5'-terminal oligopyrimidine tract for subsequent translational control by mTOR (53). The latter may account for the difference in temporal kinetic of ERK and mTOR activation upon neuritin stimulation. Nonetheless, the dual requirements on Kv4.2 induction suggest that neuritin activates both ERK and mTOR signalings.

Although previous studies implied that neuritin may function as a ligand, few investigation identified the receptor by which neuritin transduces its signal (2, 54). Our results show that neuritin activates both ERK and mTOR signalings for Kv4.2 induction. Activation of both ERK and mTOR by neuritin

displays similar mechanism of the insulin receptor pathway. Indeed, insulin also increases I_A and Kv4.2 expression. Mechanistically, we show that neuritin elicits phosphorylation of IR in CGNs and HeLa cells within 15 min, suggesting a direct effect of neuritin on activation of IR. In addition, Tyr-phosphorylated IRS-1, the immediate downstream effector of IR, was found upon neuritin stimulation. Plausibly, neuritin, as a neurotrophic hormone, is acting analogously to other insulin ligands (e.g. IGF-1 or insulin-like factors) to stimulate ERK and mTOR via phosphorylation of IR and IRS-1.

Although neuritin elicits activation of IR, the effect of neuritin on potentiation of I_A and Kv4.2 expression is not sensitive to IGF-1 receptor inhibitor PQ-401. In addition, administration of neuritin raises Tyr phosphorylation of IR, but not the IGF-1 receptor. Possibly, the extent of glycosylation of the IR and differential expression of IR α isoforms in neurons, compared with glial cells or peripheral tissues (55), offer a wide range of affinity and selectivity to account for the unique activation of IR in CGNs by neuritin.

Insulin receptor signaling, which has been studied extensively in peripheral organ systems, has recently been shown to play important roles in the central nervous system (55). Especially, brain insulin receptor signaling reportedly plays diverse roles in the CNS, including regulation of synaptic plasticity, neuronal survival, learning, and memory (56). Given the neurotrophic role of neuritin, future studies to investigate whether neuritin exploit distinct neuronal insulin receptors for neuroprotection are warranted.

In conclusion, we show that neuritin potentiates I_A amplitudes via increasing expression of Kv4.2. The effect of neuritin

on Kv4.2 expression is channeled through the IR to activate ERK and mTOR pathways. Thus, the neuritin → insulin receptor → Kv4.2 → I_A axis may play an important role in neuronal development and protection.

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