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In the previous study, we demonstrated that a glucocorticoid corticosterone (CORT) and a glutamate receptor agonist kainic acid (KA) promote neurite outgrowth in axotomized rat dorsal root ganglion (AX-DRG) neurons. In this study we further elucidate the possible mechanisms involved in these synergistic effects. Immunofluorescence staining also revealed that CORT+KA increased GAP-43 immunoreactivities especially along neurites and growth cones of DRG neurons. Another therapeutically used glucocorticoid methylprednisolone also showed profound synergy with KA in increasing GAP-43 expression. In addition, CORT+KA-increased GAP-43 expression was reduced by protein kinase C (PKC) inhibitor RO-318220, glucocorticoid receptor (GR) antagonist RU486, and mineralcorticoid receptor (MR) antagonist spironolactone. The neuite growth rate promoted by the CORT+KA treatment was completely abolished by both RU486 and the AMPA/KA receptor antagonist CNQX, and was partially attenuated by spironolactone. Furthermore, PKC inhibitor attenuated the CORT+KA-increased neurite growth when applied immediately before the KA treatment. We compared the expression of two proteins, growth-associated protein (GAP-43) and a myelin-associated growth inhibitory protein NogoA receptor (NgR) in DRG neurons. Western blot analysis showed that combined treatment of CORT and KA results in increased expression of GAP-43 and decreased expression of NgR in DRG neurons. Taken together, these results suggest that CORT and KA activate their specific receptors to promote neurite growth synergistically, which might involve counterbalanced expressions of GAP-43 and NgR via a PKC-dependent signaling pathway.

Key words: Corticosterone, Kainic Acid, Glucocorticoid Receptor, Glutamate receptor, Protein kinase C, Neurite outgrowth, Dorsal Root Ganglion

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CORT is the main adrenal glucocorticoids induced by stress in rats. Therapeutic use of high concentration of synthetic glucocorticoids in clinical treatment of spinal cord injury suggests that pharmacological action of glucocorticoids might be beneficial for nerve repair (Bracken et al., 1990; Chen et al., 1996). KA, a glutamate receptor (GluR) agonist acting on AMPA/KA receptors, has been shown to influence several aspects of neuronal function in developing nervous system such as growth, differentiation, synaptogenesis, and neurotransmission (Zafra et al., 1990; Lee et al., 2000). CORT has been reported to enhance GluR-mediated cell signaling in hippocampal neurons (Elliott and Sapolsky, 1993; Sapolsky, 1986). In the previous study, we also demonstrated that combined treat CORT and KA synergistically promoted neurite outgrowth in AX-DRG neurons (Tsai et al., 2002). However, what critical signal trigger the synergistic effects remain unclear.

The growth-associated protein (GAP-43) is a presynaptic protein. Its express is largely restricted to the nervous system. GAP-43 bind to calmodulin, when the local calcium level rise, calmodulin is liberated from it. This can trigger GAP-43 phosphorylation by PKC to regulate neuritogenesis, synaptic plasticity, and nerve regeneration (Oestreicher et al., 1997). Nogo has been identified as a component of the central nervous system (CNS) myelin that interacts with Nogo-66 receptor (NgR); both proteins are widespread in the adult mouse CNS and spinal cord. Nogo/ NgR system activation has been suggested to play an important role in limiting axonal growth, and the inhibition might through the down regulation or blockade of the activity of proteins critical for growth cone expansion such as GAP-43. (Chen et al., 2000; Grand-Pre et al., 2000; Cherry and Bor, 2002).

Although the expression of GAP-43 was found increased by NGF and kainic acid under both in vitro and in vivo conditions (Meberg et al.,1993; Levi-Montalcini, 1987), little is known about the transcription factors that directly promote GAP-43 gene transcription (Eggen et al., 1994; Nedivi et al., 1992; Starr et al., 1994). On the other hand, there seems to be a stabilization mechanism of the GAP-43 transcript to control its steady-state expression, which is mediated by the PKC and NGF signaling pathways (Perrone-Bizzozero et al., 1993; Cao et al., 1997). Recent studies have found a neuron-specific RNA binding protein HuD, which binds to a U-rich regulatory element of the 3' untranslated region (3'UTR) of the GAP-43 mRNA and stabilizes it in embryonal cortical neurons and embryonic stem cell-derived neurons in a PKC-dependent manner (Anderson, 2001). Another stabilizing factor for GAP-43 mRNA, namely cAMP-regulated phosphoprotein-19 (ARPP-19), was also found bind to the 3' end of GAP-43 mRNA and stabilize it in PC12 cells in a protein kinase A-dependent manner (Irwin, 2002).

Regarding the signaling cascades synergistically triggered by GC and KA, PKC seems to be the potential candidate especially for its close correlation with increasing GAP-43 expression. GCs have been shown to upregulate PKC activities in the anterior pituitary gland, cerebral cortex, and hippocampus for various physiological functions (John et al., 2002; Dwivedi and Pandey, 1999). On the other hand, accumulating evidence has demonstrated that KA or activation of the KA receptor result in activation of PKC in hippocampus and cerebral cortex (Melyan et al., 2002; Kaasinen et al., 2002; McNamara and Lenox, 2000). Although other signaling pathways, such as protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) cascades, were also involved in the GAP-43 expression (Irwin, 2002, Yuan et al., 2001), they seems to be not as cohesive to both GC and KA signaling as the PKC pathway. As we also described in the previous paragraph that PKC is important in mRNA stabilization and phosphorylation of GAP-43, it is rational to initiate the signaling study on the PKC pathway.

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In this study, we demonstrate the possible mechanisms of CORT+KA-promoted neurite growth, that might involve counterbalanced expressions of GAP-43 and NgR via a PKC-dependent signaling pathway.

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Primary culture of dorsal root ganglion (DRG) neurons

Primary culture of the rat DRG neurons was prepared from adult male Sprague Dawley rats (weighing around 250g). Animals were sacrificed following overdose anesthetization with 60 mg/kg body weight of sodium pentobarbital. Approximately 35 to 40 DRGs from cervical to sacral levels were dissected from the spinal column, chopped with blade, and dissociated with 0.15% collagenase in F12 medium by being shaken in a 37°C water bath for 40 min. The dissociated tissue was spun down at 1000 rpm for 5 min, washed twice with Hepes Hanks Balanced Saline Solution (HHBSS), and further dissociated with 0.25% trypsin in HHBSS in 37°C water bath for 15 min. The digestion was stopped by adding 0.05% trypsin inhibitor, and the cell suspension was fractionated by 30% Percoll and centrifuged at 1500 rpm for 5 min. The pellet, which was enriched with DRG neurons, was washed twice with F12 medium containing 10% fetal bovine serum (FBS) and plated onto poly-L-lysin-coated 35 mm culture dishes and cultured in F12/10% FBS medium in a humidified tissue culture incubator with 5% CO_2 and maintained at 37°C. The plating day was counted as the zero day in vitro (0 DIV). Animal experiments were carried out according to the NIH Guidelines for Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Determination of neurite outgrowth

Cultured DRG neurons at 1 DIV were treated with 10 μM CORT for 16-24 hrs, followed by 1 mM KA treatment. Vehicle solutions of CORT and KA were added to the control groups. Neurite length was measured everyday from 1 DIV to 5 DIV by randomly selecting neurites and visualizing their length with a microruler. About 20 to 30 neurites in a single batch were measured, and at least 5 batches of experimental data were collected for statistical analysis. .

Immunofluorescent staining

Cultured DRG neurons at 5 DIV were washed 2 times with an Earle's Balance Salt solution (EBSS), followed by incubation with fixation solution (4% formaldehyde in 20 mM PBS) for 30 min at room temperature and permeabilized cells with pre-cold EtOH/CH_3COOH (95%: 5%) for 15 min at -20¢J. Then incubated sequentially with blocking solution, primary antibody, and biotinylated secondary antibody, the anti-GAP43 antibody and anti-NgR antibody were properly diluted for primary antibody incubation. Fluorescence development of the immunoreactive cells was performed using FITC-conjugated avidin reagent. Immunomicrograph was pictured using Olympus DP50 digital camera (Olympus, Tokyo, Japan).

Western blot analysis

Cultured DRG neurons at 5 DIV with designated treatment were harvested with ice-cold lysis buffer (50 mM Tris-HCl containing 1% glycerol, and 1% IGEPAL CA-630). Cells were sonicated to obtain total cell lysate. To detect he expression of GAP-43 and NgR, 80 μg of the protein was separated onto 10% SDS-PAGE, transferred to Hybond ECL nitrocellulose membrane, and probed with the anti-GAP 43 or NgR antibodies overnight at 4°C. The immune complex was further probed with HRP-conjugated anti-mouse or goat IgG, visualized by HRP-reactive chemiluminescence reagents, and developed on autoradiographic film. The relative density of the protein band in the Western blot was further analyzed with an electrophoresis image analysis system (Eastman Kodak Co., Rochester, NY).

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Sequential effect of glucocorticoid and KA on promoting neurite extension in AX-DRG

To investigate the neuritis outgrowth of axotomized DRG neurons in culture, CORT (10 μM) and KA (1 mM) were applied at 1 DIV or 2 DIV, the neurites growth of DRG from 1 to 6 DIV were shown in Figure 1. The synergistic effects of CORT and KA on neuritis outgrowth were observed from 3 DIV to 5DIV. Either CORT or KA treatments had no influence on the basal level of neuritis growth rate (Fig.1A). The possible mechanism of this action seems to be via activation of NGF receptors TrkA and increase of GAP-43 expressions (Tsai et al., 2002). Only application of CORT and KA at 1 and 2 DIV (C+K) sequentially can promote neuites extension, reversed the administrated order (K+C) or combined treatment of CORT and KA at 1DIV(CK1) did not act the same effect as C+K-treated group (Fig. 1B). These results reveal that CORT may prime the gene (or protein) expression necessary for KA to promote neurite outgrowth.

Blockade of GR, MR, or AMPA/KA receptor activity attenuated CORT+KA-enhanced neurotrophic actions

To examine if the synergistic effect of CORT and KA on neurite outgrowth of AX-DRG is via specific receptors, we used RU486, spironolactone, or CNQX to block the glucocorticoid, mineralcorticoid, or AMPA/KA receptor activity. As shown in Figure 2A, RU486 (1 μM), sironolactone (1 μM), or CNQX (50 μM) application alone had no significant effect on the basal level of the neurite growth, but significantly reduced the CORT+KA–enhanced neurite growth to near the basal level in all three groups (Fig. 2B-D). These results suggest that CORT and KA may activate via specific receptors to induce the neurotrophic effect. Growth of neurites often results from increased expression of growth-associated proteins GAP-43 for membrane fusion to extend the growing neurites. Immunofluorescent staining showed that GAP-43 immunoreactivity was strongly present at cell membranes and neurites in DRG neurons of both control and the CORT+KA groups, whereas the GAP-43 immunostaining on neurites and growth cones were stronger in the CORT+KA group than staining in the control group (Fig. 3). On the other hand, we used a therapeutic glucocorticoid methylprednisolone combined treatment with KA also shown a profound increased of GAP-43 expression (Fig. 5). Blockade of GR and AMPA/KA receptor activity did attenuate CORT+KA-enhanced GAP-43 expression especially at growth cones, and GAP-43 expression was strongly reduced by pre-treated MR antagonist (Fig.3). These results suggest that CORT+KA may activate through specific receptors to regulate GAP-43 espression.

PKC activity was involved in CORT+KA-enhanced neurotrophic signaling

Recently studies reveal the GAP-43 gene stability and protein phosphorylation depend on PKC activation (Oestreicher et al., 1997). We applied PKC inhibitor RO-318220 (1 μM) at different time point to evaluate the role of PKC activity in CORT+KA-triggered neurotrophic signaling. Blockade of PKC activity by RO-31822 at 1 DIV (PKCI-1) or 2DIV (PKCI-2) significantly reduced the neurite extension, and completely abolished CORT+KA–promoted neurite growth when immediately inhibited PKC activity before KA application (Fig. 4A). Immunofluorescence signal also showed that CORT+KA–enhanced GAP-43 expression was reduced by pre-blockade of PKC activity (Fig. 4B).

Counterbalanced expressions of GAP-43 and NgR in CORT+KA-treated AX-DRG

Western blot analysis showed that either CORT or KA-treated group had no significantly changes in GAP-43 expression but lightly decreased in NgR expression. However, combined treatment of CORT and KA results in increased expression of GAP-43 and decreased expression of NgR in DRG neurons (Fig. 6). These results suggest that CORT+KA-promoted neurite growth might involve counterbalanced expressions of GAP-43 and NgR.

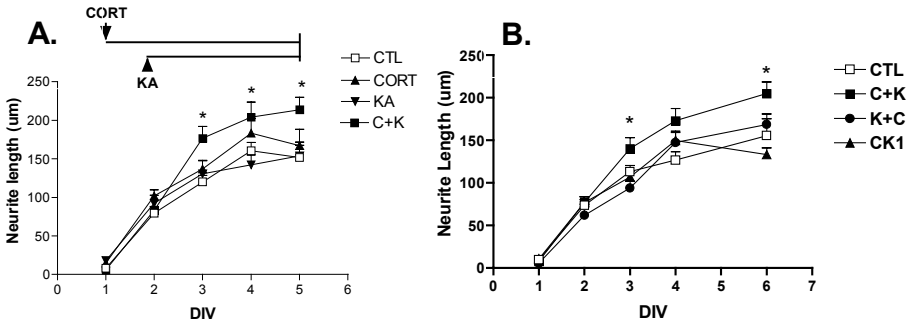

Figure 1. Sequential effect of CORT and KA on promoting neurite extension in AX-DRG. (A) Cultured dorsal root ganglion neurons were pretreated with CORT (10 μM) at 1 DIV, followed by application of KA (1 mM) at 2 DIV. **(B)** Changing of CORT and KA application sequence; control (CTL); ; CORT, 1DIV+ KA, 2DIV (C+K); 1DIV+CORT, 2DIV (K+C); CORT, 1DIV+ KA, 1DIV (CK1). Neurite length of DRG neurons was measured once a day from 1 DIV to 5 DIV under the microscope with a microruler. The data was expressed as the mean ±S.E.M. (n=50-100). **p*<0.05 as compared with the other three groups by One-way ANOVA with Newman-Keuls multiple comparison posttest.

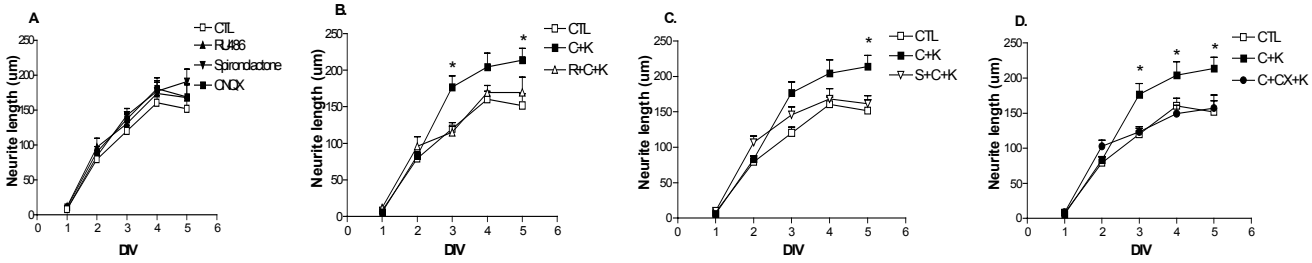

Figure 2. Blockade of GR, MR, or AMPA/KA receptor action attenuated CORT+KA-enhanced neurite outgrowth. Cultured DRG neurons at 1 DIV were treated CORT (10 μM) or combined treatment with GR antagonist RU486 (1uM) or MR antagonist spironolactone (1uM) for 30 min before CORT, 2DIV with AMPA/KA receptor antagonist CNQX (50 μM) for 30 min followed by KA (1 mM) application. **A-D** show effects of varied antagonists treat alone or combine with CORT+KA on neurite growth. The data was expressed as the mean ± S.E.M. (n=8-15). **p*<0.05 as compared with the other groups by One-way ANOVA with Newman-Keuls multiple comparison posttest. Control (CTL); CORT + KA (C+K); RU486+ CORT+KA (R+C+K); Spironolactone+CORT+KA (S+C+K); CORT+CNQX+KA (C+CX+K).

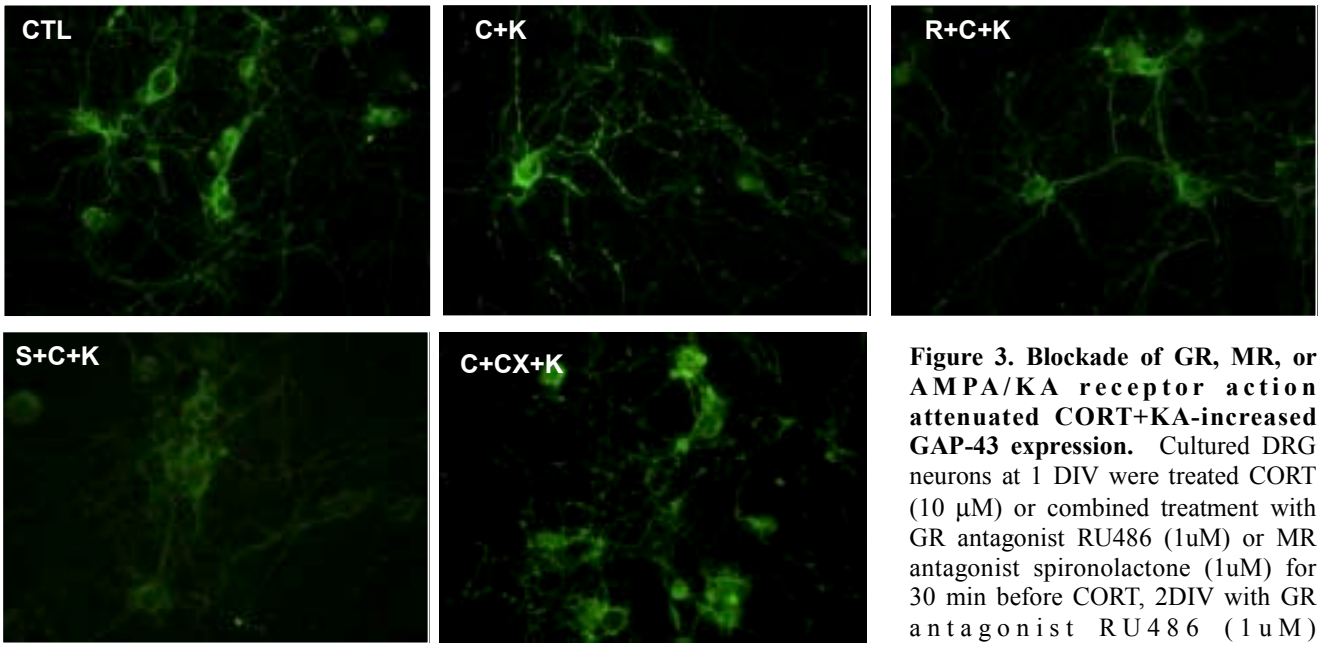

Figure 3. Blockade of GR, MR, or AMPA/KA receptor action attenuated CORT+KA-increased GAP-43 expression. Cultured DRG neurons at 1 DIV were treated CORT (10 μM) or combined treatment with GR antagonist RU486 (1uM) or MR antagonist spironolactone (1uM) for 30 min before CORT, 2DIV with GR antagonist RU486 (1uM)

or MR antagonist spironolactone (1uM) for 30 min before CORT, 2DIV with AMPA/KA receptor antagonist CNQX (50 μM) for 30 min followed by KA (1 mM) application. Immunofluoresence signal of GAP-43 were detected on DRG neurons at 5 DIV. White arrowhead indicates growth cones on neurite stained with GAP-43 immunoreactivities. Control (CTL); CORT + KA (C+K); RU486+ CORT+KA (R+C+K); Spironolactone+CORT+KA (S+C+K); CORT+CNQX+KA (C+CX+K)**.**

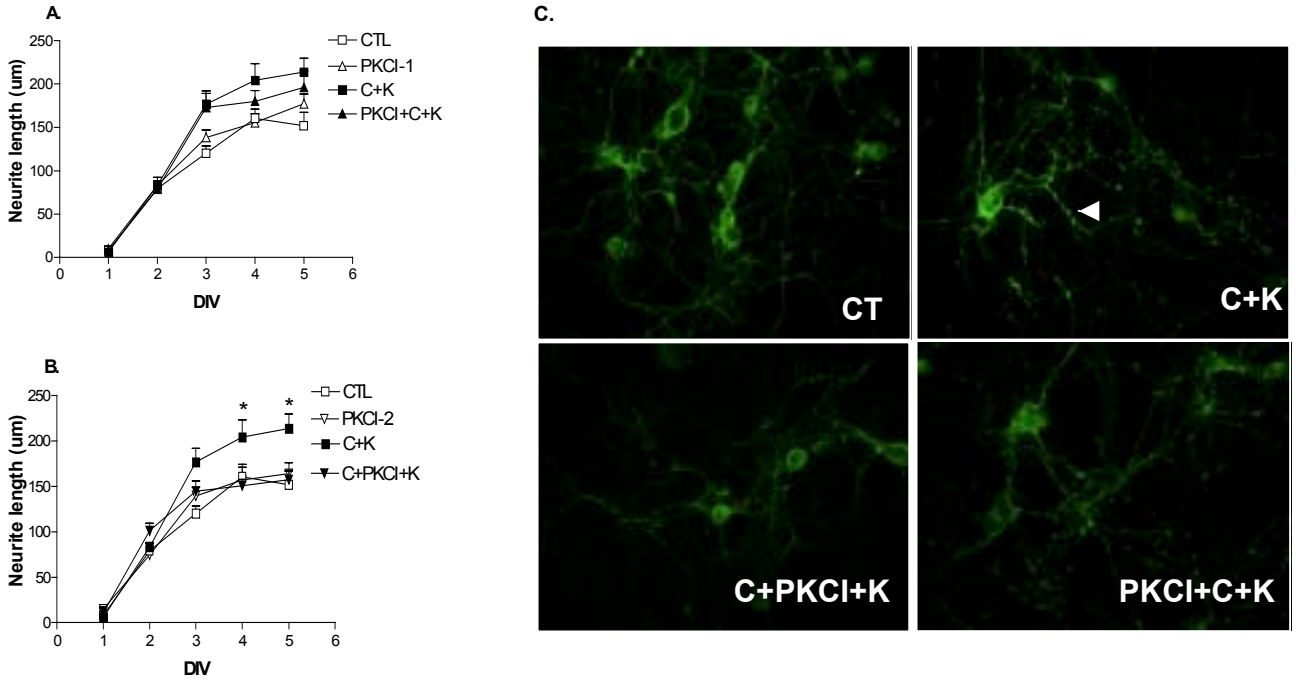

Figure 4. Blockade of PKC activity attenuated CORT+KA-enhanced neurite outgrowth and GAP43 expression in AX-DRG neurons. Cultured DRG neurons at 1 DIV were treated CORT (10 μM) or PKC inhibitor RO-318220 (1uM) for 30 min before CORT, 2DIV with RO-318220 for 30 min followed by KA (1 mM) application. **A and B** show the effects of combined treatment with PKC inhibitors and CORT+KA on neurite growth. Application of PKC inhibitor at 1DIV or 2DIV indicated as PKCI-1 or PKCI-2. The data was expressed as the mean ± S.E.M. (n=8-15). *p<0.05 as compared with the other groups by One-way ANOVA with Newman-Keuls multiple comparison posttest. **C** shows immunofluoresence signal of GAP-43 detected on DRG neurons at 5 DIV: White arrowhead indicates growth cones on neurite stained with GAP-43 immunoreactivities.

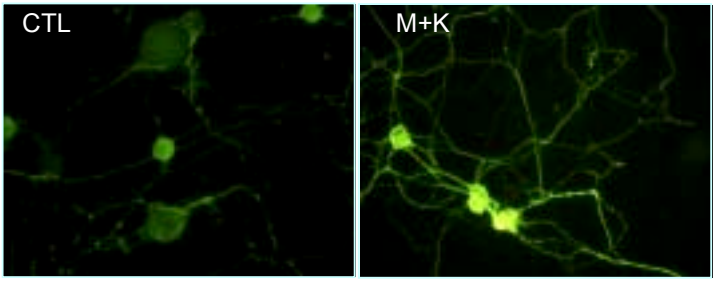

Figure 5. Methylprednisolone also increase GAP-43 expression in AX-DRG neurons. Cultured DRG neurons at 5 DIV were immunostained with anti-GAP43 antibody. Control (CTL) was vehicle-treated culture, and M+K was methylprednisolone (10 μM at 1DIV)+KA (1 mM, 2DIV)-treated culture. Immunoreactivity signal was amplified using avidin-biotin system, and was visualized using FITC fluorescence development

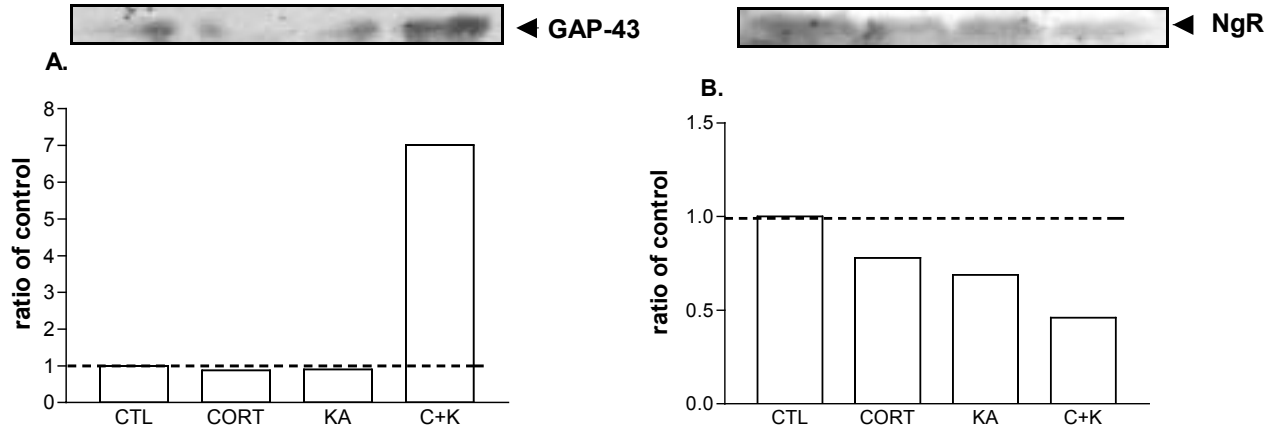

Figure 6. Western blot analysis of GAP43 and NgR expressions in cultured DRG neurons. DRG were pretreated with CORT (10 μM) at 1 DIV, followed by KA (1 mM) treatment at 2 DIV. Neurons were harvested at 5 DIV, and the total cell lysate was Western blotted with anti-GAP43 antibody **(A)** and anti-NgR antibody **(B).** Immunoblots were analyzed by densitometry, and values were given as the ratio of control (CTL) values.

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