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EXPRESSION OF G PROTEIN β SUBUNITS IN RAT SKELETAL MUSCLE AFTER NERVE INJURY: IMPLICATION IN THE REGULATION OF NEUREGULIN SIGNALING

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Abstract—Tight regulation of gene transcription is critical in muscle development as well as during the formation and maintenance of the neuromuscular junction (NMJ). We previously demonstrated that the transcription of G protein β1 (Gβ1) is enhanced by treatment of cultured myotubes with neuregulin (NRG), a trophic factor that plays an important role in neural development. In the current study, we report that the transcript levels of Gβ1 and Gβ2 subunits in skeletal muscle are up-regulated following sciatic nerve injury or blockade of nerve activity. These observations prompted us to explore the possibility that G protein subunits regulate NRG-mediated signaling and gene transcription. We showed that overexpression of Gβ1 or Gβ2 in COS7 cells attenuates NRG-induced extracellular signal-regulated kinase (ERK) 1/2 activation, whereas suppression of Gβ2 expression in C2C12 myotubes enhances NRG-mediated ERK1/2 activation and *c-fos* transcription. These results suggest that expression of Gβ protein negatively regulates NRG-stimulated gene transcription in cultured myotubes. Taken together, our observations provide evidence that specific heterotrimeric G proteins regulate NRG-mediated signaling and gene transcription during rat muscle development.

Key words: G protein, ErbB receptor, MAP kinase, neuromuscular junction, nerve activity.

The neuregulins (NRGs) are a family of growth factors that play functional roles in proliferation, differentiation, migration and survival of a number of cell types (Esper et al., 2006). The biological functions of NRG are mediated by ErbB receptors, and NRG-ErbB signaling has been implicated in myogenesis, survival of Schwann cell precursors, maturation of Schwann cells, differentiation of the postsynaptic muscle cell at the neuromuscular junction (NMJ) and muscle spindle formation (Falls, 2003). Importantly, NRG has been suggested to induce the gene expression of utrophin and acetylcholine receptor (AChR) subunits in myotube cultures or of immediate early genes in myoblasts (Gramolini et al., 1999; Kim et al., 1999; Rimer, 2003; Jacobson et al., 2004). Upon binding to ErbBs, NRG induces receptor autophosphorylation followed by the activation of Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling (Buonanno and Fischbach, 2001). Interestingly, NRG-mediated signaling and gene transcription can be regulated by cyclin-dependent kinase (Cdk) 5, a member of the Cdk family (Fu et al., 2001, 2004). Our work on delineating the plethora of NRG-regulated gene transcripts previously revealed that the transcript level of a guanine nucleotide binding protein (G protein) subunit, Gβ1, and a G protein-coupled receptor, RDC1, is up-regulated following NRG treatment of cultured myotubes (Fu et al., 1999a). These findings raise the interesting possibility that specific G proteins might be involved in modulating NRG-ErbB signaling in muscle.

Heterotrimeric G proteins are associated with various cellular responses such as cell proliferation and differentiation. There are 20 distinct G protein α (Gα) subunits identified to date. Together with five types of Gβ subunits and 12 Gγ subunits, these subunits constitute a diverse array of heterotrimeric G proteins. Gβ and Gγ subunits form stable functional complexes which play important roles in mediating proliferation and survival signals (Schwindinger and Robishaw, 2001). G$\beta\gamma$ can regulate a number of effectors ranging from ion channels, enzymes, to various kinases. The ability of G$\beta\gamma$ subunits to regulate MAPKs provides a link to transcriptional regulation. Overexpression of G$\beta\gamma$ dimers in HEK 293 cells has been reported to activate MAPKs such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (Ito et al., 1995; Coso et al., 1996; Yamauchi et al., 1997). Insulin-like growth factor-I has been shown to activate a pertussis toxin (PTX)-sensitive G protein, leading to G$\beta\gamma$-mediated and Ras-dependent MAPK stimulation in rat 1 fibroblasts (Luttrell et al., 1995) and human intestinal smooth muscle cells (Kuemmerle and Murthy, 2001). Moreover, the PTX-sensitive G proteins also appear to participate in the activation of ERK by nerve growth factor in PC12 cells (Rakhit et al., 2001). These and numerous other studies attest to the fact that substantial crosstalk exists between receptor tyrosine kinases and G protein-regulated signal transduction pathways (Lowes et al., 2002). Our previous observation on the upregulation of Gβ1 by NRG (Fu et al., 1999a) suggests a potential involvement of G proteins in NRG-mediated signaling in muscle development. Indeed, repression of terminal differ-

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Abbreviations: AChR, acetylcholine receptor; Cdk, cyclin-dependent kinase; CGRP, calcitonin gene-related peptide; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; Gα, G protein α; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NMJ, neuromuscular junction; NRG, neuregulin; PTX, pertussis toxin; SHP2, phosphotyrosine phosphatase; TTX, tetrodotoxin.

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entiation of skeletal muscle cells by fibroblast growth factor appears to be mediated via G$\beta\gamma$ subunits (Fedorov et al., 1998).

The aim of the present study is to investigate the regulation of Gβ subunits in skeletal muscle after nerve injury and during development, and its potential involvement in NRG-regulated gene expression. Here, we report the up-regulation of Gβ1 and Gβ2 mRNA in rat muscle after nerve injury or *in vivo* application of tetrodotoxin (TTX). Moreover, we provide evidence that overexpression of Gβ subunits modulates NRG-mediated downstream signaling, i.e. activation of MAPKs and expression of immediate early genes, such as *c-fos*. Our findings reveal a new regulatory mechanism for NRG-ErbB signaling in muscle, providing additional insights into the crosstalk between RTK and G protein signaling.

EXPERIMENTAL PROCEDURES

All experiments were performed in accordance with the guidelines of the Animal Care Facility at the Hong Kong University of Science and Technology, in conformance with international guidelines on the ethical use of animals. All efforts were taken to minimize the number of animals used and their suffering.

Chemicals, constructs and antibodies

Recombinant fusion protein encoding the EGF domain of NRG-β1 was purified as previously described (Fu et al., 1999a). CGRP was obtained from Calbiochem (La Jolla, CA, USA) and TTX from Sigma (St. Louis, MO, USA). Antibodies specific for Gβ1 (c-16), Gβ2 (c-16), and ErbB4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific for p-ERK1/2, p-MEK1/2, p-JNK, and MEK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA), while antibodies against ERK1/2 and FLAG were purchased from Upstate (Lake Placid, NY, USA) and Sigma, respectively. The pcDNA3/ErbB4 plasmid was kindly provided by Dr. L. Mei (University of Georgia, Athens, GA, USA).

Cloning of cDNA fragments, total RNA extraction and Northern blot analysis

The cDNA probes for Gβ1–5 used in Northern blot analysis were cloned by RT-PCR. Forward and reverse primers for rat Gβ1–5 subunits were designed as previously described [Gβ1 (Fu et al., 1999a); Gβ2–5 (Betty et al., 1998)]. For Gβ1–4, cDNA fragment of 3′ untranslated region was amplified while for Gβ5, cDNA for the coding region was amplified. Primer sequences were as follows: Gβ1; 5′-CAGTAGCAGGTGGATG-3′ and 5′-AATGCATCAGTGACAGTCAGA-3′ (586 bp, 1062–1647 nt; U88324), Gβ2; 5′-GGCCCAGGCAGGGAGCAG-3′ and 5′-AGTTGGAAGTGGTTCCTTTATGGA-3′ (350 bp; 106–456 nt; AF022084), Gβ3; 5′-GGCTGGAGGAAGAGGTGGGAA-3′ and 5′-AGGTAATAAGAGAGAACAAAA-3′ (394 bp, 1104–1500nt; L29090), Gβ4; 5′-TTG CAGATGAAGTTCTTCTATTGAGG-3′ and 5′-TTGTGCCAGTTGAATGGATGAGTT-3′ (898 bp, 149–1047 nt; AF022085) and Gβ5,5′-AGCCTCAAGGGCAAGCTAGAGGAG-3′ and 5′-ACGTCAGCCCCATGGCCATGGAAG-3′ (532 bp, 33–565 nt; AF022086). Single-stranded cDNA was prepared from 2 μg of rat adult brain total RNA using Superscript II RNase H$^-$ reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the supplier's instruction. For analysis of chick Gβ1 and CKM transcripts, rat cDNA probes were used since Gβ1 and CKM gene share >80% identity in nucleotide sequence between rat and chick, whereas a chick AChRα probe was used for AChRα transcripts (Ip et al., 2000b).

Total RNAs from C2C12 myotubes and rat muscle were prepared by guanidinium thiocyanate extraction and lithium chloride/urea extraction methods (Ip et al., 1996; Fu et al., 1999a). Twenty micrograms of total RNAs were electrophoresed on a 1% agarose–formaldehyde gel, transferred onto a nylon membrane, and cross-linked by UV irradiation. Northern blot analysis was performed as previously described (Ip et al., 1995). The DNA probes were purified and labeled with [α-^{32}P]dCTP using Megaprime labeling kit (GE Health Care). Nylon filters were then hybridized at 65 °C with radiolabeled probes in 0.5 M sodium phosphate buffer (pH 7), 1% bovine serum albumin, 7% SDS, 1 mM EDTA, and 20 μg/ml sonicated salmon sperm DNA. Filters were washed at 65 °C with 2× SSC/0.1% SDS and exposed to X-ray film with intensifying screen at −80 °C.

Denervation and *in vivo* paralysis by TTX

Procedures for nerve denervation and *in vivo* TTX paralysis were as previously described (Fu et al., 2002b). Briefly, adult rats were anesthetized and the upper thigh of animals was opened and a small segment (~0.5 cm) of sciatic nerve was removed in the nerve cut experiment. Nerve crush was performed by pinching the sciatic nerve with a pair of fine forceps for at least 5 s. Animals were killed at different times following surgery and the gastrocnemius muscles were collected for analysis. For *in vivo* TTX paralysis, an osmotic pump containing 180 μg/ml TTX in Hanks' solution supplemented with penicillin and streptomycin was implanted s.c. and fixed below the rib cage of anesthetized rats. TTX was delivered to the sciatic nerve by Silastic tubing at a rate of 4.5 mg/day. Muscle paralysis was induced by the action of TTX which blocks the nerve-evoked contraction of the gastrocnemius muscle. Control experiment was performed using Hanks' solution.

Cell culture, primary chick muscle culture, transient transfection

COS7 cells were cultured in DMEM with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37 °C with 5% CO_2 atmosphere. Mouse C2C12 myoblasts were maintained in DMEM supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin as previously described (Fu et al., 1997). Differentiation of myoblasts to myotubes was induced by switching the culture medium to DM (DMEM supplemented with 2% horse serum). Cultured C2C12 myotubes were treated with recombinant NRG (4 nM) for 48 h prior to preparation of RNA. Primary chick muscle cultures were prepared from the hind limbs of E11 chick embryos as previously described (Fu et al., 1999b). Suspended muscle cells (5×10^5) were plated onto collagen-coated 35 mm dishes, and maintained in Eagle's minimal essential medium containing 10% horse serum, 2% chick embryo extract, 100 U/ml penicillin and streptomycin. Myoblasts began to fuse by 3 days after plating and 10 μM cytosine arabinoside was added and treated for 1 day.

COS7 cells were transiently transfected with ErbB4 receptor (0.5 μg), FLAG-tagged Gβ (0.5 μg) or Gγ (1 μg) plasmids as indicated using Lipofectamine Plus reagents (Invitrogen). Each transfection was performed with 3×10^6 cells in 60 mm culture dish. Twenty-four hours after transfection, the medium was replaced by serum-free DMEM medium and 16 h later, COS7 cells were treated with NRG (4 nM) for various durations followed by protein extraction. A chemically modified siRNA targeting mouse Gβ2 subunit was designed according to the manufacturer's instruction using the Stealth RNAi technology (Invitrogen). Its corresponding scramble siRNA was used as the controls. C2C12 myotubes were differentiated for 2 days and transfected with the siRNA using Lipofectamine 2000 (Invitrogen).

Protein extraction, Western blot analysis and immunohistochemical analysis

COS7 cells and rat muscle tissues were lysed with RIPA lysis buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS; with protease inhibitors including 1 mM PMSF, 1 mM sodium orthovanadate and 10 μg/ml leupeptin and aprotinin) and then incubated for 30 min at 4 °C. The lysates were centrifuged at 20,800×*g* at 4 °C for 10 min. The supernatants were saved and the pellets were discarded. Western blot analysis was performed as previously described (Fu et al., 2001). Briefly, all samples were separated on SDS-PAGE and subsequently transferred onto nitrocellulose membranes which were then incubated with the primary antibody indicated, washed and incubated with the appropriate secondary antibody. Signals were detected using the SuperSignal® West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA).

Adult rat muscle sections, or denervated muscle sections taken from various periods after sciatic nerve cut (10 μm), were fixed with 2% paraformaldehyde/5% sucrose in PBS for 15 min at room temperature, washed and permeabilized with 0.4% Triton X-100. Double staining was performed by incubating the sections with rhodamine-conjugated α-bungarotoxin (10 nM; Molecular Probes, Eugene, OR, USA) and primary antibodies specific for Gβ1 and Gβ2 at 4 °C overnight followed by FITC-conjugated goat anti-rabbit antibody in DMEM/10% FBS for 1 h at 37 °C as described (Ip et al., 2000a). The sections were then washed and mounted for fluorescence microscopy.

Statistical analysis

Results in the current study were analyzed by two-tailed Student's *t*-test. All experiments have been repeated for at least three times. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Upregulation of Gβ1 and Gβ2 subunits in rat skeletal muscle after nerve injury and by neural activity

Despite being a critical component of G protein–regulated signaling pathways, the expression of Gβ subunits in skeletal muscle has not been well characterized. We therefore began our studies by examining the expression of different Gβ isoforms in the adult rat skeletal muscle. Among the five Gβ subunits examined, Gβ1, Gβ2, and Gβ5 transcripts could be detected by Northern blot analysis using Gβ isoform-specific probes. Given that denervation increases subsynaptic gene transcription in muscle (Ip et al., 1996; Schaeffer et al., 2001), we asked if the expression of Gβ subunits is similarly regulated. The mRNA expression of Gβ subunits in muscle following nerve injury was first examined. The sciatic nerve was damaged by either nerve cut or nerve crush, and the levels of mRNA expression of Gβ1, Gβ2 and Gβ5 subunits in skeletal muscle following nerve injury were compared using Northern blot analysis. We found that Gβ1 mRNA expression was increased by ~twofold at day 4 following nerve cut and remained elevated until day 20, while the transcript was slightly up-regulated at day 10 after nerve crush and returned to the basal level by day 20 (Fig. 1A). For Gβ2, mRNA level increased by ~threefold at day 4 following either nerve cut or crush, and remained elevated through day 20 (Fig. 1A). The expression of Gβ5 transcript was found to be relatively

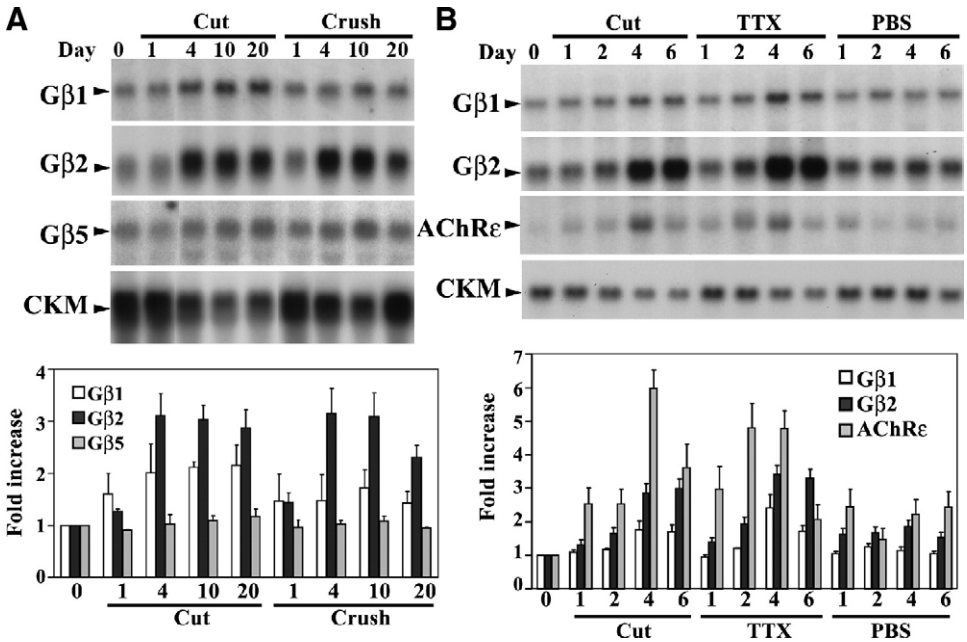

Fig. 1. The transcript levels of Gβ1 and Gβ2 subunits were up-regulated in muscle after nerve injury or *in vivo* TTX application. (A) Northern blot analysis for Gβ1, Gβ2 and Gβ5 in muscle after nerve cut and nerve crush (upper panels). Muscle creatine kinase (CKM) transcripts were included as controls. Arrowheads indicate the detectable transcripts. Fold increase of mRNA transcripts was quantified by densitometry and depicted as mean±S.D. (bottom panel). (B) The mRNA expression of Gβ1, Gβ2 and AChRε subunits in muscle was up-regulated following blockade of neural activity (upper panels). Total RNA from gastrocnemius muscle after nerve cut (Cut), *in vivo* TTX treatment (TTX) or buffer treatment (PBS) was collected. CKM transcripts were included as control. The fold change in the intensity of transcript is normalized to that of CKM at corresponding time points (mean±S.D.; bottom panels).

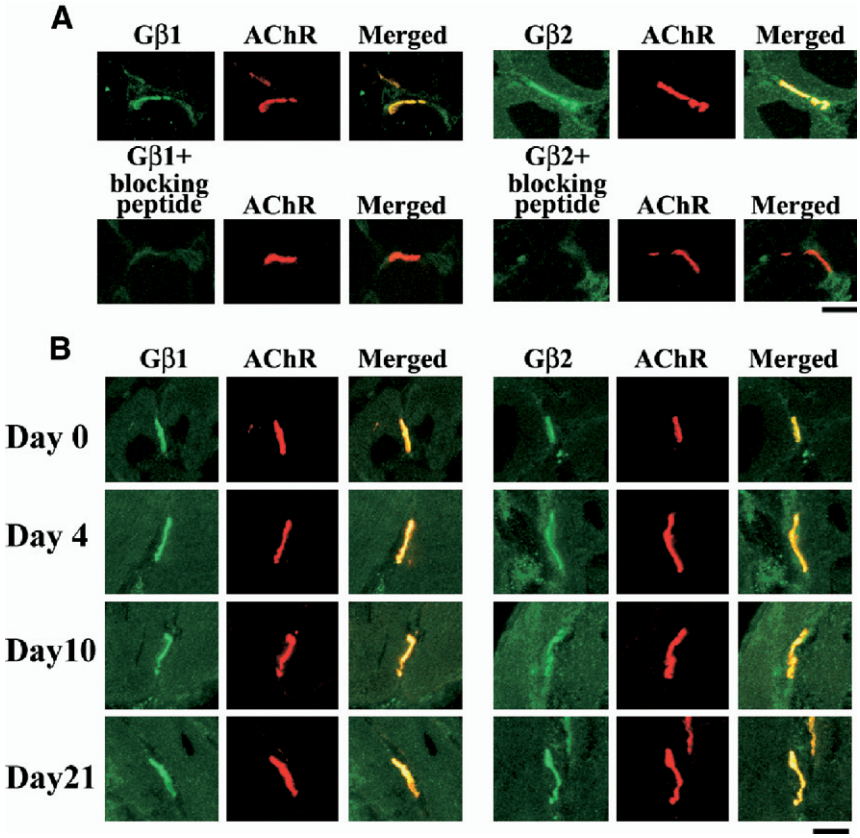

Fig. 2. Gβ1 and Gβ2 subunits remained localized at the NMJs after nerve injury. (A) Localization of Gβ1 and Gβ2 subunits to adult rat NMJs. Double immunostaining indicated the co-localization of Gβ1 or Gβ2 and AChR. Gβ subunits were detected with Gβ1 or Gβ2 specific antibodies followed by FITC-conjugated secondary antibodies while AChRs were stained with rhodamine-conjugated α-bungarotoxin. The specific staining for Gβ subunits at NMJ could not be detected when the antibodies were pre-incubated with their corresponding blocking peptides. (B) Double immunostaining was performed on adult rat gastrocnemius muscle sections at days 4, 10 and 21 after sciatic nerve cut using antibodies against Gβ1 (left panels) or Gβ2 (right panels) and rhodamine-conjugated α-bungarotoxin. Normal muscle section (day 0) was included as control. Scale bar=20 μm.

unchanged in muscle after nerve injury. Our observations suggest that denervation increases the transcription of Gβ1 and Gβ2 subunits in skeletal muscle.

Since Gβ1 and Gβ2 subunits were differentially regulated after nerve injury, it is of interest to further delineate if the upregulation of Gβ subunits transcription was due to loss of neural activity, or loss of trophic factor support. To examine if loss of nerve activity is sufficient to induce the upregulation of Gβ subunits transcription, an effective blocker of voltage-dependent sodium channel, TTX, was continuously delivered to muscle via an auto-osmotic pump implanted in the gastrocnemius muscle of an adult rat (Fu et al., 2002a). We found that the level of Gβ1 transcript increased by ~threefold after 4-day TTX treatment *in vivo*, comparable to the increase observed in muscle after nerve cut. Similarly, the changes in mRNA expression for Gβ2 after TTX treatment (~fivefold) paralleled that observed following nerve cut (Fig. 1B). The transcription of AChRε subunit was also up-regulated at day 4 after nerve cut and TTX treatment. The relative slight change in the mRNA levels of Gβ1, Gβ2 and AChRε in muscle after PBS treatment may be induced by the implantation of Silastic tubing. Together, these findings suggest that the increase in the expression of Gβ subunits observed following nerve injury was, at least in part, caused by the loss of neural activity.

Gβ1 and Gβ2 were localized to the post-synaptic region of NMJ following nerve injury

Since the regulatory profile of Gβ1 and Gβ2 transcripts is similar to that of most synaptic genes, we examined whether Gβ1 and Gβ2 subunits are indeed localized to the post-synaptic site. Frozen sections of the gastrocnemius muscle were subjected to immunohistochemical analysis. It has been demonstrated that the axon terminal at the NMJ degenerates by day 4 following nerve section (Lai et al., 2001). Comparison of Gβ1 and Gβ2 localization in normal and denervated muscle will therefore help to identify if the immunoreactivity observed is located mainly at the post-synaptic muscle membrane or presynaptic nerve terminal. At normal NMJ, Gβ1 and Gβ2 immunoreactivity was co-localized with AChR at the NMJs in adult rat gastrocnemius muscle (Fig. 2A). We found that up to 21 days post–nerve injury, Gβ1 and

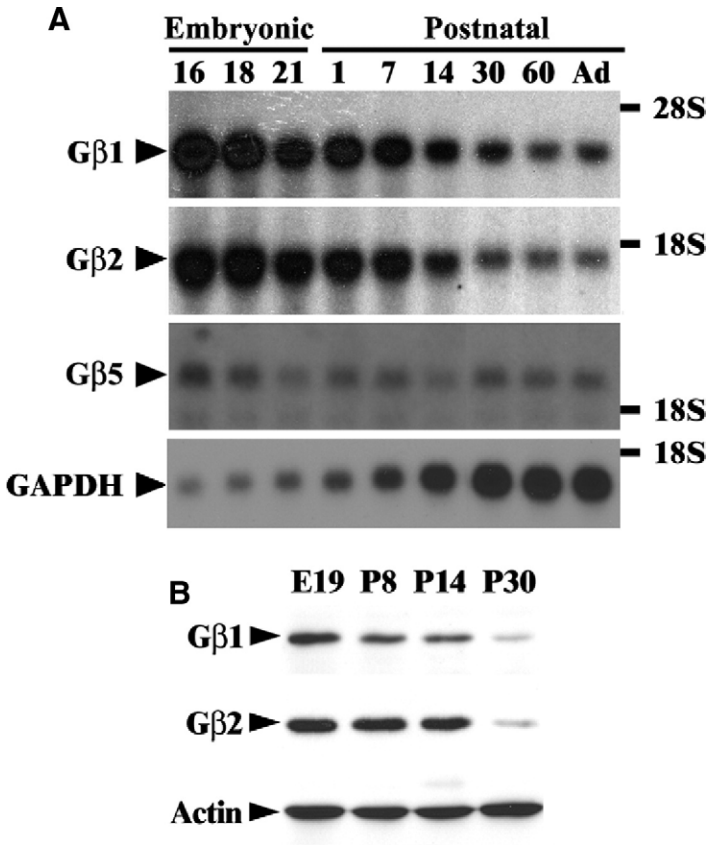

Fig. 3. The mRNA and protein expression of Gβ1 and Gβ2 subunits was developmentally regulated in rat skeletal muscle. (A) The transcripts of Gβ1 and Gβ2 were prominently expressed in rat muscle during E stages and its expression gradually decreased along development. Arrowheads indicate the detectable transcripts. The relative positions of ribosomal RNA bands (18S and 28S) are indicated on the right. Northern blot analysis for GAPDH was included as control. (B) Western blot analysis of Gβ1 and Gβ2 using rat muscle membrane fractions. Actin served as loading control. E, embryonic; P, postnatal.

Gβ2 (Fig. 2B) immunoreactivities remained concentrated and co-localized with that of AChR. The postsynaptic compartmentalization of Gβ1 and Gβ2 proteins is consistent with a potential role of Gβ1 and Gβ2 in regulating the development/functions of NMJ.

Developmental expression of Gβ1 and Gβ2 subunits in rat skeletal muscle

To further characterize the regulation of Gβ subunits at postsynaptic muscle, the expression of Gβ1, Gβ2 and Gβ5 transcripts and proteins at various developmental stages was determined. A prominent level of Gβ1 ($\sim$3.6 kb) and Gβ2 ($\sim$1.6 kb) transcripts was detected in rat muscle (Fig. 3A), especially in late embryonic stages (E16 to E18) during the period of NMJ formation. The expression of these transcripts was down-regulated from P14 and remained at a low level until adulthood, while Gβ5 mRNA was detected in E16 muscle and was relatively unchanged along the course of development. Similarly, Western blot analysis revealed that Gβ1 and Gβ2 proteins were prominently expressed in membrane fractions of E19 rat muscle, maintained at high level during postnatal stages of P8 and P14, and decreased at P30 (Fig. 3B).

TTX and NRG treatment up-regulated the mRNA expression of Gβ1 and Gβ2 subunit in myotube cultures

Similar to Gβ1 transcript, the mRNA expression of Gβ2 in C2C12 myotubes was also induced by NRG treatment [(Fu et al., 1999a); Fig. 4A]. Using chick primary muscle culture system, we further confirmed that either blockade of neural activity or NRG could up-regulate the expression of the Gβ1 subunit. Northern blot analysis was performed to determine the expression profile of Gβ1 mRNA after treatment of chick myotube culture with TTX or NRG (Fig. 4B). Since extensive studies have been conducted on the regulation of AChRα mRNA in cultured chick myotubes treated with pharmacological agents (Klarsfeld and Changeux, 1985), the mRNA expression of AChRα was simultaneously examined as control. Treatment with TTX resulted in a rapid and complete cessation of spontaneous contractions of cultured myotubes. The level of Gβ1 transcripts was induced by $\sim$twofold in TTX-treated myotubes. Experiments performed with two nerve-derived trophic factors, NRG and calcitonin gene-related peptide (CGRP), revealed that Gβ1 transcript was up-regulated by NRG but not by CGRP (Fig. 4B).

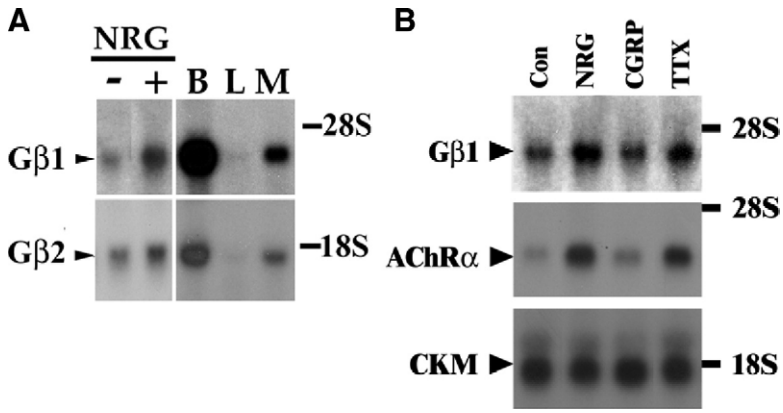

Fig. 4. The up-regulation of Gβ transcripts in primary muscle culture after NRG or TTX treatment. (A) The mRNA expression of Gβ1 and Gβ2 was up-regulated after NRG treatment. Total RNA of C2C12 myotubes with or without NRG treatment for 48 h was collected (+, NRG treated; −, Control). Northern blot analysis for Gβ1 and Gβ2 subunits was performed. L, liver; B, brain; M, muscle. (B) Regulation of Gβ1 mRNA transcripts in primary chick M culture by TTX or trophic factors. Day 4 chick myotube culture was treated with TTX (1 μM) or trophic factors, NRG (10 nM) and CGRP (100 μM), for 48 h. The Northern blots were hybridized with chick AChRα, rat Gβ1 and CKM. Arrowheads indicate the transcripts detected by the cDNA probes. Ribosomal RNA bands (18S and 28S) are indicated on the right.

Overexpression of G$\beta\gamma$ subunits negatively regulated NRG-induced MEK-ERK activation in COS7 cells

NRG has been demonstrated to induce the transcription of synapse-specific genes or immediate-early genes in myotubes via activation of MAPK signaling pathway (Si et al., 1999). The up-regulation of Gβ subunits by NRG suggests that G protein–mediated signaling may take part in modulating NRG-regulated gene transcription in myotubes. Since G$\beta\gamma$ acts as a functional monomer where the β and γ subunit never dissociates from each other, we expressed Gγ2 with Gβ1 or Gβ2 subunit to examine how G$\beta\gamma$ proteins modulate ERK1/2 and JNK signaling pathways downstream of NRG. COS7 cells were transfected with ErbB4 receptor, and FLAG-tagged Gγ2 subunit and Gβ1 or Gβ2 subunits as indicated, and then treated with NRG for 5–30 min (Fig. 5). The phosphorylation of ERK1/2 was up-regulated in pcDNA3, Gβ1γ2 or Gβ2γ2-overexpressed COS7 cells following NRG treatment (Fig. 5A). However, the observed increase of ERK1/2 phosphorylation was attenuated in Gβ1γ2 or Gβ2γ2-transfected COS7 cells, where phospho-ERK1/2 was reduced to the basal level after 30 min of NRG treatment (Fig. 5A). Thus, overexpression of Gβ1γ2 or Gβ2γ2 in COS7 cells reduced NRG-stimulated ERK activation. Similar to that observed with ERK1/2, NRG-induced phosphorylation of the upstream regulator of ERK1/2, MEK1/2, was up-regulated at 5 min and maintained until 30 min. The increase of MEK1/2 phosphorylation was found to be reduced in COS7 cells overexpressing Gβ1γ2 or Gβ2γ2 following NRG treatment (Fig. 5B). Similarly, phosphorylation of JNK was increased by NRG, but the increase was not attenuated by the G$\beta\gamma$ expression (Fig. 5C). It is interesting to note that the basal phosphorylation of JNK was elevated in cells overexpressing G$\beta\gamma$ proteins. The phosphorylation of p38, another MAP kinase downstream of Ras, was not affected by NRG treatment (data not shown). Re-probing the membranes with anti-FLAG antibody confirmed that exogenous Gβ subunits were indeed expressed in G$\beta\gamma$-transfected cells (Fig. 5D). Expression of ErbB4 was comparable, suggesting that the differences observed were not due to differential amount of ErbB4 expressed. Interestingly, we found that overexpression of Gβ subunit alone could attenuate NRG-stimulated ERK1/2 activation to a similar extent as that observed with G$\beta\gamma$ overexpression (Fig. 5E), suggesting that Gβ subunit confers G$\beta\gamma$ specificity in coupling ErbB4-activated downstream signaling events. Together, our results showed that overexpression of Gβ subunits attenuates the NRG-stimulated phosphorylation of MEK1/2 and ERK1/2, but not the phosphorylation of JNK.

Suppression of Gβ2 expression increased the NRG-mediated ERK activation and *c-fos* transcription in C2C12 myotubes

To further investigate whether Gβ signaling is involved in regulating NRG-induced gene transcription, we examined the ability of NRG to induce the transcription of an immediate early gene, *c-fos*, in cultured myotubes with suppressed Gβ2 expression. The activation of MEK-ERK signaling and induction of *c-fos* transcription was observed in C2C12 myotubes after NRG stimulation (Si et al., 1999). Since Gβ2 expression is higher than that of Gβ1 in muscle and overexpression of Gβ1 or Gβ2 exerts similar effect on the NRG-mediated signaling (Fig. 5), we examined whether knockdown of Gβ2 regulates the NRG-stimulated gene regulation in C2C12 myotubes. Transfection of C2C12 myotubes with the siRNA targeting Gβ2 resulted in reduced expression of Gβ2 mRNA and protein (~60%; Fig. 6D and E), whereas the protein expression of Gβ1 remained unchanged (Fig. 6D). In agreement with the results of the overexpression studies in COS7 cells, suppressing Gβ2 expression in C2C12 myotubes led to an increase in the NRG-mediated MEK and ERK1/2 activation (at 30 min; Fig. 6A and B). Furthermore, the NRG-induced mRNA expression of *c-fos* in these myotubes with reduced Gβ2 expression was increased when compared with that

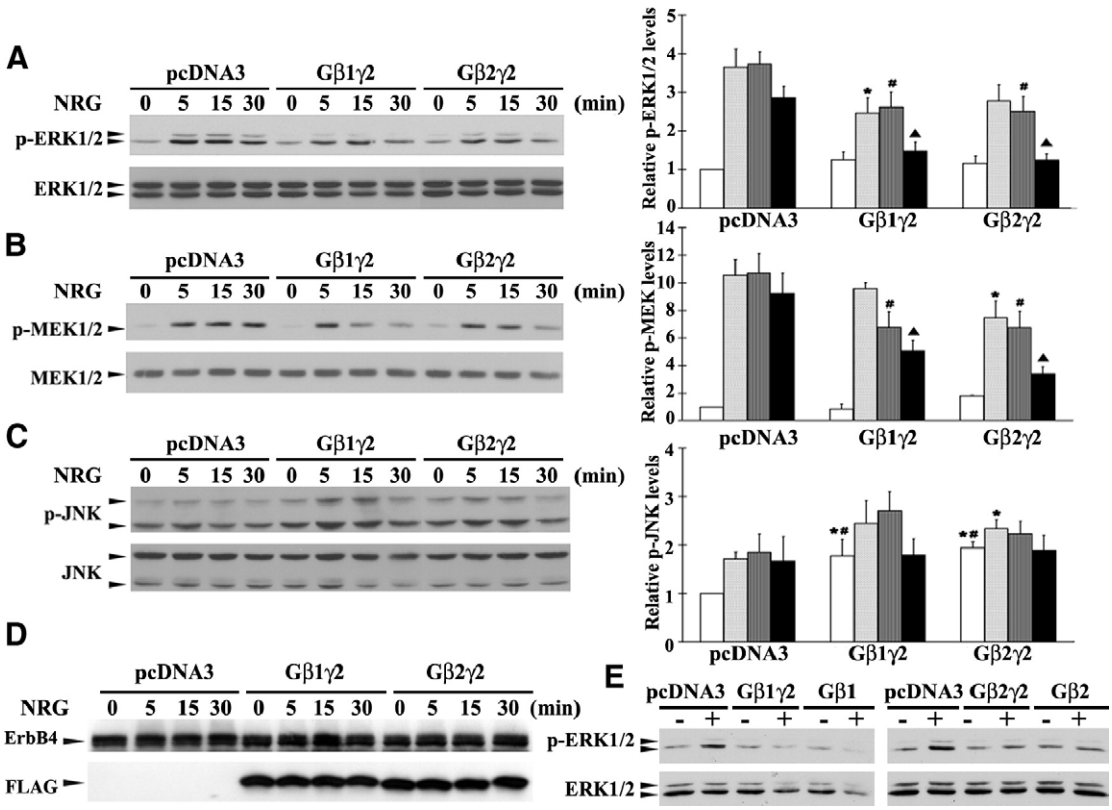

Fig. 5. Overexpression of Gβ subunits attenuated NRG-induced MAPK phosphorylation in COS7 cells. COS7 cells were transfected with ErbB4 together with expression constructs as indicated. Western blot analyses for phosphorylated and total ERK1/2, MEK1/2 and JNK were performed (A, B and C). The Gβγ-overexpressing cells were treated with NRG for 5–30 min. Quantitative analysis was depicted in the right panels and all results are presented as mean±S.D. of three independent experiments. *#, *, #, ▲ $P<0.05$ versus pcDNA3-transfected cells at corresponding time intervals, i.e. 0, 5, 15 and 30 min following NRG treatment. (D) Expression of exogenous ErbB4 receptor and FLAG-tagged Gβγ subunits was detected by ErbB4 and FLAG specific antibodies. (E) Overexpression of Gβ subunits similarly attenuated ERK activation as that observed for Gβγ subunits. Protein lysates were collected from transfected cells treated with or without NRG for 30 min (+, NRG treated; −, Control).

transfected with the control siRNA (Fig. 6E). Together, these results suggest that Gβ2 subunit negatively regulates NRG-mediated signaling and transcription in cultured C2C12 myotubes.

DISCUSSION

Our findings provide the first extensive analysis on the regulation of transcript levels of G protein β subunits in rat muscle during development, and in adult muscle following nerve injury. Among five Gβ subunits, transcripts of Gβ1, Gβ2 and Gβ5 can be detected in muscle, but only the transcription of Gβ1 and Gβ2 is up-regulated after nerve injury. In addition, we found that Gβ1 and Gβ2 are localized to the post-synaptic compartment, suggesting that Gβ-mediated signaling may be important for synapse formation and/or function.

The accumulation of Gβ1 and Gβ2 subunits in the post-synaptic compartments may be attributed by two mechanisms, either the proteins are synthesized in the subsynaptic nuclei or being synthesized at distinct sites and transported subsequently to synapses. Further analysis is required to examine whether the mRNA of Gβ subunits is concentrated at subsynaptic nuclei. However, the regulation of Gβ transcripts is similar to that of most synaptic genes. It has been shown that the synaptically enriched transcripts, such as AChR subunits, N-CAM and MuSK, share distinct features: their abundance in muscle decreases upon development and increases after denervation. Consistent with this notion, we found that the transcripts of Gβ1 and Gβ2 share a similar regulatory pattern. Nerve activity represses synaptic gene expression in the extrasynaptic areas of skeletal muscle during development or in adult. Thus, the up-regulation of Gβ transcript levels is likely representative of an increase of Gβ transcripts in the extrasynaptic regions, reminiscent of the ability of neural activity to suppress transcription of AChR subunits in the extrasynaptic region (Goldman et al., 1988). Our *in vitro* culture studies provide further evidence that the expression of both Gβ1 and Gβ2 can be regulated by neural activity or NRG. This observation is consistent with existing mechanisms implicated in the regulation of gene transcription at synapses. Accumulating evidence indicates that the post-synaptic apparatus is organized by signals from the pre-synaptic nerve terminal through two distinct mechanisms. First, electrical activity from motor neuron represses transcription of AChR genes in the extrajunctional

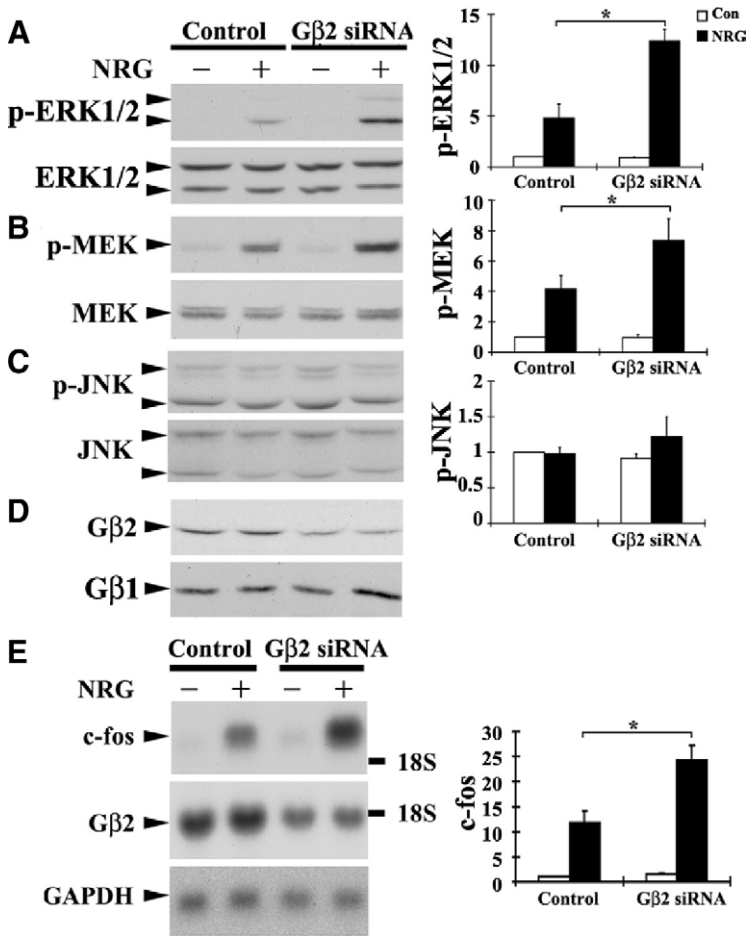

Fig. 6. Suppressing Gβ2 expression increased NRG-stimulated MEK-ERK activation and *c-fos* transcription in C2C12 myotubes. C2C12 myotubes were transfected with Gβ2 siRNA and treated with NRG. Western blot analysis for phosphorylated and total ERK1/2 (A), MEK1/2 (B) and JNK (C). Quantification is shown at the right panels. NRG was added to the myotube culture for 30 min to stimulate the MEK-ERK activation. Change of relative phosphorylation level refers to the fold change of phosphorylated signaling protein normalized with the total protein level. * $P<0.05$ versus the control (mock-transfected) cells after NRG treatment. All results are presented as mean±S.D. (D) Protein expression of Gβ2 and Gβ1. (E) Northern blot analysis for *c-fos*. Quantitative analysis is shown at the right panels. Gβ2 and GAPDH served as loading controls. Arrowheads indicate the detectable transcripts.

area of muscle fiber (Goldman et al., 1988). Second, trophic factors such as agrin or NRG, induce the AChR expression and rearrange AChR and other cytoskeletal proteins at the post-synaptic domain. Upon binding of NRG to its receptors, ErbB tyrosine kinase receptors become phosphorylated and stimulate downstream signaling pathways including Ras-Raf-MEK-ERK, JNK, Cdk5 and PI3 kinase. The rapid and transient activation of ERK and JNK induces the expression of two immediate early genes, *c-fos* and *c-jun* and other critical genes in muscle (Sunesen and Changeux, 2003; Krag et al., 2004). Although recent studies examining the role of NRG on NMJ development using mice deficient in NRG or ErbB cast doubt on the requirement of NRG in NMJ development *in vivo* (Yang et al., 2001; Escher et al., 2005), NRG has been observed to regulate a myriad of genes in myotubes, including sodium channels, utrophin and various members of the early growth response family of transcription factors (Corfas and Fischbach, 1993; Gramolini et al., 1999; Jacobson et al., 2004). These findings suggest that NRG-mediated gene transcription may take part in regulating other aspects of muscle maturation and synaptic functions at the NMJ, such as muscle spindle development (Leu et al., 2003).

In addition to the upregulation of Gβ1 and Gβ2 expression by NRG, we observed that G-proteins can in turn attenuate signaling and gene transcription downstream of NRG signaling. Previous studies have identified several proteins including phosphotyrosine phosphatase (SHP2) and erbin which could also negatively regulate NRG signaling in muscle (Tanowitz et al., 1999; Huang et al., 2003). Interestingly, like Gβ subunits, SHP2 is also upregulated in myotubes in response to NRG (Fu et al., 1999a; Tanowitz et al., 1999). These observations collectively suggest that signaling proteins, the transcript levels of which are regulated by NRG, are able to regulate NRG-mediated downstream signaling.

Previous studies have demonstrated that overexpression of G$\beta\gamma$ subunits can robustly stimulate the activation of ERK1/2 and JNK (Faure et al., 1994; Ito et al., 1995;

Coso et al., 1996). However, we found that when ErbB4 receptor was co-transfected with G$\beta\gamma$ subunits in COS7 cells, differential effects were observed for the regulation of phosphorylation status of ERK1/2 and JNK. While overexpression of G$\beta\gamma$ complexes significantly reduced the NRG-stimulated activation of ERK1/2 and MEK1/2, an elevated basal activity for JNK, but not ERK, was observed. The detailed mechanisms underlying the attenuation of ERK1/2 and MEK1/2 activation are unclear. Nonetheless, our identification of an important role of G$\beta\gamma$ in NRG-mediated ERK1/2 signaling adds it to the list of cross-talks observed between receptor tyrosine kinases and G proteins. For example, G$\beta\gamma$ subunits, associated with insulin-like growth factor 1 (IGF1) receptor, are involved in IGF-1 mitogenic signaling (Luttrell et al., 1995; Dalle et al., 2001). Gα_s subunits can be directly activated by the epidermal growth factor (EGF) receptor upon EGF treatment (Poppleton et al., 1996; Sun et al., 1997). Future studies directed toward understanding the detailed mechanism of G$\beta\gamma$ in NRG-mediated synapse-specific gene transcription in myotubes are required to delineate the functional roles of G$\beta\gamma$ subunits in NRG-regulated muscle differentiation or NMJ formation.

Since different G$\beta\gamma$ complexes have differential abilities in coupling Gα subunits to receptors, or regulating effectors, the selective regulation of Gβ subunits during nerve injury may contribute to the triggering of specific signaling cascades in muscle following nerve denervation. For example, the endothelin B receptor couples effectively to both Gα_i and Gα_q subunits in the presence of the G$\beta1\gamma2$ dimer, but to Gα_q alone in the presence of G$\beta5\gamma2$ (Lindorfer et al., 1998). In terms of regulating downstream effectors, Gβ1 and Gβ5 exert differential effects on the MAPK and JNK pathways in COS cells (Zhang et al., 1996). Therefore, Gβ1 and Gβ2 may be specifically required in recruiting signaling molecules at the post-synaptic region after nerve injury. Our study reveals the synaptic localization of Gβ1 and Gβ2 subunits at the NMJ. Such subcellular compartmentalization of G proteins may facilitate interactions between proteins expressed in the same cell. In particular, the Gβ1 and Gβ2 may be required to target specific Gα subunits to the post-synaptic membrane region at synapses, which, in turn allows the Gα subunits to activate signaling in muscle.

CONCLUSION

In summary, we have shown that both transcripts and proteins of Gβ subunits are specifically localized at the NMJ, indicating the possible involvement of G protein–regulated pathways in muscle. G proteins can perhaps participate in synaptic functions via crosstalk with RTK signaling pathways. Using the RNAi approach, we have shown that Gβ proteins negatively regulate NRG-mediated MEK-ERK activation and affect subsequent immediate gene transcription. However, the precise roles of Gβ in muscle and/or NMJ, and the possible signaling crosstalk between RTKs and heterotrimeric G proteins at the synapse remain to be determined.

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REFERENCES

Betty M, Harnish SW, Rhodes KJ, Cockett MI (1998) Distribution of heterotrimeric G-protein beta and gamma subunits in the rat brain. Neuroscience 85:475–486.

Buonanno A, Fischbach GD (2001) Neuregulin and ErbB receptor signaling pathways in the nervous system. Curr Opin Neurobiol 11:287.

Corfas G, Fischbach GD (1993) The number of Na^+ channels in cultured chick muscle is increased by ARIA, an acetylcholine receptor-inducing activity. J Neurosci 13:2118–2125.

Coso OA, Teramoto H, Simonds WF, Gutkind JS (1996) Signaling from G protein-coupled receptors to c-Jun kinase involves beta gamma subunits of heterotrimeric G proteins acting on a Ras and Rac1-dependent pathway. J Biol Chem 271:3963–3966.

Dalle S, Ricketts W, Imamura T, Vollenweider P, Olefsky JM (2001) Insulin and insulin-like growth factor I receptors utilize different G protein signaling components. J Biol Chem 276:15688–15695.

Escher P, Lacazette E, Courtet M, Blindenbacher A, Landmann L, Bezakova G, Lloyd KC, Mueller U, Brenner HR (2005) Synapses form in skeletal muscles lacking neuregulin receptors. Science 308:1920–1923.

Esper RM, Pankonin MS, Loeb JA (2006) Neuregulins: versatile growth and differentiation factors in nervous system development and human disease. Brain Res 51:161–175.

Falls DL (2003) Neuregulins and the neuromuscular system: 10 years of answers and questions. J Neurocytol 32:619.

Faure M, Voyno-Yasenetskaya TA, Bourne HR (1994) cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. J Biol Chem 269:7851–7854.

Fedorov YV, Jones NC, Olwin BB (1998) Regulation of myogenesis by fibroblast growth factors requires beta-gamma subunits of pertussis toxin-sensitive G proteins. Mol Cell Biol 18:5780–5787.

Fu AK, Cheung WM, Ip FC, Ip NY (1999a) Identification of genes induced by neuregulin in cultured myotubes. Mol Cell Neurosci 14:241–253.

Fu AK, Smith FD, Zhou H, Chu AH, Tsim KW, Peng BH, Ip NY (1999b) Xenopus muscle-specific kinase: molecular cloning and prominent expression in neural tissues during early embryonic development. Eur J Neurosci 11:373–382.

Fu AK, Fu WY, Cheung J, Tsim KW, Ip FC, Wang JH, Ip NY (2001) Cdk5 is involved in neuregulin-induced AChR expression at the neuromuscular junction. Nat Neurosci 4:374–381.

Fu AK, Fu WY, Ng AK, Chien WW, Ng YP, Wang JH, Ip NY (2004) Cyclin-dependent kinase 5 phosphorylates signal transducer and activator of transcription 3 and regulates its transcriptional activity. Proc Natl Acad Sci U S A 101:6728–6733.

Fu AK, Ip FC, Lai KO, Tsim KW, Ip NY (1997) Muscle-derived neurotrophin-3 increases the aggregation of acetylcholine receptors in neuron-muscle co-cultures. Neuroreport 8:3895–3900.

Fu WY, Fu AK, Lok KC, Ip FC, Ip NY (2002a) Induction of Cdk5 activity in rat skeletal muscle after nerve injury. Neuroreport 13:243–247.

Fu WY, Wang JH, Ip NY (2002b) Expression of Cdk5 and its activators in NT2 cells during neuronal differentiation. J Neurochem 81:646–654.

Goldman D, Brenner HR, Heinemann S (1988) Acetylcholine receptor alpha-, beta-, gamma-, and delta-subunit mRNA levels are regulated by muscle activity. Neuron 1:329–333.

Gramolini AO, Angus LM, Schaeffer L, Burton EA, Tinsley JM, Davies KE, Changeux JP, Jasmin BJ (1999) Induction of utrophin gene expression by heregulin in skeletal muscle cells: role of the N-box motif and GA binding protein. Proc Natl Acad Sci U S A 96: 3223–3227.

Huang YZ, Zang M, Xiong WC, Luo Z, Mei L (2003) Erbin suppresses the MAP kinase pathway. J Biol Chem 278:1108–1114.

Ip FC, Fu AK, Tsim KW, Ip NY (1995) Cloning of the alpha component of the chick ciliary neurotrophic factor receptor: developmental expression and down-regulation in denervated skeletal muscle. J Neurochem 65:2393–2400.

Ip FC, Fu AK, Tsim KW, Ip NY (1996) Differential expression of ciliary neurotrophic factor receptor in skeletal muscle of chick and rat after nerve injury. J Neurochem 67:1607–1612.

Ip FC, Glass DG, Gies DR, Cheung J, Lai KO, Fu AK, Yancopoulos GD, Ip NY (2000a) Cloning and characterization of muscle-specific kinase in chicken. Mol Cell Neurosci 16:661–673.

Ip FCF, Glass DG, Gies DR, Cheung J, Lai K-O, Fu AKY, Yancopoulos GD, Ip NY (2000b) Cloning and characterization of muscle-specific kinase in chicken. Mol Cell Neurosci 16:661.

Ito A, Satoh T, Kaziro Y, Itoh H (1995) G protein beta gamma subunit activates Ras, Raf, and MAP kinase in HEK 293 cells. FEBS Lett 368:183–187.

Jacobson C, Duggan D, Fischbach G (2004) Neuregulin induces the expression of transcription factors and myosin heavy chains typical of muscle spindles in cultured human muscle. Proc Natl Acad Sci U S A 101:12218–12223.

Kim D, Chi S, Lee KH, Rhee S, Kwon YK, Chung CH, Kwon H, Kang M-S (1999) Neuregulin stimulates myogenic differentiation in an autocrine manner. J Biol Chem 274:15395–15400.

Klarsfeld A, Changeux JP (1985) Activity regulates the levels of acetylcholine receptor alpha-subunit mRNA in cultured chicken myotubes. Proc Natl Acad Sci U S A 82:4558–4562.

Krag TOB, Bogdanovich S, Jensen CJ, Fischer MD, Hansen-Schwartz J, Javazon EH, Flake AW, Edvinsson L, Khurana TS (2004) Heregulin ameliorates the dystrophic phenotype in mdx mice. Proc Natl Acad Sci U S A 101:13856–13860.

Kuemmerle JF, Murthy KS (2001) Coupling of the insulin-like growth factor-I receptor tyrosine kinase to Gi2 in human intestinal smooth muscle: Gbetagamma-dependent mitogen-activated protein kinase activation and growth. J Biol Chem 276:7187–7194.

Lai KO, Ip FC, Cheung J, Fu AK, Ip NY (2001) Expression of Eph receptors in skeletal muscle and their localization at the neuromuscular junction. Mol Cell Neurosci 17:1034–1047.

Leu M, Bellmunt E, Schwander M, Farinas I, Brenner HR, Muller U (2003) Erbb2 regulates neuromuscular synapse formation and is essential for muscle spindle development. Development 130: 2291–2301.

Lindorfer MA, Myung CS, Savino Y, Yasuda H, Khazan R, Garrison JC (1998) Differential activity of the G protein beta5 gamma2 subunit at receptors and effectors. J Biol Chem 273:34429–34436.

Lowes VL, Ip NY, Wong YH (2002) Integration of signals from receptor tyrosine kinases and g protein-coupled receptors. Neurosignals 11:5–19.

Luttrell LM, van Biesen T, Hawes BE, Koch WJ, Touhara K, Lefkowitz RJ (1995) G beta gamma subunits mediate mitogen-activated protein kinase activation by the tyrosine kinase insulin-like growth factor 1 receptor. J Biol Chem 270:16495–16498.

Poppleton H, Sun H, Fulgham D, Bertics P, Patel TB (1996) Activation of Gsalpha by the epidermal growth factor receptor involves phosphorylation. J Biol Chem 271:6947–6951.

Rakhit S, Pyne S, Pyne NJ (2001) Nerve growth factor stimulation of p42/p44 mitogen-activated protein kinase in PC12 cells: role of G(i/o), G protein-coupled receptor kinase 2, beta-arrestin I, and endocytic processing. Mol Pharmacol 60:63–70.

Rimer M (2003) Neuregulins: primary or secondary signals for the control of synapse-specific gene expression. J Neurocytol 32:665–675.

Schaeffer L, de Kerchove d'Exaerde A, Changeux JP (2001) Targeting transcription to the neuromuscular synapse. Neuron 31:15–22.

Schwindinger WF, Robishaw JD (2001) Heterotrimeric G-protein betagamma-dimers in growth and differentiation. Oncogene 20: 1653–1660.

Si J, Wang Q, Mei L (1999) Essential roles of c-JUN and c-JUN N-terminal kinase (JNK) in neuregulin-increased expression of the acetylcholine receptor epsilon-subunit. J Neurosci 19:8498–8508.

Sun H, Chen Z, Poppleton H, Scholich K, Mullenix J, Weipz GJ, Fulgham DL, Bertics PJ, Patel TB (1997) The juxtamembrane, cytosolic region of the epidermal growth factor receptor is involved in association with alpha-subunit of Gs. J Biol Chem 272:5413–5420.

Sunesen M, Changeux JP (2003) Transcription in neuromuscular junction formation: who turns on whom? J Neurocytol 32:677–684.

Tanowitz M, Si J, Yu D-H, Feng G-S, Mei L (1999) Regulation of neuregulin-mediated acetylcholine receptor synthesis by protein tyrosine phosphatase SHP2. J Neurosci 19:9426–9435.

Yamauchi J, Nagao M, Kaziro Y, Itoh H (1997) Activation of p38 mitogen-activated protein kinase by signaling through G protein-coupled receptors. Involvement of Gbetagamma and Galphaq/11 subunits. J Biol Chem 272:27771–27777.

Yang X, Arber S, William C, Li L, Tanabe Y, Jessell TM, Birchmeier C, Burden SJ (2001) Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron 30:399–410.

Zhang S, Coso OA, Lee C, Gutkind JS, Simonds WF (1996) Selective activation of effector pathways by brain-specific G protein beta5. J Biol Chem 271:33575–33579.

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