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The Complex of Ciliary Neurotrophic Factor-Ciliary Neurotrophic Factor Receptor α Up-Regulates Connexin43 and Intercellular Coupling in Astrocytes via the Janus Tyrosine Kinase/Signal Transducer and Activator of Transcription Pathway[D]

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Cytokines regulate numerous cell processes, including connexin expression and gap junctional coupling. In this study, we examined the effect of ciliary neurotrophic factor (CNTF) on connexin43 (Cx43) expression and intercellular coupling in astrocytes. Murine cortical astrocytes matured in vitro were treated with CNTF (20 ng/ml), soluble ciliary neurotrophic factor receptor α (CNTFRα) (200 ng/ml), or CNTF-CNTFRα. Although CNTF and CNTFRα alone had no effect on Cx43 expression, the heterodimer CNTF-CNTFRα significantly increased both Cx43 mRNA and protein levels. Cx43 immunostaining correlated with increased intercellular coupling as determined by dye transfer analysis. By using the pharmacological inhibitor α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide (AG490), the increase in Cx43 was found to be dependent on the Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway. Immunocytochemical analysis revealed that CNTF-CNTFRα treatment produced nuclear localization of phosphorylated STAT3, whereas CNTF treatment alone did not. Transient transfection of constructs containing various sequences of the *Cx43* promoter tagged to a LacZ reporter into ROS 17/2.8 cells confirmed that the promoter region between −838 to −1693 was deemed necessary for CNTF-CNTFRα to induce heightened expression. CNTF-CNTFRα did not alter Cx30 mRNA levels, suggesting selectivity of CNTF-CNTFRα for connexin signaling. Together in the presence of soluble receptor, CNTF activates the JAK/STAT pathway leading to enhanced Cx43 expression and intercellular coupling.

INTRODUCTION

Gap junctions are intercellular channels between adjacent cells that permit the passage of various substances <1.2 kDa in size (Kumar and Gilula, 1996). Such junctions are formed when each cell provides a connexon, which itself is composed of six connexins (Cxs) (Kumar and Gilula, 1996). Cx43, the prominent Cx isoform expressed in the central nervous system (CNS), is highly expressed in astrocytes (Yamamoto *et al*., 1990; Dermietzel *et al*., 1991; Giaume *et al*., 1991), neuronal precursors (Rozental *et al*., 1998; Bittman and LoTurco, 1999), and possibly neurons (Bruzzone and Ressot, 1997; Vaney, 1999; SiuYi *et al*., 2001; Rouach *et al*., 2002). Several studies using in vitro and in vivo models have demonstrated that impediment of gap junctional coupling and/or connexin43 expression amplifies cell death and tissue damage in the wake of injuries and diseases (Blanc *et al*., 1998; Naus *et al*., 2001; Siushansian *et al*., 2001; Ozog *et al*., 2002b; Lin *et al*., 2003; Nakase *et al*., 2003). Thus, design of an intervention to up-regulate Cx43 expression and intercellular communication may lead to novel therapies against pathological disturbances.

Gap junction expression and activity can be altered on a short-term or long-term basis (reviewed by Giaume and McCarthy, 1996; Rouach and Giaume, 2001). Short-term regulation is the result of posttranslational processing such as phosphorylation or channel blocking. Long-term regulation, however, entails modification of gene transcription and turnover of gap junctions. Although numerous agents modulating short-term gap junction regulation have been identified (reviewed by Dhein, 1998), long-term regulators have not been extensively studied.

Several cytokines influence long-term gap junction regulation. Cx43 in astrocytes is up-regulated by insulin-like growth factor-1 (Aberg *et al*., 2003) or transforming growth factor (TGF)-β1 (Robe *et al*., 2000), whereas it is down-regulated with either interleukin (IL)-1β (John *et al*., 1999) or TGF-β3 (Reuss *et al*., 1998). The cytokine fibroblast growth

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factor (FGF)-2 has opposing effects on different cells of the CNS; it has been reported to increase Cx43 in neurons (SiuYi *et al*., 2001) but decrease this gap junction component in astrocytes (Reuss *et al*., 1998, 2000).

Ciliary neurotrophic factor (CNTF) is a member of the IL-6 family that is produced as a nonsecreted cytosolic cytokine by astrocytes within the CNS and has its specific receptor, termed ciliary neurotrophic factor receptor α (CNTFRα), located on neuronal cell membranes (Lin *et al*., 1989; Stockli *et al*., 1989; Ip and Yancopoulos, 1992; Patterson, 1992). This protein-receptor organization suggests that the function of CNTF is restricted to brain injury such that only when the astrocytes' membrane becomes compromised can CNTF diffuse out and bind neuronal CNTFRα. Further supporting an association with brain injury, astrocytes enter a *reactive* state after CNS disturbances and are distinguished, at least in part, by heightened CNTF levels and initiation of CNTFRα expression (Ip *et al*., 1993; Rudge *et al*., 1995).

CNTFRα exists in two forms, membrane bound and soluble. The glycosyl phosphatidylinositol linkage of CNTFRα to the cell membrane can be cleaved by phospholipases releasing CNTFRα to act as a soluble protein (Taga *et al*., 1989). Although most soluble receptors for cytokines and growth factors act antagonistically with their membrane-bound counterparts, soluble CNTFRα retains its ability to bind CNTF and act as an agonistic autocrine or paracrine factor (Marz *et al*., 1999). The heterodimer of CNTF and soluble CNTFRα (hereinafter termed "Complex") can trans-signal cells, independent of endogenous CNTFRα expression, by binding with the required β subunits glycoprotein 130 (gp130) and leukemia inhibitory factor receptor β (LIFRβ) (Davis *et al*., 1993a; Rose-John and Heinrich, 1994; Stahl *et al*., 1994). Complex-mediated heterodimerization of the transmembrane β subunits transduces the CNTF signal inside the cell via the Janus kinase/signal transducers of activated transcription (JAK/STAT) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways (reviewed by Monville *et al*., 2001).

One potential target gene of CNTF signaling is *Cx43*. Our previous work demonstrated that Complex can up-regulate Cx43 and gap junctions in communication-deficient cancer cells (Ozog *et al*., 2002a), but two major questions remained elusive. First, does Complex elicit a similar effect in nontumorigenic, communication-competent cells, i.e., can Complex increase Cx43 expression above and beyond basal levels in cells that are already highly coupled? Second, what is the signaling mechanism responsible for Complex-induced up-regulation of Cx43? In the current study, we demonstrate that CNTFRα is required for CNTF-induced Cx43 up-regulation in normal astrocytes and occurs in a JAK/STAT-dependent manner. The localization of increased Cx43 to the periphery of the cells in conjunction with increased dye passage between the cells demonstrates enhanced formation of gap junctions induced by Complex. Furthermore, CNTF-CNTFRα Complex regulation of murine *Cx43* is dependent on a specific region in the promoter that contains putative CNTF-response elements (i.e., STAT3 binding sites).

MATERIALS AND METHODS

Astrocyte Cultures

Primary cultures of murine cortical astrocytes were prepared in a manner similar to that described by Fedoroff and Richardson (1997). Briefly, brains were removed from 1-d-old CD-1 mouse pups and subsequently freed of meninges. Cortices were isolated, placed into growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin, and 10 μg/ml streptomycin; Invitrogen, Burlington, ON, Canada), and mechanically dissociated using a serological pipette. The cell suspension was then passed through a 70-μm cell strainer (Falcon, VWR International, Mississauga, ON, Canada) and subsequently diluted with growth medium at a ratio of 5 ml/cortex. Cells were plated onto 60-mm dishes (3 ml of cell suspension/dish) or 100-mm dishes (10 ml of cell suspension/dish) and maintained in a humidified incubator at 37°C in 95% air/5% CO_2. Medium was changed every 3 d thereafter in addition to shaking the cultures. After 6 wk, cultures were maintained in medium I (54 ml of neurobasal medium [Invitrogen]), 36 ml of DMEM/F-12 [Invitrogen], D-glucose [0.6%], insulin [10 μg/ml], transferrin [20 μg/ml], putrescine-HCl [62 μM], progesterone [20 nM], sodium selenite [30 nM], 10 U/ml penicillin, and 10 μg/ml streptomycin) for 1 wk. All experiments with the astrocytes were performed in medium I.

ROS 17/2.8 Cultures

The osteosarcoma cell line ROS 17/2.8 was grown in α-minimal essential medium (MEM) (Invitrogen) supplemented with FBS (10%), penicillin (10 U/ml), and streptomycin (10 μg/ml). Twenty-four hours before experiments with ROS 17/2.8 cells, their medium was switched to CELOX defined medium (CELOX Laboratories, St. Paul, MN) without serum. All experiments with ROS 17/2.8 cells were performed in CELOX defined medium.

Exposure to CNTF and CNTFRα

Astrocytes were treated with either vehicle (phosphate buffered saline; PBS), CNTF (20 ng/ml; R&D Systems, Minneapolis, MN), soluble CNTFRα (200 ng/ml; R&D Systems), or Complex (20 ng/ml CNTF + 200 ng/ml CNTFRα, which is about a 1:5 M ratio; excessive soluble receptor favored the protein to be in CNTF-CNTFRα heterodimer form). For expression of Cx43 protein, cells were treated with agents in fresh medium I every 24 h for a total of 3 d. For examination of mRNA levels, cells were treated with agents for 24 h. For assessment of STAT3, ERK1 (p44 MAPK), or ERK2 (p42 MAPK) phosphorylation, cells received a medium change and 24 h later agents were added for 5, 15, and 60 min. The STAT3 and MAPK/ERK pathways were inhibited by α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide (AG490; 50 μM; Hodge *et al*., 2002; Calbiochem, La Jolla, CA) or 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126; 10 μM; Favata *et al*., 1998; Promega, Madison, WI), respectively, 45 min before treating the cells with vehicle or Complex for 15 min.

Protein Isolation and Immunoblot Analysis

Astrocyte monolayers were rinsed twice with PBS and harvested in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) supplemented with protease inhibitors (mini-Complete; Roche, Laval, QC, Canada) by using a rubber policeman. The lysate was sheared using a 22-gauge needle and centrifuged at 10,000 × *g* for 10 min at 4°C. Although total Cx43 can theoretically be measured by summing the signal from all bands, the large difference in quantities between the nonphosphorylated and phosphorylated forms usually results in underexposure and overexposure, respectively, and thus limits quantification. To overcome this problem, selected samples were treated with alkaline phosphatase (calf intestinal; Roche) to produce only one band representing total Cx43 protein. Protein concentration of total cell lysate was determined using the bicinchoninic acid protein assay kit (Pierce Endogen, Rockford, IL). Protein samples (50 μg each) and molecular weight markers (Bio-Rad, Mississauga, ON, Canada) were subjected to 10% SDS-PAGE and subsequently electrotransferred onto a nitrocellulose membrane for 1 h at 80 W/110 cm^2. The membrane was immunoblotted using appropriate primary antibodies (Cx43; Sigma-Aldrich Canada, Oakville, ON, Canada; and phospho-STAT3 and phospho-p44/42 MAPK; Cell Signaling Technology, New England Biolabs, Mississauga, ON, Canada) and subsequently incubated in secondary antibodies tagged with horseradish peroxidase (CedarLane Laboratories, Hornby, ON, Canada). The blots were then incubated in Supersignal (Pierce Endogen) and exposed to Kodak X-Omat x-ray film to visualize antibody binding. To normalize protein loading, membranes were stripped of antibodies using 2-mercaptoethanol (10 mM), SDS (2%), and Tris (62.5 mM, pH 6.7) for 30 min at 65°C and subsequently probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; CedarLane Laboratories), STAT3 (Cell Signaling Technology), or ERK1 and ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA).

RNA Isolation

Cytoplasmic RNA was isolated from astrocytes by using the phenol-chloroform-isoamyl alcohol method outlined by Sambrook *et al*. (1989). Briefly, cells were washed with PBS and then lysed using lysis solution (0.2 M NaCl, 20 mM $MgCl_2$, and 20 mM Tris, pH 8.8). After centrifugation at 16,000 × *g* for 30 s, the supernatant was added to phenol solution (0.75 g of phenol, 3.4 mM 8-hydroxyquinoline, 4.2 M guanidine thiocyanate, 26.4 mM sodium citrate, 0.5% sarcosyl, 0.4% 2-mercaptoethanol, and 100 mM sodium acetate) followed by chloroform (10%). The mixture was then centrifuged at 16,000 × *g* for 15 min, and the aqueous layer was added to an equal volume of isopropanol.

Table 1. Primer sequences used for PCR analysis of cDNA

Target	Primers	Expected product size (bp)	Reference
CNTF	Forward 5′-GGAAGATTCGTTCAGACCTGAC-3′ Reverse 5′-CCCATCAGCCTCATTTTCAGGG-3′	350	Malgrange *et al.* (1998)
CNTFRα	Forward 5′-CCACATGCTGCCATTGATCC-3′ Reverse 5′-CCACATGCTGCCATTGATCC-3′	430	Malgrange *et al.* (1998)
Cx43	Forward 5′-CCCCACTCTCACCTATGTCTCC-3′ Reverse 5′-ACTTTTGCCGCCTAGCTATCCC-3′	500	Naus *et al.* (1997)
Cx30	Forward 5′-AATGTGGCCGAGTTGTGTTA-3′ Reverse 5′-CCAAGGCCCAGTTGTCAC-3′	399	Dahl *et al.* (1996)
GAPDH	Forward 5′-AATGCATCCTGCACCACCAA-3′ Reverse 5′-GTAGCCATATTCATTGTCATA-3′	515	Yang *et al.* (2001)
gp130	Forward 5′-CCTCACACTCCTCCAAGGCAC-3′ Reverse 5′-CTGGATTCATGCTGACTGCAG-3′	473	Zaheer *et al.* (1995)
LIFRβ	Forward 5′-TCCTGGAATCTCGGTCGATAG-3′ Reverse 5′-GAATCAAGAATTGCCTGGAATTG-3′	483	Nakashima *et al.* (1999)

The RNA was pelleted from the mixture, washed with 90% ethanol and quantified by spectrophotometry at 260 nm.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Semiquantitative RT-PCR

RT-PCR was performed on the RNA as described previously (Ozog *et al.*, 2002a). Briefly, 3 μg of RNA was pretreated with DNase, and subsequently, reverse transcribed in a thermal cycler. For semiquantitative RT-PCR, 25 cycles were used to amplify the cDNA to avoid saturation (that was evident at 30 cycles). Primer sets used for amplification are provided in Table 1.

The amplified cDNA was run on a 1.8% agarose gel containing 12% ethidium bromide along with a 1-kb DNA standard (Invitrogen). One method used to rule out false positives was performing parallel experiments in the absence of the reverse transcriptase. Functionality of all primers was confirmed by performing RT-PCR on RNA isolated from brain cortex of adult CD-1 mice. Images of amplified products were visualized and captured using Kodak ds 1D digital software (Mandel Scientific, Guelph, ON, Canada).

Northern Blot Analysis

Cells were exposed to either vehicle, CNTF, CNTFRα, or Complex in fresh medium I for 24 h. RNA was then isolated as described above. Denatured RNA samples (20 μg) were resolved in a 1% agarose/formaldehyde gel and subsequently transferred onto a nylon membrane (BrightStar-Plus; Ambion, Austin, TX) by capillary diffusion. Blots were prehybridized in 5× Denhardt's solution, 5× saline-sodium phosphate-EDTA, 50% formamide, 1% SDS, and 100 μg/ml denatured salmon sperm DNA for 4 h at 40°C. Subsequently, blots were hybridized with ^{32}P-labeled Cx43 (Beyer *et al.*, 1987) or 18S cDNA (Ambion) overnight at 40°C. The blots were washed 30 min each with 2× SSC (40°C), 1× SSC (40°C), and 0.1× SSC with 0.1% SDS (42°C) and then exposed to Kodak Bio-Max film at −80°C for 6–48 h by using intensifying screens.

Immunocytochemistry

Astrocytes were rinsed twice with PBS and subsequently fixed with 4% formaldehyde for 10 min. Cells were then washed with PBS and blocked for nonspecific binding by using 10% normal goat serum in PBS. Subsequently, cells were incubated with primary antibody according to company recommendations (glial fibrillary acidic protein (GFAP) polyclonal antibody and Cx43 polyclonal antibody, Sigma-Aldrich; and phospho-STAT3, Cell Signaling Technology). After three washes with PBS, cells were incubated in Alexa-conjugated secondary IgG (Molecular Probes, Eugene, OR) for 1 h, stained with Hoechst 33342 dye (Sigma-Aldrich) where indicated, and then mounted with Vectashield mounting medium (Vector Laboratories, Burlington, ON, Canada). One method to confirm specificity of antibody binding was performed by omitting the primary antibody from the immunolabeling protocol. Immunostaining was viewed using a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY), and images were captured using Northern Eclipse, version 5 (Empix Imaging, Mississauga, ON, Canada) or AxioVision (Carl Zeiss).

Dye Transfer Assays

Scrape-Loading. The scrape-loading method, described by el-Fouly *et al.* (1987), was used to characterize intercellular coupling. This method was chosen over the preloading (see below) and microinjection methods because the matured, quiescent astrocytes are characterized by resistance to gentle trypsinization and exhibit a flattened morphology. Briefly, the medium was aspirated from the cultures, and the cells were bathed in 50 μl of dye solution (0.1% carboxyfluorescein and 0.1% dextran tetramethylrhodamine [Molecular Probes] in PBS) while scraped using a surgical blade. After 90 s, cells were washed several times with PBS containing the gap junction blocker carbenoxolone (100 μM; Sigma-Aldrich). Carbenoxolone was used to halt the rapid progression of carboxyfluorescein throughout the highly coupled astrocytes so that differences between the pretreatments could be identified and quantified. The extent of coupling was determined by measuring the extent of carboxyfluorescein diffusion among the cells. An increase in the number of functional gap junctions would allow more extensive diffusion of the gap junction-permeable dye. Cells were immediately examined using an Axioskop microscope (Carl Zeiss). The distance of dye spread was measured (in reference to the scrape) from the proximal edge of the scraped cell (dual labeled with dextran tetramethylrhodamine and calcein) to the distal edge of the farthest cell (perpendicular to the scrape) that could be visually identified as containing carboxyfluorescein.

Preloading. Intercellular coupling between ROS 17/2.8 cells was analyzed using a modified version of the preloading method described previously (Ozog *et al.*, 2002). Briefly, ROS 17/2.8 cells were seeded into 35-mm plates and when they reached 75% confluence, their medium was switched to CELOX defined medium without serum. One day later, the ROS 17/2.8 cells were treated with vehicle, CNTF, CNTFRα, or Complex every 24 h for 3 d. Donor ROS 17/2.8 cells from sister cultures, which received the same treatment, were preloaded with dye solution (5 μM calcein-AM [Molecular Probes] and 10 μM DiI [Sigma-Aldrich] in an isotonic [0.3 M] glucose solution) for 20 min in a humidified incubator (37°C; 5% CO_2/95% air). Donor cells were then rinsed several times with isotonic glucose solution, trypsinized, seeded onto recipient sister cultures at a ratio of 1:500, and maintained for 3 h in the incubator. Cells were examined with a photomicroscope (Axiovert 10; Carl Zeiss) supplemented with differential interference contrast (DIC). Gap junctional coupling was assessed by the passage of calcein from donor cells to, and among, recipient cells. Only donor cells that were coupled to at least one recipient cell were counted.

Cx43 Promoter Regulation

Plasmids containing various deletion constructs spanning the 5′ upstream promoter region of the mouse *Cx43* gene were transfected into ROS 17/2.8 cells in a manner similar to that described by Chatterjee *et al.* (2003). ROS 17/2.8 cells were chosen to investigate the regulation of *Cx43* by its promoter due to previous success with *Cx43* promoter activity and growth factors in this cell line (Chatterjee *et al.*, 2003) and because these cells endogenously express Cx43 (Steinberg *et al.*, 1994). Before transfection, cells were plated into 24-well plates at a concentration of 8×10^4 cells/well. Twenty-four hours postseeding, the cells were cultured in CELOX defined medium without serum for 1 d. Transfection of the plasmids designated pHXPL, dB4.9, dP, and dH (6691, 1693, 838, and 446 base pairs of the Cx43 promoter upstream from the initiation site, respectively, tagged to a LacZ reporter) was performed using Lipofectamine 2000 (3 μl of 1 mg/ml; Invitrogen) in 200 μl of OPTI-MEM (Invitrogen) containing 1:200 ratio of *Renilla* luciferase reporter plasmid (pRL-SV40; Promega.) at 5 μg/well. After 7 h, 1 ml of CELOX defined medium was added with vehicle, CNTF, CNTFRα, Complex, or FBS (5%) and maintained for 48 h. Cells were subsequently lysed, and lysates were stored at −70°C for 1 wk. Reporter expression was analyzed using β-galactosidase enzyme assay system and *Renilla* luciferase assay system (Promega). β-Galactosidase and luciferase activities were corrected by subtracting background

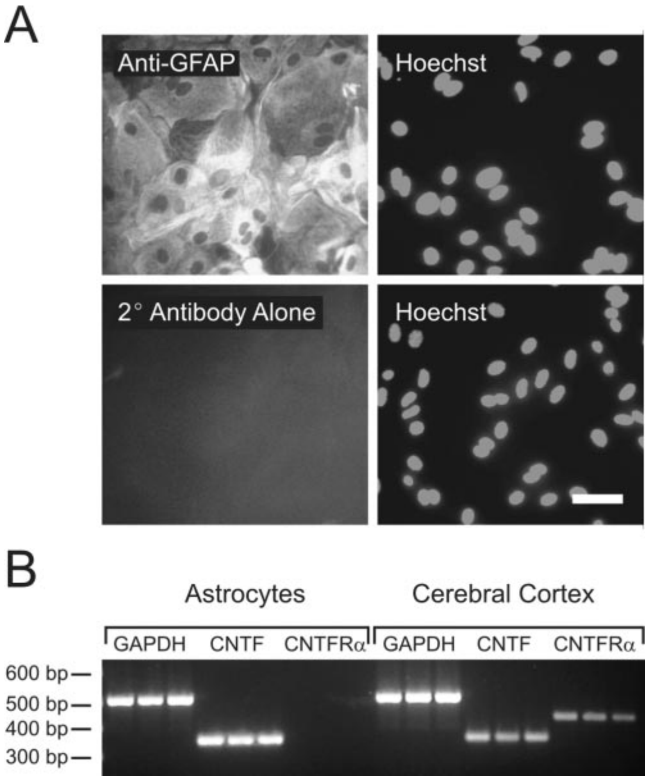

Figure 1. Mature cortical astrocytes express GFAP protein and mRNA for CNTF but not for CNTFRα in vitro. (A) An example of the primary cortical astrocytes used in experiments. Top row demonstrates immunoreactivity to anti-GFAP and nuclear staining with Hoechst. Omission of the GFAP antibody demonstrates its specificity to the astrocytes (bottom row). Bar, 50 μm. (B) RT-PCR analysis revealed that 7-wk-old astrocytes express CNTF mRNA, whereas they do not express mRNA for the receptor subunit CNTFRα. GAPDH was used as a positive control for RNA presence. Functionality of all primer sets was confirmed by performing RT-PCR on RNA isolated from cerebral cortex of adult mice. Representative gel shows triplicate amplifications for each primer set with RNA derived from three separate experiments.

activity as determined by mock-transfected samples. β-Galactosidase transfection efficiency was subsequently normalized using the luciferase activity from each sample. All transfections and assays were carried out in quadruplicate.

Data Analysis

All experiments were performed on four or more culture preparations from individual litters of mice. Densitometric analysis of immunoblots, Northern blots, and RT-PCR samples were performed using Scion Image software (Scion, Frederick, MD). Data are presented as means ± SEM. Comparisons between means were analyzed using one-way analysis of variance with the Tukey's comparisons test. A P value of <0.05 was considered significant.

RESULTS

Mature Astrocytes Express CNTF but Not CNTFRα In Vitro

Immunocytochemical analysis of the primary murine cortical astrocytes matured in culture and used for the following experiments revealed that 95% of the cells express the astroglial marker GFAP (Figure 1A). Although CNTFRα expression in the CNS is normally restricted to neurons, astrocytes begin to express CNTFRα after CNS disturbances. Astrocytes enter a reactive state after injury and are distinguished, at least in part, by CNTFRα expression (Ip *et al.*, 1993; Rudge *et al.*, 1995). Newly cultured astrocytes are believed to be in a reactive state (Wu and Schwartz, 1998); therefore, the cells used in these experiments were cultured for an extended period of time to minimize reactivity. RT-PCR analysis of RNA isolated from cortical astrocytes matured in vitro revealed that the cells expressed the transcript for CNTF but did not transcribe mRNA for CNTFRα (Figure 1B). Confirmation of primer functionality was assessed by performing RT-PCR on RNA isolated from the cerebral cortex; both CNTF and CNTFRα were detected at their expected product sizes (Figure 1B). No transcripts were observed when the same RNA samples were processed in the absence of reverse transcriptase (our unpublished data). Thus, GFAP-positive astrocytes matured in culture exhibited a CNTF/CNTFRα phenotype comparable with astrocytes in the normal/nonreactive state. Furthermore, CNTF should only be able to activate these cells when coadministered with soluble CNTFRα.

CNTF Complex Increases Cx43 Protein Expression and Intercellular Coupling

To determine whether CNTF altered Cx43 protein expression in astrocytes, cells were treated with vehicle, CNTF, CNTFRα, or Complex (CNTF:CNTFRα in a 1:5 M ratio) for 3 d and analyzed by immunoblotting (Figure 2). Consistent with previous findings (Giaume *et al.*, 1991; Li and Nagy, 2000), the astrocytes matured in vitro in this study expressed Cx43. Treatment of the astrocytes with CNTF or CNTFRα alone caused no detectable alteration in total Cx43 protein levels compared with vehicle (Figure 2, A and B). However, treatment of the astrocytes with Complex increased total Cx43 levels by 68 ± 11% (Figure 2, A and B). The phosphorylated states of Cx43 migrate at different rates through the gel and results in banding on the immunoblot (Musil *et al.*, 1990). When the phosphorylated forms of Cx43 were examined after treatment of the cells with the agents, Complex increased phosphorylated Cx43, compared with other treatments (Figure 2C). Quantitative analysis of phosphorylated Cx43 revealed that only Complex mediated a significant increase in the protein (our unpublished data). However, the ratio of phosphorylated Cx43 to unphosphorylated Cx43 was similar between all treatments.

Northern blot analysis was used to examine whether the increase in Cx43 was the result of increased mRNA for Cx43. Examination of RNA collected after 24-h treatment with the agents revealed that although neither CNTF nor CNTFRα alone caused a change in Cx43 mRNA levels compared with vehicle, Complex increased the level of Cx43 mRNA (Figure 3A). When normalized for RNA loading by using 18S mRNA levels, an increase of 107 ± 35% in Cx43 mRNA over that of vehicle treatment was induced by Complex (Figure 3B).

Localization of the Cx43 protein was examined by immunocytochemistry after 3-d treatment of the astrocytes with vehicle, CNTF, CNTFRα, or Complex. As is typical for Cx43 in astrocytes, the protein distributed intracellularly as well as to the periphery of the cells (Figure 4). Compared with vehicle treatment, no noticeable difference in either immunostaining intensity or localization of the Cx43 was detected in cells treated with CNTF or CNTFRα (Figure 4). However, astrocytes treated with Complex showed a dramatic increase in Cx43 immunolabeling (Figure 4). This Complex-induced increase in Cx43 could be detected within the cytoplasm as well as at the periphery of the cells. No differences were identified between the treatments in regard to astrocyte cell shape or size.

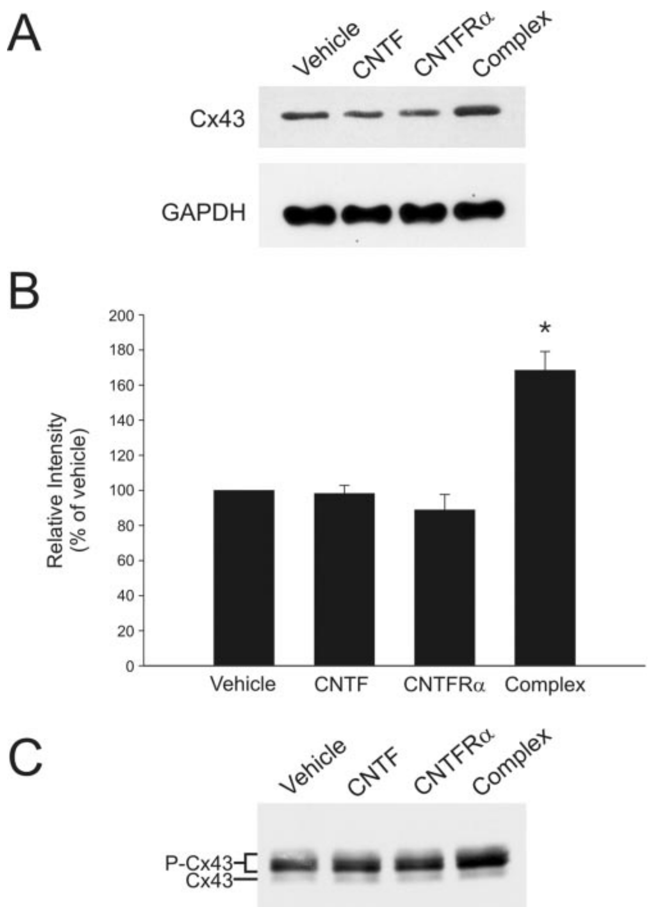

Figure 2. CNTF increases Cx43 protein levels in cultured astrocytes only when administered with soluble CNTFRα. (A) Representative immunoblot of cells treated with vehicle (PBS), CNTF (20 ng/ml), CNTFRα (200 ng/ml), or Complex (CNTF + CNTFRα) and immunoblotted for total Cx43. Immunoreactivity to GAPDH antibodies on the same immunoblot was used to normalize equal protein loading. (B) Densitometric analysis of all immunoblots revealed that Complex induced a significant increase in Cx43 protein levels compared with vehicle, CNTF, and CNTFRα. Data are mean ± SEM calculated from four independent experiments. *$p < 0.001$, compared with all other agents. (C) Representative immunoblot of astrocyte extracts treated with phosphatase inhibitors and immunoblotted with Cx43 antibodies. The Complex-induced increase in Cx43 could be detected in the nonphosphorylated form (Cx43) as well as in the phosphorylated forms (P-Cx43) of the protein.

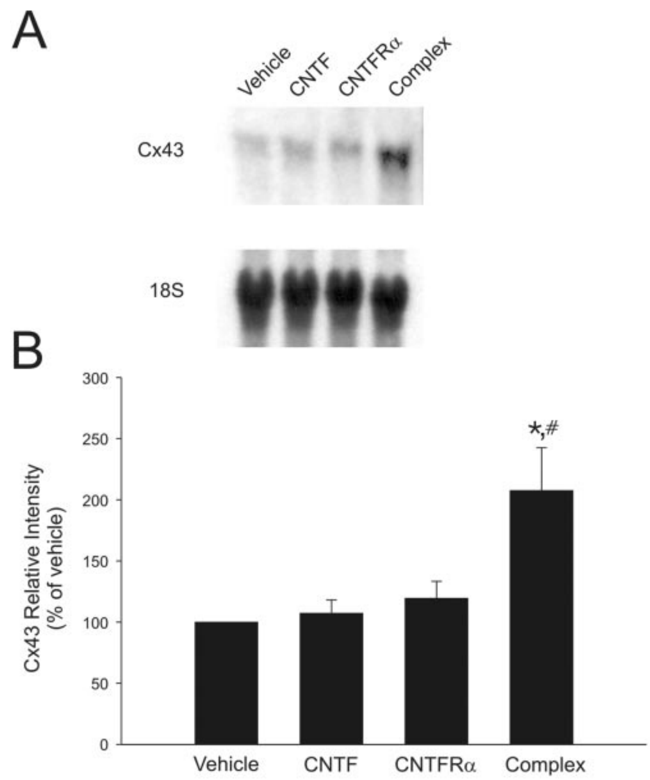

Figure 3. Complex induced an increase in Cx43 mRNA. (A) Representative Northern blot of RNA isolated from matured astrocytes treated with vehicle (PBS), CNTF (20 ng/ml), CNTFRα (200 ng/ml), or Complex (CNTF + CNTFRα). The blot was hybridized with a cDNA probe for Cx43 followed by a probe for 18S. (B) Densitometric analysis of the Northern blots was performed by normalizing RNA loading to 18S and then comparing Cx43 mRNA expression between treatments. Data are mean ± SEM calculated from four independent experiments. *$p < 0.05$ compared with CNTF and CNTFRα, #$p < 0.01$ compared with vehicle.

Intercellular coupling between the astrocytes was examined by the scrape-loading/dye transfer technique after 3-d treatment of the cells with vehicle, CNTF, CNTFRα, or Complex. Due to the extensive intercellular coupling between astrocytes in vitro, scrape-loading with time-specific addition of the gap junction blocker carbenoxolone was the method chosen to examine heightened coupling. Neither CNTF nor CNTFRα caused an increase in dye transfer between cells compared with vehicle. Complex, however, increased the spreading of the gap junction-permeable dye carboxyfluorescein (Figure 5A). When quantified from the scrape to the distal edge of the furthest cell exhibiting fluorescence, the distance over which the intercellular spread of carboxyfluorescein could visually be detected was significantly greater when cells were treated with Complex, compared with vehicle, CNTF, or CNTFRα alone (Figure 5B).

Complex-Induced Increase in Cx43 Expression Is Mediated by the JAK/STAT Pathway

Two known intracellular signaling pathways activated by CNTF in other cell systems are the MAPK/ERK pathway and the JAK/STAT pathway. When examining phosphorylation states of both pathways, total ERK1/2 and total STAT3 immunoreactivity were subsequently screened to allow normalization of protein loading between the 12 lanes. Experiments were repeated four independent times, and the results showed consistent activation/phosphorylation of the pathway constituents in each trial. When astrocytes were treated with vehicle, CNTF, CNTFRα, or Complex for 15 and 60 min, only Complex caused a detectable increase in the phosphorylation of ERK1 and ERK2 at all time points (Figure 6A). Although phosphorylation of ERK1/2 was detectable at the 5-min time point, Complex induced greater phosphorylation after longer treatments. CNTF alone caused a noticeable increase in phosphorylation of ERK1 and ERK2 only after 60-min treatment (Figure 6A). Phosphorylation of STAT3 was induced in the astrocytes by both CNTF and Complex treatments (Figure 6B). This activation of STAT3 was most dramatic at the 5-min time point and decreased with longer durations.

To determine the pathway mediating the increase in Cx43 by Complex, inhibitors of the MAPK/ERK pathway and the JAK/STAT pathway were used. Astrocytes were treated

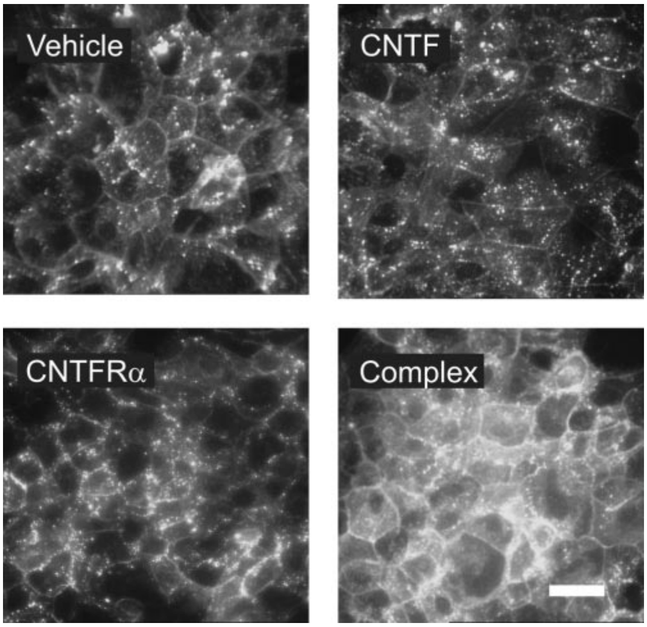

Figure 4. The increased Cx43 induced by Complex localized to both cytosolic and membrane areas of the astrocytes. Confluent monolayers of matured cortical astrocytes were treated with vehicle (PBS), CNTF (20 ng/ml), CNTFRα (200 ng/ml), or Complex (CNTF + CNTFRα) for 3 d and immunostained with Cx43 antibody. The Complex-induced increase in Cx43 immunostaining is identified both within the cytoplasm and at the cell periphery. Bar, 100 μm.

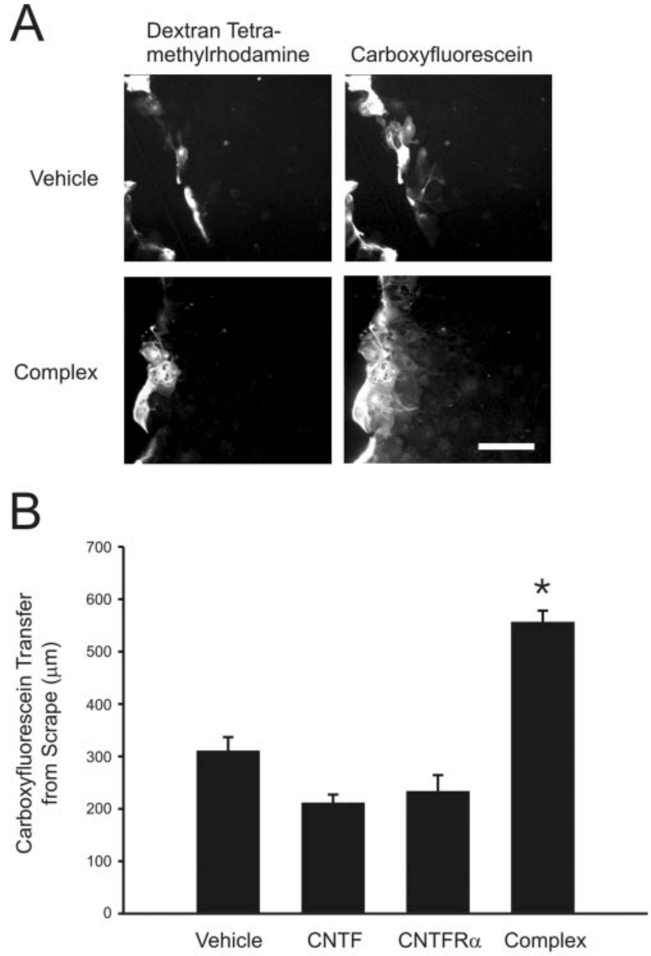

Figure 5. Complex increases intercellular coupling in astrocytes. Confluent monolayers of matured cortical astrocytes were treated with vehicle (PBS), CNTF (20 ng/ml), CNTFRα (200 ng/ml), or Complex (CNTF + CNTFRα) for 3 d, scrape-loaded, and examined for dye transfer. (A) Representative plate showing dye transfer in vehicle-treated (similar results to both CNTF and CNTFRα treatments) and Complex-treated cells. Intercellular coupling was detected by passage of carboxyfluorescein (gap junction permeable) from scraped cells (colabeled with the gap junction-impermeable dye dextran tetramethylrhodamine). Bar, 150 μm (B) When quantified, the distance over which carboxyfluorescein spread did not differ in cells treated with vehicle, CNTF, or CNTFRα. However, Complex significantly increased the distance over which the dye spread between cells compared with all other treatments. Data are mean ± SEM calculated from four independent experiments. *$p < 0.001$ compared with vehicle, CNTF, and CNTFRα.

with the ERK-activating kinase (MEK) 1/2 inhibitor U0126 for 45 min before and throughout the addition of Complex. U0126 has been shown to noncompetitively inhibit MEK1 and MEK2 (Duncia *et al*., 1998) and inhibited ERK1/2 phosphorylation induced by Complex in these experiments (Figure 7A). In parallel experiments, astrocytes were pretreated with the JAK inhibitor AG490. AG490 is a tyrphostin known to block JAK2/3, and to a lesser degree JAK1 (De Vos *et al*., 2000). Inhibition of JAK activation can prevent the subsequent phosphorylation and activation of STATs. AG490 inhibited nearly all STAT3 phosphorylation activated by Complex treatment (Figure 7A). Failure of AG490 to completely halt STAT3 phosphorylation (trace amounts detectable on immunoblots) may be credited to incomplete JAK1 or TYK2 (another member of the JAK family) activation or insufficient amount of blocker used. Immunoblot analysis revealed that acute exposure (24 h) of the astrocytes to either U0126 or AG490 alone did not alter Cx43 levels (our unpublished data). RT-PCR analysis revealed that Complex still increased Cx43 mRNA levels in the presence of dimethyl sulfoxide (DMSO; solvent of AG490 and U0126) and in the presence of U0126 (Figure 7, B and C). However, the Complex-induced increase in Cx43 mRNA was not observed when cells were treated with AG490 (Figure 7, B and C).

Although both CNTF and Complex induced phosphorylation of STAT3, only Complex caused an up-regulation of Cx43. Immunocytochemical analysis of cells treated with CNTF or Complex for 15 min revealed a difference in cellular localization of the phosphorylated STAT3. In cells treated with CNTF alone, the majority of phosphorylated STAT3 remained cytosolic, and nuclear translocation was markedly limited (Figure 8A). However, treatment of the astrocytes with Complex resulted in the majority of phosphorylated STAT3 translocating to the nucleus within the same exposure time (Figure 8A). No immunostaining for phosphorylated STAT3 was detected when cells were treated with vehicle or CNTFRα (Figure 8A).

Immunoblot analysis revealed that the dramatic increase in STAT3 phosphorylation induced by Complex was acute (Figure 6). However, increased Cx43 expression and intercellular coupling was readily identified after treating the cells with Complex every 24 h for 3 d. When levels of phosphorylated STAT3 were examined by immunoblot analysis on total protein isolated from astrocytes after the 3-d treatment, phosphorylated STAT3 could still be detected with CNTF and Complex treatments (Figure 8B). Immunocytochemical staining against phosphorylated STAT3 in sister cultures revealed that CNTF induced extensive diffuse

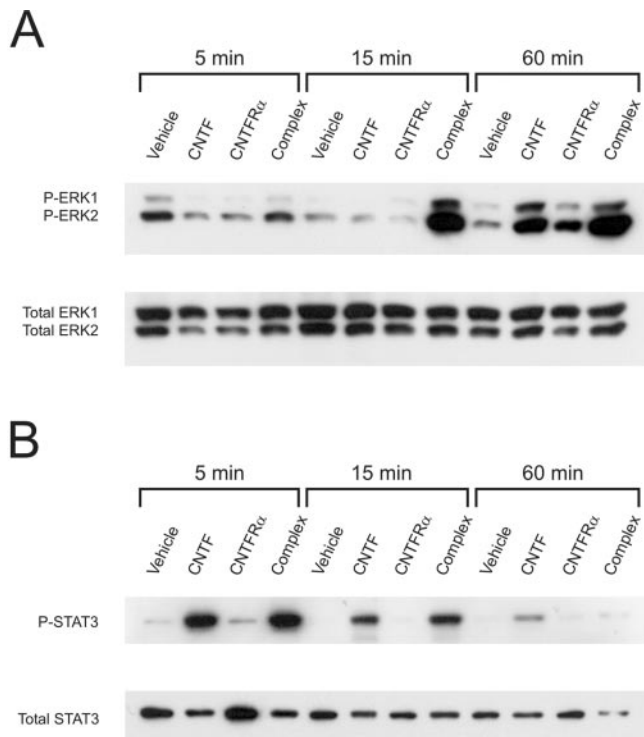

Figure 6. Both the MAPK/ERK and the STAT signaling transducers are phosphorylated in astrocytes after exposure to Complex. Representative immunoblots of astrocyte extracts after treatment with vehicle (PBS), CNTF (20 ng/ml), CNTFRα (200 ng/ml), or Complex (CNTF + CNTFRα) for 5, 15, and 60 min. Immunoblots were incubated with antibodies against phosphorylated ERK1/2 (P-ERK1 and P-ERK2) followed by total ERK1 and total ERK2 (A) or phosphorylated STAT3 (P-STAT3) followed by total STAT3 (B).

staining with some localization at the periphery of the cell (Figure 8C). Complex treatment caused phosphorylated STAT3 to localize throughout the cell, at the cell's periphery, and to the nucleus (Figure 8C).

Modulation of Cx43 Promoter Activity by Complex

To analyze the *Cx43* promoter for regions that may respond to regulation by Complex, deletion mutation constructs of the *Cx43* promoter tagged to a LacZ reporter were transfected into a cell model previously used to evaluate *Cx43* promoter activity, the ROS 17/2.8 cell line (Chatterjee *et al*., 2003). ROS 17/2.8 cells endogenously express Cx43 (Steinberg *et al*., 1994) and transcribe mRNA for CNTF, gp130, and LIFRβ but not for CNTFRα mRNA (as determined by RT-PCR analysis; Figure 9A). Four LacZ reporter constructs containing various lengths of the Cx43 promoter upstream from the initiation site (designated pHXPL, dB4.9, dP, and dH; Figure 9B) were cotransfected with *Renilla* luciferase into ROS 17/2.8 cells. Treatment of the cells with vehicle, CNTF, and CNTFRα mediated little or no β-galactosidase activity in the cells transiently transfected with the four constructs (Figure 9C). Treatment with Complex, however, increased β-galactosidase activity in cells transfected with the pHXPL and dB4.9 plasmids but not with dP or dH (Figure 9B). This finding confirms the requirement of CNTFRα for CNTF to activate Cx43 expression and that the *Cx43* promoter region containing CNTF regulatory elements (-1693 to -838 base pairs) is crucial for CNTF to activate transcription of this gene.

Heightened Intercellular Coupling Induced by Complex Is Not Restricted to Glia

The ability of Complex to activate the Cx43 reporter constructs in ROS 17/2.8 cells suggested that this osteosarcoma cell line may respond to Complex in a similar manner as the astrocytes. Analysis of alterations in intercellular coupling in the ROS 17/2.8 cells was performed using the preloading/dye transfer technique. When treated with CNTF or CNTFRα alone, the extent of dye passage was similar to that of vehicle treated cells (Figure 10A). However, when ROS 17/2.8 cells were treated with Complex, calcein passed to a great deal more recipient cells from the single donor cell, indicative of heightened gap junctional coupling (Figure 10A). Complex treatment caused no detectable morphological changes in the ROS 17/2.8 cells. When the number of recipient cells coupled to a single donor cell was quantified, neither CNTF nor CNTFRα caused significant changes from vehicle-treated cells (Figure 10B). In addition, the number of recipient cells coupled to one donor cell when treated with either vehicle, CNTF, or CNTFRα ($\sim$12) is similar in value to that previously determined by Lecanda *et al.*(1998), who used a microinjection/dye transfer technique on ROS 17/2.8 cells. Complex treatment increased the number of recipient cells coupled to an individual donor cell by more than threefold compared with the other treatments (Figure 10).

DISCUSSION

Understanding the regulation of gap junction expression by different agents, including cytokines, may prove productive when devising potential therapies against cytotoxic disturbances. In vivo, Cx43 expression and gap junction formation is likely regulated by the coordinated release of various cytokines and the receptor expression profile of each cell type. Administration of exogenous cytokines could enhance the intervention in the pathological progression of various diseases and disturbances. Whereas CNTF may elicit specific responses to CNTFRα-presenting cells, the Complex form of this cytokine may allow CNTF's cytoprotective properties, i.e., via up-regulated intercellular coupling mediated by Cx43 expression, to extend to non-CNTFRα–expressing cell types.

Collectively, previous studies have demonstrated that astrocytes respond inconsistently to exogenously administered CNTF, and this may be a consequence of the experimental model used. Because astrocytes can be found in "normal" and "reactive" states both in vitro and in vivo (reviewed by Wu and Schwartz, 1998), the physiological condition of these cells may confound the observed responses to CNTF. Whereas normal astrocytes lack CNTFRα expression, reactive astrocytes express both mRNA and protein for CNTFRα (Ip *et al*., 1993; Rudge *et al*., 1994; Duberley *et al*., 1995). Therefore, unlike their normal counterparts, reactive astrocytes may be responsive to CNTF. Attention should be given to the state of the astrocytes when examining CNTF's effects in vitro because astrocytes isolated and cultured from neonatal animals are typically in the reactive state (Langan and Slater, 1992). However, if maintained in culture for a long duration, astrocytes enter a nonreactive/normal state. In this study, the astrocytes cultured from neonatal mice were matured in vitro for 7 wk before experiments. These astrocytes demonstrated a mature, nonreactive phenotype distinguished, at least in part, by the absence of CNTFRα

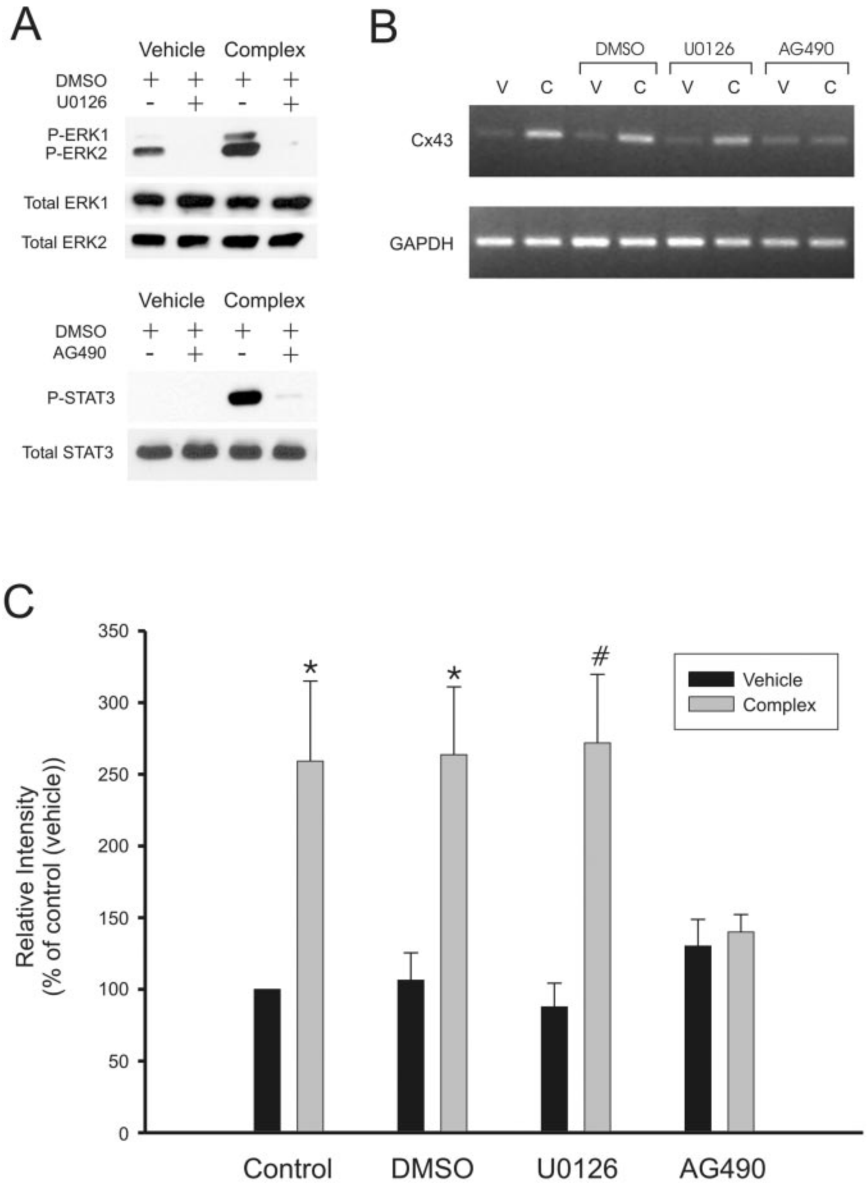

Figure 7. The increase in Cx43 protein induced by Complex is mediated through the JAK/STAT pathway and not the MAPK/ERK pathway. Astrocytes were pretreated with the MEK1/2 (upstream to ERK1/2) inhibitor U0126 (10 μM) or with the JAK2/3 (upstream to STAT3) inhibitor AG490 (50 μM). (A) Representative immunoblot demonstrating U0126 prevents the Complex-induced phosphorylation of both ERK1 (P-ERK1) and ERK2 (P-ERK2) when examined 15 min after treatment with Complex. Confirmation of protein presence and equal lane loading was determined by immunolabeling total ERK1 and total ERK2. Lower immunoblots demonstrate the inhibition of Complex-induced phosphorylation of STAT3 (P-STAT3) by AG490 when examined 15 min after treatment with Complex. Confirmation of protein loading was determined by immunolabeling total STAT3. (B) RT-PCR analysis of Cx43 revealed that compared with vehicle (V) treatment, Complex (C) increased Cx43 mRNA in the presence of DMSO and U0126 but not AG490. (C) When normalized to GAPDH levels, quantitative RT-PCR analysis demonstrates that neither DMSO nor U0126 affected the Complex-induced increase in Cx43 mRNA. However, AG490 prevented Complex from significantly increasing Cx43 mRNA levels. Data are mean ± SEM calculated from four independent experiments. *$p < 0.05$ and #$p < 0.01$ compared with vehicle.

expression but positive for Cx30 expression (as determined by RT-PCR analysis). The Cx30 isoform is not found in astrocytes freshly isolated from neonates but rather is associated with astrocytes of both adult mice (Dahl *et al*., 1996; Nagy *et al*., 1999) and those that have been cultured for extensive periods (Nagy *et al*., 1999; Dermietzel *et al*., 2000).

Our results indicate that in the absence of its specific receptor, CNTF has no effect on Cx43 expression in mature astrocytes. However, when the Complex of CNTF with soluble CNTFRα was administered, the astrocytes responded with an increase in Cx43. This finding supports the requirement of its specific receptor for CNTF to influence Cx43 expression in mature, nonreactive astrocytes. Furthermore, the Complex-induced increase in Cx43 protein was likely due to heightened availability of mRNA template for translation, as supported by analysis of total RNA content.

The cellular localization and phosphorylation state of the Complex-induced increase in Cx43 protein in conjunction with enhanced dye transfer supports increased gap junctional communication in the treated astrocytes. The heightened level of Cx43 was detected within the cytoplasm as well as at the periphery of the cells. At the plasma membrane, Cx43 becomes phosphorylated, forms a connexon, and subsequently combines with an adjacent cell's connexon to produce the gap junction (reviewed by Musil and Goodenough, 1991; Lampe and Lau, 2000). The unchanged ratio of phosphorylated to unphosphorylated Cx43 between treatments suggests that the de novo Cx43 induced by Complex was phosphorylated. Functionality of the overexpressed gap junctions was demonstrated by the intercellular diffusion of gap junction-permeable dyes.

The Complex-induced regulation of Cx43 is dependent on the JAK/STAT pathway and not the MAPK/ERK pathway. Although Complex was capable of activating both signaling pathways, only inhibition of the JAK/STAT pathway prevented the increase in Cx43. AG490 did not alter basal levels of Cx43, at either the mRNA or protein level, but it did prevent Complex from causing sufficient STAT3 phosphorylation and the subsequent amplification of Cx43 mRNA.

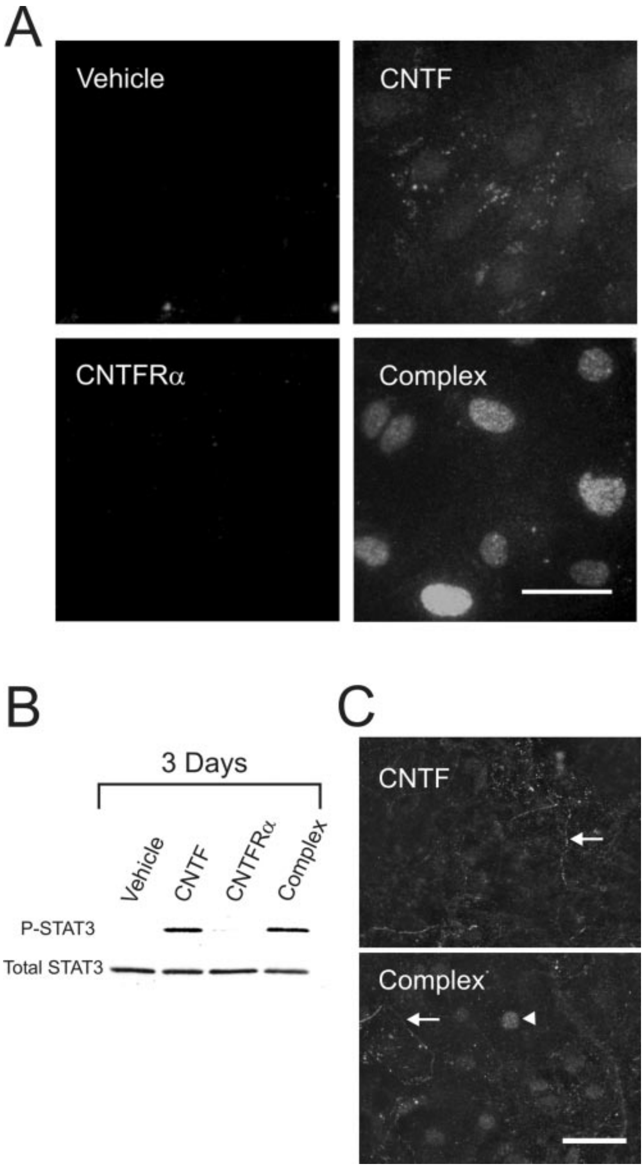

Figure 8. Immunocytochemical analysis reveals that Complex induces nuclear localization of phosphorylated STAT3. (A) Sample immunocytochemical staining of phosphorylated STAT3 in astrocytes after treatment with agents for 15 min. Although vehicle and CNTFRα show limited background immunostaining, CNTF induced phosphorylated STAT3 that localized to the cytoplasm. Complex treatment, however, caused nearly all detectable phosphorylated STAT3 to localize to the nucleus. Bar, 30 μm. (B) Immunoblot analysis of protein isolated from astrocytes treated every 24 h for 3 d revealed that both CNTF and Complex still caused detectable levels of phosphorylated STAT3 (P-STAT3). Subsequent screening of the same immunoblot for total STAT3 shows nearly equal protein loading between the lanes. (C) After 3-d treatment with CNTF, phosphorylated STAT3 immunolabeling is diffuse throughout the cell with some localization to the periphery of the cells (arrow). Three-day treatment with Complex induced diffuse immunolabeling, localization to the cell periphery (arrow), and nuclear localization (arrowhead). Bar, 50 μm.

The inability of the MAPK/ERK inhibitor to prevent the Complex-induced increase in Cx43 levels suggests that regulation of this gene by Complex occurs independently of this pathway.

In the astrocytes, both CNTF and Complex elicited similar JAK/STAT signaling responses, yet only Complex influenced Cx43 expression. In the absence of its specific receptor, CNTF administration induced STAT3 phosphorylation in mature astrocytes. In other cell models, CNTF has been shown to elicit LIF-like effects, including STAT3 phosphorylation, by nonspecifically interacting with IL-6 receptors or LIFRβ (Baumann *et al.*, 1993; Davis *et al.*, 1993b; Nesbitt *et al.*, 1993; Gearing *et al.*, 1994). Because astrocytes express LIFRβ but not IL-6 receptors (Alderson *et al.*, 1999; Monville *et al.*, 2001), it is likely that in this study CNTF mediated a STAT3 response by promiscuous interaction with LIFRβ. This also may explain the CNTF-mediated increased ERK1/2 phosphorylation at the later time point examined. However, the CNTF-induced STAT3 phosphorylation did not translocate to the nucleus as effectively as Complex treatment, suggesting that CNTF treatment alone neither produces the essential nuclear translocation signals (via STAT3 dimerization) nor activates the appropriate nuclear shuttling protein(s). Thus, CNTFRα may be required for CNTF to induce sufficient nuclear translocation and subsequent activation of genes that contain CNTF-response elements.

Exposure of Complex to the matured astrocytes leads to a dramatic, immediate-early phosphorylation of STAT3 yet modifications in Cx43 are readily detectable after 1 (mRNA levels) to 3 d (protein and coupling). There are two possible reasons to explain the differences between the time course of the signaling response and that of the modified gene expression. First, DiStefano *et al.* (1996) have shown that there is a nonequivalence between the biochemical desensitization and pharmacological activity of CNTF. Despite down-regulation of the CNTF signal transduction cascades to repeated administration of CNTF, there is no functional desensitization to this cytokine. CNTF-induced signals continue to be recognized and interpreted by the cell. When we applied Complex every 24 h for 3 d, phosphorylated STAT3 was still at detectable levels indicating that our cells did not become desensitized to the cytokine. Furthermore, the phosphorylated STAT3 after the 3-d treatment was identified at the cell periphery (where it becomes activated), throughout the cell, and consistently within the nucleus (again, not seen with CNTF treatment).

The second main link between the time course differences of signaling cascades and Cx43 expression may be explained by possible CNTF-induced changes in expression of other genes. Albrecht *et al.* (2002) have shown that CNTF stimulates astrocytic production and release of FGF-2. In accordance to this finding, Song and Ghosh (2004) identified that FGF-2 induces chromatin remodeling in astrocytes, which regulates CNTF-mediated gene expression. Thus, in addition to directly activating various genes containing CNTF responsive elements, CNTF may set up a positive feedback loop by altering production and release of other gene products (i.e., FGF-2) that act in an autocrine manner (i.e., induce chromatin remodeling) and further amplify the CNTF response (by facilitating STAT binding within the promoter regions).

Specific genes containing CNTF-response elements (binding sites for STAT3 dimers that contain base sequences $TTCCN_{3–5}AA$) within their promoter are influenced by CNTF administration (Bonni *et al.*, 1993; Schindler *et al.*, 1995; Seidel *et al.*, 1995; Bonni *et al.*, 1997). In astrocytes, functional CNTF-response elements have been identified in the gene for the intermediate filament GFAP (Clatterbuck *et al.*, 1996). In the promoter region of *Cx43* (Chen *et al.*, 1995), we can identify three putative CNTF-response elements that advocates for the ability of Complex to increase this gene's

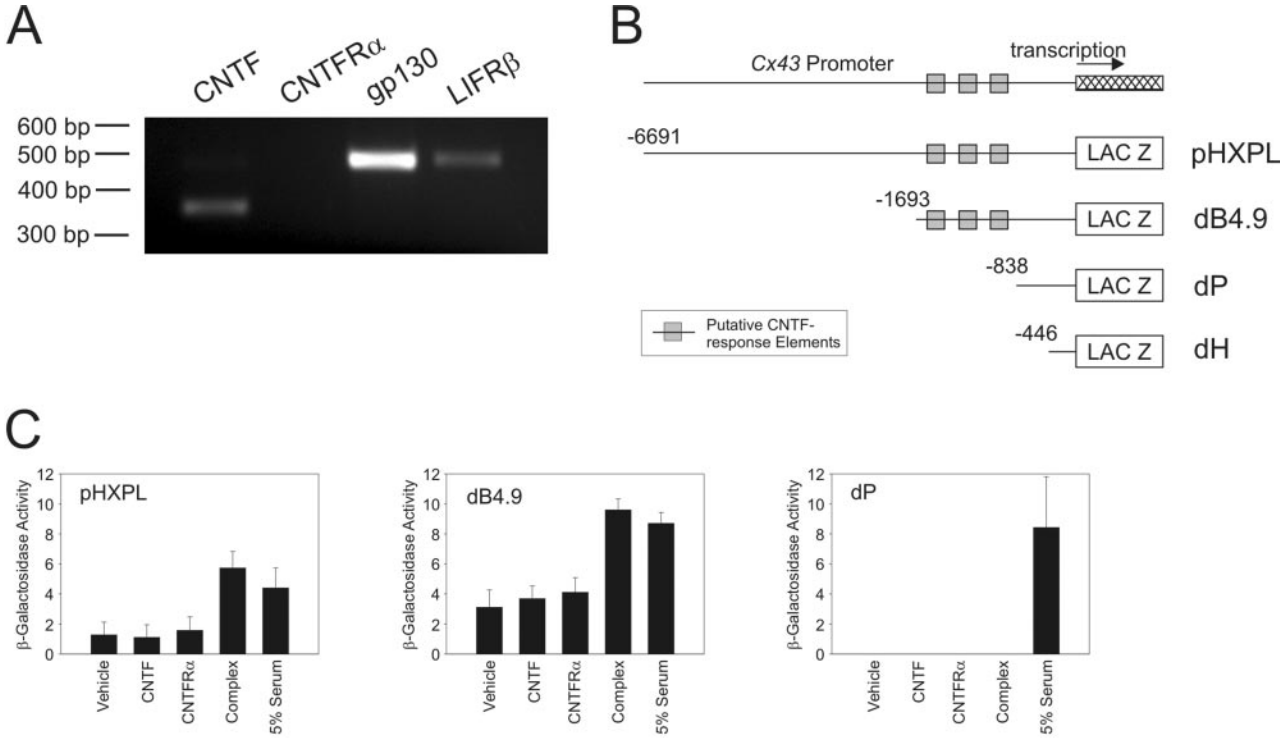

Figure 9. Complex activation of *Cx43* is dependent on a promoter region that contains CNTF-response elements. (A) RT-PCR analysis on total RNA isolated from ROS 17/2.8 cells indicate that the cell line endogenously expresses transcripts for CNTF, gp130, and LIFRβ but lacks CNTFRα. (B) Schematic of the plasmid constructs used to examine Complex regulation of *Cx43*. Plasmid pHXPL contained 6.7 kb of the murine Cx43 promoter inserted in front of a LacZ expression cassette. Plasmids dB4.9, dP, and dH are deletion constructs derived from pHXPL. Three putative CNTF-response elements are located at −893, −1179, and −1510. (C) When transiently transfected into ROS 17/2.8 cells, vehicle, CNTF, and CNTFRα treatment caused little or no β-galactosidase activity with any of the constructs. Complex induced activity from pHXPL and dB4.9 constructs, whereas 5% FBS serum induced notable activity in pHXPL, dB4.9, and dP. None of the agents tested caused detectable β-galactosidase activity in the dH construct.

expression in mature astrocytes. These three elements, located at regions −1510, −1179, and −893, are all situated within the region deemed essential for *Cx43*-regulated expression by Complex as determined using the dB4.9 and dP reporter plasmid constructs in this study. Although it is beyond the focus of this article, each of these sites, or in various combinations with one another, may directly bind STAT3 and enhance Cx43 expression.

The ability of the CNTF pathway to increase Cx expression may be selective to the Cx isoform. Although Complex was capable of increasing Cx43, levels of Cx30 were not altered (as determined by RT-PCR analysis). The ability of signals initiated by Complex to target selective Cx isoforms may be due to the presence or absence of CNTF-response elements. Although putative CNTF-response elements are contained within the promoter region of *Cx43*, it is absent from *Cx30*. Further supporting the importance of specific regions within the promoter (i.e., putative STAT3-binding sites) is our finding that Cx43 expression was dependent on the JAK/STAT pathway and on the promoter region containing such sites. Thus, the ability of Complex to affect Cx43 and not Cx30 suggests that the Complex-mediated effects of CNTF are selective.

Although Complex had not yet been identified in vivo, circumstantial evidence suggests that this injury-release–regulated CNTF-soluble CNTFRα dimer occurs and potentially exerts its actions on the astrocytes. It is well known that CNTF is only released, at least in the mammalian system, after injury with the loss of membrane integrity. In addition, increased levels of soluble CNTFRα have been detected after injury, suggesting that the activity of CNTFRα as a released factor is of physiological importance. Finally, increased Cx43 immunostaining has been identified after ischemia, suggesting regulated expression by this gene.

Long-term up-regulation of gap junctions via Complex administration may prove therapeutically beneficial against many disturbances and disorders particular, but not restricted, to the CNS. In the brain, astrocytes are typically highly coupled to one another by gap junctions, and this coupling plays a pivotal role under normal and pathophysiological conditions. The abolition of gap junctional communication has been shown to heighten neuronal vulnerability to various disturbances (Blanc *et al.*, 1998; Naus *et al.*, 2001; Ozog *et al.*, 2002b). Furthermore, Cx expression, both dependent and independent of gap junctional communication, also has been shown to be protective against cellular injury (Siushansian *et al.*, 2001; Lin *et al.*, 2003; Nakase *et al.*, 2003, 2004; Theis *et al.*, 2003). These studies included the ubiquitous disruption of Cx43 expression as well as astrocyte-specific Cx43 knockout animal models. Specifically, Theis *et al.* (2003) have identified that astrocytic-specific Cx43-deficient mice are characterized by increased spreading depression upon a CNS disturbance. Sohl *et al.* (2000) have recognized that even a small decrease in astrocytic Cx43 can perturb proper spa-

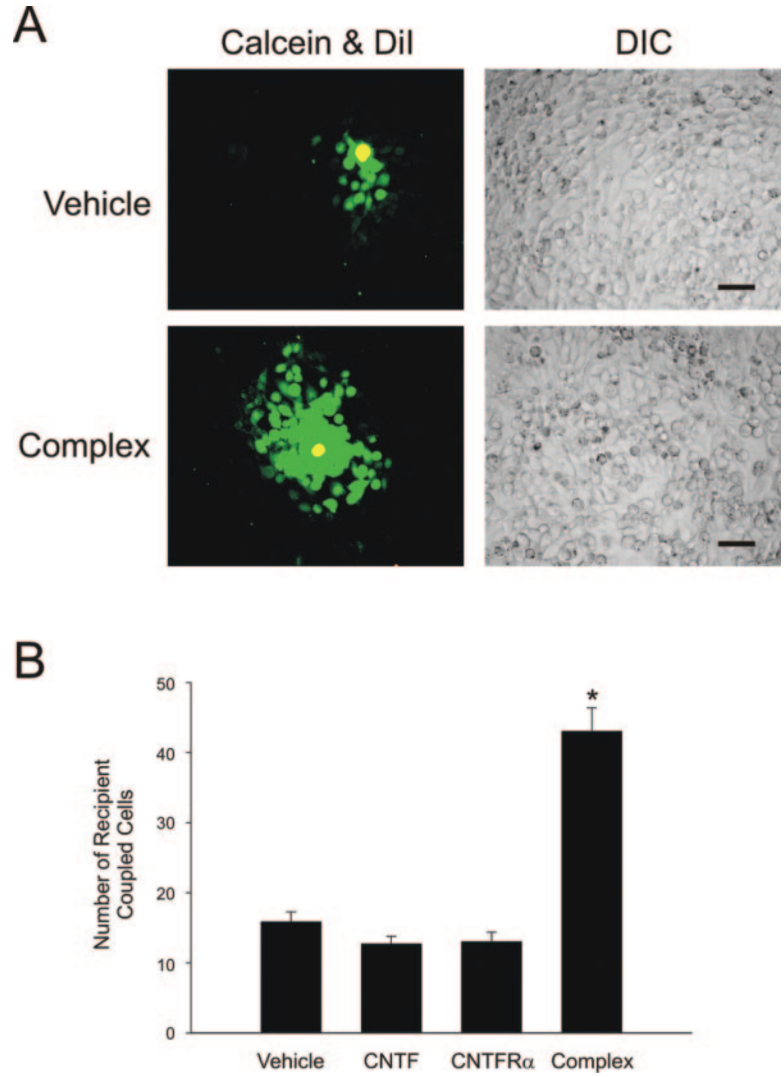

Figure 10. ROS 17/2.8 cells respond to Complex treatment by increasing intercellular coupling. (A) Donor ROS 17/2.8 cells preloaded with both calcein (green) and DiI (red), overlayed together as yellow, pass the gap junction-permeant dye calcein to, and among, neighboring recipient cells (which are only labeled green). Pretreatment of the cells with Complex enhances the intercellular passage of the calcein among recipient cells. DIC images show confluence of the cultures, which were similar in morphology between treatments. Bar, 50 μm. (B) Analysis of the number of recipient cells that received calcein from one donor cell indicates that Complex treatment significantly enhanced intercellular communication, compared with all other treatments. Data are mean ± SEM calculated from four independent experiments (for a total of 40 samples per treatment). *$p < 0.001$.

tial buffering of K^+, leading to heightened spreading depression and ultimately neuronal death.

Aberrations in astrocytic Cx43 and intercellular coupling that lead to increased tissue damage suggest two potential therapeutic roles for Complex. First, its application may increase intercellular communication in the CNTFRα-lacking astrocytes above and beyond basal levels, and thus extend cytoprotective actions. Although astrocytes are normally highly coupled, the negative prognosis associated with aberrations in Cx43 expression and intercellular coupling suggests that amplification of this gene may thwart tissue damage, at least under certain pathophysiological conditions. A second therapeutic role of Complex may not rely on amplifying Cx43 beyond basal levels, but rather amplifying Cx43 to maintain the basal level. A multitude of factors released within the vicinity of tissue damage have been shown to decrease Cx43 or intercellular communication, including epidermal growth factor (McDonough *et al.*, 1999), FGF-2, FGF-5, FGF-9 (Reuss and Unsicker, 1998; Reuss *et al.*, 2000), IL-1β (John *et al.*, 1999), nerve growth factor (Mayerhofer *et al.*, 1996), and others. Rather than individually antagonizing the possible negative Cx43 actions of these molecules, Complex application may provide a Cx43 up-regulation–down-regulation balance to maintain the important intercellular coupling.

It should not go without mention that other studies support the notion that gap junctions are detrimental to cells of the CNS in the wake of injury or disease (Rawanduzy *et al.*, 1997; Saito *et al.*, 1997; Cotrina *et al.*, 1998; Rami *et al.*, 2001; Frantseva *et al.*, 2002). The conflicting notions of gap junctions as beneficial and detrimental agents may, however, be a product of the research model used, including animal species, injury type and Cx isoform impeded.

As mentioned above, the therapeutic implication of Complex may not be restricted to the CNS. Complex may be able to influence other organ systems in which Cx43 and intercellular communication is pivotal. Because the β-subunits of the CNTF receptor heterocomplex are ubiquitously expressed, the application of CNTF in the presence of soluble CNTFRα may extend its properties to other tissues, including bone. Gap junctional intercellular communication mediated by Cx43 has been shown to be critically important in a variety of fundamental processes in human and rodent bone physiology, including osteoclastogenesis, osteogenesis, and normal osteoblast function, and active hematopoiesis (Krenacs and Rosendaal, 1998; Lecanda *et al.*, 1998; Montecino-Rodriguez and Dorshkind, 2001). The effects of CNTF in bone cells has gone relatively uninvestigated due to its restricted actions to CNTFRα-expressing cells. However, in the current experiments we identified that Complex, and not CNTF alone, activates the Cx43 promoter and heightens intercellular coupling in an osteosarcomma cell line. This finding opens a potential field for CNTF-related therapeutics in bone formation, bone marrow transplantations, and blood formation.

In accordance with the protective theory of Cxs, both dependent and independent of gap junctions, novel therapies against CNS disturbances may incorporate use of the CNTF Complex to heighten both Cx43 expression and intercellular communication. Finally, the ability of Complex to stimulate non-CNTFRα–expressing cells gives promise that CNTF's inherent cytoprotective properties may have applications beyond the CNS.

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