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

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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-benzylicinnamamide (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 (Rozenfeld et al., 1998; Bitman and LoTurco, 1999), and possibly neurons (Bruzzone and Ressot, 1991; Giaume et al., 1998; Bittman 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
factor (FGF)-2 has opposing effects on different cells of the CNS; it has been reported to increase Cx43 in neurons (Siu Yi 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 (CNTF-Rα), 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 CNTF-Rα. 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 CNTF-Rα expression (Ip et al., 1993; Rudge et al., 1995).

CNTF-Rα exists in two forms, membrane bound and soluble. The glycosyl phosphatidylinositol linkage of CNTF-Rα to the cell membrane can be cleaved by phospholipases releasing CNTF-Rα 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 CNTF-Rα 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 CNTF-Rα (hereinafter termed “Complex”) can trans-signal cells, independent of endogenous CNTF-Rα expression, by binding with the required β subunits glycoprotein 130 (gp130) and leukemia inhibitory factor receptor β (LIF-Rβ) (Davis et al., 1995a; 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).

A 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 nonmalignant, 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 CNTF-Rα 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-CNTF-Rα 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 seeded onto 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% CO2. Astrocytes were tested every 3 d thereafter in addition to shaking the cultures. After 6 wk, cultures were maintained in medium 1 (54 ml of neurobasal medium [Invitrogen]), 36 ml of DMEM-F/12 [Invitrogen], n-glucose [0.6%), insulin [10 μg/ml], transferrin [20 μg/ml], putrescine-HCl [62 μM], proline [20 mM], sodium selenite [30 nM], and sodium selenite [30 nM], and streptomycin [100 μg/ml streptomycin] for 1 wk. All experiments with the astrocytes were performed in medium 1.

**ROS 17/2.8 Cultures**

The osteosarcoma cell line ROS 17/2.8 was grown in a 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 CEFLOx defined medium (CELOX Laboratories, St. Paul, MN) without serum. All experiments with ROS 17/2.8 cells were performed in CEFLOx defined medium.

**Exposure to CNTF and CNTF-Rα**

Astrocytes were treated with either vehicle (phosphate buffered saline; PBS), CNTF (20 ng/ml; R&D Systems, Minneapolis, MN), soluble CNTF-Rα (200 ng/ml; R&D Systems), or Complex (20 ng/ml CNTF + 200 ng/ml CNTF-Rα) which is about a 1.5 M ratio; excessive soluble receptor favored the protein to be in CNTF-CNTF-Rα heterodimer form). For expression of Cx43 protein, cells were treated with agents in fresh medium 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 15, 30, and 60 min. The STAT3 and MAPK/ERK pathways were inhibited by α-cyano-(3,4-dihydroxy)-N-benzylcinnaminate (AG490; 50 μM; Hodge et al., 2002; Calbiochem, La Jolla, CA) or 1,4-diamino-2,3-dicyano-1,4-bis[2-amino-phenethyl]butoxadiene (U0126; 10 μM; Fava 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) to remove one band representing phosphorylated Cx43. Protein concentration of total cell lysate was determined using the bicinchoninic acid protein assay kit (Bio-Rad, Mississauga, ON, Canada), subjected to 10% SDS-PAGE and subsequently electrotransferred onto a nitrocellulose membrane for 1 h at 80 W/110 cm². The membrane was immunoblotted using appropriate primary antibodies (Cx43; Sigma-Aldrich Canada, Oakville, ON, Canada; and phospho-STAT3 and phospho-p44/p42 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, 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 cm². The membrane was immunoblotted using appropriate primary antibodies (Cx43; Sigma-Aldrich Canada, Oakville, ON, Canada; and phospho-STAT3 and phospho-p44/p42 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, Rockford, IL) and exposed to Kodak X-Omat x-ray film to visualize antibody binding. To normalize protein loading, membranes were stirred with 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 using the phenol-chloroform-isooamyl alcohol method outlined by Sambrook et al. (1989). Briefly, cells were lysed with PBS and then lysed using lysis solution (0.2 M NaCl and 1 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, and 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.
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, 5 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 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 calcine) to the distal edge of the farthest cell (perpendicular to the scrape) that could be visually identified as containing carbosylfluorescein.

### Table 1. Primer sequences used for PCR analysis of cDNA

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Expected product size (bp)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>CNTF</td>
<td>5'-GGAGATCTCTACCGACTTGC-3'</td>
<td>5'-CTTGGGCTTCTCTTCAC-3'</td>
<td>350</td>
<td>Malgrange et al. (1998)</td>
</tr>
<tr>
<td>CNTFRα</td>
<td>5'-CCACATGCTGGCAGGATC-3'</td>
<td>5'-TTGGGTGTTGCTTTCAC-3'</td>
<td>430</td>
<td>Malgrange et al. (1998)</td>
</tr>
<tr>
<td>Cx43</td>
<td>5'-CCGTCCTCCACGGTGCTC-3'</td>
<td>5'-GTTGCTTCACCTCTTCTC-3'</td>
<td>500</td>
<td>Naus et al. (1997)</td>
</tr>
<tr>
<td>Cx30</td>
<td>5'-AATGTTGCCAGGTTGCTTGA-3'</td>
<td>5'-CCAAGGCCCCAGGTAC-3'</td>
<td>399</td>
<td>Dahl et al. (1996)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GTACCATTATGACAGGCCA-3'</td>
<td>5'-CTGGATTCTAGCTGACGTAC-3'</td>
<td>515</td>
<td>Yang et al. (2001)</td>
</tr>
<tr>
<td>gp130</td>
<td>5'-CTCTCACTCTGCAACCCA-3'</td>
<td>5'-CCATGATGGTCACTGAG-3'</td>
<td>473</td>
<td>Zaheer et al. (1995)</td>
</tr>
<tr>
<td>LIFRβ</td>
<td>5'-TGCCGAAATCCTGCATGATG-3'</td>
<td>5'-GGAAGATTCGTTCAGACCTG-3'</td>
<td>483</td>
<td>Nakashima et al. (1999)</td>
</tr>
</tbody>
</table>

**Northern Blot Analysis**

Cells were exposed to either vehicle, CNTF, CNTFRα, or Complex in fresh medium 1 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 with 5% Denhardt's solution, 5% saline-sodium phosphate-EDTA, 50% formamide, 1x SDS, and 100 µg/mL denatured salmon sperm DNA for 4 h at 40°C. Subsequently, blots were hybridized with 32P-labeled Cx43 (Beyer et al., 1987), was used to characterize intercellular coupling. This method was modified from the scrape-loading method described previously (el-Fouly et al., 1998), was used to characterize intercellular coupling. This method was 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 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 calcine) to the distal edge of the farthest cell (perpendicular to the scrape) that could be visually identified as containing carbosylfluorescein.

**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 Axiosvision (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 modified from the scrape-loading (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% carboxylfluorescein 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 carb oxolone (100 µM; Sigma-Aldrich). Carb oxolone was used to halt the rapid progression of carboxylfluorescein 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 carboxylfluorescein 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 calcine) to the distal edge of the farthest cell (perpendicular to the scrape) that could be visually identified as containing carbosylfluorescein.

**Preloading.** Intercellular coupling between ROS 17/2.8 cells was analyzed using a modified version of the preloading method described previously (Ozog et al., 2002b). 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% CO2/95% air). Donor cells were then rinsed several times with isotonic saline 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 calcine 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 pXFLX, pJ4B9, pJ, and DH (6991, 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 (5 µl of 1 µg/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 then washed twice 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 Axiosvision (Carl Zeiss).
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/non-reactive 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 immunochemistry 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.
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/H9251, 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/H9251 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).

![Figure 2](image1.png)

**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.

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/H9251, 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...
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 CNTF/Ag (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
Increased activity in the cells transiently transfected with the four Cx43 (H11002) phosphorylated STAT3 (P-STAT3) followed by total STAT3 (B). (P-ERK1 and P-ERK2) followed by total ERK1 and total ERK2 (A) or were incubated with antibodies against phosphorylated ERK1/2 (Figure 8C). Complex treatment caused phosphorylated STAT3 to localize throughout the cell, at the cell’s periphery, (Figure 8C). 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 (~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).

**Modulation of Cx43 Promoter Activity by Complex**

To analyze the Cx43 promoter for regions that may respond to regulation by Complex, deletion mutagenesis 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 Ronilla 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.

**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.
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 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.
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. (1993; Gearing 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_{5}–5AA) 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 advocate for the ability of Complex to increase this gene’s
expression in mature astrocytes. These three elements, located at regions \(-1510, -1179, \) and \(-893\), are all situated within the region deemed essential for \(C_x43\)-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 \(C_x43\) expression.

The ability of the CNTF pathway to increase \(C_x\) expression may be selective to the \(C_x\) isoform. Although Complex was capable of increasing \(C_x43\), levels of \(C_x30\) were not altered (as determined by RT-PCR analysis). The ability of signals initiated by Complex to target selective \(C_x\) 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 \(C_x43\), it is absent from \(C_x30\). Further supporting the importance of specific regions within the promoter (i.e., putative STAT3-binding sites) is our finding that \(C_x43\) expression was dependent on the JAK/STAT pathway and on the promoter region containing such sites. Thus, the ability of Complex to affect \(C_x43\) and not \(C_x30\) 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 CNTFRA 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 CNTFRA have been detected after injury, suggesting that the activity of CNTFRA as a released factor is of physiological importance. Finally, increased \(C_x43\) 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, \(C_x\) 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 \(C_x43\) expression as well as astrocyte-specific \(C_x43\) knockout animal models. Specifically, Theis et al. (2003) have identified that astrocytic-specific \(C_x43\)-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 \(C_x43\) can perturb proper spa-
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 pathophysiologic 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 Dorskind, 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 exper-
iments we identified that Complex, and not CNTF alone, activates the Cx43 promoter and heightens intercellular coupling in an osteosarcoma cell line. This finding opens a potential field for CNTF-related therapies 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-CNTF receptor-expressing cells gives promise that CNTF’s inherent cytoprotective properties may have applications beyond the CNS.

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REFERENCES


