Changes in Neurotrophic Factor Expression and Receptor Activation Following Exposure of Hippocampal Neuron/Astrocyte Cocultures to Kainic Acid

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Neurotrophic factor expression in the adult mammalian CNS is largely neuronal. However, upon traumatic injury reactive astrocytes express a number of neurotrophic factors including ciliary neurotrophic factor (CNTF), fibroblast growth factor (FGF), and NGF. In this study, we examined whether the upregulation of neurotrophic factors in reactive astrocytes and cultured astrocytes is a consequence of separation from their neuronal counterparts, and whether neurotrophic factor levels can be regulated by placing astrocytes into coculture with neurons. We show that reintroduction of rat hippocampal neurons to rat hippocampal astrocytes in vitro leads to a time dependent downregulation in astrocytes of the neurotrophic factors CNTF, NGF, and neurotrophin 3 (NT-3). In contrast, brain-derived neurotrophic factor (BDNF) mRNA, which is only expressed in neurons in these cultures is slightly increased. Once neurotrophic factor levels in cocultures had reached a steady state in the neuron/glia cocultures, we initiated a traumatic event with the excitotoxin kainic acid. BDNF protein was rapidly upregulated within 24 hr after lesion, whereas CNTF protein upregulation was delayed reaching maximal levels by 3 d. Despite the endogenous upregulation of both of these trophic factors, no activation of their respective receptors, as measured by tyrosine phosphorylation, was detectable following kainate administration. However, following addition of exogenous CNTF at any time point up to 24 hr after kainate administration, the β components of the CNTF receptor (LIFRβ and gp130) could be phosphorylated. Furthermore, although activation of neuronal LIFRβ and gp130 by exogenous CNTF declined during the period of neuronal death, these receptors reappeared on astrocytes and could be activated by CNTF. In contrast, phosphorylation of TrkB by exogenous BDNF was undetectable by 24 hr and could not be reactivated at this point. These data suggest that the intimate association of astrocytes and neurons in the CNS serves to suppress astrocyte-derived neurotrophic factor expression and that neuronal loss leads to a derepression of neurotrophic factor synthesis in astrocytes. However, the upregulation of endogenous BDNF and CNTF observed after excitotoxic lesion in this culture model is insufficient to activate signal transduction and protect against neuronal loss.

[Key words: brain-derived neurotrophic factor, ciliary neurotrophic factor, signal transduction, epilepsy, ischemia, excitotoxicity, receptor regulation]

Astrocytes have been implicated in a wide variety of supportive functions for their partner neurons in the CNS, such as neuronal guidance during development and nutritional and metabolic support throughout life (Rakic, 1971; Varon and Somjen, 1979; Shao and McCarthy, 1994). Astrocytes have also been suggested to provide neurotrophic factors essential for neuronal maintenance and survival (Lindsay, 1979; Banker, 1980; Manthorpe et al., 1986; Rudge, 1993). This hypothesis has been fostered by numerous studies where cultured "type 1" astrocytes release trophic activities that promote survival and differentiation of a number of different neuronal types in vitro. Confirming these observations, astrocytes have been found to express mRNA and protein for a number of defined neurotrophic factors such as members of the neurotrophin family, pleiotropic factors such as ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF) (for reviews, see Manthorpe et al., 1986; Rudge, 1993). Due to their capacity to express neurotrophic factors in vitro, it was therefore puzzling when in situ hybridization studies revealed that expression of several neurotrophic factors, such as NGF, was almost completely neuronal (Ayer-LeLievre et al., 1988; Whittemore et al., 1988; Bandtlow et al., 1990; Ermnors et al., 1990; Gall et al., 1991). Although there are some exceptions, such as expression of NGF in astrocytes of the early developing optic nerve (Lu et al., 1991), and expression of CNTF in the mature optic nerve (Stockli et al., 1991) and glia limitans of the adult rat brain (Ip et al., 1993b), neurotrophic factor production is not a property of most astrocytes within normal adult brain parenchyma. This situation changes dramatically following brain injury.

Traumatic injury to the adult mammalian CNS leads to a well documented reactive gliosis (Berry et al., 1983) in which reactive astrocytes are capable of expressing NGF (Lu et al., 1991; Altar et al., 1992; Lindholm et al., 1992; Yoshida and Gage, 1992), CNTF (Ip et al., 1993; Asada et al., 1995) bFGF (Frautschy et al., 1991), and, as yet, undefined biological activities, as assessed by actions on cultured neurons (Nieto-Sampedro et al., 1983). Suggestive of increased endogenous neurotrophic activity in the brain after injury is the finding that lesioning of the CNS prior to transplantation of embryonic striatum into the entorhinal...
cortex resulted in dramatically improved survival of the transplants at the peak period of glial reaction and neurotrophic factor production (Nieto-Sampedro et al., 1983). Thus, we became interested in the mechanisms that control neurotrophic factor expression in astrocytes in vivo and the functional consequences of this regulation.

In this study, we have used a defined model of cultured "type 1" astrocytes, which can be obtained at greater than 95% purity, and characterized their expression of neurotrophic factors (Rudge et al., 1992). Type 1 astrocytes have been shown to express NGF (Norrgren et al., 1980; Furukawa et al., 1986; Yamakuni et al., 1987), brain-derived neurotrophic factor (BDNF; Zafra et al., 1990), neurotrophin-3 (NT-3; Rudge et al., 1992), neurotrophin-4/5 (NT4/5; Condorelli et al., 1994), CNTF (Stockli et al., 1989), and hFGF (Ferran et al., 1988). The levels of these neurotrophic factors can also be regulated independently of GFAP as has been shown for NGF (Furukawa et al., 1986; Friedman et al., 1990; Carman-Krzan et al., 1991), BDNF (Zafra et al., 1992), NT-3 (Condorelli et al., 1994), and CNTF (Carroll et al., 1994; Rudge et al., 1994a). However, the high level of expression of these factors in astrocytes in vitro is more reminiscent of a reactive astrocyte population than a resting astrocyte population in vivo. In order to develop a model where the levels of neurotrophic factor expression in astrocytes are more reflective of their levels in situ, we have cocultured embryonic hippocampal neurons with hippocampal astrocytes and examined the regulation of neurotrophic factors over time in coculture. We find that there is a time dependent downregulation of neurotrophic factor expression when neurons are cocultured with astrocytes, which can be rapidly reversed if the neurons are injured by an excitotoxic lesion. However, the upregulation of endogenous BDNF and CNTF proteins after trauma does not result in phosphorylation of their respective receptors, suggesting that there are further levels of regulation controlling functional stimulation of neurons after trauma. Indeed, exogenously administered CNTF can stimulate its receptor up to 24 hr after lesion resulting in a functional response that is biphasic as receptor phosphorylation declines as neurons die but then is reinduced on the astrocytes that remain. In contrast, phosphorylation of TrkB by exogenous BDNF is lost as neurons die but then is never regained.

**Materials and Methods**

**Hippocampal neuron/astrocyte coculture.** Astrocytes were cultured from postnatal day 1 rat hippocampi as described previously (Rudge et al., 1992, 1994b). After 28 d in vitro and one passage, the confluent monolayer of astrocytes was seeded with E18 rat hippocampal neurons prepared as described previously (Ip et al., 1991; Rudge et al., 1994b). The neurons were seeded at a density of 4 × 10^6 cells/100 mm dish or 0.8 × 10^6 cells/35 mm dish in DME containing 10% fetal calf serum. DME containing N2 supplements; 50% of this medium was changed twice a week for the next 2 weeks. The neurons were seeded at a density of 4 × 10^6 cells/35 mm dish in DME containing 10% fetal calf serum.

Northern analysis of neurotrophic factor levels in neuron/astrocyte coculture. Total astrocyte RNA was prepared from hippocampal astrocyte/neuron cocultures by extraction in 4 M guanidinium thiocyanate, followed by phenol/chloroform extraction. Total RNA was extracted from adult rat brain tissue using 0.3 M LiCl/6 M urea followed by phenol/ chloroform extraction. Ten micrograms of RNA were electrophoresed on a formaldehyde agarose gel and transferred to a nylon membrane (MSI, Westboro, MA), and hybridized to 32P probes labeled by random oligo priming (Stratagene, La Jolla, CA). Equal loading of RNA samples was confirmed by ethidium-stained gels.

The probes used to examine gene expression and regulation included cDNA fragments to CNTF (600 bp coding region), GFAP (2.7 kb, a kind gift of Dr. Ronald Liem), BDNF (800 bp), NT3 (800 bp), and NGF (1.3 kb). Bands were quantified on a Fuji Bioimager using PDQ software and visualized by autoradiography on Biomax film (Kodak, Rochester, NY).

**Immunoblotting.** Using a 1% NP40 lysis buffer containing protease inhibitors (Ip et al., 1992), lysates of astrocyte/neuron cocultures were prepared with at 1, 7, 14, and 21 d after neurons were seeded onto a confluent monolayer of astrocytes. Lysates were then boiled in sample buffer and electrophoresed on 12% (CNTF and GFAP) or 7.5% (200 kDa neurofilament) polyacrylamide gels. Gels were blotted to Immobilon and then probed with the following antibodies: CNTF (RG0036; Friedman et al., 1986), NT3 (Zafra et al., 1990), BDNF (Wang et al., 1989), and GFAP (N2; Boehringer Mannheim, Indianapolis, IN). Primary antibodies were visualized using secondary antibodies linked to HRP followed by the enhanced chemiluminescent reaction (Amersham, Arlington Heights, IL). Bands for CNTF (24 kDa), GFAP (41 kDa), and neurofilament (200 kDa) were quantified using a Perkin-Elmer scanning densitometer. Levels of CNTF were further quantified by running control lanes with recombinant rat CNTF to obtain a standard curve. The levels of TrkB present in cocultures after the addition of kainate were determined by extracting cultures at time 0 (with and without treatment with 50 ng/ml BDNF) and at 2 hr, 6 hr, 18 hr, 24 hr, and 48 hr after treatment with kainate. Glycoproteins containing an N-acetyl glucosamine residue, of which TrkB is one, were precipitated with wheat germ agglutinin (Knuessel et al., 1994), washed and bound using reagents on Western blots. The blots were probed with a specific antibody to TrkB (Signal Transduction labs 1/2000) and visualized using the ECL reaction.

**Cytotoxicity assay.** To examine the time course over which kainic acid was cytotoxic to neurons in the rat hippocampal neuron/astrocyte coculture, we added acridine orange (final concentration, 0.25 ng/ml) and propidium iodide (final concentration, 2.5 ng/ml) to the cultures. Acridine orange was taken up by viable cells and fluoresced green under fluorescent optics on a Zeiss Axiopt SOL inverted stage microscope, and propidium iodide was taken up by dead cells and fluoresced orange under rhodamine optics. These two reagents were added for 30 min, either prior to the addition of 50 μM kainic acid or to sister cultures at 3, 6, 8, 16, 20, 24, 30, and 48 hr after addition of kainate. The percentage of dead cells was determined by counting the number of propidium iodide stained cells at these time points and expressing the number obtained as a percentage of the maximal number of dead cells obtained at 48 hr.

Two site BDNF ELISA. Astrocytes or neuron/astrocyte cocultures were treated with 50 μM kainic acid and then after 0, 2, 6, 18, 24, and 48 hr, conditioned medium was removed and stored at −80°C prior to assay. At the same time points, the cells were extracted in lysis buffer (50 mM Tris pH 7.5, 0.3% NaCl, 0.1% Triton X-100, 1% BSA, 1 mM benzamidine, 0.1 M benzamidine chloride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF). The BDNF two site ELISA was carried out as defined previously (Acheson et al., 1995). A monoclonal antibody to recombinant human BDNF (RP 43-01) was bound to Immulon 4 microtiter plates as a capture antibody. After washing, test samples were added in a 1:1 dilution of lysis buffer and PBS + 1% BSA. A biotinylated polyclonal antibody specific for BDNF (C. A. Altar and S. Radica, unpublished observations) was then added as a reporter. Following incubation, bound antibody was visualized using a horseradish peroxidase–avidin D conjugate followed by TMB substrate. The resulting color change was read at 450 nm on an Applied Biosystems 96 well plate reader and expressed in ng/ml of BDNF relative to a standard curve of known rhBDNF concentrations. The range of the standard curve was 1 mg/m to 0.008 ng/ml and the sensitivity of the assay was set at a concentration of the BDNF standard which was 2× the OD of the blank.

**Immunocytochemistry.** Cocultures of hippocampal astrocytes and neurons were fixed in 4% paraformaldehyde in PBS at the following time points: 1 d after the neurons were seeded onto the astrocyte monolayer, 14 d after coculture, and 3 d after addition of 50 μM kainate to cocultures (17 d of coculture). Cells were then placed in blocking buffer (0.3% Triton X-100, 1% BSA, 50 mM Tris HCl pH 7.5) and then incubated overnight with a rabbit polyclonal antibody directed against GFAP (Chemicon, AB1890), a rabbit polyclonal antibody directed against rat CNTF (RG0036) or a mouse monoclonal against neurofilament protein (Boehringer Mannheim RT97). The cells were then rinsed four times, 10 min each, in PBS containing 0.02% Triton X-100 and 0.25% bovine serum albumin, and incubated for 1 hr in biotinylated goat anti-rabbit or anti-mouse secondary anti-

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Figure 1. Differential regulation of CNTF mRNA and GFAP mRNA expression over time in hippocampal neuron/astrocyte coculture. A, Northern analysis. E18 rat hippocampal neurons were seeded onto a confluent monolayer of rat hippocampal astrocytes (28 d in vitro) and RNA extracted from the coculture after 4 hr, 7 d, 14 d, and 21 d. Northern blots were run and probed for CNTF mRNA, GFAP mRNA, and GAPDH mRNA. B, Quantitation. Northern blots (n = 3–5) were quantified by exposing the blots to the image plate of a Fuji Bioimager and the bands quantified using PDQ software with the values obtained at 4 hr set as an initial level of 1.0 for each probe. Differential loading was corrected for by comparing values obtained for CNTF mRNA and GFAP mRNA to the value obtained for GAPDH mRNA.

Results

The presence of neurons decreases the levels of CNTF mRNA and CNTF protein in cultured astrocytes

CNTF mRNA. Confluent astrocytes in vitro are known to possess high levels of CNTF mRNA and GFAP mRNA (Stockli et al., 1989; Rudge et al., 1992), whereas neither CNTF mRNA nor GFAP mRNA are present in cultured hippocampal neurons as measured by Northern analysis (Rudge et al., 1992). However, when astrocytes were cultured with embryonic hippocampal neurons, CNTF message (1.2 kb) was downregulated over 21 d in coculture reaching a level that was only 20% of that observed after a 4 hr incubation with neurons (Fig. 1A,B). The level of CNTF mRNA at 21 d of coculture was similar to that found in a sample of adult rat brain run in an adjacent lane on the same Northern blot (data not shown). GFAP mRNA was also downregulated by 21 d of coculture, but more gradually, falling by 21 d to 50% of that observed 1 d after neurons were seeded onto the astrocytes. Although there was an evident change in their morphology during this time period, the number of astrocytes in culture did not change (data not shown). This is in agreement with the findings of Hatten et al. who showed that coculture of astrocytes with neurons inhibited astrocyte proliferation (Hatten, 1985).

CNTF protein. To determine whether the changes in RNA for body at a dilution of 1:1,500, followed by a 60 min rinse in KPBS. After a 1 hr incubation in avidin–biotin–peroxidase complex (Vector Elite Kit, Vector, Burlingame, CA), the cells were rinsed in acetate-imidazole and the HRP visualized with diaminobenzidine (DAB) intensified with nickel sulfate.

Protein tyrosine phosphorylation. As a measure of the phosphorylation state of TrkB, 14 d cocultures were extracted in lysis buffer (Ip et al., 1992) either prior to addition of kainate or at 2 hr, 6 hr, 18 hr, 24 hr, and 48 hr after kainate exposure. With the same time points, a parallel set of cultures were treated with BDNF (50 ng/ml) for 5 min prior to extraction. Samples were immunoprecipitated with a pan-Trk antibody (RG145) and the resultant Western blots probed with an antibody to phosphotyrosine (UBI 4G10). The level of sensitivity of the assay was determined by adding BDNF to 14 d cocultures at 50, 5, 0.5, and 0.05 ng/ml for 5 min, extracting the cells in lysis buffer, immunoprecipitating with RG145 and probing the blot with the anti-phosphotyrosine antibody. Concentrations of BDNF less than 5 ng/ml did not result in detectable phosphorylation of TrkB above background.

As a measure of CNTF receptor activation, and thus a measure of the functional status of the receptor, cocultures were examined for their endogenous level of phosphorylation prior to the addition of kainic acid and 2 hr, 6 hr, 1 d, 2 d, 3 d, and 7 d afterwards. Phosphorylation of the CNTF receptor β components (LIFRβ and gp130) was measured by preparing protein lysates in 1%NP40 lysis buffer (supplemented with proteinase and phosphatase inhibitors) as previously described (Ip et al., 1992) followed by immunoprecipitation with a specific LIF receptor β antibody (RG134, Regeneron Pharmaceuticals) and protein G conjugated to sepharose (Pharmacia, Piscataway, NJ). Immunoprecipitates were then electrophoresed on 7.5% polyacrylamide gels, immunoblotted with anti-phosphotyrosine antibodies as previously described (Ip et al., 1992) and specific proteins detected by enhanced chemiluminescence (ECL, Amersham).

To determine the capacity of the cells to respond to CNTF over the above time course, cultures were treated with 50 ng/ml recombinant rat CNTF for 15 min at 0, 2 hr, 6 hr, 1 d, 2 d, 3 d, and 7 d following kainate administration. The samples were then processed in the same way as described above for assessment of receptor phosphorylation.
Table 1. Regulation of neurotrophic factor mRNA after 14 d in hippocampal neuron/astrocyte coculture

| Neurotrophic factor (transcript size) | Fold control levels (4 hr after seeding = 1.0) |
|--------------------------------------|-----------------------------------------------|
| NGF (1.3 kb)                         | 0.39 ± 0.01                                   |
| BDNF (4.0 & 1.6 kb)                  | 1.47 ± 0.24                                   |
| NT3 (1.4 kb)                         | 0.53 ± 0.11                                   |
| CNTF (1.2 kb)                        | 0.2 ± 0.01                                    |

Table 2. Regulation of CNTF mRNA levels in neuron/astrocyte coculture by exogenous agents

| Additive               | Fold control levels |
|------------------------|---------------------|
| Control                | 1.0 ± 0.16          |
| Clonidine (10 μM)      | 1.29 ± 0.05         |
| γ interferon (30 ng/ml)| 0.99 ± 0.21         |
| IL 1β (10 ng/ml)       | 0.82 ± 0.08         |
| TNFα (100 ng/ml)       | 0.84 ± 0.12         |
| TGFβ1 (10 ng/ml)       | 0.95 ± 0.14         |
| Kainate (50 μM)        | 2.81 ± 0.56         |

CNTF and GFAP in coculture were also reflected in a change in protein levels, we used Western blotting with specific antibodies for each of these proteins. In addition, we used an antibody to the 200 kDa subunit of neurofilament protein as a quantiative measure of neuronal survival and growth. We found that CNTF protein decreased from 120 ng/mg total protein to 20 ng/mg total protein over 14 d in coculture (Fig. 2A). In contrast, neurofilament protein increased by 40-fold over 21 d in culture reflecting very extensive neurite outgrowth (Fig. 2B). The decrease in CNTF protein over time in coculture could possibly be due to a dilution effect produced by an increase in the amount of neuronal protein contributing to total protein. However, when CNTF levels were recalculated as ng/well rather than ng/mg protein, a similar decrease was observed (data not shown). Over time in coculture, GFAP protein levels remained fairly constant, despite a 50% decrease in GFAP mRNA, suggesting that GFAP protein is slowly turned over.

NGF, NT3, and CNTF mRNA decrease over time in coculture whereas BDNF mRNA increases

To determine whether CNTF is regulated differently from other neurotrophic factors in this system, we also examined the regulation of NGF, BDNF and NT-3 mRNA after 14 d of coculture. Table 1 shows that NGF mRNA and NT-3 mRNA were decreased relative to their levels at the start of the coculture, whereas BDNF mRNA levels were increased to about 1.5-fold the level at the start of the coculture. We have shown previously, cultured hippocampal astrocytes express NGF mRNA, NT-3 mRNA, and CNTF mRNA but not BDNF mRNA whereas cultured hippocampal neurons possess BDNF mRNA and NT-3 mRNA but not NGF mRNA or CNTF mRNA (Rudge et al., 1992). The differential regulation observed in this study may be explained by a decline in astrocyte-derived neurotrophic factors and an increase in neuron-derived neurotrophic factors. This has already been suggested for NGF in a cortical neuron/astrocyte coculture system (Vige et al., 1992).
CNTF mRNA and BDNF mRNA are upregulated in neuron/astrocyte coculture after kainate addition

In order to determine whether CNTF mRNA levels could be regulated in neuron/astrocyte coculture, we applied purified factors which are known to regulate the levels of CNTF or IL6 in purified astrocyte cultures (Fig. 3A).

We have shown previously that the α2 adrenergic agonist clonidine upregulates CNTF mRNA by twofold in rat hippocampal astrocyte cultures (Rudge et al., 1994a), as can γ interferon (Carroll et al., 1994). In addition, Norris and Benveniste (1993) have shown that expression of the pleiotropic cytokine IL6, which shares receptor components with CNTF can be regulated by IL1β and TNFα.

Of the different agents tested in this study, only kainic acid caused an increase in neurotrophic factor mRNA and protein. BDNF mRNA was elevated 6 hr after kainate administration returning to baseline by 3 d (data not shown), similar to previous observations using purified hippocampal neurons (Zafra et al., 1992). In contrast, CNTF mRNA showed a more delayed elevation, increasing by 2.5-fold, 24 hr after kainate addition (Fig. 3A). Closer examination of the time course for CNTF mRNA showed a slight increase in CNTF mRNA at 6 hr, with maximal levels of five- to sixfold being reached after 48 hr (Fig. 3B). Levels of GFAP mRNA increased by 1.5-fold after 24 hr, but increased only slightly thereafter (Fig. 3B). The level of CNTF protein was also increased, showing a three- to fourfold increase (20 to 60–80 ng CNTF/mg protein), 3 d after kainate lesion (Fig. 3C). When calculated on a per well basis, the increase in CNTF protein was 2.3-fold, 3 d after kainate lesion (data not shown). GFAP protein was unchanged after kainate whereas the 200 kDa subunit of neurofilament protein fell precipitously to undetectable levels, 24 hr after kainate addition (Fig. 3C).

Neuronal loss correlates with the kainate induced upregulation of CNTF mRNA and protein

As shown in Figure 3A, kainic acid was the only exogenous additive that increased the level of CNTF mRNA. However, examination of the cultures indicated that this was accompanied by neuronal cell loss suggesting that the increase in CNTF was due to a removal of the repressive influence of neurons on astrocyte expression of neurotrophic factors. This is in agreement with our findings in vivo where CNTF was found to be upregulated in reactive astrocytes after neuronal injury resulting, either from an aspiration lesion of the dorsal hippocampus and overlying cortex (Ip et al., 1993) or an excitotoxic lesion of the adult rat hippocampus produced by systemic administration of kainic acid (Rudge et al., 1995). To further characterize the kainic acid lesion in vitro, we examined the time course of neuronal cell damage following the addition of 50 μM kainic acid to the neuron/glial cocultures. Incorporation of propidium iodide into dead cells was used as a measure of cytotoxicity. Staining of
BDNF protein is upregulated in coculture after kainic acid lesion

Although it has previously been shown that kainic acid administration to cultured rat hippocampal neurons causes a dramatic upregulation of BDNF mRNA (Zafra et al., 1992), it has not yet been shown how this affects BDNF protein levels. We have replicated the finding of induction of BDNF mRNA following kainate exposure and extended our study to also look at BDNF protein synthesis and TrkB signal transduction. In close agreement with the study of Zafra et al. (1992) in purified neuronal cultures, we observed a maximal five- to sixfold increase in BDNF mRNA, 6 hr after exposure to 25–50 μM kainic acid. This similarity in dose response for the actions of kainate on purified neurons compared to coculture was also observed for neuronal loss and TrkB phosphorylation, suggesting that the protective effect of astrocytes on glutamate toxicity in culture (Rosenberg et al., 1992) is not applicable to kainate. The increase in BDNF mRNA in this culture system was followed by an increase in BDNF protein in extracts of the cocultures (Fig. 5A). The increase in BDNF protein reached maximal levels between 6 and 18 hr after the addition of kainate and declined thereafter. However, at no time could we detect BDNF protein in the conditioned medium (within the limit of assay sensitivity, 8 pg/ml) before or after kainate addition. Interestingly, when astrocytes cultured alone, before or after kainate addition were examined, no BDNF was detectable in either the cell extract or the medium (data not shown) suggesting that the 0.3 ng/ml of BDNF in the cell extract, 48 hr after lesion, may be a lesion-induced expres-

![Figure 4. Development of the excitotoxic effect after administration of kainic acid to neuron/astrocyte coculture](image)

![Figure 5. Regulation of BDNF protein and TrkB signal transduction after kainic acid lesion in neuron/astrocyte coculture. A, two-site ELISA. Conditioned media and cell extracts were taken prior to kainate (50 μM) addition and then 2, 6, 18, 24, and 48 hr afterwards. These samples were frozen at −80°C prior to assay with a specific BDNF two-site ELISA. B, TrkB Western. Fourteen day cocultures were either extracted in lysis buffer (0−) or treated with BDNF (50 ng/ml) (0+) for 5 min prior to extraction. The remaining cocultures were treated with kainic acid (50 μM) and extracted in lysis buffer at 2 hr, 6 hr, 18 hr, 24 hr, and 48 hr. Samples were precipitated with wheat germ agglutinin bound to agrose and bound material run on a Western blot. The blot was then probed for TrkB using a TrkB specific polyclonal antibody. C, TrkB phosphorylation on BDNF treated cocultures before and after excitotoxic lesion. A similar set of cultures to that outlined in Figure 4B was immunoprecipitated with a pan-Trk antibody and the resulting Western blot probed for phosphotyrosine. The only observable phosphorylation of TrkB was in BDNF treated cultures prior to the addition of kainate. D, TrkB phosphorylation on BDNF treated cocultures before and after excitotoxic lesion. A similar set of cultures to that outlined in Figure 4B was treated with vehicle (0−) or with BDNF (50 ng/ml) for 5 min at 0+, 2, 6, 18, 24, and 48 hr after kainic acid exposure and then immunoprecipitated with a pan-Trk antibody and the resulting Western blot probed for phosphotyrosine. The only observable phosphorylation of TrkB was in BDNF treated cultures prior to the addition of kainate.](image)
Figure 6. Morphological change and CNTF localization in astrocytes after kainate treatment of hippocampal neuron/astrocyte cocultures. Astrocytes stained with an antibody to GFAP show a stellate morphology, 1 d after coculture with neurons (A), this is more pronounced after 14 d in coculture (B). Three days after the addition of kainate astrocytes have numerous vacuoles and a return to the flat epithelioid morphology indicative of astrocytes grown without neurons (C). Neurons stained with a 200 kDa neurofilament antibody are very sparse after 1 d in coculture (D), but after 14 d there is a dense neuritic network covering the astrocyte monolayer (E). There is a dramatic change 3 d after kainate addition with very few neuronal profiles observable (F). CNTF staining is very evident in a population of astrocytes responding to the presence of neurons 1 d after
length TrkB was lost over the same time course as the neurons died following exposure to kainate (see Fig. 4). Prior to the addition of kainate, full length TrkB (gp145TrkB) was capable of being phosphorylated by the addition of exogenous BDNF (50 ng/ml) (Fig. 5C,D, 0+ BDNF). However, after the addition of kainate, gp145TrkB was not phosphorylated by the endogenously upregulated BDNF at any time point examined (Fig. 5C) although the sensitivity of the phosphorylation assay would require greater than 5 ng/ml of BDNF in the medium to elicit a phosphorylation signal over background (data not shown).

Moreover, following the excitotoxic insult, the addition of exogenous BDNF (50 ng/ml) was also unable to elicit phosphorylation of gp145TrkB at any time points after 6 hr of treatment with kainic acid (Fig. 5D) even though detectable levels of gp145TrkB were present up to 24 hr after kainate addition. Also, increasing the excitotoxic dose of kainic acid to 500 μM dramatically reduced phosphorylation of TrkB by exogenous BDNF (data not shown).

Changes in astrocyte morphology and CNTF expression after kainate lesion

In order to assess how astrocytes respond to the presence and subsequent loss of neurons and to relate this to CNTF expression in astrocytes, we stained the cultures directly for GFAP, neurofilament protein and CNTF (Fig. 6). As described previously by Hatten and colleagues (Hatten, 1985; Gasser and Hatten, 1990), neurons induced stellation of astrocytes in coculture (Fig. 6A,B). However, 3 d after exposure to kainic acid with resulting neuronal loss, the majority of astrocytes reverted to a polygonal, epithelioid shape (Fig. 6C). Identifying the neurons by expression of the 200 kDa neurofilament subunit showed that very little neurofilament had been produced by 1 d after seeding neurons onto the astrocytes (Fig. 6D). However, by 14 d there was a robust network of neurite processes and cables extending across the astrocyte surface (Fig. 6E). This was reflected in the 40-fold increase in neurofilament protein shown in Figure 2B. By this criterion, excitotoxic lesion with kainic acid (50 μM) was very effective in killing the neurons (Figs. 6F, 5B). The staining pattern for CNTF was interesting in that coculturing with neurons caused the astrocytes to assume a stellate morphology and revealed very strong staining of a subpopulation of the astrocytes (Fig. 6G) (Stockli et al., 1991; Rudge et al., 1994a). Astrocytes cease to express CNTF immunoreactivity after 14 d of coculture (Fig. 6H). In agreement with the Western blotting data (Fig. 3C), CNTF levels rose dramatically after kainate lesions. By immunocytochemical staining, this pattern was amorphous and did not fill the cytoplasm but surrounded phagocytic vacuoles which contained neuronal debris (Fig. 6I).

Does the rise in CNTF after kainate lesion result in phosphorylation of the CNTF receptor?

As we see such a dramatic rise in CNTF mRNA and protein after neuronal damage both in the present study and after lesion in vivo (Ip et al., 1993), we addressed the question of whether the CNTF receptor β components might be phosphorylated on tyrosine during the period of excitotoxic neuronal death in culture.
to produce NTFs at times when they are required during development and after trauma? (3) Is there a time window after trauma but prior to their eventual loss in which neurons are still functionally responsive to neurotrophic factor administration? (4) Are NTFs as a whole broadly upregulated or are there signals which allow differential neurotrophic factor upregulation? (5) Are there contexts in which neurons are unable to respond to neurotrophic factors, despite the presence of their cognate receptors? (6) What are the consequences of simultaneous binding and/or activation of astrocytic and neuronal neurotrophic factor receptors? To begin to address these questions, we have utilized a defined neuronal/astrocyte culture system in which we can study the molecular mechanisms underlying neuronal control of astrocyte NTF expression and elucidate how this might be relevant under traumatic conditions.

A good example of a neurotrophic factor which may give some insight into the questions raised above is CNTF. This cytokine is present at very low levels in the adult CNS but is dramatically upregulated in astrocytes after traumatic injury (Ip et al., 1993). CNTF is also present at high levels in cultured astrocytes, so high, in fact, that astrocytes were used as the source for cloning rat CNTF (Stockli et al., 1989). Thus, CNTF was an ideal candidate to ask the question of whether the presence of neurons would be sufficient to suppress astrocyte expression of this neurotrophic factor.

In this study, we show that hippocampal neurons downregulate CNTF mRNA and CNTF protein expression in coculture as a dense neuritic network spreads out over the astrocytic monolayer. By 21 d in coculture, CNTF mRNA levels resemble those observed in adult rat CNS. Attempts to upregulate CNTF in coculture by agents known to augment CNTF expression in astrocytes cultured alone (Rudge et al., 1994a) were unsuccessful, suggesting a stringent regulation of CNTF synthesis when neurons are in intimate contact with astrocytes. However, when neurons were damaged by administration of kainic acid, CNTF mRNA was increased in astrocytes in response to the dying neurons, reaching maximal levels 48 h after kainate administration. CNTF protein was also increased reaching maximal levels 3 d after lesion. To ascertain the functional consequences of this increase, we examined receptor phosphorylation. At no time during neuronal death and CNTF upregulation were the β components of the CNTF receptor phosphorylated suggesting that during this period neither CNTF nor its family members which act through these receptors were released by dying neurons or astrocytes. However, when CNTF was exogenously applied, receptor phosphorylation could be induced at early stages after kainate addition. This was followed by a dormant period and then a return of inducible receptor phosphorylation on the remaining astrocytes.

Astrocytes downregulate neurotrophic factor expression upon sustained contact with neurons in culture

The close association between neurons and astrocytes during development involves a complex interchange of signals between these two cell types where macromolecules can be transferred from neuron to glia (Lasek et al., 1977) and astrocytes can develop a differentiated phenotype upon contact with neurons in culture (Gasser and Hatten, 1990). During development, diffusible signals in embryonic forebrain can control the identity of radial glial cells (Hunter and Hatten, 1995) which then act as supports for neuronal migration both in vitro (Gasser and Hatten, 1990) and in vivo (Rakic, 1971). The radial glial cells can then transform into astrocytes that are capable of providing energy substrates to more mature neurons under stress by controlling glycogenolysis (Sorg and Magistretti, 1991, 1992; Pellerin and Magistretti, 1994). In the same vein, the expression of neurotrophic factors by astrocytes in culture has led to the hypothesis that astrocytes in vivo may be capable of supplying their partner neurons with the appropriate factors necessary for their survival and maintenance (Varon et al., 1979; Manthorpe et al., 1986; Rudge 1993).

However, studies in vivo suggest that the neuron/glial relationship may be more dynamic where neurotrophic factors are involved. Under normal conditions in the adult CNS, the apparent lack of neurotrophic factor expression in astrocytes supports the view that neurotrophic factors are a means of communication between neurons signaling homeostasis in an efficiently functioning system. Neurotrophic factor release by astrocytes could be potentially detrimental to this system and upset the fine balance between neurons and their targets in the CNS. However, after injury or during disease, neuronal homeostasis is disrupted and the astrocyte begins to manufacture and secrete factors that support neurons under stress. Thus, reactive astrocytes in vivo and astrocytes in vitro deprived of their normal environment appear to react to the insult by synthesis of a number of supportive factors for the neuron, of which neurotrophic factors may only be a subset.

CNTF has been shown to possess a number of pleiotropic properties ranging from acute regulatory actions on synaptic function (Stoop and Poo, 1995), potent neurotrophic effects on injured dopaminergic substantia nigra neurons (Hagg and Varon, 1993) and cholinergic and noncholinergic septal neurons in vivo (Hagg et al., 1992), to actions on a number of non-neuronal cells (Schooltink et al., 1992; Louis et al., 1993; Rudge et al., 1994b). Although exogenous CNTF is able to rescue both cholinergic and dopaminergic neurons from axotomy-induced degeneration, this factor alone is unable to sustain their neuronal phenotype (Hagg et al., 1992). This is in contrast to members of the neurotrophin family such as NGF, which can sustain both the survival and neurotransmitter phenotype of specific neurons following axotomy (Hagg et al., 1992). Thus, CNTF and the neurotrophins may have complimentary roles in the CNS after trauma that result in the maintenance of a fully functioning neuron.

In our culture system, we have found that by coculturing neurons with astrocytes, we are able to downregulate both CNTF mRNA and protein to levels similar to those observed in the parenchyma in vivo (Stockli et al., 1991). The control of CNTF downregulation in coculture is also quite stringent, as exogenous factors which are capable of upregulating CNTF mRNA in astrocytes cultured alone are unsuccessful when administered to the coculture. This type of downregulation of neurotrophic factors in coculture has also been shown by Vige et al. where NGF was downregulated in cultures of cortical neurons with cortical astrocytes (Vige et al., 1992). This effect does not appear to be exclusive to hippocampal neurons as we have also observed down-regulation of CNTF mRNA in cocultures of rat hippocampal astrocytes with chick sympathetic neurons or PC12 phaeochromocytoma cells (data not shown).

However, upon lesioning the coculture with kainic acid, we can produce an upregulation of CNTF mRNA and protein similar to that observed after an aspirative lesion of the hippocampus and overlying cortex where CNTF levels are increased in reactive astrocytes (Ip et al., 1993c).
Neuronal loss results in an upregulation of CNTF in astrocytes but this does not result in CNTF receptor activation

We have shown previously that an aspiration lesion to the dorsal hippocampus causes a dramatic upregulation in CNTF mRNA (Ip et al., 1993c) and CNTFRα mRNA (Rudge et al., 1994b) in the lesioned tissue surrounding the wound site. The expression of CNTF and its receptor in either the same cells or closely associated cells at the wound site raises the question as to whether CNTF may act in an autocrine or paracrine manner in those cells which possess functional receptors. We have shown that cultured astrocytes and meningeal fibroblasts possess fully functional CNTF receptors (Rudge et al., 1994b) and that CNTF enhances survival in cultured rat hippocampal neurons (Ip et al., 1991).

In this study, we asked whether the rise in CNTF after lesion with kainic acid was reflected by an increase in CNTF receptor activation as measured by phosphorylation of the CNTF receptor complex. We found that during the time that CNTF is increased in astrocytes and that neurons are dying, no phosphorylation of LIFRβ or gp130 was detectable within the limits of sensitivity of our assay. The conclusion from this study is that CNTF increases within the astrocyte after neuronal injury but, due to the lack of a signal sequence (Stockli et al., 1989), is not released into the extracellular space. Thus, CNTF induced in astrocytes by injury can only be released if the astrocyte is sufficiently compromised to release its cytosolic contents. This may be one explanation for the enhanced survival of transplants in vivo when their insertion into a lesion cavity is delayed by 1 week (Nieto-Sampedro et al., 1983, 1984). Interestingly, the lesioned coculture shows two phases of response to exogenous addition of CNTF: In the first phase, prior to the addition of kainate, LIFRβ and gp130 are phosphorylated by the addition of 50 ng/ml rat CNTF. After kainate addition, it is still possible to phosphorylate these receptors through exogenous CNTF addition but the response diminishes with the same time course as neuronal death, leading to a period at 48 hr after kainate where the culture is refractory to CNTF. This period is then followed by an induction of responsiveness to exogenous CNTF by the astrocytes in the culture. At this point, receptor phosphorylation reaches similar levels to that observed in astrocytes cultured alone. This switching from a neuronal expression of CNTF receptor to a non-neuronal expression also occurs in vivo after an aspiration lesion of the dorsal hippocampus and overlying cortex (Rudge et al., 1994b) and may reflect a shift for CNTF from the role of a neurotrophic factor to that of a pleiotropic cytokine after injury.

Kainic acid lesion causes BDNF upregulation in coculture but makes the neurons refractory to receptor activation

It has been established that seizure activity (Gall and Isackson, 1989; Isackson et al., 1991; Zafra et al., 1991) as well as a number of other types of insult (Zafra et al., 1990; Ballarin et al., 1991; Duzich-Djordjevic et al., 1992; Lindvall, 1992; Kokaia, 1993) are capable of causing dramatic increases in NGF mRNA and BDNF mRNA in adult rat hippocampus. The interpretation of these results was that although neurotrophin upregulation has been suggested as a protective mechanism to counteract cell death (Lindvall et al., 1994), excessive cell death still occurs in the CA1 and CA3 areas of the hippocampus despite neurotrophin upregulation. If we look at BDNF in particular, this result could be interpreted as (1) BDNF mRNA is increased but fails to translate to protein before the neuron is irreversibly compromised, (2) the neurotrophin protein is made but is sequestered by the high levels of truncated TrkB on the astrocyte (Friisen et al., 1993; Rudge et al., 1994b) and therefore never “sees” the functional TrkB on the injured neuron, or (3) the signal transduction mechanism for BDNF is somehow inactivated by the injury so that the increase in BDNF is ineffective. The coculture model that we have developed has allowed us to address the issues raised above.

We show that BDNF expression is upregulated after kainic acid lesion of hippocampal neurons in coculture, although the increase is observed only in the cell extract and never in the conditioned medium. However, regardless of any increase that occurs in BDNF protein, we also show that full length TrkB is not phosphorylated by endogenous BDNF (within the limits of sensitivity of our detection system) at any time after the addition of kainic acid. Thus, although injured neurons and their partner astrocytes respond to an excitotoxic insult by upregulating their levels of endogenous neurotrophic factors, this is clearly insufficient to protect the neurons from excitotoxic injury and death. This may be an abortive attempt at neuroprotection for neurons at the focal point of the injury but, in vivo, could possibly make a difference to neurons in the lesion penumbra. However, for the neurons that are directly affected, there is a narrow window, up to 6 hr after the initiation of the kainic acid injury, in which both BDNF and CNTF receptors can be activated. Thus, it may be possible to pharmacologically administer BDNF and/or CNTF within this time frame, or alternatively induce the astrocytes to express and/or release neurotrophic factors, to stabilize calcium levels within the neuron and ameliorate neuronal damage. We are in the process of addressing these questions in this system.

Conclusions

We have shown in this study that the intimate association of neurons with astrocytes can suppress astrogial expression of neurotrophic factors in vitro. This places the astrocyte under stringent controls such that addition of factors which are capable of upregulating neurotrophic factors in astrocytes cultured alone are unable to do so in coculture. However, excitotoxic lesion of the coculture with kainic acid results in a rapid upregulation of BDNF and a delayed upregulation of CNTF in astrocytes caused by removal of the neuronal repression. Although these neurotrophic factors are substantially upregulated, at no time do they initiate a functional response as measured by phosphorylation of their respective receptors. However, exogenously administered BDNF and CNTF can activate their receptors up to 6 hr after excitotoxic injury providing a narrow window in which administration of these trophic factors may be able to ameliorate the excitotoxic insult.

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