The Regulation and Role of Cytokines in Models of Synaptic Activity and Plasticity

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ABSTRACT

While many functions have been proposed for cytokines in the CNS, most research has focused on injury and infection. Initially described as protein signaling molecules between cells of the immune system, many cytokines and their receptors have since been localized to both the central and peripheral nervous systems. Our experiments explore the contribution of cytokines to the fundamental brain functions, neuronal activity and synaptic plasticity.

Since cytokines are up-regulated by brain trauma, we first asked if cytokine mRNA expression is affected by the procedures used to make hippocampal slices and to study long term potentiation (LTP) *in vivo*. Indeed, expression of many cytokines is altered in the preparation of hippocampal slices, and we find even more dramatic cytokine changes with electrode penetration *in vivo*. We therefore turned to a chronic *in vivo* preparation in which the effects of injury are eliminated by a three week recovery period between electrode placement and electrophysiological stimulation. In this preparation, we find that brain derived neurotrophic factor expression is decreased by low-frequency test stimuli. More dramatically, interleukin-6 (IL-6) is up-regulated specifically by LTP induction. Since IL-6 can regulate neuronal activity, this finding suggests a role for IL-6 in the control of LTP.

We also studied the regulation of neuropoietic cytokines by neuronal activity in the hippocampus using pilocarpine-induced seizure. Using a semi-quantitative RNase protection assay, we find that leukemia inhibitory factor (LIF) and oncostatin M are strongly up-regulated by seizure activity. Each cytokine is, however, induced in discrete cell populations with a distinct time course, suggesting particular roles in both the seizure and in its pathological consequences.

The relatively rapid induction of LIF in hippocampal astrocytes suggested that this cytokine could control astrocyte activation in response to seizure. Using the electroconvulsive shock (ECS) model of seizure in LIF knockout and wild type mice, and

glial fibrillary acidic protein (GFAP) induction as the marker of astrocyte activation, we find that LIF is required for GFAP up-regulation.

In sum, certain cytokines are regulated by particular patterns of neuronal activity, including those thought to be involved in learning and memory. Moreover, one cytokine, LIF, is required for astrocytic activation, a key process in subsequent hippocampal pathology.

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LIST OF ABBREVIATIONS

%BL	Percent baseline
ACh	Acetylcholine
ACSF	Artificial cerebrospinal fluid
apTBL-1	*Aplysia* tolloid/BMP-1 like protein
BDNF	Brain derived neurotrophic factor
BME	Beta-mercaptoethanol
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CA	Cornu Ammonis
CaMK	Calcium/calmodulin-dependent kinase
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
ChAT	Choline acetyl transferase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
cpm	Counts per minute
CSF	Cerebral spinal fluid
CT-1	Cardiotrophin-1
dB	Decibel
DDRT-PCR	Differential-display RT-PCR
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Dinucleotide triphosphate
DRG	Dorsal root ganglia

DTT	Dithiothreitol
DYN	Dynorphin
E-	Embryonic day
ECS	Electroconvulsive shock
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
ENK	Enkephalin
EPSP	Excitatory post-synaptic potential
ERK	Extracellular signal regulated protein kinase
FC	Frontal cortex
FGF	Fibroblast growth factor
GABA	Gamma-amino-butyric acid
GAPDH	Glyceraldehye-3-phosphate dehydrogenase
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
gp130	Glycoprotein 130
GPA	Growth promoting activity
GS	Glutamine synthetase
HB-EGF	Heparin-binding epidermal growth factor-like factor
icv	Intracerebroventricular
IFN	Interferon
IL	Interleukin
KA	Kainate or Kainic acid
KO	Knock-out
LC	Locus coeruleus
LIF	Leukemia inhibitory factor
LTP	Long-term potentiation

MECS	Maximal electroconvulsive shock seizure
MF	Mossy fiber
NAc	Nucleus accumbens
NDF	Neu differentiation factor
NF	Neurofilament
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
NT-3	Neurotrophin-3
OD	Optical density
OSM	Oncostatin M
P-	Postnatal day
PBS	Phosphate buffered saline
PBT	Phosphate buffered saline with 0.1% Triton X
PC	Pheochromocytoma
PCR	Polymerase chain reaction
PNS	Peripheral nervous system
PPF	Paired pulse facilitation
PPI	Paired pulse inhibition
PTP	Post-tetanic potentiation
PTZ	Pentylenetetrazol
-R	Receptor
RA	Receptor antagonist
RNA	Ribonucleic acid
RNAse	Ribonuclease
RNasin	RNAse inhibitor
RPA	RNAse protection assay

RT-PCR	Reverse-transcription polymerase chain reaction
SCG	Superior cervical ganglia
SDS	Sodium dodecyl sulfate
SN	Substantia nigra
SOM	Somatostatin
SP	Substance P
SSC	Sodium chloride/Sodium citrate buffer
SSPE	Sodium chloride/sodium phosphate/EDTA buffer
STP	Short-term potentiation
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
TI	Tris-Imidizole
TNF	Tumor necrosis factor
UV	Ultraviolet
VIP	Vasoactive intestinal peptide
VRC	Vanadyl ribonucleoside complex
VTA	Ventral tegmental area
WT	Wild-type

Chapter 1

Introduction: Cytokines in the CNS, their endogenous regulation and exogenous affects on LTP and seizure

Joanna L. Jankowsky and Paul H. Patterson

1. CYTOKINES DEFINED

Cytokines are a diverse group of soluble protein factors, numbering over 100 members by some definitions (Mire-Sluis and Thorpe, 1998). Generally small proteins, ranging in size from 8-26 kD, cytokines can be produced by a wide variety of cell types (Zhao and Schwartz, 1998). Additionally, many cytokines have more than one property, and often can mediate an extensive range of functions in diverse settings. Given their diversity and broad distribution, investigation of the physiological importance and functions of cytokines is well justified.

Cytokines have been subdivided into several families, often grouped by functional similarity rather than by sequence homology. The hematopoietic cytokines comprise one of the largest cytokine families, including most of the interleukins 1 though 18. Interleukin-1 (IL-1) was one of the first identified cytokines, and is still one of the best studied, with roles ranging from hematopoietic cell differentiation to fever induction (Colotta et al., 1998). In addition to roles in other systems, most members of the hematopoietic family act as intercellular signals between cells of the immune system.

The transforming growth factor beta (TGFβ) superfamily comprises another large subgroup of cytokines. Family members do share limited sequence homology (Kingsley, 1994), and often have similar effects on specific cell types (Mehler and Kessler, 1995). The TGFβ superfamily is subdivided into the TGFβ family, including TGFβs 1-5, the activin family, including inhibin A, inhibin B and nodal, the decapentaplegic (dpp) family, comprised of bone morphogenetic protein 2 (BMP2), BMP4 and dpp, the 60A family, including BMPs 5-8 and 60A, the BMP3/osteogenin, BMP9, and BMP12 subgroups, and the dpp- and Vg1-related (DVR) family, which is a large group that includes the dpp and 60A subfamilies as well as five additional members (Kingsley, 1994; Mehler et al., 1997). Additional TGFβ superfamily members, such as glial-derived neurotrophic factor (GDNF), share such limited homology with other TGFβ factors that they have not been classified into any of these groups (Kingsley, 1994). In addition to their roles in hematopoietic

signaling, TGFβ superfamily members are critically important in the early development of the nervous and skeletal systems (Kingsley, 1994; Mehler and Kessler, 1995; Mehler et al., 1997).

The neuropoietic family includes a diverse group of cytokines: ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM) and cardiotrophin-1 (CT-1), as well as two interleukins, IL-6 and IL-11. These factors are grouped into the neuropoietic family not by sequence homology, but by common secondary structure, functional similarity and the shared use of the signal transducing receptor subunit glycoprotein 130 (gp130) (Murphy et al., 1997; Taga and Kishimoto, 1997). The neuropoietic family acts on many systems in the body, with effects ranging from the prevention of embryonic stem cell differentiation *in vitro*, to cardiac muscle trophic support, to activation of the hepatic acute phase response following infection (Taga and Kishimoto, 1997).

Additional cytokines such as the fibroblast growth factors and epidermal growth factor are generally classified simply under the heading "growth factors." Others, such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) are not grouped into families at all. However, the functional effects of each, despite nomenclature or grouping, has expanded well beyond the feature for which they were originally identified (Mire-Sluis and Thorpe, 1998).

Because the number of cytokines is so great, I have chosen to focus this Introduction on a select few, with an emphasis on their regulation and roles in the nervous system. Like the neurotrophins (nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and others), many cytokines can affect neuronal survival, morphology and gene expression (Merrill, 1992; Bartfai and Schultzberg, 1993; Logan and Berry, 1993; Kingsley, 1994; Schobitz et al., 1994; Mehler and Kessler, 1995; Rothwell and Hopkins, 1995; Sei et al., 1995; Mehler et al., 1997; Murphy et al., 1997; Benveniste, 1998; Ebendal et al., 1998; Flanders et al., 1998; Mire-Sluis and Thorpe, 1998). Unlike the cytokines,

neurotrophins have also been extensively studied for their role in neuronal activity and plasticity (Lo, 1995; Berninger and Poo, 1996; Lu and Figurov, 1997; Schuman, 1999). Because these two sets of protein factors share so many functional effects in common, it was reasonable to suspect that, like the neurotrophins, cytokines may also play important roles at the synapse. My thesis work addresses this possibility by investigating the regulation of specific cytokine expression in two models of synaptic activity: hippocampal long term potentiation (LTP) and seizure. Once activity-responsive factors were identified, a role for one of these cytokines, LIF, was investigated using knock-out (KO) mice.

2. LOCALIZATION OF CYTOKINES IN THE CNS

Although many cytokines are expressed in the nervous system, I will focus on those cytokines studied in the course of my thesis. While several of these proteins are thought to play important roles in neural development, their localization in the adult central nervous system is most relevant to their potential role in synaptic activity and plasticity, and will comprise the bulk of this section. For the sake of completeness, a few paragraphs on the effects of each factor on several aspects of neuronal biology follows the description of the localization of the cytokine and its receptor components in the CNS.

2.1 IL-1

Interleukin-1 is the term for two polypeptides, IL-1α and IL-1β, which are encoded by separate genes and share only about 30% similarity. Nonetheless, these proteins have nearly identical biological activities (Benveniste, 1992). Within the CNS, immunohistochemical studies have revealed IL-1β expression in neurons of the hypothalamus, hippocampus and olfactory tubercle (Bartfai and Schultzberg, 1993; Schobitz et al., 1994). Under normal conditions, IL-1β mRNA expression is relatively low in the CNS, but it has been detected in the cerebellum, olfactory bulb, hypothalamus, and the pyramidal cell layer and dentate gyrus of the hippocampus (Bandtlow et al., 1990;

Schobitz et al., 1994). Under basal conditions, IL-1α expression is even lower than that of IL-1β; in one study, IL-1β mRNA was detected by PCR in the pituitary, cerebellum and cerebral cortex, while little IL-1α could be detected in any brain area tested (Gabellec et al., 1995). Our work demonstrates that although not abundant, IL-1α mRNA is present in the rodent hippocampus (Chapter 2). In addition to neurons, IL-1 can also be produced by astrocytes and microglia in culture (Bartfai and Schultzberg, 1993). Indeed, it is believed that microglia are the main source of IL-1 in the brain (Sei et al., 1995).

A third member of the IL-1 "family," a highly selective IL-1 receptor antagonist, is also present in the adult CNS. This polypeptide exists in three forms, two intracellular and one secreted, which result from differential splicing (Hirsch et al., 1996). All three forms have the ability to inhibit IL-1 effects in cultured cells (Hirsch et al., 1996). Expression of IL-1RA mRNA has been detected by RT-PCR in the rodent cerebellum and cerebral cortex (Gabellec et al., 1995), and by *in situ* hybridization in the hypothalamus, dentate gyrus of the hippocampus, the cerebellum, and the choroid plexus (Licinio et al., 1991). While IL-1RA expression was localized to neurons in the dentate gyrus, the cell types responsible for expression in other IL-1RA-positive areas have not been defined.

Two forms of the IL-1 receptor have been identified; both IL-1 receptor type I (IL-1R1) and IL-1R type II bind all three forms of IL-1 (IL-1α, IL-1β and IL-1RA), albeit with somewhat different affinities (Colotta et al., 1998). Localization of IL-1 receptors in the brain has been studied by ligand-binding. Receptors are present at highest density in the dentate gyrus of the hippocampus, specifically on dentate granule cells (Otero and Merrill, 1994). Additional IL-1 binding sites are found in the choroid plexus, pituitary, and meninges (Otero and Merrill, 1994). *In situ* hybridization experiments confirmed the expression of IL-1R type I mRNA in the hippocampus, choroid plexus, raphe nucleus, and pituitary (Cunningham et al., 1991; Otero and Merrill, 1994; Schobitz et al., 1994). Thus, both the IL-1 receptor(s) and ligands are present in the adult brain, often with overlapping

patterns of localization, allowing the potential for functional participation of this cytokine in the mammalian CNS.

Perhaps the best-studied role for IL-1 in the CNS is the induction of fever (Sundgren-Andersson et al., 1998). IL-1β was the first endogenous pyrogen discovered, and remains among the most potent (Sundgren-Andersson et al., 1998). In addition, exogenously applied IL-1 can induce slow-wave sleep and anorexia (Ban et al., 1991; Faggioni et al., 1995). Another prominent action of IL-1 is its control of the hypothalamic-pituitary-adrenal axis (HPA); IL-1 induces secretion of adrenocorticotropin hormone (ACTH) and glucocorticoids, and release of various pituitary hormones both *in vitro* and *in vivo* (references cited in Ban et al., 1991; Schobitz et al., 1994). Interleukin-1 also induces the release of several other important intercellular mediators, including TNFα, TGFβ1, and IL-6 (Sei et al., 1995), and is itself up-regulated in most models of peripheral infection (Schobitz et al., 1994). Combined with its effects on thermogenesis, IL-1 thus acts to coordinate many aspects of the brain's response to corporal events such as infection.

Additionally, IL-1 also affects many aspects of neuronal biology that have implications for synaptic plasticity. Exogenously applied IL-1β alters membrane potential in several different populations of neurons, and in one area, the supraoptic nucleus, it can also affect neuronal firing rate (Miller et al., 1991; Sawada et al., 1991; Li et al., 1992; Yu and Shinnick-Gallagher, 1994). Exogenous IL-1 may alter membrane potential though its effects on several ionic currents, including a decrease in voltage-gated calcium conductance and an increase in the GABA receptor-mediated chloride current (Koller et al., 1997). These actions of IL-1 are discussed in more detail in the following section (Section 3.4). Furthermore, IL-1 application to hippocampal slices prior to tetanic stimulation can prevent the induction of LTP (Schneider et al., 1998), and intracerebroventricular (icv) injection of IL-1 in the behaving animal impairs performance in spatial learning tasks the following day (Oitzl et al., 1993). The effects of IL-1 on LTP are presented in further detail in Section 4.

IL-1 also affects neuronal survival both *in vitro* and *in vivo* (Bartfai and Schultzberg, 1993; Rothwell and Relton, 1993). In this regard, the effects *in vitro* are somewhat contradictory to those observed *in vivo*, and may reflect differences in the neurons or the models studied. In dorsal root ganglia (DRG)/sciatic nerve organotypic cultures, addition of an anti-IL-1β antibody results in increased neuronal degeneration (Bartfai and Schultzberg, 1993), suggesting that endogenous IL-1 produced by these cells acts in a neuroprotective manner. *In vivo*, however, physiological amounts of IL-1 can dramatically increase neuronal death induced by ischemia, excitotoxins, or traumatic injury (Rothwell, 1998). Moreover, cellular damage normally seen after several of these insults can be attenuated by injection of IL-1RA (Rothwell, 1998).

Finally, IL-1 may also act to enhance neurite outgrowth *in vitro* through its effects on the synthesis and stability of NGF (Merrill, 1992). This effect may be direct, or may act through an intermediary factor such as IL-6, which can be induced by IL-1 in astrocytes, and in turn acts to increase NGF release by these cells (Merrill, 1992).

2.2 TGFβ

Five isoforms of TGFβ have been described (TGFβ1-5), although only TGFβ1-3 are found in mammals (Logan and Berry, 1993). Each TGFβ is encoded by a separate gene, but all share a similar sequence indicating the presence of a common cysteine knot structure (Kingsley, 1994). Within the mammalian CNS, the TGFβ2 and TGFβ3 isoforms are most prevalent; mRNA for both genes was found in all brain areas tested, including cortex, hippocampus, striatum, cerebellum and brainstem (Unsicker et al., 1991). Immunoreactivity for TGFβ2 and TGFβ3 is also widespread in the adult CNS. The patterns of expression for TGFβ2 and TGFβ3 are virtually identical, and the only difference noted is in the intensity of staining. Both neurons and glia are TGFβ-positive, with areas of highest staining in the motor nuclei of the brainstem and spinal cord, as well as pyramidal neurons of the cortex and hippocampus. *In situ* hybridization for TGFβ2

confirmed the synthesis of this factor within CNS neurons; mRNA is localized within Purkinje cells of the cerebellum and neurons of the hippocampus (Krieglstein et al., 1995).

In contrast to the abundant expression of TGFβ2 and TGFβ3 in the CNS, relatively little expression of TGFβ1 is detected in under normal conditions in the rodent CNS. Immunostaining for TGFβ1 in the adult rat brain is found only in the meninges and choroid plexus (Unsicker et al., 1991). Several studies of TGFβ1 mRNA within the CNS under normal conditions failed to detect expression using *in situ* hybridization in any brain area examined (Lindholm et al., 1992; Logan et al., 1992; Weißner et al., 1993; Lehrmann et al., 1995). By turning to Northern hybridization, Lindholm et al. (1992) were able to detect very low basal levels of TGFβ1 expression in the rat cerebral cortex. In addition, we detect basal expression of TGFβ1 mRNA in the rat hippocampus (Chapter 2). Although the cell types responsible for this expression *in vivo* have not been identified, astrocytes, microglia, and oligodendrocytes can be activated to express TGFβ1 *in vitro* (Benveniste, 1998). Additionally, transient ischemia or cerebral injury can up-regulate TGFβ1 expression in areas near the injury site, overlapping with areas of astrocytic and microglial activation, consistent with TGFβ1 expression in these cells *in vitro* (Lindholm et al., 1992; Logan et al., 1992; Weißner et al., 1993; Lehrmann et al., 1995).

Receptors for the TGFβs have been classified into three types, I-III, all of which may be used in a single heteromeric TGFβ receptor (Mehler and Kessler, 1995). Within each group, multiple receptors bind TGFβ *in vitro*, and several of these receptors have been localized in the adult CNS *in vivo*. Expression of the TGFβ-receptor type I (TβR-I) is found in the dentate gyrus and habenula of the adult mouse brain (Soderstrom et al., 1996). In addition, TβR- II expression is also localized to the hippocampus, as well as the cortex, midbrain, cerebellum and brain stem (Bottner et al., 1996). Another type I receptor, Habrec 1, is also found in the adult CNS (Lorentzon et al., 1996). Strongest expression is in neurons of the cerebellum, cortex, and striatum, with lower mRNA expression in the hippocampus, amygdala, olfactory bulb and hypothalamus. Taken

together, these overlapping patterns of expression demonstrate that at least some of the necessary receptors are present together with their ligands to permit functional signaling in the normal, adult CNS.

As indicated by the strong up-regulation of TGFβ expression by cortical injury (Lindholm et al., 1992; Logan et al., 1992; Weißner et al., 1993; Lehrmann et al., 1995), one of the best-studied functions of TGFβ is regulating the immune response to insult or infection. TGFβ functions include suppressing proliferation of B and T cells, inhibiting production of pro-inflammatory cytokines such as TNFα and IL-1, and down-regulating expression of MHC proteins (Benveniste, 1998). The importance of TGFβ in the immune response is most clearly demonstrated by early postnatal death resulting from multifocal inflammatory disease observed in mice mutant for TGFβ1 (Benveniste, 1998; Ruscetti et al., 1998).

The TGFβs exert significant effects on neuronal survival *in vitro*, although these effects are only revealed in the presence of complex culture conditions such as co-culture on an astrocytic monolayer (Krieglstein et al., 1995). *In vitro*, TGFβs can enhance the survival of both motor and sensory neurons (Krieglstein et al., 1995). The effects of TGFβ on sensory neuron survival were blocked by anti-NGF antibodies, suggesting that NGF may mediate the actions of TGFβ on these cells (Krieglstein et al., 1995). Because of the structural and functional homology to GDNF, the effects of TGFβ have been best studied on midbrain dopaminergic neurons, with an eye towards potential therapeutic use of these factors in neurodegenerative diseases such as Parkinson's disease. Exogenous TGFβ not only provides substantial trophic support for these dopaminergic neurons, but also protects them from MPP+ toxicity (Krieglstein et al., 1995). The effects of the TGFβs on different neuronal populations under various conditions is more complex than one might suspect from the actions described thus far, and a more detailed account of the effects of TGFβ on neuronal survival in vitro can be found in Section 3.1.

Beyond supporting neuronal survival *in vitro*, the TGFβs have also been found to enhance axonal growth. TGFβ1 and β2 increases neurite number and elongation in cultures of hippocampal neurons, and has similar effects on neurons from the dorsal root ganglia (Krieglstein et al., 1995).

2.2 BMPs 2 and 6

These two BMPs share less homology with each other than they do with other members of the TGFβ superfamily, and so have been grouped into separate subfamilies. BMP2 is a member of the dpp subfamily, with greatest structural similarity to BMP4 and dpp, while BMP6 is a member of the 60A subfamily, which includes BMPs 5-8 and 60A. Despite belonging to distinct subfamilies, BMP2 and BMP6 are able to form a functional heterodimer, which supports neuronal survival and neurite outgrowth (Mehler and Kessler, 1995). These two BMPs are also active as separate homodimers (Mehler and Kessler, 1995).

The expression of BMP2 *in vivo* has not been well documented. Only one study has addressed the expression pattern in the developing and adult CNS. Using an RNAse protection assay, Zang et al. (1994) detected BMP2 transcripts in the murine brain from embryonic day 12 through adulthood. We find BMP2 mRNA in the adult hippocampus (Chapter 2), although expression levels are moderately low. *In vitro*, BMP2 transcripts have been localized to several cell types, including microglia, neurons and astrocytes (Mehler et al., 1997).

Much more information is available on the distribution of BMP6 *in vivo*. By contrast to BMP2, expression of BMP6 is relatively high in early embryonic, postnatal and adult brain (Mehler and Kessler, 1995). Several studies have identified BMP6 immunostaining in neurons of the adult hippocampus and cerebellum, with additional staining in the choroid plexus and meninges (Sauermann et al., 1992; Wall et al., 1993). *In situ* hybridization confirmed the neuronal localization of BMP6 in the adult

hippocampus, and additionally found high levels of mRNA expression in neurons of the neocortex (Tomizawa et al., 1995).

Receptors for the BMPs have been classified into two groups, with characteristics similar to the type I and II receptors for the TGFβs (Kingsley, 1994; Mehler et al., 1997). One of the type II receptors, BMPRII, is found in the adult brain (Soderstrom et al., 1996; Lewen et al., 1997). BMPRII mRNA is localized to neurons of the cortex, striatum, cerebellum, dentate gyrus, hippocampus, habenula and the substantia nigra (Soderstrom et al., 1996). Interestingly, expression of this receptor is down-regulated by contusion injury to the cortex (Lewen et al., 1997). Expression of other BMP-receptor subunits BMPRI-A and BMPRI-B are below detection in the adult brain by *in situ* hybridization (Soderstrom et al., 1996). Further studies with more sensitive techniques will be necessary to determine if the type I subunits needed to form a functional heteromeric BMP receptor are actually present in the adult CNS.

The BMPs are recognized primarily for their importance in embryonic development. In the nervous system, BMPs function in multiple events, including primary neural induction, dorsal-ventral patterning of the neural tube, regionalization of the brain, eye development, apoptosis, and lineage determination in the PNS (Hogan, 1996). BMPs also affect several aspects of neuronal biology at later times in development. Application of individual BMPs (2-6) or their heterodimers to cultures of E17-E18 brain regions leads to increased neuronal survival and neurite outgrowth (Mehler et al., 1997). BMP2 and BMP6 also induce dendritic growth in cultured sympathetic neurons (Guo et al., 1998). Further, several BMPs significantly affect gene expression in cultures of early postnatal sympathetic neurons. BMP2 and BMP6 each induce expression of dynorphin (DYN), neuropeptide Y (NPY), somatostatin (SOM) and vasoactive intestinal peptide (VIP) in a dose-dependent manner (Fann and Patterson, 1994). Interestingly, the effects of each of these factors is differentially affected by neuronal depolarization in high potassium medium (Fann and Patterson, 1994).

2.3 Activin βA, βB, and Inhibin α

Activins are homo- or heterodimers of two subunits, activin βA and activin βB. These two proteins are much more closely related to each other than to other members of the TGFβ superfamily, and so are grouped as their own activin subfamily. In addition to forming activin dimers, the βA and βB subunits can also bind to a third protein, inhibin α, to form inhibin heterodimers. The activins and inhibins have opposing biological effects in many systems, demonstrating how dimerization of different partners can have functional consequences.

The mRNA expression of all three subunits in the adult brain by RNAse protection analysis was demonstrated shortly after their identification and cloning (Meunier et al., 1988). Northern analysis localizes all three transcripts to the hippocampus (Tretter et al., 1996). *In situ* hybridization for βA also reveals expression in neurons of the cortex and amygdala. Expression of βA mRNA is strongly induced in response to neurotoxic lesion, ischemia and seizure (Andreasson and Worley, 1995; Lai et al., 1996; Tretter et al., 1996). Induction of βA in each of these insults is most obvious in neuronal cell body layers of the hippocampus and cortex. Where examined, early induction of βA mRNA occurs in the absence of up-regulation of either βB or inhibin α transcripts, indicating that perhaps only activin βA homodimers are involved in the neuronal response to injury (Lai et al., 1996; Tretter et al., 1996).

Activin receptors of both type I and type II groups have been identified in the adult CNS. Using RT-PCR, Tretter et al. (1996) identified activin receptor IA (ActRIA) and ActRIB in the hippocampus; using *in situ* hybridization, Soderstrom et al. (1996) localized this expression to the neuronal cell body layers, in agreement with an earlier study of ActRIA (Bengtsson et al., 1995). Expression of ActRII is also found in the dentate gyrus, with labeling extending into the entorhinal cortex and amygdala (Bengtsson et al., 1995; Soderstrom et al., 1996). In a separate study, Cameron et al. (1994) found a wider

distribution of ActRII in the adult CNS, with mRNA expressed not only in the hippocampus and amygdala, but also in the cortex, hypothalamus and brainstem. Additionally, this study also found that ActRIIB is expressed in a pattern almost identical to, though at lower levels than, ActRII. Thus, subunits for several functional activin receptors are present in overlapping areas of the adult brain.

Activins not only respond to neuronal activation and insult, but can also affect neuronal properties such as gene expression. Expression of activin βA mRNA is significantly up-regulated both by seizure activity and by mechanical injury to the brain (Andreasson and Worley, 1995; Lai et al., 1996; Tretter et al., 1996). Further, homodimers of this subunit in the form of activin A substantially affect the expression of neurotransmitter enzyme and neuropeptide mRNAs in cultures of sympathetic neurons (Fann and Patterson, 1994; Fann and Patterson, 1994b; Fann and Patterson, 1995). Several neuropeptides and neurotransmitter enzymes, including cholecystokinin, choline acetyltransferase (ChAT), SOM, NPY, DYN, and calcitonin gene-related peptide (CGRP), are up-regulated by exposure to activin A (Fann and Patterson, 1994). Similar to observations with BMP exposure, the effects of activin A on neuropeptide and neurotransmitter gene expression are altered by neuronal depolarization, suggesting that different patterns of neuronal activity in combination with individual cytokine factors could be used to regulate neuronal phenotype (Fann and Patterson, 1994).

2.4 LIF

LIF was originally purified and cloned for its ability to induce differentiation of the M1 monocytic leukemic cell line *in vitro* (Hilton and Gough, 1998). The same factor was independently isolated by several other groups based on a variety of alternative functions, possibly the most striking of which was the ability to induce a phenotypic switch in sympathetic neurons from noradrenergic to cholinergic (Yamamori et al., 1989). Subsequently, LIF has been found to have a wide range of effects on cultured neurons of

both the peripheral and central nervous systems (Murphy et al., 1997; Hilton and Gough, 1998), and LIF transcripts and binding sites are found in the developing and adult brain (Lemke et al., 1996; Qiu et al., 1997).

Studies using RNase protection assays or RT-PCR, detect LIF expression in the cortex, hippocampus and mesolimbic dopamine system (Banner et al., 1997; Chapter 2, 3, and Appendix herein). Using low stringency *in situ* hybridization, LIF mRNA is found in neurons throughout the cortex, cerebellum, striatum, and hippocampus (Lemke et al., 1996; Lemke et al., 1997). Studies using high stringency *in situ* hybridization do not detect LIF mRNA in any brain area examined (Banner et al., 1997; Chapter 3). Induction of LIF expression following cortical injury or seizure is restricted to non-neuronal cells. The LIF^+ cells are predominantly astrocytes, although occasional microglia are found following cortical injury (Banner et al., 1997; Chapter 3).

The LIF receptor is composed of two distinct subunits, the ligand binding subunit LIFRβ and the signal transducing subunit gp130. Expression of both of these subunits has been detected in various regions of the adult brain. Using *in situ* hybridization, LIFRβ mRNA has been localized to the hippocampus, cerebellum, cortex, thalamus and hypothalamus (Yamakuni et al., 1996). In several of these areas, LIFRβ mRNA appears to be expressed predominantly in neurons. These results confirm earlier studies that demonstrated widespread LIFRβ expression in the CNS by Northern blot analysis (Ip et al., 1993). Like LIFRβ, gp130 mRNA is found in every adult brain area tested, including the olfactory bulb, cortex, hippocampus, thalamus, midbrain, hindbrain, cerebellum, and spinal cord. Thus, mRNA expression for both subunits that are required to form a functional LIF receptor are present throughout the adult brain. In addition, a study of LIF binding sites in the developing and adult rat brain demonstrated the presence of LIF binding in all areas examined (Qiu et al., 1997). That study did, however, note binding only in the dentate subfield of the hippocampus, with undetectable levels of binding in other subfields.

Within the nervous system, LIF appears to serve three primary functions. First, LIF is up-regulated in response to injury or trauma, indicating a role in the inflammation response. Consistent with this finding, LIF KO mice display a diminished astrocytic and microglial responses to cortical injury, and a diminished inflammatory response to sciatic nerve injury (Sugiura et al., submitted). Second, LIF is required for maintenance of uninjured spinal motor neurons in maturity (Sendtner et al., 1996), a function that was only revealed in the combined absence of both LIF and CNTF. Third, LIF plays a role in the regulation of astrocytic glial fibrillary acidic protein (GFAP). *In vitro*, LIF can stimulate the expression of astrocyte-specific markers in glial progenitor cells (Murphy et al., 1997), and *in vivo*, LIF KO mice display decreased GFAP immunostaining in both the early postnatal and adult brain (Bugga et al., 1998; Koblar et al., 1998).

In addition, LIF affects several other aspects of neuronal biology *in vitro*. LIF acts as a potent survival factor for several populations of embryonic and postnatal neurons, including DRG, nodose, and trigeminal ganglia (Murphy et al., 1997). Under different conditions, however, LIF induces neuronal death in a dose-dependent manner (Murphy et al., 1997). Exogenous LIF acts to promote survival of postnatal hippocampal neurons, and slightly alters their dendritic morphology *in vitro* (Gadient et al., 1998). Application of LIF to sympathetic neurons produces quite a different response; these neurons retract their dendrites when LIF is added to the culture medium, leading to an 80% reduction in the size of the arbor, a result of antagonism between LIF and the BMPs present in the culture system (Guo et al., 1999). Thus the effects of LIF on both survival and morphology are at least in part subject- and context-dependent.

LIF exerts dramatic effects on neuropeptide and neurotransmitter gene expression in both sympathetic and sensory neurons *in vitro* and *in vivo*. Over 20 years ago, LIF, in the form of heart-cell conditioned medium, was shown to induce a phenotypic switch in neurons cultured from early postnatal superior cervical ganglia (Patterson, 1978). The LIF-exposed cells went from expressing noradrenergic markers, such as tyrosine

hydroxylase (TH), to expressing largely cholinergic markers, such as ChAT. In addition, the cohort of neuropeptides expressed by these cells switches in conjunction with the neurotransmitter phenotype (Nawa et al., 1991). While it was later found using LIF KO mice that LIF is not the factor responsible for this phenotypic switch *in vivo*, it was demonstrated that LIF is critical for a similar change in gene expression that occurs in sympathetic and sensory neurons in response to axotomy (Rao et al., 1993). LIF mRNA is quickly up-regulated after injury both in the SCG and in the sciatic nerve of wild-type mice (Banner and Patterson, 1994; Sun and Zigmond, 1996), and soon thereafter altered expression of neuropeptide mRNAs is seen. In the absence of functional LIF expression, this change in gene expression following injury is not observed, demonstrating that LIF plays a critical role in regulating neuropeptide expression following neuronal insult in the PNS (Rao et al., 1993; Corness et al., 1996; Sun and Zigmond, 1996). The regulation of gene expression by LIF in several neuronal populations is discussed in further detail in Section 3.3.

2.5 CNTF

Although CNTF is now recognized for a variety of effects on a wide range of neural cells in culture, its ability to promote the survival of cells from the chick ciliary ganglia was the original test of CNTF function (Stockli et al., 1991; Richardson, 1994; Sendtner et al., 1994; Murphy et al., 1997). Within the adult CNS, highest levels of CNTF expression are seen in the optic nerve and the olfactory bulb (Stockli et al., 1991). Immunostaining for CNTF in both of these areas indicates that the $CNTF^{+}$ cells were likely to be astrocytes (Stockli et al., 1991). Dissociated cell culture of the olfactory bulb provided further evidence that astrocytes in the CNS are capable of producing high amounts of CNTF mRNA. This is consistent with the glial localization of CNTF in the PNS. In the sciatic nerve, CNTF staining is restricted to Schwann cells. Other areas of the brain also express CNTF mRNA, including the brain stem, hippocampus, and cerebellum

(Stockli et al., 1991). The expression levels in these areas, however, are only a fraction of the amounts found in optic nerve and olfactory bulb. In partial disagreement with this finding, Ip et al. (1993) used Northern blots to demonstrate high levels of CNTF expression in the spinal cord, thalamus/hypothalamus, midbrain, hindbrain and olfactory bulb. Consistent with the results of Stockli et al. (1991), much lower, though clearly detectable, signal was found in the cortex, hippocampus and cerebellum.

Roder and coworkers (Henderson et al., 1994; Seniuk-Tatton et al., 1995) have provided *in situ* hybridization and immunostaining evidence for a very different CNTF expression pattern than previously demonstrated by Ip et al. (1993) and Stockli et al. (1991). The Roder group detected almost pan-neuronal expression within the hippocampus and cerebellum (Seniuk-Tatton et al., 1995), two areas that show very low CNTF levels by Northern analysis (Stockli et al., 1991; Ip et al., 1993). Immunostaining for CNTF is strongest in neurons, and is found at high levels in the hippocampus and cerebellum, in addition to several other brain areas. Because of the disagreement of these expression patterns with previous results, it has been suggested that this neuronal localization be interpreted with caution (Sendtner et al., 1994).

The receptor for CNTF is heteromeric, and like other members of the neuropoietic family, is composed of a specific ligand binding subunit (CNTFRα) and the signal transducing subunit gp130. The CNTF receptor also contains a third subunit, LIFRβ. Both gp130 and LIFRβ expression are widespread in the adult brain (Section 2.4). Unlike gp130 and LIFRβ, which are expressed throughout the body, CNTFRα expression is restricted to the nervous system and skeletal muscle (Ip et al., 1993). Within the adult brain, CNTFRα levels are relatively high, and are localized to neuronal cell bodies within the hippocampus, cortex, thalamus, substantia nigra, several brain stem nuclei and the ependymal zone of the lateral ventricle (Ip et al., 1993). Western analysis of CNTFRα protein in various areas of the adult brain reveals significant expression only in the olfactory bulb and the cortex, however (Kirsch and Hofmann, 1994).

Due to the receptor subunits shared by both, CNTF and LIF have nearly identical effects on cells in culture (Fann and Patterson, 1994b; Murphy et al., 1997). In sympathetic neurons, the neuropeptide and neurotransmitter pattern induced by CNTF is identical to that induced by LIF (Fann and Patterson, 1994b). In cultured DRG neurons, however, CNTF does not alter levels of ChAT, VIP, substance P (SP), or SOM (Murphy et al., 1997), findings different from those reported for LIF (Nawa et al., 1991). In addition, depolarization blocks the effect of LIF on DRG cells, but has no effect on gene expression up-regulated by CNTF (Murphy et al., 1997).

Like LIF, CNTF is also up-regulated in response to injury *in vivo* (Ip et al., 1993; Banner et al., 1997), albeit with a relatively slower course. As in the case of LIF induction following injury, the $CNTF^{+}$ cells are believed to be astrocytes and Schwann cells. However, the function of CNTF following injury *in vivo* has not been defined, and no studies of CNTF KO mice have been published in this regard.

In vitro, CNTF acts as a trophic factor for at least some members of all classes of peripheral neurons (Richardson, 1994; Sendtner et al., 1994). CNTF also affects the survival of cultures from a significant number of CNS regions, including embryonic hippocampus, cerebellum, cortex, brainstem, and spinal cord (Richardson, 1994; Sendtner et al., 1994). The trophic action of CNTF on certain neuronal populations has also been demonstrated *in vivo*; like LIF, it is required for maintenance of normal motor neurons in the adult rodent (Sendtner et al., 1996).

The effects of CNTF on neuronal morphology have also been studied both *in vitro* and *in vivo*. In primary dissociated cultures of five embryonic brain regions, CNTF induces moderate process outgrowth (Mehler and Kessler, 1994). *In vivo*, CNTF promotes sprouting by motor neurons innervating the adult mouse gluteus muscle (Murphy et al., 1997). These effects are similar to observations in molluscan neurons in which cells survive in the absence of CNTF, but rapidly produce neurites on the addition of CNTF to the medium (Richardson, 1994).

2.6 IL-6

Initially known as the B-cell stimulatory factor-2, IL-6 has diverse biological actions in the hematopoietic system. Recently, IL-6 has also been implicated as an important regulator of communication between cells of the nervous system (Gruol and Nelson, 1997). Low levels of IL-6 mRNA are detected throughout the brain, with significant levels in the hippocampus, cerebellum, cortex and hypothalamus (Schobitz et al., 1993; Gadient and Otten, 1994). Expression is restricted to neurons in the hippocampus and cerebellum (Gadient and Otten, 1994), although non-neuronal expression is detected in white matter areas such as the internal capsule, which contains only fibers and glia (Schobitz et al., 1993). *In vitro*, astrocytes and microglia are both capable of producing IL-6 (Gruol and Nelson, 1997; Benveniste, 1998). Interestingly, IL-6 expression in the CNS is developmentally regulated, with highest mRNA levels seen in the adult brain (Gadient and Otten, 1997).

Like other members of the neuropoietic family, the IL-6 receptor is heteromeric, including both a ligand-specific subunit, IL-6R, and (two molecules of) the signal transducing subunit gp130 (Taga and Kishimoto, 1997). As described earlier, expression of gp130 is widespread in the adult CNS (Section 2.4). Similarly, IL-6R mRNA is expressed in many brain regions (Schobitz et al., 1993; Gadient and Otten, 1997), including the hippocampus, cerebellum, hypothalamus and cortex. In fact, localization of the IL-6R precisely overlaps the pattern of IL-6 expression in every brain area examined (Yan et al., 1992; Schobitz et al., 1993; Schobitz et al., 1994). Both transcripts are localized to neurons within the hippocampus and cerebellum, with additional, less-intense, non-neuronal signal detected in white matter areas (Schobitz et al., 1993). Little information is available to correlate protein expression of the IL-6R to the mRNA distribution. Some reports indicate that contrary to the high levels of mRNA, neurons do not appear to express appreciable levels of the membrane-bound IL-6R protein, and thus

may depend on another form of the ligand-binding subunit, the soluble IL-6R, to respond to this factor (Benveniste, 1998).

Analysis of mice in which the IL-6 gene has been disrupted emphasizes the fact that IL-6 is a critical component of the immune response. Although IL-6 KO mice appear to develop normally, they fail to control challenge with endotoxin or virus (Ryffel, 1995). Injury to the CNS is also regulated by IL-6. In the absence of IL-6, KO mice display reduced astrocytic and microglial response to axotomy of CNS neurons (Klein et al., 1997; Penkowa et al., 1999). Other forms of cortical insult, including several neurodegenerative diseases, are associated with elevated levels of IL-6 (Gruol and Nelson, 1997; Gadient and Patterson, 1999). Transgenic mice over-expressing IL-6 in the brain display severe astrogliosis and microgliosis (Campbell et al., 1993; Brett et al., 1995; diSanto et al., 1996). Additionally, IL-6 is a potent pyrogenic agent, and may help control the immune response by elevating body temperature during infection (Gruol and Nelson, 1997).

IL-6 is also neuroprotective under a variety of conditions both *in vitro* and *in vivo*. IL-6 can support the survival of cholinergic neurons of the basal forebrain and septum and sensory neurons from the periphery in culture (Gadient and Otten, 1997). Addition of IL-6 to the medium of cultured midbrain catecholaminergic neurons results in a higher proportion of mature TH^+ cells surviving in culture, suggesting that IL-6 acts as a selective trophic factor for this neuronal population (Gruol and Nelson, 1997). Further, IL-6 can protect many types of neurons from toxic insult *in vitro*; pretreatment of hippocampal neurons with IL-6 attenuates cell death in response to glutamate-induced excitotoxicity. IL-6 also protects striatal cholinergic cells from NMDA-induced excitotoxicity (Gadient and Otten, 1997). The effects of IL-6 are not always positive, however, as adding IL-6 to midbrain-derived neuronal cultures during the first two days *in vitro* (div) actually results in fewer surviving neurons. After 2 div, IL-6 promotes TH^+ neuronal survival as noted above. *In vivo*, IL-6 protects several neuronal populations from the effects of various

neurotoxic insults, including ischemia, nerve injury, and MPP^+ exposure (Gadient and Otten, 1997).

Significant effects on neuronal morphology have also been observed after the addition of IL-6 *in vitro*. Over a decade ago, IL-6 was shown to induce neurite extension from PC12 cells in a manner similar to NGF (Satoh et al., 1988). IL-6 also enhances the neurite structure of many types of embryonic and postnatal primary neurons (Hama et al., 1991; Kushima et al., 1992; Sarder et al., 1996); these effects are discussed further in Section 3.2.

Interestingly, IL-6 treatment of developing CNS neurons can alter responses to NMDA receptor activation. Chronic treatment of cultured cerebellar granule neurons with IL-6 enhances the intracellular calcium signal evoked by brief application of either NMDA or glutamate (Gruol and Nelson, 1997). The effect is specific to the NMDA receptor; no differences in calcium current are observed in the presence of kainate receptor agonists, or in response to membrane depolarization in high potassium (Gruol and Nelson, 1997). These results suggest that exposure to IL-6 leads to an increase in NMDA-receptor number or a change in receptor function. IL-6 may also affect the expression of other ion channels; in addition to stimulating neurite extension in PC12 cells, application of IL-6 results in an increased number of sodium channels (Schobitz et al., 1994).

2.7 OSM and CT-1

Although identified more than a decade ago (Shoyab et al., 1998), little is known of the tissue distribution of OSM. A few cell types have been shown to synthesize and secrete OSM, among them U-937 histiocytic lymphoma cells, macrophages, and T-cells, which produce OSM only after exogenous stimulation (Shoyab et al., 1998). Cells derived from AIDS-associated Kaposi's sarcoma are the only cells that had been known to secrete OSM without provocation (Shoyab et al., 1998) until the recent discovery that OSM is strongly expressed in the developing testis and may function in spermatogenesis (de Miguel et al.,

1997). Our work (Chapter 3) provides the first demonstration that OSM is also expressed in the nervous system. Specifically, OSM mRNA is found in the adult rat hippocampus and its expression is strongly up-regulated by seizure activity in the brain. Following seizure, increased levels of OSM appear to be localized to neurons, most likely GABA-ergic interneurons, within the cortex, hippocampus, and amygdala (Chapter 3).

Two high-affinity OSM receptors have been described in human tissue, one composed of an OSM-specific ligand binding subunit with the signal transducing subunit gp130, and the other a receptor shared with LIF, composed of LIFRβ and gp130 (Ichihara et al., 1997). Murine OSM, however, signals only through the receptor containing its specific binding subunit (Ichihara et al., 1997; Lindberg et al., 1998). The distribution of gp130 in the adult rodent brain is widespread (Section 2.4). The distribution of the OSM-specific subunit in the brain has not been investigated.

The *in vitro* effects of OSM are similar to several other members of the neuropoietic family, including LIF and CNTF. When exogenously applied to cultured sympathetic neurons, OSM induces the same pattern of neuropeptide and neurotransmitter expression as do LIF and CNTF (Fann and Patterson, 1994b). In addition, OSM can induce the expression of IL-6 in endothelial cells and fibroblasts (Brown et al., 1991; Richards and Agro, 1994), suggesting that it may act as an immediate early gene to regulate later IL-6 production in other cell systems. OSM also affects neurite outgrowth and terminal branching of cultured CNS neurons in a manner and extent similar to LIF and CNTF, all of which induce moderate enhancement in neurite structure (Mehler and Kessler, 1994).

Cardiotrophin-1 is the most recently identified member of the neuropoietic family (Pennica et al., 1995). Despite its cloning as a trophic factor for cardiac myocytes (Pennica et al., 1995), it is expressed in a wide variety of tissues *in vivo*. Low, but detectable expression is observed in adult mouse brain by Northern analysis, while little or no CT-1 is found in human brain (Pennica et al., 1995; Pennica et al., 1996). We find CT-1 mRNA expression in the hippocampus of adult rats (Chapter 3). The level of CT-1 mRNA

observed in this region is moderately low, consistent with the earlier Northern analysis of total brain extract (Pennica et al., 1995). No additional information is available on CT-1 expression in other areas of the brain, nor is there information on the localization of CT-1 protein in the CNS.

CT-1 signals through a tripartite receptor composed of the LIFRβ, gp130, and a CT-1-specific subunit, CT-1Rα (Robledo et al., 1997). Both LIFRβ and gp130 are expressed throughout the adult brain (see Section 2.4). The distribution of CT-1Rα has not yet been described *in vivo*, however.

CT-1 has effects on several types of cultured neurons. Consistent with its high expression levels in the developing limb bud, CT-1 is a potent survival factor for embryonic spinal motor neurons during the time of axonal projection to the periphery (Pennica et al., 1996). At later times in development, exogenously applied CT-1 can protect sciatic motor neurons from axotomy-induced degeneration (Pennica et al., 1996). This cytokine also regulates gene expression in cultured sympathetic neurons in the same way as the other neuropoietic cytokines, inhibiting tyrosine hydroxylase activity and stimulating choline acetyltransferase activity, thus modulating the neuronal phenotype of these cells (Pennica et al., 1995; Cheng et al., 1997).

3. CYTOKINES EFFECTS ON NEURONAL PROPERTIES, IN DEPTH

To convey a sense of the wide range of effects cytokines have on neuronal cells, as well as the number of factors that can elicit these changes, this section will illustrate by example the effects of cytokines on four significant facets of neuronal biology. Each section will focus on the spectrum of functions displayed by one cytokine, and cite references for similar actions of additional factors.

3.1 Cytokines and neuronal survival: TGFβ

The mammalian TGFβs 1-3 affect the survival of many different types of embryonic neurons *in vitro* (Krieglstein and Unsicker, 1994; Flanders et al., 1998). Trophic effects of the TGFβs are generally not seen in highly purified neuronal cultures, but rather require more complex conditions such as growth on an astrocytic monolayer to allow these cytokines to affect neuronal survival (Krieglstein et al., 1995).

Martinou et al. (1990) were the first to show that TGFβ1 could act as a potent survival factor for embryonic neurons *in vitro*. In their cultures of E14 motor neurons on astrocytic monolayers, TGFβ1 increased neuronal survival by 2-fold. TGFβs also affects survival of embryonic sensory neurons *in vitro*, but only in combination with other factors, the neurotrophins NT-3 and NT-4 (Chalazonitis et al., 1992; Krieglstein and Unsicker, 1996). Additionally, in DRG cultures treated only with the neurotrophins, a neutralizing antibody to TGFβ decreases neuronal survival, suggesting that endogenous TGFβ in these cultures affects the action of the neurotrophins. Contrary to these survival-promoting effects of TGFβ, Flanders et al. (1991) found that in the presence of chick eye extract, TGFβ2 and β3 *inhibits* the survival of cultured chick ciliary neurons. Thus, the net effect of TGFβ on neuronal survival depends in part on the environment in which it is presented.

Because of their structural and functional similarities to GDNF, the TGFβs have been carefully examined for their trophic effects on another neuronal cell population, the midbrain dopaminergic neurons, for which GDNF is a potent survival factor. These cells are specifically affected in certain human neurological conditions such as Parkinson's disease, and so factors that would enhance survival of midbrain dopaminergic neurons may have important applications as therapeutic agents. All three TGFβs promote survival of embryonic midbrain dopaminergic neurons *in vitro*, with effects matching that of FGF-2 (Krieglstein and Unsicker, 1994; Poulsen et al., 1994; Krieglstein et al., 1995). In addition, the TGFβs also protect dopaminergic neurons from the toxic effects of MPP^+, to which these neurons are specifically vulnerable (Krieglstein et al., 1995; Krieglstein et al., 1995).

In addition to the ability of the TGFβs to protect neurons against MPP^+ toxicity, these cytokines also promote neuronal survival following several other forms of injury. All three TGFβ isoforms are protective against hypoxia induced by addition of sodium cyanide (Prehn et al., 1993). Two of the TGFβs, β1 and β3, can also protect neurons against short-term, N-methyl-D-aspartate (NMDA)- or glutamate-induced excitotoxicity (Prehn et al., 1993; Prehn and Miller, 1996). Conversely, application of TGFβ2 to neuronal cultures exposed to NMDA actually *increases* excitotoxic cell death (Kane et al., 1996), an effect also found for TGFβ1 in cultures exposed to long-term (24 hour) excitotoxic insult (Prehn and Miller, 1996), indicating that the action of each of these factors can vary significantly with context. Another form of injury that has been examined is X-irradiation; here TGFβ1 protects neurons against irradiation-induced apoptotic death. Consistent with these results is the finding that TGFβ1 not only prevents calcium entry after NMDA or calcium ionophore exposure, but also increases neuronal expression of the apoptotic-protective Bcl2 protein (Prehn et al., 1994).

Two other interesting possibilities for the mechanism by which TGFβs affect neuronal survival have been proposed. Evidence *in vitro* suggests that the TGFβs up-regulate the expression or potentiate the action of NGF in mixed neuronal and glial cultures (Chalazonitis et al., 1992; Buchman et al., 1994). In one set of experiments, addition of an anti-NGF antibody eliminated the neurotrophic effect of TGFβ1, although treatment with this factor did not alter NGF mRNA expression nor change the amount of NGF in the medium (Chalazonitis et al., 1992). However, in co-cultures of a different neuronal population and their target cutaneous cells, Buchman et al. (1994) found somewhat different results; in their system, all three TGFβs were able to increase NGF mRNA expression. In both studies, the effects of TGFβ on neuronal survival may indeed be mediated through a separate factor, although the details and conditions of each system emphasize the effects of environment on the specific actions of these factors *in vitro*.

The second mechanism by which TGFβs may alter neuronal survival is through their effects on astrocytes in the mixed culture systems. Astrocytes have been extensively implicated in protecting neurons from the effects of excitotoxicity, and factors that adversely affect astrocytic function may decrease the neuronal protection they provide. Loss of the astrocytic enzyme glutamine synthetase (GS) is associated with increased extracellular glutamate concentration and neurotoxicity following ischemia, and Chao et al. (1992) found that both TGFβ1 and TGFβ2 significantly inhibit GS activity in mixed neuronal/astrocytic cultures. The presence of TGFβ2 results in increased neuronal death after exposure of these mixed astrocytic/neuronal cultures to NMDA (Chao et al., 1992; Kane et al., 1996). Indeed, this would be consistent with a reduction in astrocytic GS activity, and concomitant decrease in glutamate breakdown after release from the synapse, thus increasing neuronal exposure to high levels of this transmitter.

Many other cytokines also have significant effects on neuronal survival, both *in vitro* and *in vivo*. References for papers reviewing these functions are listed below:

BMPs	Mehler and Kessler, 1995 Hogan, 1996 Mehler et al., 1997
CNTF	Patterson, 1994 Sendtner et al., 1994 Richardson, 1994 Mehler and Kessler, 1995 Murphy et al., 1997
CT-1	Murphy et al., 1997

IL-1	Rothwell and Relton, 1993 Schobitz et al., 1994 Rothwell and Hopkins, 1995 Rothwell et al., 1996 Lawrence et al., 1998 Rothwell, 1998
IL-6	Frei et al., 1989 Schobitz et al., 1994 Rothwell et al., 1996 Gadient and Otten, 1997 Gruol and Nelson, 1997
LIF	Patterson, 1994 Mehler and Kessler, 1995 Murphy et al., 1997

3.2 Cytokines and neuronal morphology: IL-6

Seminal studies of neuronal differentiation and neurite extension were performed on the pheochromocytoma cell line PC12. Their characteristic transformation from round cells without neurites to cells forming a profusely branched network of extended processes was classically induced by addition of the neurotrophin NGF (Greene and Tischler, 1976). Additional trophic factors were later found that could induce a similar phenotypic change in these cells, including IL-6 (Satoh et al., 1988). In the presence of IL-6, Satoh et al. (1988) demonstrated that PC12 cells extend a network of long neurites after approximately 6 days *in vitro*, displaying a similar morphology and developmental time course as cultures treated with NGF. Later work with several variants of the PC12 cell line revealed that only certain subclones of these cells, and not the original cell line, responded to IL-6 (Wu and

Bradshaw, 1996; Wu and Bradshaw, 1996; Marz et al., 1998). The original PC12 cells do, however, up-regulate expression of GAP43 in the presence of IL-6. In addition, neurite extension in response to NGF or EGF can be induced at lower concentrations after the addition of IL-6 (Wu and Bradshaw, 1996; Marz et al., 1998). Following the demonstration that the original PC12 cells could extend processes in response to IL-6 when provided in combination with soluble IL-6 receptor, it was suggested that the original cell line expressed more gp130 than IL-6R, and that the extent of activated gp130 molecules determined the quality of the response (Marz et al., 1998).

IL-6 can also affect the morphology of primary neurons when added *in vitro*. Exogenous IL-6 increases neurite length in cultures of several neuronal populations, including embryonic hippocampal neurons, as well as tyrosine hydroxylase (TH) positive neurons from E16, P13 and P15 midbrain (Hama et al., 1991; Kushima et al., 1992; Sarder et al., 1996). Moreover, application of IL-6 to TH^+ neurons results in better developed neurites with more varicosities (Hama et al., 1991; Kushima et al., 1992). IL-6 also supports the survival of these neurons in serum-free media, and enhances survival in serum-containing media.

The effects of several other cytokines on neuronal morphology have been examined. A brief list with both primary and review references follows:

BMPs	Kreiglstein et al., 1995 Mehler and Kessler, 1995 Mehler et al., 1997 Guo et al., 1998
CNTF	Richarson, 1994 Murphy et al., 1997
CT-1	Pennica et al., 1996

IL-1	Fagan and Gage, 1990 Merrill, 1992 Schobitz et al., 1994
LIF	Murphy et al., 1997 Gadient et al., 1998 Blesch et al., 1999
TGFβs	Ishihara et al., 1994 Krieglstein et al., 1995

3.3 Cytokines and neuronal gene expression: LIF

Like the original neurotrophin NGF, the effects of one cytokine, LIF, on neuronal cultures was studied long before the gene was cloned and the protein identified. During the 1970's it was found that exogenous application of heart-cell conditioned medium to cultures of early postnatal sympathetic neurons induced a phenotypic switch in neurotransmitter expression that mimicked the change in gene expression these cells would normally undergo *in vivo* on contact with their target tissue in the foot pad (Patterson, 1978). In the presence of conditioned medium, sympathetic neurons dramatically up-regulated expression of ChAT, synthesis of acetylcholine, and formation of cholinergic synapses between cells. At the same time, sympathetic neurons down-regulated expression of noradrenergic properties, including catecholamine synthesis. Identification of the active factor in the conditioned medium as the cytokine LIF opened the way for molecular analysis of the effects of this cytokine on neuronal gene expression. Soon it was found that LIF controls acetylcholine and catecholamine synthesis by causing simultaneous up-regulation of ChAT and down-regulation of TH mRNA expression (Nawa et al., 1991). In addition, LIF also alters the neuropeptide phenotype of sympathetic neurons (Yamamori et al., 1989; Nawa et al., 1991). LIF up-regulates the mRNAs for SOM, substance P (SP),

cholecystokinin (CCK), enkephalin (ENK) and VIP, while down-regulating expression of NPY (Nawa et al., 1991; Fann and Patterson, 1993). Interestingly, LIF also induces this noradrenergic-to-cholinergic switch in sympathetic neurons *in vivo* when mis-expressed in the pancreas under control of the insulin promoter (Bamber et al., 1994). Importantly, the neurons innervating the pancreas never undergo this switch under normal conditions *in vivo*.

Another interesting aspect of the effect of LIF on gene expression in sympathetic neurons *in vitro* is its sensitivity to neuronal depolarization. Depolarization in high potassium medium largely blocks the effect of exogenous LIF on gene expression in sympathetic neurons (Walicke et al., 1977; Raynaud et al., 1987). Similarly, exposure to veratridine or direct electrical stimulation also prevents the effects of LIF (Walicke et al., 1977).

While LIF is not the cholinergic differentiation factor for early postnatal sweat gland neurons *in vivo*, this cytokine is critical for the phenotypic switch that occurs in adult sympathetic neurons in response to injury (Rao et al., 1993). Following axotomy of the superior cervical ganglia (SCG) or its removal to organ culture, sympathetic neurons up-regulate a suite of neuropeptides including VIP, galanin (GAL) and neurokinin A (NK) (Sun and Zigmond, 1996). Experiments performed in LIF KO mice demonstrated that LIF is critical for this phenotypic injury response; the up-regulation of neuropeptide expression was significantly diminished in the absence of LIF (Rao et al., 1993). Further work with the LIF KO mice revealed that this factor also contributes to the correlate down-regulation of TH and NPY that are normally expressed at high levels in healthy SCG neurons (Sun and Zigmond, 1996), thus confirming *in vivo* the full spectrum of its impact on gene expression *in vitro*.

Similar changes in neuropeptide gene expression occur in the sensory neurons of the sciatic nerve in response to injury. In the sciatic nerve, as in the SCG, LIF expression is quickly up-regulated after transection (Banner and Patterson, 1994; Sun and Zigmond,

1996). Knockout mice were again used to demonstrate that LIF is required for the changes in neuropeptide expression seen after axotomy (Corness et al., 1996; Sun and Zigmond, 1996). In the LIF KO mice, there is less up-regulation of GAL after sciatic nerve lesion (Corness et al., 1996; Sun and Zigmond, 1996), and additional decreases in regulation of NPY and galanin-message-associated-protein (GMAP) (Corness et al., 1996).

Within the CNS, there are reports that indicate LIF can regulate the neurotransmitter phenotype in spinal cord and motor neurons (Murphy et al., 1997). Here as in the periphery, LIF induces largely cholinergic properties.

Many other members of the neuropoietic family as well as several members of the TGFβ superfamily have been assayed for their regulation of neurotransmitter and neuropeptide expression in sympathetic neurons, and several key references for these factors are included below. Analysis of a number of additional factors did not reveal any significant effects on gene expression in sympathetic neurons (Fann and Patterson, 1994b), and an understanding of their potential to regulate neuronal mRNA expression will await future studies with other neuronal populations or target genes.

Activin A	Fann and Patterson, 1994b Fann and Patterson, 1995 Mehler and Kessler, 1995
BMP 2 and 6	Fann and Patterson, 1994 Mehler and Kessler, 1995
CNTF	Rao et al., 1992 Lewis et al., 1994 Sendtner et al., 1994 Richardson et al., 1994 Rudge et al., 1996 Murphy et al., 1997

CT-1	Cheng et al., 1997
IL-1	Lapchak et al., 1993
IL-6, IL-11	Fann and Patterson, 1994b
OSM	Rao et al., 1992 Shoyab et al., 1998
TGFβ	Krieglstein et al., 1995

3.4 Cytokines and neuronal physiology: IL-1β

The effects of IL-1β on various types of neuronal preparations have been extensively studied, although the results of these experiments are not always consistent. In two separate studies on cultured neurons, one in dissociated guinea pig hippocampal CA1 cells, the other in snail suboesophageal ganglia, application of recombinant human IL-1β (rhIL-1β) leads to decreased calcium current through voltage gated calcium channels (Plata-Salaman and Ffrench-Mullen, 1992; Szucs et al., 1992). Later studies in rodent synaptosome preparations added additional layers of complexity to the effect of IL-1β on calcium current. Murray et al. (1997) found that the decreased calcium influx in response to potassium chloride stimulation is age dependent: IL-1β has a significant effect on synaptosomes prepared from four month old rats, but no effect on preparations from 22 month old rats. In addition, Campbell and Lynch (1998) demonstrated that the effect of IL-1β is biphasic and dose-dependent; IL-1β decreases calcium influx at low concentrations (3.5 ng/ml), and increases calcium flux at high concentrations (100 ng/ml). The action of IL-1β at each dose is also differentially sensitive to downstream signaling inhibitors, indicating that different second messenger pathways are activated at each dose.

Another effect of IL-1β observed in several studies is its ability to induce neuronal hyperpolarization. The proposed cause of the decreased membrane potential, however, differs with the preparation studied. Exogenous rhIL-1β causes hyperpolarization of

Aplysia neurons by inducing a slow outward current associated with a decrease in sodium conductance (Sawada et al., 1991). In neuronal or synaptosomal preparations from adult rats, a different mechanism of IL-1β-induced hyperpolarization was identified. Application of rhIL-1β to cortical synaptosomes leads to increased postsynaptic γ-aminobutyric $acid_A$ ($GABA_A$) receptor function, and consequent increases in chloride transport (Miller et al., 1991). Support for this mechanism *in vivo* was provided by experiments showing increased threshold for pentylenetetrazol-induced seizures in IL-1β-treated mice (Miller et al., 1991). Neuronal hyperpolarization is also found in brain slice preparations of the basolateral amygdala, and again, a GABA-mediated mechanism is implied (Yu and Shinnick-Gallagher, 1994). However, the failure of IL-1β to depress responses to directly applied GABA or glutamate receptor agonists led these authors to conclude that IL-1β hyperpolarizes the membrane by indirectly enhancing endogenous GABA action.

Effects of IL-1β on glutamate release have also been documented, although again not all studies agree in their results. Murray et al. (1997) observed a reduction in glutamate release from potassium chloride (KCl)-stimulated hippocampal synaptosomes prepared from young but not aged rats, concluding that the effects of IL-1β are presynaptic. Contrary to this, Allan et al. (1998) found no effect of IL-1β or IL-1RA on glutamate release in rat striatal synaptosomes also stimulated with KCl, indicating that the effects of IL-1β are unlikely to be presynaptic. Between these two extremes, Yu and Shinnick-Gallagher (1994) reported that exogenous application of IL-1β inhibits both excitatory and inhibitory postsynaptic potentials evoked by stimulation of neurons projecting to the basolateral amygdala, and as mentioned above, IL-1β does not depress responses to GABA or glutamate agonists applied to these neurons, leading these authors to propose a presynaptic effect of IL-1β. Thus, the proposed site of IL-1β action shifts from one side of the synapse to the other with each set of conditions examined.

In contrast to the predominantly inhibitory actions of IL-1β on many preparations described thus far, when rhIL-1β is applied to neurons in the supraoptic nucleus, the firing

rate of these neurons *increases* (Li et al., 1992). Using intracellular recording in rat brain slices, Li et al. found that in the majority of neurons examined, exogenous IL-1β also *depolarizes* the membrane. The depolarization remains in the presence of tetrodotoxin, but is abolished by sodium salicylate, suggesting that prostaglandins may be involved in the effects of IL-1β on these neurons. Indeed, many mechanisms of IL-1β action may be required to mediate its varying effects on different neuronal population.

Exogenous application of IL-1β and inhibition with IL-1RA can substantially alter both *in vitro* and *in vivo* models of learning and memory. Several groups have demonstrated that bath application of IL-1β can prevent induction of LTP in all three excitatory synapses of the hippocampal slice (Katsuki et al., 1990; Bellinger et al., 1993; Cunningham et al., 1996). Intraventricular infusion of IL-1β also inhibits LTP *in vivo* (Murray and Lynch, 1998). In addition, exogenous IL-1β can alter learning and memory in awake behaving animals; experimental manipulation of IL-1β levels in rodents significantly affects performance in spatial learning tasks such as the Morris water maze (Oitzl et al., 1993; Gibertini et al., 1995). These effects of IL-1β on LTP and behavioral tasks will be discussed in more detail in Section 4.

Although the effects of IL-1β on neuronal activity are probably the best studied of the cytokines, several others have been examined in various systems. Examples of both primary and review papers describing the effects of exogenous cytokine application on neuronal physiology are listed below. Effects on long-term potentiation will be covered in the following section.

Activin A	Oliet et al., 1995
CNTF	Cheng et al., 1996
IL-1α	Mimura et al., 1994

IL-6	Steffensen et al., 1994 Gadient and Otten, 1997 Gruol and Nelson, 1997 Koller et al., 1997

4. CYTOKINES AND LONG TERM POTENTIATION

This section discusses the regulation of endogenous cytokine expression following the induction of LTP both *in vitro* and *in vivo*, as well as the effects of exogenous cytokine application on LTP in slice preparations. Because BDNF is the best-characterized trophic factor in this context, I will begin by reviewing the case for BDNF in synaptic modulation, and will follow with what is known to date for the cytokines.

4.1 BDNF and LTP

The role of BDNF in synaptic plasticity and its regulation by neuronal activity have been extensively examined. The regulation of BDNF expression by high levels of synaptic activity was originally demonstrated in several models of epileptic seizure (Zafra et al., 1990; Ernfors et al., 1991; Isackson et al., 1991). This neurotrophin is also regulated by the more physiological form of synaptic activity that is used to induce LTP (Patterson et al., 1992). The levels of BDNF mRNA were assayed in hippocampal slices before and several hours after the induction of LTP in the Schaffer collateral pathway. BDNF expression, determined by radioactive *in situ* hybridization, is increased roughly 2.5-fold by LTP induction, only in the area affected by the tetanizing stimulus, demonstrating that the effect is largely input-specific. The same type of up-regulation occurs following LTP induction *in vivo*. Interestingly, although LTP was induced by unilateral perforant path stimulation, *bilateral* induction of BDNF mRNA is observed (Castren et al., 1993; Dragunow et al., 1993). Increased BDNF expression is limited to the dentate gyrus, again demonstrating the

input specificity of the mRNA alterations. Both the extent and time course of up-regulation are similar *in vitro* and *in vivo*, reaching levels several-fold higher than control values, and peaking within a few hours of LTP induction. The receptor for BNDF, trkB, is also up-regulated by LTP induction in the perforant path. Where examined, increased trkB expression is limited to the dentate gyrus of the stimulated hemisphere, and increases less than 2-fold over basal levels (Bramham et al., 1996; Dragunow et al., 1997), indicating that the regulation of trkB and BDNF occur independently.

In a series of complementary experiments, it was further shown that application of exogenous BDNF to slice preparations enhances electrically-induced LTP. In the rodent hippocampus, the ability to produce LTP does not develop until roughly two weeks of age (Patterson et al., 1996). Application of BDNF to slices from animals only 12-13 days old promotes the induction of LTP by tetanic stimulation in these slices, which in the absence of BDNF, show only short-term potentiation (Figurov et al., 1996). BDNF-enhanced LTP has also been reported in preparations from adult animals; low doses (20 ng/ml) of exogenous BDNF increase the amplitude of potentiated responses by more than 20% in slices of the rat visual cortex (Akaneya et al., 1997).

Significantly, experiments designed to test the presence or magnitude of LTP in the absence of BDNF function also point to a critical role for this neurotrophin in synaptic plasticity. Preincubation of adult hippocampal slices with the trkB-receptor blocking fusion protein trkB-IgG reduces the neuronal response to tetanus and substantially decreases the magnitude of LTP induced following tetanic (theta-burst) stimulation (Figurov et al., 1996). Similarly, exposure to trkB-IgG completely blocks LTP induction in visual cortical slices prepared from young rats (Akaneya et al., 1997). This sensitivity to trkB-IgG in hippocampal slices is largely dependent on the temporal pattern of stimulation used to induce LTP (Kang et al., 1997). The trkB-IgG-treated slices show significant impairment in LTP only when theta-burst or pairing stimulation is used; LTP induced by several trains of tetanic stimulation is normal despite similar exposure to the trkB antagonist

(Kang et al., 1997). Moreover, addition of trkB-IgG to previously untreated slices reverses LTP (by bringing potentiated responses back to baseline levels) when applied up to one hour after tetanus, demonstrating distinct temporal phases of BDNF-dependence in this form of synaptic plasticity (Kang et al., 1997).

Another way to examine synaptic plasticity in the absence of BDNF is to study LTP in BDNF KO mice. Although these animals do not survive longer than 4 weeks after birth, LTP was examined in slices prepared from early postnatal animals. Korte et al. (1995) found that hippocampal LTP is significantly impaired in the absence of BDNF. Both the magnitude of potentiation, and the success rate of LTP induction are decreased in BDNF homozygous as well as heterozygous mutants. In an independent strain of BDNF KOs, Patterson et al. (1996) demonstrated that the mutant animals display defects in basal synaptic function as well as deficits in certain evoked responses, such as paired-pulse facilitation. Both BDNF homozygous and heterozygous mutants show equally deficient LTP. Recognizing that experiments with KO animals may be complicated by cumulative developmental defects, both groups tested the acute effects of BDNF in rescue experiments. Korte et al. (1996) reintroduced BDNF to postnatal KO hippocampal slices using virus-mediated gene transfer, while Patterson et al. (1996) directly applied recombinant protein in the perfusion bath. Reintroduction of BDNF rescues the induction of LTP in the BDNF KO slices, demonstrating by yet another approach that BDNF has an acute role in synaptic plasticity.

In addition to its role in electrically-evoked LTP, exogenous application of BDNF also induces a novel form of synaptic potentiation. First found in developing neuromuscular synapses (Lohof et al., 1993), BDNF also potentiates synaptic transmission in the adult hippocampus (Kang and Schuman, 1995). In both systems, exogenous BDNF application results in enhanced evoked responses, at least partially through presynaptic mechanisms. This effect is specific in that application of BDNF and NT-3 results in enhanced synaptic transmission, while NGF is without effect (Lohof et al., 1993; Kang

and Schuman, 1995). In the hippocampal slice preparation, BNDF-induced potentiation is distinct from electrically-evoked LTP. Following the addition of BNDF and subsequent enhancement of synaptic responses, LTP can still be induced in these slices by tetanic stimulation. Importantly, LTP was induced by 100 Hz tetanic stimulation, which is a trkB-independent form of LTP (Kang et al., 1997). Conversely, slices in which LTP is induced still respond to BNDF with further enhancement of synaptic transmission (Kang and Schuman, 1995). BDNF-induced potentiation is also observed in several other preparations, including cultured hippocampal neurons (Levine et al., 1995), entorhinal/hippocampal slices (Scharfman, 1997), and visual cortical slices (Akaneya et al., 1997; Carmignoto et al., 1997). Moreover, exogenous BDNF appears to have the same effect *in vivo* as observed *in vitro*. Infusion of BDNF into the hippocampus of anaesthetized rats leads to enhanced evoked responses in the ipsilateral dentate gyrus (Messaoudi et al., 1998). As in hippocampal slices, this effect requires several hours to reach maximal levels, and *in vivo* the effects are observed for as long as 10 hours after a brief (25 minute) infusion of BNDF.

Studies of the mechanisms by which BDNF enhances synaptic strength have predominantly focused on intracellular calcium. Application of BDNF and NT-3, but not NGF, results in a 10-fold increase in intracellular calcium (Berninger et al., 1993). Li et al. (1998) found that BNDF acts at a presynaptic site to strengthen glutamatergic transmission in cultured hippocampal neurons, and the accompanying rise in intracellular calcium requires release from IP_3-gated stores. Not all groups have observed the effects of BDNF on synaptic enhancement, however, and several possible experimental differences have been proposed to explain the discrepancies (Schuman, 1999). In two experiments where BDNF potentiation was not observed, results indicate that BDNF decreases inhibitory transmission rather than augmenting excitatory transmission (Tanaka et al., 1997; Frerking et al., 1998), suggesting yet another mechanism by which BDNF may affect synaptic responses.

Finally, BDNF is one of the few proteins that has been examined for its role in behavioral tasks that require the hippocampus. In experiments of one-way inhibitory avoidance learning, Ma et al. (1998) found that BNDF mRNA levels are increased in the hippocampus from one to six hours following training. Prevention of this BDNF induction using antisense oligonucleotides injected directly into the dentate gyrus prior to training markedly impairs later retention performance in this avoidance task (Ma et al., 1998). In addition, injection of the antisense oligonucleotides reduces the amplitude and slope of the excitatory postsynaptic potential (EPSP) seen after induction of LTP *in vivo*. Thus, not only does BDNF play a role in evoked synaptic plasticity, but it may also be important for synaptic function during learning in the behaving animal. As with other aspects of BDNF action in synaptic plasticity, not all experiments agree on the importance of BDNF in hippocampal-dependent behavioral tasks. Fischer et al. (1994) tested several neurotrophins for the ability to reverse spatial memory impairments in aged rats. Under these conditions, BDNF is the only factor tested that *does not* improve performance in the water maze task. It is possible that BDNF does not diffuse as well as the other proteins tested, or that the role of BDNF at the synapse changes with age. In addition, there may be many differences in the synaptic activity required by each of these tasks, and these must be understood before the role of BNDF in the functioning animal can be fully elucidated.

4.2 Regulation of endogenous cytokine expression by LTP

Despite the relative wealth of information on neurotrophins in LTP, relatively little is known about the role of cytokines in this paradigm. Recent work has described the regulation of three cytokines, activin βA, IL-1β, and Neu differentiation factor (NDF) by synaptic plasticity. Our work described in Chapter 1 identifies a fourth cytokine, IL-6, which is up-regulated following LTP induction *in vivo*. Finally, the regulation of a cytokine-related protein, BMP-1, by synaptic facilitation in *Aplysia* suggests another,

indirect, mechanism by which endogenous changes in gene expression may enhance the effects of cytokines at the synapse.

4.2.1 Activin βA

Using differential and subtractive cloning techniques, Andreasson and Worley (1995) identified activin βA to be an activity-induced gene in hippocampal tissue from animals subjected to maximal electroconvulsive shock seizure (MECS). *In situ* hybridization showed intense up-regulation of activin βA in the granule cell layer of the hippocampus as early as one hour after MECS. Expression peaks between 2 and 4 hours, and returns to nearly basal levels by 24 hours. Increased mRNA levels were confirmed by Northern analysis, and the up-regulation could be blocked with the protein synthesis inhibitor cycloheximide. Importantly, chronically implanted, behaving animals, display increased βA activin mRNA in response to the more physiological stimulus used to induce LTP in the hippocampus. The temporal pattern of expression following tetanic stimulation (and subsequent LTP) is similar to that seen after MECS. Like LTP itself, blockade of the NMDA receptor with systemic MK801 also prevents the induction of activin βA. Andreasson and Worley also tested the regulation of activin βA expression by intrinsic synaptic activity. Using tetrodotoxin (TTX) injected into one eye to interrupt afferent input to the visual cortex, they found that normally high neuronal expression of activin βA in layers II/III and V/VI is significantly decreased in the contralateral cortex. This indicates that the regulation of activin βA may be important not only in experimentally induced synaptic plasticity, but also in the synaptic changes that normally mold neuronal connections *in vivo*.

An independent screen for activity-induced genes using seizures induced by kainate (KA) or pentylenetetrazol (PTZ) confirmed the finding that activin βA is up-regulated by synaptic activity (Inokuchi et al., 1996). Northern blot analysis of hippocampal RNA showed that activin βA expression is induced by KA after 3 and 6 hours of seizure, and

that cycloheximide blocks this induction. *In situ* hybridization analysis revealed that PTZ-induced seizures, like MECS, up-regulate activin βA expression mainly in the granule cell layer of the dentate gyrus. Activin βA expression is also increased in the dentate gyrus following induction of LTP in the perforant path *in vivo*. Using urethane-anesthetized animals, Inokuchi et al. found highest expression of activin βA at 3 hours after tetanic stimulation, even in experiments where LTP lasts for more than 10 hours. Again, activin βA induction is prevented by administration of MK-801. Interestingly, the related cytokine subunit, inhibin α, is not affected by synaptic activity (Inokuchi et al., 1996).

4.2.2 IL-1β

In addition to its roles in neuronal activity and calcium homeostasis (see Section 3.4), IL-1β is implicated in LTP as well. In hippocampal slices, IL-1β mRNA levels steadily increase with time after LTP induction in the Schaffer collateral pathway (Schneider et al., 1998). Increased expression is evident after 1 hour of tetanic stimulation, and reaches levels nearly 16-fold higher than control within 2-3 hours. Furthermore, in one slice displaying stable LTP for 24 hours, elevated IL-1β expression persisted for the duration of the experiment. An important point in the protocol of Schnieder et al. was incubation of the slices for more than 5 hours after dissection before doing electrophysiology. Levels of IL-1β increase rapidly after slice preparation, and a long recovery time is required for return to basal levels before induction of LTP.

Schneider et al. (1998) also showed that IL-1β expression is elevated by LTP induction *in vivo*. Eight hours after tetanic stimulation of the perforant path, IL-1β mRNA levels are increased 10-fold over basal levels only in the stimulated hippocampus; this induction is prevented by icv injection of the NMDA receptor antagonist AP-5. In addition, animals receiving the same tetanic stimulation but developing only short-term (2-3 hour) potentiation display no significant changes in IL-1β expression. Moreover, the induction of IL-1β expression by stimulation of the perforant path *in vivo* and similar up-regulation

following activation of the Schaffer collateral pathway *in vitro* indicate that IL-1β-regulation by LTP is not pathway-specific, but rather represents a more widespread mechanism, at least within the hippocampus.

Perturbation experiments using the IL-1 specific antagonist IL-1RA provided insight into the role of endogenous IL-1 in synaptic plasticity (Schneider et al., 1998). Applied to hippocampal slices 90 minutes after the induction of LTP, IL-1RA substantially decreases the amplitude of evoked responses for the duration of the experiment (3 hours after washout of the antagonist). Moreover, administration of IL-1RA *in vivo* by icv injection 90 minutes after induction of LTP significantly decreases the evoked response amplitude. Interestingly, administration of the antagonist 30 minutes before or immediately after tetanization has no effect on subsequent LTP. That the IL-1RA can reverse previously established LTP, but is without effect when administered before or coincident with the LTP-inducing stimulus suggests that IL-1 signaling plays a significant role in LTP maintenance but not in LTP induction. This conclusion contradicts previous studies in which exogenous application of IL-1β to hippocampal slices prior to (and during) tetanic stimulation inhibits the induction of LTP (Katsuki et al., 1990; Bellinger et al., 1993; Cunningham et al., 1996). One reason suggested for the disparate actions of IL-1β may be the dose used for bath application *in vitro*. The concentration used for exogenous application may be closer to the high levels of IL-1β observed *in vivo* during pathological conditions such as infection rather than the actual (exceedingly low) levels of IL-1β present in the brain under normal circumstances.

4.2.3 NDF

Neu differentiation factor (NDF), a member of the neuregulin family of cytokine factors, has recently been shown to be up-regulated by synaptic activity *in vivo*. Eilam et al. (1998) demonstrated that NDF mRNA is up-regulated in several limbic areas following kainic acid-induced seizure, as is one subunit of its receptor complex, erbB-4. Using

acutely implanted animals, this study also demonstrated the regulation of NDF expression by LTP. Levels of NDF mRNA are substantially increased in neurons of the dentate gyrus and area CA3 within one hour of perforant path stimulation, an effect that is blocked by injection of the NMDA-antagonist CPP prior to tetanic stimulation. No induction is seen in area CA1, nor are alterations in the receptor subunits erbB-3 and erbB-4 observed one hour after LTP induction. It is interesting to note, however, that NDF mRNA is elevated in both the target subfield (the dentate gyrus), and in an area of the hippocampus (CA3) in which LTP is not directly induced.

4.2.4 BMP-1

Unlike other BMP's, BMP-1 is not a member of the TGFβ superfamily. Instead, BMP-1 is a metalloproteinase that may enhance the activity of TGFβ proteins (references in Kessler et al., 1996). For this reason, the induction of BMP-1 by synaptic facilitation will be covered in this section. Using differential display (DD) RT-PCR, Liu et al. (1997) identified a gene in sensory neurons of *Aplysia* that is induced by treatment with serotonin, which leads to long-term changes in synaptic efficacy analogous to LTP. The cloned fragment is 41-45% identical to *Drosophila tolloid* and human BMP-1, and so is named *Aplysia* tolloid/BMP-1-like protein (apTBL-1). Levels of apTBL-1 mRNA increase 80% by the end of a 1.5 hour incubation with serotonin, and return to basal levels within several hours of washout. Moreover, the same apTBL-1 up-regulation can be induced by behavioral shock training that leads to long-term enhancement in the defense response and presynaptic facilitation in the pleural sensory neurons. Liu et al. propose that apTBL-1 enhances synaptic facilitation by processing immature TGFβ into the active form, which influences the responses of nearby neurons. Indeed, results from exogenous application of TGFβ support this mechanism, as presented in the following section.

4.3 Regulation of LTP by exogenous cytokine application

4.3.1 TGFβ

To test the hypothesis that apTBL-1 enhances synaptic strength by activating TGFβ at the synapse, Zhang et al. (1997) tested the response of *Aplysia* neurons to recombinant TGFβ. They found that TGFβ increases the amplitude of evoked responses in cultured ganglia tested 24 hours later. Furthermore, when tested again 24 hours after complete washout of the cytokine, responses are even greater, a pattern similar to that observed after serotonin-induced facilitation. While TGFβ is able to mimic the effects of serotonin on the long-term facilitation of these neurons, it is not able to reproduce the fast effects on neuronal excitability induced by serotonin exposure. Moreover, sequential application of serotonin and TGFβ results in facilitation no greater than observed after treatment with serotonin alone, suggesting that TGFβ acts downstream of serotonin in producing facilitation. Indeed, application of a soluble TGFβ-receptor antagonist for 24 hours during and after serotonin treatment significantly decreases serotonin-induced facilitation. In addition, Zhang et al. (1997) found that application of the TGFβ antagonist prevents facilitation induced by electrical stimulation of the afferent nerves. These findings strongly support the hypothesis that serotonin and TGFβ induce long-term facilitation through a common pathway, or that TGFβ is, in fact, required for serotonin-induced facilitation.

4.3.2 EGF

Terlau and Seifert (1989) performed some of the earliest experiments testing the effects of growth factors on hippocampal synaptic transmission and LTP. While application of low concentrations of EGF to hippocampal slices has very little effect on basal synaptic properties such as membrane potential or paired pulse facilitation (PPF), it increases the magnitude of LTP following tetanic stimulation. Abe et al. (1991) demonstrated that EGF promotes induction of LTP from short tetanic stimuli that produce only short-term potentiation (STP) in untreated slices.

Importantly, EGF induces similar effects *in vivo*. When injected icv in anaesthetized rats, EGF promotes the generation of LTP from sub-threshold (short duration, low frequency) tetanic stimulation (Ishiyama et al., 1991). As *in vitro*, infusion of EGF has no effect on basal synaptic properties. However, unlike the slice result, EGF does not further enhance LTP evoked by strong tetanic stimulation *in vivo*, indicating that synaptic connections not present *in vitro* may contribute to attenuating the effects of electrical or chemical (EGF) stimuli *in vivo*. In a related experiment, Abe et al. (1992) tested the effects of EGF infusion on LTP induction in fimbria-fornix (FF)-lesioned animals. Transection of the FF normally results in decreased LTP in the hippocampus as well as marked impairment of spatial learning and memory. Administration of EGF effectively reverses the LTP deficits resulting from FF-lesion, significantly facilitating LTP induction.

EGF may promote LTP induction by affecting intracellular calcium concentration. Using cultured hippocampal neurons loaded with fura-2, Abe and Saito (1992) found that application of EGF specifically increases calcium influx following stimulation with glutamate. Dissection of this response with subtype specific agonists revealed that EGF selectively enhances the NMDA-receptor mediated calcium influx.

4.3.3 FGF

Experiments by Terlau and Seifert (1990) also paved the way for investigation of the effects of basic fibroblast growth factor (bFGF) on hippocampal synaptic transmission and LTP. As with EGF, bath application of bFGF to hippocampal slices has no influence on basal synaptic properties, but significantly increases the magnitude of potentiated responses. Moreover, bFGF enhances LTP induction *in vivo* in a manner similar to EGF. Infusion of bFGF icv allows the induction of LTP from sub-threshold stimuli that would only produce STP in untreated animals (Ishiyama et al., 1991; Hisajima et al., 1992).

Basic FGF also increases the success of LTP induction in FF-lesioned animals (Abe et al., 1992).

Despite the functional similarities between EGF and bFGF, the mechanism by which bFGF enhances post-tetanic responses is different from that of EGF. Unlike EGF, bFGF does not alter fura-2 monitored calcium influx in cultured hippocampal neurons (Tanaka et al., 1996). Instead, bFGF decreases the amplitude of spontaneous calcium oscillations, and the GABA-antagonist bicuculine masks these effects. Thus, bFGF may enhance LTP by modulating GABAergic rather than glutamatergic transmission.

An alternative form of FGF, acidic FGF, also affects the induction and extent of LTP, both *in vitro* and *in vivo*. In hippocampal slices, aFGF reduces the amplitude of evoked responses prior to tetanus in a dose-dependent manner (Sasaki et al., 1994). Correspondingly, aFGF also enhances paired-pulse facilitation by decreasing the amplitude of the initial response. Following sub-threshold tetanic stimulation that induces only STP in control slices, application of aFGF increases both the magnitude of STP and the frequency of developing LTP. These effects are only observed when aFGF is applied 10 - 30 minutes before delivering tetanic stimulation, however. This factor has no effect on STP or LTP when administered with or after the tetanus, indicating that the effect on post-tetanic responses may be indirect.

Since food intake increases the aFGF levels in the cerebrospinal fluid a thousand-fold within 2 hours (references in Sasaki et al., 1994), this provides a natural, if non-specific, route for FGF manipulation *in vivo*. Using fasted and non-fasted animals to produce different levels of endogenous aFGF in the brain, Hisajima et al. (1992) found that icv administration of recombinant aFGF differentially enhances post-tetanic responses to sub-threshold stimulation. Infusion of aFGF promotes the generation of LTP by a 60 Hz tetanus only in fasted animals; even at the highest doses, it was without effect on non-fasted animals. Hisajima et al. (1992) proposed that the long duration reduction in aFGF

concentration in CSF may have led to an up-regulation (or increased sensitization) of aFGF receptors, thereby enhancing sensitivity to exogenous aFGF.

4.3.4 IL-1β

A series of papers have reported the effects of IL-1β on LTP in all three excitatory synapses of the hippocampal formation. In each case, exogenous application of the cytokine to slices prior to tetanic stimulation prevents the induction of LTP (Katsuki et al., 1990; Bellinger et al., 1993; Cunningham et al., 1996). At all three synapses, the effect on evoked EPSP is apparent within minutes after tetanus. Where examined, the inhibitory action of exogenous IL-1β is partially (Cunningham et al., 1996) or fully (Katsuki et al., 1990) reversed by co-application of IL-1-specific antagonists. In general, inhibition of LTP occurs without affecting basal synaptic transmission. However, when IL-1β perfusion occurs well before tetanus, a significant decline in the slope and amplitude of evoked responses is observed (Bellinger et al., 1993). Within 30 minutes of washout, the basal population spike (PS) amplitude returns to control levels, although the excitatory post synaptic potential (EPSP) slope remains low.

In an interesting twist on the standard "exogenous application" experiment, Bellinger et al. (1993) tested whether IL-1β exposure affects synaptic responses long after the original application. Given the transient effects of other growth factors and cytokines, it is striking that they found that a 8-12 minute perfusion of IL-1β significantly reduces the magnitude of LTP induced by tetanic stimulation 50 minutes later. Both the PS amplitude and the EPSP slope are substantially decreased in a dose-dependent manner, while heat-inactivated IL-1β, has no effect. Thus, exposure to IL-1β affects synaptic activity long after the factor is removed.

Exogenous application of IL-1β also reduces LTP *in vivo*. Intraventricular infusion of IL-1β 30 minutes prior to tetanic stimulation prevents the induction of LTP in the perforant path (Murray and Lynch, 1998). Animals treated with IL-1β show no

potentiation of post-tetanic responses. Pre-tetanus responses *in vivo* were also decreased by IL-1β exposure. In contrast to effects observed *in vitro*, IL-1β does not alter the basal EPSP slope *in vivo*, but significantly decreases the response amplitude. Consistent with reduced LTP magnitude observed in older (22 month) animals, endogenous IL-1β levels in the hippocampus increase with age (Murray and Lynch, 1998). Furthermore, both increased IL-1β and decreased LTP magnitude can be reproduced in rats exposed to isolation stress. In seeking a mechanism for this observation, Murray and Lynch (1998) found that both *in vitro* and *in vivo* IL-1β induces production of reactive oxygen species and consequent lipid peroxidation in the hippocampus. In addition, icv infusion of IL-1β decreases membrane arachidonic acid concentration. These changes in reactive oxygen species, lipid peroxidation and arachidonic acid are also associated with aging. Thus, it is proposed that IL-1β mediates both the membrane and glucocorticoid hypotheses of aging. It will be critical to test this notion using IL-1 RA and IL-1 KO mice.

Contrary to these studies in which IL-1β application inhibits LTP, Schneider et al. (1998) found that blocking IL-1β action reduces LTP (see Section 4). In their experiments, application of the antagonist IL-1RA following LTP induction *in vitro* and *in vivo* significantly, though reversibly, reduces post-tetanus evoked responses. In contrast, administration of IL-1RA prior to or coincident with tetanic stimulation does not affect the induction of LTP, suggesting that IL-1β does not act during LTP induction, but is required for LTP maintenance. While there are differences in reagents and experimental design between these studies, the results of Schneider et al. raise the possibility that the endogenous function of IL-1β in synaptic plasticity may differ from the effects it evokes when exogenously applied at higher doses.

Exogenous IL-1β administration can also affect performance in a spatial memory task. Intracerebroventricular injection of IL-1β 60 minutes before training in a Morris water maze task significantly impairs spatial navigation performance the following day (Oitzl et al., 1993). The role of IL-1β-induced cognitive impairment in sickness behavior

was elegantly demonstrated in a series of experiments comparing the spatial performance of mice made ill with administration of *Legionella* bacterium and mice co-administered *Legionella* and anti-IL-1β antibodies (Gibertini et al., 1995). Mice treated only with *Legionella* performed poorly in the water maze, and the IL-1β-neutralizing antibodies normalized learning, even though the illness continued. Thus, cytokine-mediated cognitive impairment may be a significant side effect of the response to sickness.

One mechanism by which IL-1β may affect LTP and behavior is by decreasing calcium influx following neuronal stimulation. Consistent with this hypothesis, Cunningham et al. (1996) found that radiolabeled calcium concentration in tissue homogenates prepared from hippocampal slices following LTP induction was altered by exposure to IL-1β prior to tetanus. The normal elevation in calcium concentration was almost completely prevented by treatment with IL-1β. Co-administration of IL-1RA did not significantly alter the IL-1β-induced calcium decrease, however. Additional work with neuronal cultures and synaptosomal preparations has provided further evidence for decreased calcium currents following chemical stimulation (Plata-Salaman and Ffrench-Mullen, 1992; Szucs et al., 1992; Murray et al., 1997; Campbell and Lynch, 1998), consistent with the notion that IL-1β may prevent LTP by a calcium-mediated mechanism. Moreover, application of IL-1β induces neuronal hyperpolarization *in vitro* (Sawada et al., 1991), and may also have effects on both GABAergic and glutamatergic transmission (Miller et al., 1991; Yu and Shinnick-Gallagher, 1994; Murray et al., 1997; Allan et al., 1998). These effects of IL-1β on neuronal properties are discussed further in Section 3.4.

4.3.5 IL-2

Several cytokines such as TNFα and IFN (D'Arcangelo et al., 1991; Tancredi et al., 1992) generally reduce or inhibit LTP evoked in their presence. IL-2 provides another example of such effects. Unlike many of the previous proteins described, the influence of IL-2 on LTP has been reported only once, and its effects have been examined only *in vitro*.

When bath applied to the hippocampal slice, IL-2 has no effect on basal synaptic transmission, but significantly reduces post-tetanic potentiation and prevents the generation of STP and LTP (Tancredi et al., 1990). The inhibitory effects on LTP are reversible, however. Following a 2-3 hour washout, all components of potentiation can be induced in slices previously exposed to IL-2. Possibly the most interesting effect of IL-2 is its ability to reverse previously established LTP. IL-2 applied to the slice after induction of LTP reduces the slope of evoked responses nearly to basal levels within 5-10 minutes. Again, the effects are reversible, and potentiation returns to normal levels within several hours of washout.

Application of IL-2 alters the conductance of several ion channels in hippocampal neurons, which may contribute to its ability to prevent and reverse LTP at the hippocampal CA1 synapses. IL-2 reduces both voltage gated calcium and sodium currents in dissociated hippocampal neurons (Zona et al., 1990; Plata-Salaman and Ffrench-Mullen, 1993). In addition, IL-2 reduces evoked neurotransmitter release in hippocampal slices. Hanisch et al. (1993) demonstrated that IL-2 has a biphasic effect on potassium chloride (KCl)-evoked acetylcholine (ACh) release. At the highest concentration tested (1 nM), IL-2 inhibits ACh release by more than 50%. The effects are observed within 20 minutes of IL-2 exposure, and last for up to an hour. At very low concentrations ($\leq$ 0.1 pM), however, exposure to IL-2 *increases* KCl-evoked ACh release from hippocampal slices. Thus, the evoked ACh release can be modified in either direction by IL-2 depending on the concentration. Such a biphasic response pattern has also been observed in the membrane potential of hippocampal neurons exposed to varying concentrations of IL-2 (Wang et al., 1994). Similar to its effects on ACh release, high concentrations of IL-2 reduce neuronal excitability in culture, causing neuronal hyperpolarization. However, at low IL-2 concentrations, half of the neurons recorded have depolarized membrane potentials, and display bursting activity.

Effects of IL-2 on other neurons have also been described. Although without effect on resting membrane potential, IL-2 reduces both ACh-induced potassium current and

GABA-induced chloride current in isolated *Aplysia* neurons (Sawada et al., 1992; Sawada et al., 1992). *In vivo*, icv infusion of IL-2 affects the firing rate of neurons in several brain nuclei (Bindoni et al., 1988), and evokes epileptiform discharges (Hanisch and Quirion, 1996). Thus, the mechanism by which IL-2 prevents induction of LTP in hippocampal slices may depend largely on what concentrations are applied and what neuronal population is examined.

4.3.6 IL-6

In order to examine the role of IL-6 in disease-related memory impairments, hippocampal slices from transgenic mice over-expressing IL-6 under the control of the GFAP promoter (GFAP-IL-6 mice) were examined for several synaptic parameters, including LTP (Bellinger et al., 1995). While evoked synaptic transmission mice (PS and EPSP amplitudes, PPF and PPI) appears normal in these transgenic mice, LTP is significantly impaired. Tetanic stimulation of the perforant path leads to potentiation >60% of baseline in all control mice, but no significant potentiation is seen in slices from the transgenic animals. Overall, the magnitude of LTP is reduced more than 50% in the GFAP-IL-6 animals, with greater deficits at later times after tetanus. Bellinger et al. (1995) suggest that IL-6 can be a contributing factor to cognitive disorders associated with infection and inflammation, which involve high levels of IL-6.

The IL-6 transgenic mice also exhibit behavioral abnormalities, and display severe pathology in the hippocampus and cerebellum (Campbell et al., 1993). The extent of architectural changes is substantial, including neuronal damage, astrocytosis and astrogliosis, and inappropriate angiogenesis. Significant neuronal and dendritic changes occur in the cerebellum and in the hippocampus, including collapsed and vacuolized processes, with an overall decreased complexity. The morphology of the cerebellum is described as being similar to "Swiss-cheese," which is attributed to the great increase in angiogenesis in the brains of the transgenic mice. It is no surprise then, given these

significant developmental defects, that later neurological tests revealed atypical electrophysiological properties (Steffensen et al., 1994), nor that a more subtle property such as LTP should be significantly impaired. Thus, these mice are perhaps not the best model in which to examine the effects of IL-6 on synaptic plasticity, and studies using wild-type mice have provided information on its effects without the complications of developmental alterations.

It is not that IL-6 is without effect on synaptic transmission in the adult hippocampus. One study of exogenously applied IL-6 demonstrated that as little as 10 minutes of exposure to IL-6 before and during tetanic stimulation reduces the magnitude of LTP (Li et al., 1997). At relatively high concentrations (500-2000 U/ml), IL-6 completely prevents all components of the post-tetanic response, including PTP, STP, and LTP. Even at these high doses, however, IL-6 has no effect on basal synaptic transmission. Unlike several other "inhibitory" cytokines, IL-6 does not influence previously established LTP. When applied for as long as 30 minutes at the highest concentrations tested, the magnitude of potentiation is unaffected. Thus IL-6 may indeed affect synaptic plasticity in the hippocampus, especially in light of its up-regulation following induction of LTP *in vivo* (Chapter 1).

4.3.7 IFN

The effects of interferon (IFN) were first studied in the Schaffer collateral-CA1 synapses of the hippocampal slice preparation (D'Arcangelo et al., 1991). Continuous treatment with varying doses of IFN reduces the magnitude of STP and prevents the induction of LTP in tetanized slices. In addition, application of IFN following tetanic stimulation results in a slowly-developing (15-20 minutes) decrease in the amplitude of potentiated responses. Thus it would seem that the effects of IFN were entirely inhibitory. However, subsequent work showed that IFN increases neuronal excitation in area CA3. In hippocampal slices, each of three isoforms of IFN excite CA3 pyramidal cells, decrease

evoked inhibitory responses, and eventually cause epileptiform bursting (Muller et al., 1993). The concentration of IFN used in these experiments was, however, more than 1000 times that used for examination of its effects in area CA1. Therefore, it is difficult to discern whether the disparate observations in these experiments are due to a biphasic response to IFN concentration or to differences in the neuronal populations tested. However, other experiments using high doses of IFN with cultured cerebellar neurons (Calvet and Gresser, 1979) and icv infusion *in vivo* (Dafny et al., 1985) also found neuronal hyperexcitability and behavioral evidence of seizures, suggesting that it is the concentration rather than the neuronal population that is responsible for the different responses observed in the hippocampal slice experiments.

Consistent with their contradictory findings, each set of experiments with IFN led to different conclusions about the mechanism by which it acts in the hippocampus. D'Archangelo et al. (1991) used cultured embryonic hippocampal neurons to dissect the currents affected by acute exposure to IFN. While the effects on calcium currents in voltage-clamped neurons are variable, in some cases increasing with IFN exposure, and in other cases decreasing calcium influx, IFN consistently attenuates the NMDA-activated current, and this effect may be responsible for its inhibition of LTP in hippocampal slices.

Believing that the effects of IFN on hippocampal cells were mediated at least in part by reactive oxygen intermediates, Muller et al. (1993) tested the ability of free radical scavengers to prevent the excitation caused by IFN in the CA3 pyramidal cells. Both catalase and superoxide dismutase preclude the IFN-evoked epileptiform bursting. In addition, application of hydrogen peroxide to the slices mimics the effects of IFN. Inhibitory responses are reduced by exposure to hydrogen peroxide, and epileptiform activity soon develops, supporting the hypothesis that the effects of IFN, at least at the high doses used in these experiments, are mediated in part by the generation of free radicals. Such results could have relevance for inflammatory conditions where IFN is generated at high levels.

4.3.8 TNF

The same group that provided the first demonstration of the inhibitory effects of IFN on LTP also contributed the earliest report of LTP inhibition by tumor necrosis factor a (TNF). Study of the duration and dose-dependency of TNF action in hippocampal slices revealed that bath-applied TNF can largely reduce PTP and STP and completely inhibit the generation of LTP following stimulation of the Schaffer collateral pathway (Tancredi et al., 1992). To effectively alter post-tetanic responses, however, long exposure time to TNF is required. Interestingly, despite its inhibitory effects on LTP, exposure to TNF enhances baseline synaptic transmission. The inhibition of LTP by bath application of TNF is also observed following perforant path stimulation, indicating that such effects are not specific to the CA1 synapses. In the dentate gyrus, even short applications of TNF (15 minutes) are able to completely abolish LTP (Cunningham et al., 1996). In this study, however, TNF had no effect on basal synaptic transmission.

The mechanism of TNF action at hippocampal synapses is largely unknown. In contrast to its inhibition of LTP in hippocampal slices, exposure to TNF increases the frequency of spontaneous miniature synaptic currents in cultured hippocampal neurons (Grassi et al., 1994). Although TNF can facilitate spontaneous transmitter release at both GABAergic and glutamatergic synapses, this study found no difference in intracellular calcium concentration following cytokine application. In contrast, other authors have reported enhanced calcium current in cultured neurons following TNF application (Soliven and Albert, 1992).

5. CYTOKINES AND SEIZURE

This section covers the endogenous regulation of cytokine expression in various animal models of epileptic seizure, and addresses corresponding work in humans where available. While little is known about the roles cytokines play in seizure activity and its

pathological aftermath, a small number of factors have been studied by exogenous application, and these will also be reviewed in this section. As with LTP, much more is known about the regulation and function of the neurotrophins than the cytokines in seizure models, and a discussion of BDNF will introduce the importance of trophic factors in seizures and their sequelae.

5.1 BDNF and seizure

The up-regulation of BDNF mRNA by seizure activity is found in a variety of animal models. Increased levels of BNDF mRNA following high levels of neuronal activity were first documented in kainic acid-induced seizure (Zafra et al., 1990). Induction of BDNF also follows epileptiform activity induced by electrolytic lesions (Isackson et al., 1991), kindling (Ernfors et al., 1991; Bengzon et al., 1993; Kokaia et al., 1994; Sato et al., 1996; Simonato et al., 1998), electroconvulsive shock seizure (ECS; Nibuya et al., 1995), as well as in several models of chemoconvulsant seizure, including pilocarpine- (Schmidt-Kastner et al., 1996), pentylenetetrazol- (Humpel et al., 1993), and tetanus toxin-induced events (Liang et al., 1998). Elevated BDNF mRNA expression is also seen in hippocampal tissue from human epileptic patients (Murray et al., 1994; Mathern et al., 1997), indicating that this aspect of the animal models is consistent with the human condition.

In general, the up-regulation of BNDF mRNA occurs quickly, rising to maximal levels as high as 40-fold over control by 3-6 hours after seizure initiation (Zafra et al., 1990; Ballarin et al., 1991; Ernfors et al., 1991; Gall et al., 1991; Isackson et al., 1991; Dugich-Djordjevic et al., 1992; Rocamora et al., 1992; Gwag and Springer, 1993; Humpel et al., 1993; Wetmore et al., 1994; Nibuya et al., 1995; Schmidt-Kastner et al., 1996; Rudge et al., 1998; Simonato et al., 1998). Both the extent and duration of BDNF induction depend on the type and severity of seizure induced. For example, in their study of unilateral focal electrolytic hilar lesion that leads to short-duration, intermittent behavioral

seizures of the limbic kindling type, Isackson et al. (1991) found that BDNF levels are dramatically elevated within hours of the lesion, but return to nearly basal values 2-3 days later. In contrast, the continuous seizures produced by intracranial administration of kainic acid last for up to 8-10 hours, and elevated levels of BDNF mRNA persist in the dentate gyrus for up to two weeks following the initial seizure event (Garcia et al., 1997).

Interestingly, in models in which the initiating insult is placed only in one hemisphere, such as unilateral hilar lesion or kindling, elevations in BNDF are commonly observed *bilaterally* (Ballarin et al., 1991; Ernfors et al., 1991; Isackson et al., 1991; Rocamora et al., 1992; Bengzon et al., 1993; Gwag and Springer, 1993; Kokaia et al., 1994). This may not be surprising, given the fast generalization of focally-initiated seizures. The temporal and spatial pattern of BDNF expression is similar in many disparate models of seizure. Increased BNDF *in situ* hybridization is first and most strongly seen in the dentate gyrus, with additional signal at early times in the pyramidal cell layers of area CA1 and CA3. Shortly afterwards, increased hybridization is found in areas outside of the hippocampus, including the neocortical mantle, piriform cortex, and amygdala. Such BDNF expression, with varying levels of cortical up-regulation but always with hippocampal elevation, has been observed following hilar lesion-seizures (Gall et al., 1991; Isackson et al., 1991; Rocamora et al., 1992), kainate-induced seizures (Zafra et al., 1990; Ballarin et al., 1991; Dugich-Djordjevic et al., 1992; Gwag and Springer, 1993), pilocarpine-induced seizures (Schmidt-Kastner et al., 1996) and kindling (Ernfors et al., 1991; Bengzon et al., 1993; Kokaia et al., 1994).

Surprisingly, the up-regulation of BNDF mRNA by kainate is not blocked by co-administration of NMDA-receptor antagonists. Zafra et al. (1990) tested the involvement of different glutamate receptor subtypes and found that while kynurenic acid (a broad spectrum glutamate receptor antagonist) and CNQX (a non-NMDA-receptor antagonist) effectively prevent the kainate-induced increase in BNDF mRNA levels, administration of MK-801 (an NMDA-specific antagonist) has no effect. Comparable findings were reported

by Wetmore et al. (1994). Recent work has begun to examine events downstream of membrane receptors. In a study of the action of various protein kinases following kainate-induced seizures, Murray et al. (1998) found that a specific inhibitor of calcium/calmodulin-dependent kinases (CaMK), KN-62, prevents the induction of BDNF mRNA. Further, this effect is specific to CaMK; inhibitors of both protein kinase C and cAMP-dependent kinase are without effect.

BNDF protein is also affected by epileptiform events. Indeed, elevated BDNF protein levels are reported in the kainate (Rudge et al., 1998), hilar lesion (Nawa et al., 1995), and kindling models of seizure (Elmer et al., 1998), as well as in tissue resected from human epileptic patients (Takahashi et al., 1999). In general, up-regulation of BNDF protein occurs somewhat more slowly and to a lesser extent than the BNDF mRNA induction in the hippocampus. For example, the same dose of kainic acid that causes a 15-fold elevation of mRNA levels within 6 hours, increases BDNF protein levels 3-fold in 24 hours (Rudge et al., 1998). In addition, elevated protein levels are observed long after mRNA levels return to basal values (Nawa et al., 1995; Rudge et al., 1998). The spatial pattern of BDNF immunoreactivity is similar but not identical to that of its mRNA, indicating that cellular transport (Nawa et al., 1995) or internalization from extracellular sources (Rudge et al., 1998) may occur after translation.

In addition to increased levels of BDNF mRNA and protein following seizure activity, several models reveal a corresponding up-regulation of trkB receptor. This induction follows a similar time course and spatial distribution that of BDNF mRNA after kindling (Bengzon et al., 1993; Merlio et al., 1993), ECS (Nibuya et al., 1995), pilocarpine- (Schmidt-Kastner et al., 1996), and pentylenetetrazol-induced seizures (Humpel et al., 1993). TrkB protein is also up-regulated (Merlio et al., 1993; Rudge et al., 1998). Within two hours of completing a 40-stimulation kindling protocol, trkB immunoreactivity is observed in the neuronal cell body layers of all hippocampal subfields (Merlio et al., 1993). Later work by Rudge et al. (1998) adds another layer of complexity

to the regulation of trkB protein. Following administration of kainic acid, they find no change in the level of full length trkB, but a significant increase in truncated trkB protein, which may be used to sequester the additional BDNF produced after seizure.

While these observations can suggest possible roles for BDNF in neuronal activity and aftermath, perturbation studies are required for further conclusions. Several groups have undertaken gain-of-function experiments based on infusion of exogenous BDNF during the seizure event. In the kindling model, a seven day infusion of BDNF into the hippocampus during the initial stimulation delays or prevents the development of the fully kindled state (Larmet et al., 1995; Osehobo et al., 1996; Reibel et al., 1996). Both after-discharge duration and seizure stage are reduced by treatment with BDNF. Most impressively, the influence of BNDF exposure lasts long after perfusion was completed; both seizure intensity and duration are diminished for up to two weeks after the end of treatment. In this context then, it is surprising that BDNF enhances cell loss in area CA3 when administered prior to and during the period of neuronal death caused by kainic acid (Rudge et al., 1998). Moreover, BNDF has no effect on kainate-induced seizure severity.

Loss-of-function experiments have been used to better address the role of endogenous BDNF in seizures and their sequelae. In mice heterozygous for the BDNF gene (homozygous mutant mice do not survive to adulthood), Kokaia et al. (1995) found that kindling development is markedly suppressed. The mutant mice require a greater number of stimulations to reach the same seizure milestones as wild-type controls, although no differences between mutants and controls are seen in seizure duration, latency, or threshold once those stages were reached. Confirming these results through a different approach, Binder et al. (1999) found that icv infusion of a trkB receptor body (the ligand-binding domain of trkB fused to the Fc region of human IgG1) reduces BDNF availability and delays the development of kindling. As in the BDNF heterozygous mice, no differences in other seizure parameters such as duration or threshold are observed. Thus, BDNF signaling is required for the development of kindling, in apparent contradiction to

the finding that exogenous BDNF prevents kindling. To resolve this paradox, Binder et al. (1999) proposed that exposure to increased concentrations of BNDF in the studies of exogenous infusion results in reduced trkB expression. It is also possible that BDNF exerts quite different actions at various concentrations. Regardless of the explanation, it is clear that both endogenous and exogenous BDNF exert critical effects on seizure development.

Based on its ability to promote axonal growth *in vitro*, BDNF was also suspected to play a role in the mossy fiber sprouting that occurs following prolonged or repeated seizures. Indeed, exogenous BDNF enhances both the number and length of axon outgrowth from granule cell explant cultures (Lowenstein and Arsenault, 1996), making a strong case for its potential to have the same effect *in vivo*. However, slice cultures from the hippocampus of mice lacking BNDF show no difference in deafferentation-induced mossy fiber (MF) sprouting, suggesting that this neurotrophin is not essential for this outgrowth (Bender et al., 1998). This conclusion is further supported by studies employing the trkB receptor body to block BDNF function. Using organotypic slices in which MF sprouting is induced by KA treatment, no difference in the extent of outgrowth is seen when trkB signaling is inhibited (Routbort et al., 1997). In fact, *in vivo*, Kokaia et al. (1995) observe *more* mossy fiber sprouting in fully kindled BDNF heterozygous mice than in wild-type controls. However, the heterozygotes require more stimulations to reach the fully kindled state, and the Timm's staining score (indicating the extent of mossy fiber sprouting into the inner molecular layer) correlates with the number of kindling stimulations. In addition, exogenous application of BNDF to wild-type animals during kindling has no effect on later mossy fiber sprouting (Osehobo et al., 1996). Thus, the evidence to date does not support a role for BNDF in hippocampal mossy fiber sprouting.

Consistent with its regulation by, and effects on seizure in animal models, elevated BDNF mRNA and protein is reported in brain tissue from human epileptic patients (Murray et al., 1994; Mathern et al., 1997; Takahashi et al., 1999). Using *in situ* hybridization,

Mathern et al. (1997) detect 2.5-fold higher levels of BDNF mRNA in epileptics than in tissue taken on autopsy from control, non-injured patients, confirming a previous report of similar results (Murray et al., 1994). Furthermore, the elevation of BNDF is positively correlated with the extent of mossy fiber sprouting observed in the epileptic tissue, and inversely correlated with the cell density in the damaged tissue. That is, more BDNF mRNA is found in tissue with greater axonal sprouting and less neuronal death. In addition, increased BDNF protein is observed in tissue resected from patients with intractable epilepsy (Takahashi et al., 1999). The relative increase in BDNF protein parallels the elevation of BDNF mRNA, both being increased 2.5 to 2.6-fold over control. The time course and duration of BDNF induction cannot easily be determined in the human tissue, however. Indeed, the up-regulation of both mRNA and protein, as well as its correlation with axonal sprouting and cell survival in the human tissue indicate that the animal models have more to tell us on the function of BNDF in seizure aftermath.

5.2 Regulation of endogenous cytokine expression following seizure activity

5.2.1 FGF

Up-regulation of bFGF following seizure activity is seen in several models of epilepsy, including ECS (Follesa et al., 1994), kindling (Simonato et al., 1998), hilar-lesion- (Gall et al., 1994), bicuculine- (Riva et al., 1992) and kainate-induced seizures (Humpel et al., 1993; Bugra et al., 1994; Riva et al., 1994; Gomez-Pinilla et al., 1995; Riva et al., 1995). Although best studied using kainate-induced activity, the temporal pattern of bFGF mRNA expression is similar in several of these models. Basic FGF expression is increased in the hippocampus within several hours of seizure onset, and reaches maximal levels first in the dentate gyrus, then in other areas of the hippocampus and cortex within 6-24 hours, at levels up to 6-fold higher than control (Riva et al., 1992; Humpel et al., 1993; Bugra et al., 1994; Follesa et al., 1994; Gall et al., 1994; Riva et al., 1994; Gomez-Pinilla et al., 1995). Where examined, bFGF expression generally returns to

near basal levels within several days (Gall et al., 1994), but elevated levels of bFGF mRNA are detected in area CA1 as late as two weeks in animals experiencing particularly prolonged seizures (Riva et al., 1994). The exception to this pattern is found with fully developed kindling-induced seizures, after which no elevation in bFGF is detected (Sato et al., 1996; Simonato et al., 1998). The protracted course of stimulation required to induce generalized seizures in this model may thus not affect bFGF expression in the same way as other models in which behavioral seizures are observed following a single initiating event.

The results of *in situ* hybridization experiments describing the location of bFGF up-regulation are generally not consistent, leaving its localization uncertain (Humpel et al., 1993; Bugra et al., 1994; Gall et al., 1994; Riva et al., 1994; Gomez-Pinilla et al., 1995). Seizure-induced mRNA elevation is found either throughout the brain, including the neocortex, hippocampus, amygdala, and septum (Humpel et al., 1993; Gall et al., 1994), or just within the hippocampus (Riva et al., 1994). Other reports are restricted to the hippocampus (Bugra et al., 1994; Gomez-Pinilla et al., 1995). Within the hippocampus, some studies describe bFGF expression strictly in neurons (Bugra et al., 1994; Riva et al., 1994), others, strictly in astrocytes (Humpel et al., 1993), and still others in both neurons and astrocytes (Gall et al., 1994; Gomez-Pinilla et al., 1995). These discrepancies may be due to differences in the seizure model employed (kainate vs. hilar lesion), the time point examined (3 hours versus 12-24 hours), the probe used for hybridization (200 to 1000 base pairs, where described), or the conditions of the hybridization protocol. Nonetheless, even when several of these variables are equalized, different findings have been reported. For example, Riva et al. (1994) and Humpel et al. (1993) both used partial cRNA radiolabeled probes to examine bFGF expression in coronal brain sections 6 hours after kainate-induced seizures. Yet one study describes increased hybridization in astrocytes throughout the brain, while the other finds bFGF up-regulation limited to the hippocampus and most superficial cortical layers, but present in both astrocytes and neurons.

Elevated levels of bFGF protein following seizure are also reported, primarily in astrocytes (Humpel et al., 1993; van der Wal et al., 1994; Gomez-Pinilla et al., 1995; Ballabriga et al., 1997). Increased bFGF protein is found in several areas of the brain that also display elevated mRNA levels, including the hippocampus, amygdala and cortex (Humpel et al., 1993; van der Wal et al., 1994). Moreover, the relative increase in bFGF protein correlates closely with the increase in mRNA, although peak protein levels are seen slightly later than peak mRNA expression (Humpel et al., 1993). Another study found that elevated levels of bFGF protein can persist for much longer than the mRNA, however. Ballabriga et al. (1997) detect bFGF-positive astrocytes as long as 30 days after kainic acid injection. Thus, relatively rapid changes in mRNA levels may have long-term effects on bFGF function.

Two additional FGFs, acidic FGF (or FGF-1) and FGF-5, are also up-regulated following kainate-induced seizures. In contrast to bFGF, FGF-1 mRNA levels in the hippocampus are unchanged for up to 16 hours following kainate-induced seizure. By 24 hours, however, FGF-1 mRNA levels jump to 5-fold over control (Bugra et al., 1994). Later time points were not examined, and it is not known if this represents the maximal up-regulation of this factor following seizure activity. Radioactive *in situ* hybridization shows elevated FGF-1 expression in the dentate gyrus, but the signal was relatively weak, and increases in other subfields may not have been detected. In addition to kainate-induced seizures, FGF-1 regulation was also examined in the kindling model, where no increase FGF-1 was found in any brain area examined over the same 24-hour time period (Sato et al., 1996; Simonato et al., 1998), suggesting that this factor may be differentially regulated by distinct types of seizure activity.

Radioactive *in situ* hybridization was used to quantify up-regulation of FGF-5 mRNA after kainate-induced seizures (Gomez-Pinilla et al., 1995). Expression is increased nearly 2-fold after 12 hours, but returns to basal levels shortly thereafter. In a

pattern quite different than that observed for other FGFs, elevated expression is restricted to the neuronal cell body layers of the hippocampus (other brain areas were not examined).

Seizure also up-regulates two high-affinity FGF receptors (FGFR). Using semi-quantitative RT-PCR, Bugra et al. (1994) demonstrated increased FGFR-1 (also known as flg) expression in the hippocampus of kainate-treated rats as early as 30 minutes after drug administration. Levels increase steadily to nearly 6-fold over control by the latest time point examined, 24 hours after seizure. *In situ* hybridization revealed that expression is localized to the neuronal cell body layers of CA1, CA3 and the dentate gyrus. Similar localization results were reported by Gomez-Pinilla et al. (1994). Immunohistochemistry demonstrates that FGFR-1 protein is also up-regulated by seizure activity, in both neurons and astrocytes (van der Wal et al., 1994; Gomez-Pinilla et al., 1995). Like FGFR-1 mRNA, protein expression is increased in the hippocampus within several hours of seizure activity, which then spreads to the cerebral cortex at later times. Immunohistochemistry has also been used to study the regulation of FGFR-3 following kainate-induced seizures (Ballabriga et al., 1997). Interestingly, FGFR-3 protein is found predominantly in OX-42-positive microglia, in addition to a small population of GFAP-positive astrocytes. Staining is seen throughout areas of the brain affected by kainate seizures, including the hippocampus, amygdala, piriform and entorhinal cortices, as late as 30 days after the initial seizure event. Thus, alterations in FGF signaling are likely to continue long after remission of behavioral seizures.

5.2.2 HB-EGF

Using systemic kainate administration, the seizure-induced regulation of heparin-binding epidermal growth factor like-growth factor (HB-EGF), a member of the EGF family, was recently demonstrated (Opanashuk et al., 1999). Northern blot analysis reveals significant elevation of HB-EGF mRNA levels in the hippocampus as early as 3 hours after kainate injection. Expression continues to rise until 24 hours, when it reaches

levels roughly 7-fold higher than control, then begins to decline by 48 hours. Using *in situ* hybridization, elevated mRNA expression is found within many areas of the brain, including the cortex, amygdala, thalamus and hypothalamus. The most dramatic increases are noted at early times (3-6 hours) in the dentate granule cell layer, and at later times (12-24 hours) in the CA1 subfield and in the lateral septal nucleus. Western blot analysis showed elevated HB-EGF protein as well (Opanashuk et al., 1999). Four species of HB-EGF are identified on blots of total hippocampal extracts, two of which are up-regulated over a time course similar to HB-EGF mRNA. Expression of the smallest isoform remains elevated for a longer time, suggesting that the effects of HB-EGF may outlast the up-regulation of its mRNA.

5.2.3 Activin βA

Several studies indicate that activin βA expression is increased quickly by neuronal activity (Andreasson and Worley, 1995; Inokuchi et al., 1996; Lai et al., 1996; Tretter et al., 1996). Indeed, elevated levels of βA mRNA are observed as early as 1 hour after seizure (Andreasson and Worley, 1995; Lai et al., 1996). The duration and decline of βA expression varies with the type of seizure studied, however. Electroconvulsive seizures that last for less than a minute result in peak βA expression 2-4 hours after stimulation, with mRNA levels returning to near-basal values by 24 hours (Andreasson and Worley, 1995). Other methods of seizure induction, such as kainate infusion or hypoxia, which cause prolonged neuronal hyperactivity, result in extended periods of βA up-regulation, with the decline to control levels delayed for several days (Lai et al., 1996; Tretter et al., 1996). We used an RNase protection assay to quantify the hippocampal expression of activin βA following pilocarpine-induced seizures. Activin βA mRNA levels are elevated roughly 7-fold over control values by 2 hours, and more than 25-fold by 4 hours after pilocarpine injection (J.L. Jankowsky and P.H. Patterson, unpublished observation).

In situ hybridization demonstrated that in all models examined, βA up-regulation occurs in the dentate granule cell layer (Andreasson and Worley, 1995; Inokuchi et al., 1996; Lai et al., 1996; Tretter et al., 1996). Additional hybridization is found over the pyramidal cell layers of CA1 and CA4 (Lai et al., 1996; Tretter et al., 1996). Following the most prolonged seizures, signal is also found outside of the hippocampus in the amygdala, piriform cortex, and thalamus (Lai et al., 1996). The localization of hybridization over primary cell body layers within the hippocampus indicates that βA up-regulation is largely neuronal. The rapid induction in the dentate granule cells suggested that βA may act as an immediate early gene; however, pretreatment with the protein synthesis inhibitor cycloheximide prevents βA mRNA up-regulation, indicating that it is instead regulated as a delayed early gene (Andreasson and Worley, 1995; Inokuchi et al., 1996).

Interestingly, despite the rapid and strong increase in activin βA expression, neither of its heterodimeric partners, activin βB nor inhibin α, are affected by seizure activity, suggesting that only the activin A homodimer is involved in activity-regulated functions (Andreasson and Worley, 1995; Inokuchi et al., 1996; Lai et al., 1996; Tretter et al., 1996). However, to date, no information is available on the protein translation of elevated βA mRNA, which will be needed to show the functional potential of activin A in seizure activity and aftermath.

5.2.4 TGFβ

Up-regulation of TGFβ1 by seizure activity occurs in the kainic acid model. Expression of TGFβ1 mRNA is elevated in the hippocampus within several hours of kainate administration, and continues to escalate to levels more than 5-fold higher than control values at 2 days (Morgan et al., 1993). Highest expression of TGFβ1 is detected in area CA3 and the hilus, regions associated with greatest neuronal loss after seizure. Possible up-regulation in other areas of the brain affected by seizure was not described.

Combined *in situ* hybridization and immunohistochemistry identified the TGFβ1 mRNA-positive cells as OX-42-positive microglia. Given their increased expression of IL-1 cytokines (Section 5.2.7), microglia may thus be poised to play a significant role in sculpting the extracellular environment following seizure activity.

5.2.5 GDNF

Elevated levels of GDNF mRNA are observed following seizure induction using either of two convulsant drugs, pilocarpine or kainic acid (Humpel et al., 1994; Schmidt-Kastner et al., 1994). GDNF expression is first detected in the dentate gyrus over the granule cell layer between 3 and 6 hours after drug administration. Where quantified, expression levels are 3-fold above background (Humpel et al., 1994). While GDNF mRNA reaches maximal levels in the dentate gyrus at 6 hours and declines by 24 hours, elevated expression extends to the hilus and later becomes prominent in CA1 and CA3. Following kainate-induced seizures, GDNF expression is restricted to the hippocampus; after pilocarpine-induced activity, however, increased GDNF mRNA is also found in the striatum and in the neocortex. Expression in each of these regions displays a distinct temporal pattern. Although cresyl violet counter-staining was done, the cellular localization of GDNF expression within the striatum and the neocortex is unclear. However, hybridization over the primary cell body layers in the hippocampus after both kainate- and pilocarpine-induced seizures clearly suggests that the majority of GDNF-positive cells in this region are neurons.

5.2.6 NDF

Elevation in NDF expression following seizure activity has recently been demonstrated. Within hours of systemic kainate injection, increases in both mRNA and protein are detected in limbic cortical areas, including the neocortex, hippocampus, hypothalamus and amygdala (Eilam et al., 1998). NDF is up-regulated several-fold in the

amygdala, from 1 to 4.5 hours after seizure, while in two other areas quantified, the hippocampus and the neocortex, NDF is up-regulated to a lesser extent, and displays biphasic peaks during this time. Immunohistochemical examination of brains from kainate-treated animals confirms NDF up-regulation at the protein level. In general, Eilam et al. (1998) describe a lag of approximately 3.5 hours between the peaks of mRNA and protein expression. Both NDF mRNA and protein are localized to large pyramidal neurons in the cortex and hippocampus; no staining is seen over areas rich in astrocytes, nor over small cells characteristic of microglia or oligodendrocytes.

In addition, up-regulated expression of one NDF receptor, erbB-4, is observed over a time course similar to that of NDF (Eilam et al., 1998). Significant increases in erbB-4 mRNA levels are seen over the neuronal cell bodies of all hippocampal subfields. In the hippocampus, cortex and thalamus, erbB-4 levels are elevated 2- to 5-fold over basal values, and in several areas, display a biphasic peaks timed in synchrony with those of NDF. Interestingly, expression of a second NDF receptor, erbB-3, is not altered by seizure activity in any brain area examined. Nonetheless, the presence of both NDF and one of its receptors in the same cells over the same time course indicates the potential for this factor to act in these areas during and after seizure induction.

5.2.7 IL-1

Although the regulation of IL-1 in many forms of injury causing neuronal damage or death is well established (Rothwell and Relton, 1993; Schobitz et al., 1993; Rothwell, 1998), its regulation by seizure activity (which in turn can lead to neuronal degeneration) has received less attention. Following kainic acid- or PTZ-induced seizure, IL-1β mRNA expression is up-regulated quickly, reaching peak levels by 1.5 hours after injection (Minami et al., 1990; Minami et al., 1991). Like many other cytokines and growth factors, maximal IL-1β expression is not simultaneous in all areas of the brain. One hour after kainic acid injection, increased expression is detected only in the hippocampus, whereas

maximal levels of IL-1β in the cortex, thalamus and hypothalamus occur at successively later times. Expression in all brain areas except the hippocampus returns to basal levels by 24 hours, and within 72 hours, IL-1β mRNA is no longer detected in any region. This time course is accelerated following PTZ-induced seizures, which result in faster-onset, shorter-duration behavioral convulsions than kainate. Expression of IL-1β is detected as soon as 30 minutes after injection, and decays to control levels within 3 hours.

Kainate-induced seizure up-regulates IL-1β primarily in glial cells (Yabuuchi et al., 1993). However, the IL-1β-positive cells do not co-stain for GFAP, indicating that non-astrocyte cells, possibly microglia, are responsible for increased IL-1β expression. Additional support for the microglial identification comes from studies of IL-1β expression following localized excitotoxic neuronal death. The early phase of IL-1β protein up-regulation in and around the lesion area occurs exclusively in microglia, in which elevated protein is detected as early as 60 minutes after drug injection (Pearson et al., 1999). It is worth noting that occasional IL-1β-positive neurons and vascular endothelial cells are also present following seizure, however.

Elevated expression of the other IL-1 isoform, IL-1α, is also reported in animal models of epilepsy. Using the genetically-susceptible Frings mouse strain, brief generalized seizures can be induced non-invasively by exposure to a 20 second, 110 dB sound. Analysis at various times after seizure revealed that IL-1α mRNA levels are increased within two hours of seizure, reach maximal levels several-fold over control at 6 hours, and return to basal levels by 24 hours (Gahring et al., 1997). In contrast to many other seizure models, elevated mRNA expression is *not* observed in the hippocampus. Instead, the only brain area in which IL-1α levels are affected is the hypothalamus. Both the time course and the unique localization of IL-1α expression were confirmed using a second strain of mice susceptible to sound-induced seizure, DBA/2J. Interestingly, as had been described earlier for IL-1β expression following kainic acid-induced seizures, up-

regulation of IL-1α mRNA after audiogenic seizures is inhibited by pretreatment with dexamethasone (Minami et al., 1990; Gahring et al., 1997).

Altered IL-1α expression is also found in tissue from human epileptic patients. Analysis of IL-1α immunoreactivity in surgically resected temporal lobe tissue reveals three times more IL-1α-positive cells than in tissue from control, unaffected patients (Sheng et al., 1994). The immunopositive cells have morphological characteristics of activated microglia, and they are not only greater in number, but also larger in size, more intensely stained, and display more prominent, ramified processes than do the control samples. These results provide an important link between laboratory studies of animal models and the human condition, and support the temporal and spatial correlation between cytokine alterations and epileptic neuropathology.

In addition to the up-regulation of both IL-1 agonists, expression of the endogenous IL-1RA is also affected by seizure activity. Using *in situ* hybridization to assess mRNA alterations after kainic acid injection, Eriksson et al. (1998) described elevated IL-1RA expression in the hippocampus, thalamus, amygdala, and piriform, perirhinal and entorhinal cortex. Expression is first detected at 5 hours, increases at 12 hours, and reaches maximal levels at 24 hours after seizure. Four days later, expression has returned to basal levels, and remains low thereafter. Counterstaining with Cresyl violet revealed that the majority of IL-1RA-positive cells have the morphology of microglia. Indeed, immunostaining of consecutive sections with ED1 displays a distribution of microglial cells very similar to the pattern of IL-1RA hybridization. In addition, IL-1RA-positive neurons are also found in certain cortical areas. However, the up-regulation of all three IL-1 ligands in microglia strongly supports a role for these cells in IL-1 related seizure sequelae.

There is also evidence for the up-regulation of the IL-1 receptor following kainate-induced seizures. Increased expression of the type 2 IL-1 receptor (IL-1R2) mRNA is first observed in the dentate gyrus and the basolateral amygdala 8 hours after the injection of

kainic acid (Nishiyori et al., 1997). By 12 to 24 hours, IL-1R2 hybridization is stronger in these two areas, and extends to other subfields of the hippocampus, as well as the cortex and hypothalamus. The majority of IL-1R2-positive cells are likely to be neurons as counterstaining with Cresyl violet localizes most of the mRNA in large, lightly stained cells resembling neuronal cell bodies. Expression in all areas declines to nearly basal levels by 48 hours. It has been suggested that the IL-1R2 does not act to transduce IL-1 signal, but instead, may act as a decoy receptor (Colotta et al., 1993). In this capacity, the IL-1R2 may absorb excess IL-1 signal, and modulate the functional consequences of concurrent IL-1 up-regulation in the brain following neuronal hyperactivity.

5.2.8 TNF

The regulation of TNFα mRNA expression was among the first described effects of neuronal activity on cytokine transcription in the adult CNS (Minami et al., 1991). At early times after kainate injection TNFα expression is strongly up-regulated in the cerebral cortex, hippocampus, striatum, thalamus and hypothalamus. Subsequent experiments demonstrated enhanced TNFα-like activity in the hippocampus following kainate-induced seizure. Using hippocampal slices prepared from rats treated with intra-amygdala injections of kainic acid, de Bock et al. (1996) found elevated cytotoxic activity in slice supernatants at both 2 and 7 days after seizure. Despite unilateral injection of kainate, tissue taken from both hemispheres demonstrates enhanced cytotoxic activity, suggesting that generalized seizure activity, and not local tissue damage, is responsible for the up-regulation. The cytotoxic activity was attributed to secreted TNFα; however, no blocking experiments were done to verify the action was specific to this factor.

In their characterization of TNF receptor KO mice, Bruce et al. (1996) demonstrate the cellular localization of TNFα protein in the hippocampus of both WT and mutant mice following kainate induced seizures. Four hours after unilateral injection of the convulsant, elevated immunostaining for TNFα is particularly apparent in neuronal cell body layers,

especially over areas CA1 and CA3. Immunoreactivity is noticeably greater in the injected hippocampus than in the contralateral area, suggesting that direct excitotoxicity, as well as seizure activity, may have contributed to the ipsilateral TNFα induction.

5.2.9 IL-6

Minami et al. (1991) provided the first evidence of IL-6 regulation following experimentally-induced neuronal activity. Following systemic kainate administration, they found weak IL-6 mRNA elevation in the hippocampus at 2 hours, which is markedly increased at 4 hours, along with expression in the cortex, thalamus, and hypothalamus. This initial study was performed using RT-PCR, and so no information was obtained on the relative increase nor cellular localization of mRNA upregulation. Subsequent work used a separate semi-quantitative RT-PCR assay to determine that hippocampal IL-6 expression following pilocarpine-induced seizures is elevated 6-fold over control values at 2 hours, and nearly 10-fold over control by 4 hours after drug administration (J.L. Jankowsky and P.H. Patterson, unpublished observations).

In addition, two studies have suggested that IL-6 protein is elevated after seizures, both in animal models and in human epileptics. Using hippocampal slices prepared from animals previously subjected to kainate-induced seizures, de Bock et al. (1996) find elevated IL-6 bioactivity two days after the seizure event. However, enhanced IL-6 release is only observed in the hemisphere ipsilateral to the kainate infusion, and may therefore not represent an effect of generalized bilateral seizure activity. Also, since no specific inhibitor of IL-6 was used, we cannot be sure that this activity did not represent another member of the neuropoietic cytokine family. Peltola et al. (1998) report elevated concentrations of IL-6 protein in the cerebrospinal fluid of 4 out of 15 patients with newly developed tonic-clonic seizures. Spinal fluid IL-6 concentrations in these patients range from 3 to 20-fold higher than unaffected, control levels. Interestingly, all four patients with elevated IL-6 levels were analyzed within 15 hours of seizure occurrence; other patients, in which

elevated IL-6 levels were not observed, were assessed as late as 72 hours after seizure. One interpretation of these findings is that IL-6 was consistently up-regulated by seizure activity, but in the majority of cases, decayed before lumbar puncture could be performed.

5.2.10 CNTF

Several studies have used ciliary neuron survival assays to evaluate the up-regulation of CNTF-like factors in the rodent hippocampus following kainate-induced seizures. Hippocampal homogenates were added to cultures of embryonic chick ciliary ganglia in which neuronal survival is supported by CNTF but not by unrelated neurotrophic factors, such as NGF. Cell survival is significantly improved in the presence of hippocampal extracts taken several days to 2 weeks after seizure (Nieto-Sampedro et al., 1983). Moreover, neurotrophic activity increases with time after seizure. Similar results were reported by two other groups using similar assays for CNTF activity (Heacock et al., 1986; Lowenstein et al., 1993). In none of these studies, however, were inhibitors used to show the effect is due to CNTF itself rather than a related factor with similar biological activity.

Elevated expression of CNTF mRNA following seizure activity is described for the first time in Chapter 2. We find that after a prolonged, pilocarpine-induced seizure, the CNTF response is quite delayed compared to other cytokine factors. Expression of CNTF mRNA in the hippocampus is not significantly different from saline-injected controls until 1 day after seizure, but remains elevated several-fold for the duration of the experiment. Thus, increased CNTF mRNA correlates well with elevated ciliary neuronal survival activity present in the hippocampus at later times after seizure.

5.2.11 LIF

Until recently, the only demonstration of LIF regulation by neuronal activity came from a single RT-PCR study of several factors following systemic kainate injection

(Minami et al., 1991). It was reported that LIF mRNA is expressed in the cortex and hippocampus under basal conditions, and is slightly up-regulated at early times after kainic acid. A marked, but transient elevation in LIF expression is seen in the hypothalamus after 2 hours. Work described herein (Chapter 2) provides data on the quantification of LIF induction and its cellular localization. We find that LIF mRNA is up-regulated nearly 30-fold in the hippocampus following a four-hour pilocarpine-induced seizure and is localized to GFAP-positive astrocytes within the brain parenchyma. In addition, there is a significant population of GFAP-negative cells residing outside of the blood-brain barrier that express LIF mRNA following seizure.

5.2.12 OSM and CT-1

Work presented herein provides the first description of the regulation of OSM and CT-1 following seizure activity (Chapter 2). OSM expression in the hippocampus is up-regulated in a biphasic manner by pilocarpine-induced seizures. *In situ* hybridization analysis reveals OSM-positive cells throughout the brain, and some of these can be co-labeled for pan-neuronal markers. The localization of these cells within the hippocampus indicates that they are likely to be interneurons.

CT-1 is affected much less dramatically than the other neuropoietic cytokines examined in the pilocarpine model of seizure. Its levels are elevated 40-50% over basal values, with a delayed time course in which significant differences from control are only noted several days after seizure.

5.3 Effects of exogenous cytokines or their inhibitors on seizure activity and aftermath

5.3.1 FGF

Since bFGF can be neuroprotective *in vitro* and after injury *in vivo*, the ability of bFGF to protect hippocampal neurons from seizure-induced death was tested using long-term (7 day), low-dose (2.5 ng/hr) infusion of the factor before and after kainic acid-

induced seizures (Liu et al., 1993). While bFGF has no effect on seizure latency or duration at this dose, it can prevent hippocampal cell loss. In addition, icv infusion of low-dose bFGF for 7 days before and after seizure, alters long-term behavioral performance in spatial learning, handling and open field tests (Liu and Holmes, 1997). In all assays, animals treated with 2.5 ng/hr bFGF perform better than animals treated with vehicle or a lower dose (0.5 ng/hr) of bFGF during kainate-induced seizures. These same animals were next tested for alterations in inhalant-induced seizure threshold caused by a prior kainate exposure. Again, animals treated with 2.5 ng/ml bFGF were more resistant to convulsants than animals receiving vehicle- or low-dose infusions. Finally, fewer animals infused with bFGF display hippocampal lesions characteristic of seizure damage. Thus, chronic elevation of bFGF levels in and around the time of seizure significantly improves long-term behavioral recovery, and reduces hippocampal damage.

In stark contrast to its neuroprotective effects following chronic low-dose infusion, acute bolus injection of bFGF alone *induces* seizures in otherwise untreated rats (Liu and Homes, 1997). At 25 or 50 ng, bFGF injection into the hippocampus results in immediate EEG ictal discharge and behavioral seizure in a significant fraction of animals. The effects are dose-dependent in that both the percentage of animals and the duration of seizures induced are both increased at the higher dose. These seemingly contradictory findings may result from concentration-dependent differences in the effects of bFGF. Indeed, these authors describe a preliminary study to find the appropriate dose for chronic infusion in which administration of concentrations >5 μg/ml resulted in behavioral twitching and electrographic discharges (Liu et al., 1993). In addition, acutely administered bFGF was injected directly into an epileptogenic site within the hippocampus which may have further facilitated the generation of seizure activity.

The potential of bFGF to affect other aspects of seizure pathology has been investigated *in vitro*. Using microdissected regions of the dentate gyrus and CA3 cultured in a collagen matrix, Lowenstein and Arsenault (1996) found that exogenous application of

bFGF significantly enhances both the number and length of neurites extending from dentate granule cells. Interestingly, bFGF also leads to the migration of neuron-like cells out of the explant, an effect not seen in the presence of other neurite-enhancing proteins such as BDNF. Because both axonal sprouting and neuronal migration are common consequences of prolonged or repeated seizures (Houser, 1992; Bengzon et al., 1997; Parent et al., 1997; Parent et al., 1998; Scott et al., 1998), the potential of bFGF to affect both of these processes could have substantial consequences on hippocampal reorganization. Neither of these effects of bFGF action have as yet been investigated *in vivo*, however.

Exogenous administration of another FGF isoform, aFGF, has substantial effects on both seizure activity and associated hippocampal damage. Intravenous injection of microgram levels of aFGF 10 minutes before and again 10 minutes after administration of kainic acid significantly attenuates neuronal degeneration in the hippocampus evaluated 24 hours later (Cuevas et al., 1994). Pathological alterations are limited to CA3, whereas control animals display degenerative changes throughout CA1, CA3, CA4, and the subiculum. A single intraperitoneal injection of the same amount of aFGF significantly decreases the intensity, duration and mortality of kainate acid-induced seizures (Cuevas and Gimenez-Gallego, 1996). Indeed, it is possible that the neuroprotective effect noted in the previous study reflects decreased seizure severity in aFGF-treated animals, rather than, or in addition to, a direct trophic effect.

5.3.2 IL-1

Inhibition of IL-1 action at the time of seizure initiation significantly protects hippocampal neurons from subsequent kainate-induced death. Injection of recombinant IL-1RA icv 10 minutes before and again 10 minutes after systemic kainate injection protects neurons in all subfields of the hippocampus from damage in a dose-dependent manner. This occurs in the absence of an effect of IL-1RA on seizure severity or duration, or on physiological functions such as blood pressure and body temperature (Panegyres and

Hughes, 1998). Surprisingly, the lowest dose of IL-1RA tested, 10 μg, provides the greatest amount of neuroprotection; injection of 40 μg allows as much damage as in the untreated controls. In addition, or possibly consequent to, reducing neuronal loss, IL-1RA treatment also decreases GFAP mRNA up-regulation following seizure. These findings support a role for endogenous IL-1 in seizure-associated neuronal death, an effect that does not result from attenuation of neuronal activity.

At higher concentrations found in pathological conditions, however, IL-1β *enhances* susceptibility to seizure activity. Yuhas et al. (1999) found that *Shigella*-infected mice are more sensitive to the convulsant drug pentylenetetrazol. Preinjection of antibodies against IL-1β 30 minutes prior to administration of *Shigella* prevents the infection-induced seizure susceptibility, but does not protect the mice from sickness and death. In another study of the effects of high IL-1β levels, Nistico and DeSarro (1991) observed spiking activity after icv or intrahippocampal IL-1 injection *without* the need for chemoconvulsant challenge beginning within minutes of exposure. Seizure response to IL-1β treatment has also been reported in human patients receiving cytokine therapy for carcinoma (Redman et al., 1994). Thus, elevated concentrations of IL-1β during infection or illness may contribute to neurological dysfunction.

5.3.3 TNF

In the same study that established a role for IL-1β in *Shigella*-induced seizure sensitivity, Yuhas et al. (1999) also examined the contribution of endogenous TNFα to seizure development. Preinjection with anti-TNFα antibodies prevents enhanced seizure susceptibility caused by *Shigella* infection. Mice treated with anti-TNFα antibodies do, however, have slightly shorter survival times, and greater mortality than infected controls, likely the result of inhibiting TNF function in the immune response.

Prevention of TNF function by eliminating either or both TNF-receptors in KO mice has also been used to examine the role of TNF in seizure-associated neuropathology

(Bruce et al., 1996; Gary et al., 1998). Unilateral hippocampal kainate injection results in seizures in both WT and mutant mice, with no differences in severity or duration. However, damage to hippocampal neurons is exacerbated in double TNFR KO mice. While only minimal neuronal death is observed following low-dose kainate injection in WT animals, 30-50% neuronal loss is seen in CA1, CA3 and the hilus of the mutant mice (Bruce et al., 1996). This pattern of damage is also present in the single p55 receptor KOs, but not in mice lacking only p75, suggesting that p55 receptor function is neuroprotective in WT mice (Gary et al., 1998). Conversely, the microglial response to seizure damage in the double KOs is suppressed, suggesting that neuronal damage in this model is not mediated by microglial factors, and that microglial activation requires TNF signaling.

Mice in which TNFα is over-expressed in neurons display substantial neurological disease. At 3-8 weeks of age, TNFα-transgenic mice develop chronic inflammatory demyelinating disease that is manifested by ataxia, seizures, paresis and early death (Probert et al., 1995). All of these symptoms can be completely prevented by peripheral administration of anti-TNFα antibody once a week from the time of birth, demonstrating that they are the direct consequence of TNF over-expression. The severe neuropathology in these mice prevents direct correlation to most forms of human epilepsy, however, and also complicates the interpretation of the effects of exogenous TNF on seizure activity and pathology. Overall, findings to date indicate that elevated levels of TNF enhance seizure susceptibility and consequent pathology, and endogenous TNF protects neurons from damage caused by drug-induced seizures.

5.3.4 IL-6

The neurological consequences of chronic IL-6 over-expression has also been investigated using transgenic mice (Campbell et al., 1993). Animals in which the IL-6 expression is driven by the GFAP promoter develop severe neurobehavioral deficits, characterized by tremor, ataxia, and seizure. Histological examination reveals significant

neuropathology in the hippocampus and cerebellum, with changes in connectivity and cytoarchitecture, as well as reactive astrocytosis and inappropriate neovascularization. Not surprisingly, electroencephalographic recordings from these mice are characterized by abnormalities, including hippocampal paroxysmal discharge and suppressed theta rhythm (Steffensen et al., 1994). Evoked responses are also disturbed, including increased recurrent inhibition in the dentate gyrus (despite the loss of 90% of parvalbumin-immunoreactive interneurons in the hilus), and loss of paired pulse disinhibition in the cholinergic septohippocampal projection. Acute versus developmental effects of elevated IL-6 on seizure are difficult to sort out at this point, and will await temporally-defined inhibition experiments in these mice.

5.3.5 GDNF

As described previously, GDNF expression is increased after kainic acid-induced seizure. To examine the effect of exogenous GDNF, the cytokine was infused icv 1 hour before systemic injection of kainate. This results in a significant attenuation of both seizure intensity and subsequent hippocampal cell loss (Martin et al., 1995). Moreover, improved neuronal survival in the hippocampus, thalamus and amygdala parallels the decreased seizure intensity. Although GDNF is injected unilaterally, abrogated cell loss is evident bilaterally, suggesting a possible indirect effect through suppression of neuronal activity rather than a direct action on neuronal survival. This possibility, as well as the role of endogenous GDNF on neuroprotection and seizure susceptibility will be important research topics in the future.

5.3.6 IL-2

The effects of exogenous IL-2 on both drug- and sound-induced seizures were assessed in DBA/2 mice, a strain genetically susceptible to audiogenic seizures (De Sarro et al., 1994). Intracerebroventricular injection of IL-2 facilitates the induction of seizures in

all models tested, causing increased incidence of seizures following drug or audiogenic challenge, and promoting sound-induced seizures at subthreshold volumes. Furthermore, treatment with IL-2 decreases the latency to epileptiform electrocortical discharges, and increases the duration of seizure activity. When tested in Wistar rats, infusion of high doses of IL-2 alone induce electrographic epileptiform discharges without the need for chemoconvulsant stimulation (Nistico and DeSarro, 1991). Onset of abnormal activity occurs within one minute of injection, and recurs periodically for up to 3 hours. While these results clearly demonstrate that high doses of exogenous IL-2 can promote seizure generation, the role of endogenous IL-2 is much less clear. Intracerebroventricular infusion of anti-IL-2 or anti-IL-2 receptor antibodies prior to sound-induced seizures has no effect on seizure intensity or occurrence (De Sarro et al., 1994). Antibody penetration is, of course, a possible issue in this negative result.

5.3.7 IL-3

There is one published report of the effects of IL-3 on neuronal activity. In this study, icv infusion produces characteristic spike-wave epileptiform activity within five minutes (Nistico and DeSarro, 1991). Behavioral manifestations appear as frozen immobility or, more rarely, wet dog shakes. The epileptic discharges last for 15 to 35 minutes, subsiding into behavioral sedation.

5.3.8 IFN

Because IFN is used in the treatment of several human diseases, it is one of the few cytokines for which we have a significant medical literature regarding its association with seizure. Various reports document the occurrence of seizures in patients receiving IFNa treatment for conditions such as viral hepatitis (Janssen et al., 1990; Miller et al., 1994; Shakil et al., 1996; Woynarowski and Socha, 1997), hairy cell leukemia (Dierckx et al., 1985) and multiple myeloma (Brouwers et al., 1999). Seizure types varied from partial,

photosensitive facial jerking (Brouwers et al., 1999) to generalized (Janssen et al., 1990) and grand mal (Shakil et al., 1996) as well as status epilepticus (Dierckx et al., 1985; Miller et al., 1994). In each case described, seizures remit after discontinuation of IFN. Several other medical reports of IFN therapy have listed seizures among its side effects (Morris, 1994; Fattovich et al., 1996; Iorio et al., 1997; O'Duffy et al., 1998; Perry and Wagstaff, 1998), with varying prevalence, from less than 1 in 1000 (Fattovich et al., 1996) to as high at 1.3 in 100 (Shakil et al., 1996).

Conversely, a handful of reports have described the use of IFN to stop seizures in patients with primary immune diseases. Intractable seizures associated with conditions as diverse as cytomegalovirus infection, hairy cell leukemia and Rasmussen's encephalitis have been successfully abrogated with IFN treatment (Peddinani and Savery, 1985; Kohyama et al., 1993; Dabbagh et al., 1997). Thus, the net effect of IFN on seizure occurrence may depend in part on the pre-existing condition for which it is being prescribed.

At very high concentrations, IFN can induce epileptiform bursting in chronic cerebral and cerebellar cultures (Calvet and Gresser, 1979), and in acute hippocampal slices (Muller et al., 1993). Bursting activity can be induced within 2 minutes and last for several hours (Muller et al., 1993). IFN-induced activity may result from reduction of inhibitory transmission as evoked IPSP amplitude is generally decreased prior to onset of bursting activity. In addition, Muller et al. (1993) provide evidence that the effects of IFN may be mediated in part by reactive oxygen intermediates. IFN-induced bursting is inhibited by co-application of free radical scavengers, and its effects on both evoked IPSP amplitude and epileptiform activity are mimicked by direct application of hydrogen peroxide.

5.3.9 LIF

Work described herein presents the first evidence for the requirement of cytokine signaling in the initiation of reactive astrogliosis in the hippocampus following seizure.

Using LIF KO mice, we find that the induction of GFAP mRNA is substantially attenuated in the absence of this factor (Chapter 3).

5.4 Relevance of cytokine function during and after seizure activity

The pathological alterations that occur in the hippocampus following prolonged or repeated seizures begin within hours and cause changes that last throughout life. Although a vast oversimplification, five separate, but overlapping and interrelated events define hippocampal pathology: seizure itself, neuronal death, neuronal birth, reactive gliosis and mossy fiber sprouting. When delimited in this manner, cytokines can be said to affect all aspects of seizure pathology.

A recurring theme in this discussion has been the ability of cytokines to alter neuronal physiology. Several factors affect basal synaptic transmission *in vitro* (D'Arcangelo et al., 1991; Tancredi et al., 1992; Bellinger et al., 1993), and the induction of LTP both *in vitro* and *in vivo* (see references in Section 4.3). Thus these proteins can significantly alter "physiological" neuronal properties. Moreover, in this context, almost a dozen factors influence seizure activity (see Section 5.3), and thus can also significantly modulate neuronal behavior under pathophysiological conditions. The majority of these proteins, including bFGF, IL-2 and TNFα, increase the propensity for seizure initiation, and some exacerbate behavioral and electrographic manifestations. Two factors, aFGF and GDNF, however, act in the opposite manner, and the presence of either prior to convulsant administration *decreases* the intensity and duration of seizure activity (Section 5.3). Thus the overall effect of cytokines on seizure outcome depends largely on the collection and concentration of factors present in various brain areas during the period of neuronal hyperactivity.

One of the earliest signs of pathology in the hippocampus after prolonged seizure activity is neuronal loss in area CA3 and the hilus, known as hippocampal sclerosis (Schwob et al., 1980; Ben-Ari, 1985; McNamara, 1994; Represa et al., 1995; Lynch et al.,

1996). In various animal models, cell death begins within hours of seizure, and continues for several days. Many cytokines and growth factors are up-regulated over this time period (Section 5.2), and most can affect neuronal survival *in vitro* (Sections 2 and 3.1). Evidence to support such a role is, however, available for only a handful of factors. Exogenous GDNF and aFGF reduce hippocampal cell loss by attenuating the severity and duration of the initial seizure event (Section 5.3). Several other factors, TNFα, IL-1 and bFGF, improve neuronal survival *without* affecting any parameters of the seizure itself (Section 5.3), and thus may act in a more characteristically trophic or protective fashion. Taken together, these findings support a role for cytokines and growth factors in the pattern of neuronal loss and survival that follows seizure activity.

Following the period of rapid neuronal loss induced by seizure, ongoing neurogenesis in the hippocampus is increased (Bengzon et al., 1997; Parent et al., 1997; Parent et al., 1998; Scott et al., 1998). Following pilocarpine-induced seizure, the only model in which the time course of neurogenesis has been examined, increased BrdU labeling is detected in the subgranular zone of the dentate gyrus from 3 to 13 days after seizure (Parent et al., 1997). This period corresponds to the up-regulation of several mitogenic factors (Section 5.2). For example, bFGF, one of the most potent proliferative agents for neuronal progenitors (Sensenbrenner, 1993; Temple and Alvarez-Buylla, 1999)), is up-regulated in and around the proliferative zone of the hippocampus (Section 5.2). Cytokines and trophic factors may also affect progenitors by regulating their differentiation. In the case of bFGF, some progenitors respond to bFGF by extending neurites, and developing a neuronal morphology (Basilico and Moscatelli, 1992). While many other cytokines and trophic factors have potent effects on neuronal progenitors in the hippocampus, this example raises the issue that such factors can both enhance and attenuate neuronal proliferation, and that individual factors can have both effects when examined under different conditions *in vitro*. Obviously, much more work is needed in this area to

establish the contribution of cytokine factors *in vivo* to this interesting new facet of seizure biology.

Nearly coincident with the period of accelerated neurogenesis is the glial reaction to seizure. Beginning about 3 days after epileptic activity, and reaching maximal levels shortly thereafter, the astrocytic and microglial response is a major component of hippocampal sclerosis seen in both seizure models and human patients (McNamara, 1994; Niquet et al., 1994; Beach et al., 1995; Khurgel et al., 1995; Represa et al., 1995; Khurgel and Ivy, 1996; Lynch et al., 1996; Mathern et al., 1996). The astrocytic response includes altered morphology, gene expression, and proliferation. Cytokine action can significantly contribute to this reactive process (Benveniste, 1992; Merrill and Benveniste, 1996). In fact, many aspects of astrogliosis can be induced in healthy animals simply by the injection of cytokines such as CNTF (Levinson et al., 1996) and IL-1 (Giulian et al. 1994). Moreover, results presented in Chapter 3 demonstrate a requirement for endogenous LIF in GFAP up-regulation following seizure, a key sign of astrocyte activation. Although the downstream effects of astrocyte activation are not clearly known, there are several ways in which this response may influence both short- and long-term recovery from seizures. At least a subpopulation of astrocytes is believed to prevent the passage of regenerating neurites by expression of inhibitory surface molecules (Hatten et al., 1991; Eddleston and Mucke, 1993; Fawcett, 1997; Ridet et al., 1997; Stichel and Muller, 1998). This aspect of the astrocytic reaction may contribute to the aberrant axonal growth seen in the dentate gyrus shortly after the climax of the glial response. Astrocytes in CA3 (the normal field of granule cell innervation) may present a non-permissive environment, leaving mossy fiber axons to sprout into an aberrant, but possibly more permissive, surrounding in the dentate gyrus (Represa et al., 1995). Indeed, Represa et al. have noted differences in the surface antigen profile and the rate of proliferation of astrocytes in these two areas, highlighting the fact that the glial reaction is not homogeneous.

Under normal conditions, astrocytes provide a major mechanism by which glutamate released from neurons is degraded and recycled (Benveniste, 1992; Landis, 1994). Alterations in this function could also substantially contribute seizure-associated pathology. Decreased uptake would leave higher glutamate concentrations in the extracellular space potentially leading to increased neuronal excitation and enhanced excitotoxic neuronal death. Alternatively, more efficient glutamate clearance may indirectly lead to enhanced neuronal activity via compensatory mechanisms to maintain pre-seizure levels of neurotransmitter in the synaptic clefts. More work needs to be done in this area to properly understand the potential importance of astrocytic changes, and thereby the contribution of cytokines, in the development of epilepsy.

Finally, reactive astrocytes themselves provide a rich source of cytokines after injury or insult (Benveniste, 1992; Eddleston and Mucke, 1993; Merrill and Benveniste, 1996; Ridet et al., 1997). Thus, cytokine activation of astrocytes can in turn expand the network of factors produced after seizure. Many of the factors up-regulated by astrocytes, including CNTF, IL-6, TGFβ, LIF and IL-1 (Eddleston and Mucke, 1993; Ridet et al., 1997), can affect astrocyte proliferation, migration, morphology and adhesion (Merrill and Benveniste, 1996). Furthermore, as discussed throughout this review, most of these cytokines can also influence the physiology of nearby neurons (Sections 2, 3, 4.3 and 5.3).

Microglia also respond to seizure by changing morphology, gene expression and proliferative state (Niquet et al., 1994; Represa et al., 1995). Unlike astrocytes, which after activation produce factors that promote neuronal survival, the net effect of microglial-secreted factors, at least under certain circumstances *in vitro*, has been *neurotoxic*. (Giulian, 1993; Chao et al., 1995). Cytokines can influence the activation of microglia and the profile of their secreted products (Gehrmann et al., 1995; Gebicke-Haerter et al., 1996; Spranger and Fontana, 1996). Indeed, in the absence of TNF signaling *in vivo*, the microglial response to seizure activity is substantially abrogated (Bruce et al., 1996). Thus

cytokine stimulation of these two cell types may set up a rivalry whose outcome determines the fate of nearby neurons (Giulian, 1993). It must be said, however, that much remains to be done towards understanding the role of the microglial response in seizure pathology.

One of the later changes to follow seizure is the aberrant sprouting of dentate granule cell processes back into their own dendritic field (Lynch et al., 1996; Parent and Lowenstein, 1997). Known as mossy fiber sprouting, this arborization peaks between 2 and 15 weeks after seizure (Mello et al., 1993; Okazaki et al., 1995; Vaidya et al., 1999). Due to their effects on neuronal morphology and neurite extension, cytokines and growth factors are obvious candidates for regulation of this aspect of seizure-induced remodeling in the hippocampus (Sections 2 and 3.2). Indeed, several of these proteins promote axonal sprouting from dentate granule cells *in vitro*, including BDNF and bFGF (Lowenstein and Arsenault, 1996). Furthermore, NGF promotes mossy fiber sprouting after kindling stimulation *in vivo*. Artificial elevation of NGF levels increases outgrowth (Adams et al., 1997) and, conversely, the presence of NGF-blocking antibodies decreases axonal sprouting (Holtzman and Lowenstein, 1995; Van der Zee et al., 1995). Clearly, control of post-seizure reorganization of connectivity is another area that merits more attention.

In sum, expression of many cytokines is stimulated by seizure activity and these signaling proteins can alter both the seizure itself, and the subsequent course of pathological changes in the hippocampus and elsewhere. Future work in this area has many basic, though technically challenging, questions to address. Progress on these issues will benefit both our understanding of fundamental cell interactions in the CNS, and hopefully contribute to therapeutic interventions in the future.

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Chapter 2

Cytokine responses to hippocampal slice preparation, *in vivo* electrode insertion, and LTP induction

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ABSTRACT

Because exogenous application of a number of cytokines and growth factors can alter synaptic properties, we sought to determine if endogenous cytokine expression is affected by neuronal activity. In addition, we examined whether cytokine expression is altered by the techniques used to stimulate and record from hippocampal neurons. Using semi-quantitative RNase protection and RT-PCR assays, we studied the expression of 18 cytokine, growth factor, and receptor genes in the hippocampus following the induction of Schaffer collateral-CA1 long-term potentiation (LTP). We found various cytokines are dramatically induced following both preparation of slices for *in vitro* recording, and as a result of injury following acute electrode placement *in vivo*. These increases can be eliminated *in vivo*, however, using permanent electrodes implanted 3 weeks prior to testing. In contrast to previous reports on brain derived neurotrophic factor (BDNF) and LTP, we find that BDNF mRNA is specifically down-regulated by the low frequency test stimuli used to assess synaptic responses. In contrast, interleukin-6 (IL-6) is up-regulated nearly 20-fold by the induction of LTP *in vivo*, marking the first demonstration of endogenous regulation of a neuropoietic cytokine in response to LTP. Coupled with previous results showing that exogenously applied IL-6 can prevent the induction of LTP (Li et al. 1997), this finding suggests a mechanism by which the local release of a cytokine could regulate potentiation at nearby sites.

INTRODUCTION

A variety of intercellular signaling proteins have recently been shown to modulate hippocampal long-term potentiation (LTP). Some of the best-studied examples of such factors are the neurotrophins, particularly BDNF. The mRNA for BDNF is up-regulated in response to synaptic activity both in hippocampal slices and in the intact animal (Schuman, 1999). Moreover, exogenously applied BDNF can potentiate synaptic transmission *in vitro* and *in vivo* (Kang and Schuman, 1995; Messaoudi et al., 1998) and reduction of

endogenous BDNF levels or function can impair LTP (Korte et al., 1995; Patterson et al., 1996; Kang et al., 1997).

A number of cytokine families are functionally similar to the neurotrophins, displaying the ability to affect neuronal differentiation, survival, and axonal sprouting (Loughlin and Fallon, 1993; Patterson, 1995; Gadient and Otten, 1997; Mattson et al., 1997; Murphy et al., 1997). Despite these similarities, the role of cytokines at the synapse has not been thoroughly explored. Cytokine expression can be up-regulated in response to the overt neuronal activity induced by seizure (Minami et al., 1991; Jankowsky and Patterson, 1999), and up-regulation of certain cytokines following a more physiological, LTP-inducing stimulus has also been observed (Andreasson and Worley, 1995; Inokuchi et al., 1996; Schneider et al., 1998). In addition, work from several groups has demonstrated that certain exogenously applied cytokines can affect synaptic transmission and LTP (Patterson, 1995). Together, these findings indicate parallels to the well-studied roles of neurotrophins at the synapse. Unlike the small number of neurotrophins, however, there are over 40 known cytokines, many found in the CNS (Benveniste, 1992; Sei et al., 1995). In the present experiments, we therefore took a broad approach to identifying the potential roles of cytokines in synaptic transmission by examining a wide array of cytokine ligands and receptors in several preparations used in the study of synaptic plasticity.

Recognizing that cytokines are known to function in neural injury, where they may act to mediate communication between cells of the nervous and immune systems, it was also important to look carefully for cytokine perturbations caused by the techniques used to study synaptic activity. All preparations used to study LTP require some form of mechanical intervention, either slicing the hippocampus for *in vitro* experiments or inserting electrodes *in vivo* , each of which is likely to cause injury-induced cytokine expression.

In the present study, we assayed the expression of 18 cytokine, growth factor, and receptor genes by semi-quantitative RNAse protection and RT-PCR assays before and after

LTP induction in the Schaffer collateral-CA1 pathway, both *in vivo* and *in vitro*. We find that the mechanical damage required to produce two common electrophysiological preparations can alter the expression of specific cytokines, which could, in turn, affect synaptic plasticity. Therefore, to separate the effects of neural injury from those induced by synaptic activity, we utilized a chronic *in vivo* preparation in which electrode implantation (and consequent injury) is temporally separated from the electrical stimulation. We describe changes in expression of two genes that are affected by activity in the hippocampus, one of which suggests a mechanism by which the potentiated synapse could enhance its own relative effectiveness by down-regulating its neighbors' ability to respond to input.

MATERIALS AND METHODS

Hippocampal slice electrophysiology. Young adult (6-7 week old), male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) were anaesthetized with Halothane and killed by decapitation. The brain was quickly removed, placed in ice-cold, oxygenated artificial cerebrospinal fluid (ACSF: 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 11.0 mM glucose), and both hippocampi dissected out. Transverse slices, 450μm thick, were prepared with a tissue chopper, and were allowed to recover for at least 1.5 hours in a humidified, oxygenated chamber, suspended over a dish of ACSF, prior to use for electrophysiology. Slices were then transferred to a recording chamber where they were submerged in a stream of ACSF maintained at room temperature (22-25° C) and perfused with 95% O_2/5% CO_2. Three slices were used in the recording chamber at one time. One slice was placed at the back of the recording chamber with no electrodes and was used as a control for the effects of tissue preparation ("naive"). Bipolar tungsten stimulating and glass capillary recording electrodes were placed into the stratum radiatum layer of area CA1 in each of the two remaining slices. Field EPSPs were evoked by stimulation of the Schaffer collateral-commissural afferents

once every 30 seconds and the initial (1 to 2 ms) slope was measured. Baseline responses were recorded for at least 20 minutes prior to induction of LTP by tetanic stimulation (four individual 100 Hz trains delivered for 1 s each at the test intensity with an intertrain interval of 15 s) in one of the two recorded slices ("LTP"); the other slice received only the continued low-frequency test stimuli to control for effects of electrophysiological recording ("LFS"). Field responses were measured for one hour after applying the tetanus; percent baseline values were determined from the final 10 minute interval recorded. Only those sets of slices in which the percent baseline (%BL) of the LTP slice was over 130%, and the %BL of the LFS slice was between 90 and 110% were used for mRNA analysis.

In vivo hippocampal electrophysiology. All studies used male Sprague-Dawley rats (Charles River, NC) weighing 350-375 g. Animals were housed in pairs, with food and water available *ad libitum*, and maintained on a 12:12 hour light:dark cycle, in accordance with NIH guidelines.

For studies involving both acute and chronic electrode implantation, animals were anesthetized with pentobarbital (50 mg/kg i.p.) and given booster pentobarbital injections (25 mg/kg, i.p.) at 30-45 minute intervals to maintain a surgical level of anesthesia. Body temperature was maintained at 37° C with a heating pad. Following mounting of the head in a stereotaxic frame, all surgical procedures were performed under sterile conditions. Holes were drilled in the skull above the right hemisphere with a sterile drill bit, and the overlying dura was punctured with a needle.

Bipolar stimulating electrodes, made from twisted Teflon-coated, stainless steel wire (0.012 inch outside diameter) exposed only at the tip (tip separation approximately 0.10 mm), were used to deliver current to the Schaffer collaterals. Constant current stimulation (100-500 μA biphasic pulses, 0.1 msec. duration each phase) was provided by a Grass (Braintree, MA) stimulus isolation unit. The recording electrode, a single Teflon coated wire exposed only at the tip, was placed in the stratum radiatum approximately 200

μm below the CA1 pyramidal layer of the dorsal hippocampus (AP -3.5 mm, ML 3.0 mm, DV 2.3 mm from top of brain (Paxinos and Watson, 1982). The stimulating electrode was placed about 0.5 mm posterior to the recording electrode, and slightly medial to the recording site, (AP -4.0 mm, ML 2.8 mm, DV 2.53 mm from top of brain). This corresponds with the orientation of stratum radiatum fibers in the intact brain (Rawlins and Green, 1977; Derrick and Martinez, 1994). Extracellular recordings were referenced to an indifferent site (a screw mounted on the anterior skull).

In the acute studies, responses were collected and LTP was induced shortly (30-60 min.) after surgical electrode implantation (n=7). In studies involving permanent electrode implantation (Barnes, 1979), electrode wires were attached to gold Amphenol pins (Newark Electronics, Thousand Oaks, CA), mounted in 9 pin Malino/MacIntyre (Science Technology Centre, Carleton University, Canada) sockets, and affixed to the skull with dental acrylic. Animals were given antibiotics (Bicillin, 100,000 units i.m.) and oral analgesic (Ibuprofen) and allowed to recover for 3 weeks prior to recording (n=4).

In both acute surgical studies and studies using animals with permanently-implanted electrodes, animals were anesthetized with pentobarbital prior to evoking responses. Anesthesia was necessary in animals with permanent electrodes because our preliminary studies revealed that the stimulation parameters used to induce non-decremental Schaffer-CA1 LTP (1 sec. 100 Hz trains) frequently elicited seizures and "wet dog shakes" in awake animals. Once fully anesthetized (approximately 20 min.), responses were evoked using a current intensity eliciting a response that was 50% of the amplitude (baseline to peak) of the maximally-evokable response (as determined by asymptotic amplitudes using a current ranging from 10-500 μA). This 50% current intensity was used for evoking 0.05 Hz responses and induction of LTP with high-frequency stimulation. The magnitude of the field EPSP response was measured by the EPSP slope (mV/msec) occurring between 1 and 3 msec. after response onset. Responses were collected at a rate of 0.05 Hz for a 15-20 minute period. LTP was induced by delivery of three 1 sec. trains of 100 Hz stimulation

(100 pulses), with an intertrain interval of 5 minutes. These parameters were found to be optimal for induction of non-decremental LTP in area CA1 (Frey et al., 1993). Following delivery of the trains, responses were collected every 20 seconds for 1 hour. All evoked responses were amplified on a Grass P3 series A.C. preamplifier, filtered at 0.1 Hz-10 KHz, digitized (10 KHz) and stored for off line analysis using DataWave software (Thornton, CO). Stimulation-induced changes in evoked responses (LTP) were measured by comparing slopes of responses evoked during the 5 minute period prior to tetanization with the slopes of responses evoked between 55 and 60 minutes post-tetanus.

Two control groups were included for acute surgical LTP studies: LFS and naive. Low-frequency stimulation-only (LFS) animals underwent surgery, electrode insertion, and 0.05 Hz stimulation, but received no tetanic stimulation (n=7). The naïve controls had no surgery (n=9). Because of the limited amount of tissue recovered from each animal, and the large number of cytokines assayed, not all animals were assessed for expression of each cytokine tested.

Studies involving permanent electrode implantation included three control groups: LFS (n=3), naive (n=4), and sham-operated animals (n=3) that underwent surgery and permanent electrode placement, but received no electrical stimulation. Our initial naive cohort included only three animals, two were housed together, one alone. During the course of this study, evidence that isolation stress could affect levels of IL-1β was published (Murray and Lynch, 1998); we then added one additional control animal that was singly housed for a time equivalent to the duration of a chronic LTP experiment.

RNA isolation. Four hours after the induction of LTP *in vivo* (both acute and chronic preparations), animals were euthanized by sodium pentobarbital overdose and decapitated. The brain was quickly removed, and the right hippocampus was isolated. The area of electrode impalement was identified by small blood marks left by electrode insertion; the hippocampus was trimmed to a 3 mm block composed of tissue 1.5 mm to each side

(rostral/caudal) of the electrode tracks. This hippocampal piece was further cut into three transverse slices (to aid in later dentate gyrus removal) before being quickly frozen in pre-chilled isopentane.

Hippocampal slices used for *in vitro* electrophysiology were harvested and quickly frozen on dry ice one hour after tetanus was delivered to one of the three slices in the recording chamber.

All hippocampal tissue (*in vitro* and *in vivo*) was stored at –80° C until use. On the day of RNA isolation, each slice was thawed individually in 500 µl of phosphate buffered saline (PBS) containing 10 mM vanadyl ribonucleoside complex (Gibco BRL, Gaithersburg, MD) as an RNAse inhibitor. The dentate gyrus and remaining parts of the fimbria-fornix and entorhinal cortex were dissected away, and the isolated CA3-CA1 fragment was transferred to Solution D (Chomczynski and Sacchi, 1987) and homogenized. Each animal used for *in vivo* electrophysiology was analyzed individually, while RNA from hippocampal slice tissue was extracted from groups of 10 slices to obtain enough RNA for multiple assays. The distribution of individual slices into four groups (10 experiments each) was balanced so that the average %BL in the LTP slices (163±1.47%) and the average time of recovery before use for electrophysiology (4.01± 0.01 hours) was equal in each group. Because of the large number of cytokines assayed, and the relatively small amount of RNA recovered from each slice group, not all cytokines were measured in each set of slices.

Hippocampal slices not used for electrophysiology were collected after various recovery times in the air-interface chamber (0, 2, 4.5, and 7 hours after slice preparation). The tissue was quickly frozen on dry ice and stored at -80° C until use. Four slices from each time point were pooled for RNA extraction; two sample pools were collected for each point. In order to allow direct comparison of cytokine responses in our slice preparation to results previously published for IL-1β (Schneider et al. 1998), the dentate gyrus was not removed from this tissue prior to RNA extraction.

Additional hippocampal tissue was collected intact (without slicing or electrophysiological manipulation) to determine basal cytokine expression in this area. The dentate gyrus was not removed from this tissue before RNA extraction.

Total RNA was extracted from both the isolated CA1-CA3 and the intact hippocampal tissue by a modification of the acid-phenol method (Chomczynski and Sacchi, 1987), and stored at -80° C until use.

RNAse Protection Assay (RPA). Plasmids containing portions of ciliary neurotrophic factor (CNTF), transforming growth factor β1 (TGFβ1), transforming growth factor β2 (TGFβ2), bone morphogenetic protein 6 (BMP6), interleukin-1β (IL-1β) and interleukin-1 receptor antagonist (IL-1RA) were cloned by PCR from sciatic nerve cDNA (CNTF) or adult rat brain cDNA. Polymerase chain reaction fragments were ligated into either pCRII (Invitrogen, San Diego, CA) or Bluescript (Stratagene, La Jolla, CA) vectors and sequenced to confirm their identity. Rat IL-6 cDNA was purchased from ATCC (Rockville, MD). Additional rat cDNAs were gifts from several laboratories: CNTF receptor (CNTFR) clone pSK-rCNTFR(5'-PST1) from Dr. Samuel Davis and Dr. George Yancopolous, Regeneron Pharmaceuticals; IL-6 receptor (IL-6R) (pSPT19 clone) from Dr. Reto Gadient and Dr. Uwe Otten, University of Basel, Switzerland; BMP2 from Dr. Jian Feng, University of Texas, San Antonio; activin βA, activin βB, and inhibin α from Dr. Ming-Ji Fann (Fann and Patterson, 1995) and leukemia inhibitory factor receptor (LIFR) from Dr. Lisa Banner (Banner and Patterson, 1994). The leukemia inhibitory factor clone was previously described in this laboratory (Yamamori et al., 1989). The housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control with each cytokine or receptor RPA reaction, and was a gift from Dr. Lisa Banner (Banner and Patterson, 1994). The RPA was performed as described (Patterson and Fann, 1992), using approximately 5 μg total RNA per reaction. Briefly, plasmids were linearized and ^{32}P-labeled antisense probes generated by *in vitro* transcription were hybridized to

hippocampal total RNA. After overnight hybridization at 55° C, reactions were digested with RNAse A and RNAse T1. Digestion was stopped with proteinase K and RNA extracted with phenol-chloroform. Reaction products were separated on denaturing 6% polyacrylamide gels to yield protected fragments of 266 nucleotides for CNTF, 294 nucleotides for IL-6R, 589 nucleotides for IL-1β, 222 nucleotides for IL-1RA, 430 nucleotides for CNTFR, 245 nucleotides for BMP2, 311 nucleotides for BMP6, 406 nucleotides for TGFβ1, 252 nucleotides for TGFβ2, 299 nucleotides for LIFR, 450 nucleotides for GP130, 386 nucleotides for inhibin α, 348 nucleotides for activin βA, 267 nucleotides for activin βB, 169 nucleotides for LIF, and 133 nucleotides for GAPDH. Radioactivity was measured by scanning the protected fragments on a Phosphoimager 445SI (Molecular Dynamics, Sunnyvale, CA) and quantitated with ImageQuant software. The intensity of the protected fragment for each cytokine or receptor was compared to the intensity of the protected fragment of GAPDH in that reaction and the ratio was expressed in arbitrary units. Quantitation of GAPDH mRNA levels across experimental conditions revealed that the GAPDH mRNA levels were not altered significantly by electrophysiological manipulation. Determination of statistical significance between conditions was performed by the two sample Student's t-test, assuming equal variances (two tailed p value).

Reverse-transcription polymerase chain reaction (RT-PCR). The basal levels of several cytokine mRNAs were too low to detect by RPA, requiring the more sensitive RT-PCR assay to measure changes induced by electrophysiological manipulation. Brain-derived neurotrophic factor was included among the factors tested by RT-PCR as a possible positive control for the assays: BDNF was shown to be induced by LTP both *in vivo* and *in vitro*, although under slightly different conditions than we used in our experiments (Patterson et al., 1992; Castren et al., 1993; Dragunow et al., 1993; Bramham et al., 1996). Prior to reverse transcription, hippocampal RNA was treated with 1 unit of DNAse

(Gibco BRL) in 1x DNAse buffer for 15 minutes at room temperature. The DNAse was inactivated by addition of EDTA and heated at 65° C for 10 minutes, then quickly chilled on ice. Random hexamers (0.5 μg, Gibco BRL) were used to prime the RT reaction, and were added just before denaturing the RNA at 70° C for 8 minutes. The final RT reaction mixture consisted of 1x reverse transcription buffer, 1 mM each dNTP (Boehringer Mannheim), 10 mM DTT, 20 units RNAsin (Boehringer Mannheim), and 200 units Superscript II reverse transcriptase (Gibco BRL). Reactions were incubated for 10 minutes at room temperature, followed by 2 hours at 42° C. After synthesis of cDNA was complete, reactions were diluted to a final volume of 150 μl, and separated into 10 μl aliquots placed into thin walled PCR tubes (USA Scientific, Ocala, FL) for storage at -20° C. The following primer sets, listed 5' to 3', were used to amplify specific gene products: interleukin-1α (IL-1α): 5' TCC TGA CTT GTT TGA AGA CC, 3' CTT AGC CGT CTC TTC TTC AG; interleukin-6 (IL-6): 5' TGT TCT CAG GGA GAT CTT GG, 3' TCC AGG TAG AAA CGG AAC TC (Pitossi and Besedovsky, 1996); BDNF: 5' ATG ACC ATC CTT TTC CTT ACT ATG GT, 3' TCT TCC CCT TTT AAT GGT CAG TGT AC (Zaheer et al., 1995); GAPDH: 5' ACC ACC ATG GAG AAG GCT GG, 3' CTC AGT GTA GCC CAG GAT GC (Brown et al., 1994). Amplification reactions were performed in a final volume of 100 μl, consisting of 10 μl of diluted cDNA, 1 unit of Taq polymerase (Promega, Madison, WI), 1x PCR buffer (Promega), 2.5 mM $MgCl_2$ (except IL-6: 1.5 mM), 0.2 mM of each dNTP, and one set of primers (200 ng each). The cycle programs used to amplify each gene were: IL 1α, IL-6, and BDNF: 94° C for 5 min., 58° C for 85 sec., 72° C for 1 min. (75 sec. for BDNF) 1x, followed by 39 cycles of 94° C for 30 sec., 58° C for 85 sec., and 72° C for 30 sec. (75 sec. for BDNF); GAPDH: 94° C for 5 min., 60° C for 85 sec., and 72° C for 1 min. 1x, followed by 27 cycles of 94° C for 30 sec., 60° C for 85 sec., and 72° C for 45 sec. To follow the amplification rate of each reaction and identify the linear range, PCR reactions were stopped every 4 cycles starting at

24 cycles (IL-1α, IL-6, and BDNF) or 12 cycles (GAPDH), and 5 μl aliquots were removed.

cDNA hybridization. Reaction products from each PCR cycle sampled were blotted onto Nytran 0.2 μm nylon membrane (Schleicher & Schuell, Keene, NH), and fixed to the membrane by UV cross-linking (Stratagene Stratalinker) followed by baking at 80° C for 10 min. Prior to hybridization with radiolabeled probes, membranes were prehybridized for 2 hours at 42° C in a solution of 6x SSPE, 1% SDS, 10x Denhardt's solution, 20 μg/ml tRNA (Boehringer Mannheim), and 50 μg/ml herring sperm DNA. During prehybridization, oligonucleotide probes designed to bind the PCR products were end-labeled with ^{32}P using polynucleotide kinase (Boehringer Mannheim), and purified over G25TE spin columns (Boehringer Mannheim). Oligonucleotide probes were tested for specificity by hybridization against end-stage PCR reactions run out on agarose gels and transferred to nylon membrane. Internal oligonucleotide sequences, listed 5' to 3', were: GAPDH: ATC GTG GAA GGG CTC ATG ACC ACA GTC CAT; IL-1α: TAC AGT TCT GCC ATT GAC CAT CTG TCT CTG; IL-6: CAG CGA TGA TGC ACT GTC AGA AAA CAA TCT G; BDNF: TGG GTC ACA GCG GCA GAT AAA AAG ACT GCA. After 2 hours, prehybridization buffer was removed, and replaced with 6x SSPE/1% SDS containing 500 kcpm/ml of labeled probe. Hybridization was continued for an additional 14-16 hours at 65° C. Membranes were then washed three times for 10 minutes each in 6x SSPE/ 1% SDS at room temperature, followed by a final wash in 1x SSPE/ 1% SDS for 3 minutes at 65° C.

Quantitation of RT-PCR reactions. Bound radioactivity was quantitated with a Phosphorimager 445SI using ImageQuant software. Using samples taken from regular intervals in the PCR amplification, a linear range was determined for each reaction. Over many trials, we found that the amount of PCR product generated by 28 cycles fell in the

linear range for the IL-1α, IL-6 and BNDF reactions, while GAPDH reached linear values by 16 cycles. In order to control for variations in cDNA synthesis between reactions, values for IL-1α, IL-6 and BDNF expression, taken as the intensity of hybridization of each reaction at 28 cycles, were expressed as a ratio to GAPDH expression amplified 16 cycles from the same initial cDNA reaction. Values for cytokine expression based on this ratio are expressed in arbitrary units, and statistical significance between conditions assessed by Student's t-test.

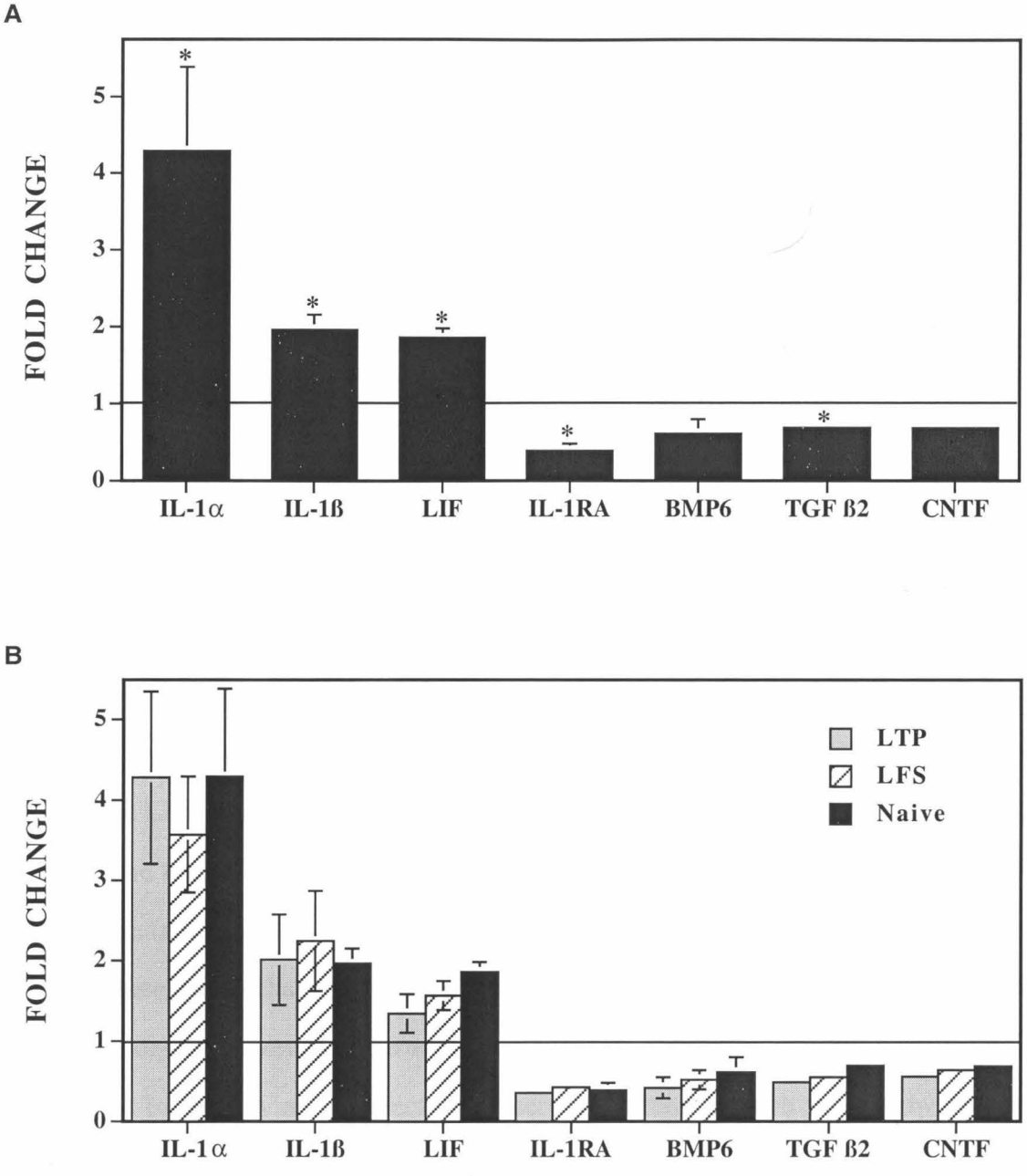
RESULTS

Cytokine expression in hippocampal slices. Because preparing the hippocampus for *in vitro* recording requires substantial mechanical damage, and cytokine expression is known to be altered in many models of neuronal injury, it was important to establish what effects cutting slices for *in vitro* experiments may have on cytokine levels. We compared cytokine mRNA levels from intact, freshly dissected hippocampus to mRNA levels from "naive" slice tissue. This tissue had been cut for *in vitro* recording, allowed to recover in an air-interface chamber for at least 90 minutes, and held (without electrodes) in the perfusion recording chamber for the duration of an LTP experiment. Of seventeen mRNAs assayed (IL-6 was not assayed in slices), seven were altered by the manipulations needed to prepare the hippocampus for *in vitro* recording (Figure 1*A*). Three cytokines, IL-1α, IL-1β, and LIF, were up-regulated 2- to 4-fold by slice preparation, while four others, IL-1RA, BMP6, TGFβ2, and CNTF, were down-regulated in the naive slices compared to the intact hippocampus. Thus, cutting hippocampal slices for *in vitro* experiments appears to alter the expression of certain cytokines.

Once the change in basal cytokine expression caused by slice preparation was determined, we asked if expression of any of these cytokines would be further altered by the induction of LTP. Comparison of the mRNA levels in naive slices to levels in slice

Figure 1. Expression of several cytokines is affected by cutting slices for *in vitro* experiments, and synaptic activity does not further alter cytokine levels. (*A*) Expression of seven cytokines is altered by the tissue damage caused by making slices. Cytokine expression in naive slices (without electrodes) are expressed relative to the internal control GAPDH and then normalized to the levels of basal cytokine expression in the intact hippocampus. Several cytokines are up-regulated by the tissue preparation (*: IL-1α, IL-1β, LIF, $p<0.05$, Student's t-test). Four cytokines, IL-1RA, BMP6, TGFβ2 and CNTF, are lower in the slice tissue than in the intact hippocampus (*: IL-1RA, TGFβ2, $p<0.05$, Student's t-test). (*B*) Cytokine expression is not further altered by electrical stimulation. Data from A, showing alterations in mRNA levels produced by slice preparation, are shown again for comparison in this figure (naive slices, *black bars*). Cytokine expression in slices used for electrophysiology (LFS, *striped bars*, and LTP, *gray bars*) is also shown normalized to basal expression levels in the intact hippocampus. No significant differences were observed between experimental conditions, raising the possibility that cytokine alterations due to injury may be masking effects due to synaptic activity.

Figure 1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BMP6
TGF ß2
CNTF

tissue one hour after the induction of LTP revealed no further differences for any of the cytokines tested (Figure 1*B*).

In addition, we examined the time course of IL-1β up-regulation following slice preparation. Under our conditions, IL-1β expression increased steadily with time after slice preparation, from an initial level nearly equal that found in the intact hippocampus, to levels 4-fold higher by 7 hours *in vitro* (Figure 2).

Cytokine expression in the acute in vivo *preparation.* Since we were unable to find differences in cytokine expression following induction of LTP using the *in vitro* preparation, we next utilized an acute *in vivo* preparation. This approach had two potential advantages. First, if cutting hippocampal slices masked effects of LTP, the *in vivo* preparation might uncover cytokine changes caused by synaptic activity. Second, a longer time course following LTP could be effectively examined *in vivo*. Surprisingly, of the 18 mRNAs examined, expression of seven genes was significantly altered simply by the placement of electrodes into the hippocampus. Activin βA, BNDF, LIF, IL-6, IL-1α, IL-1RA, and IL-1β were all substantially induced by the impalement of electrodes and delivery of only the LFS test stimuli (without tetanus; Figure 3). As with the slice experiments, we observed no further changes in cytokine expression by the induction of LTP in the *in vivo* preparation (data not shown).

The extreme changes in cytokine expression *in vivo* following electrode placement were avoidable, however. The induction of several cytokines could be substantially attenuated by modifying the electrode insertion. Using a much more slow placement technique in which each electrode is inserted only once, and approximately 10 minutes is allowed for the cells to recover/reseal between the placement of each electrode, cytokine induction could be substantially reduced (Figure 3).

Figure 2

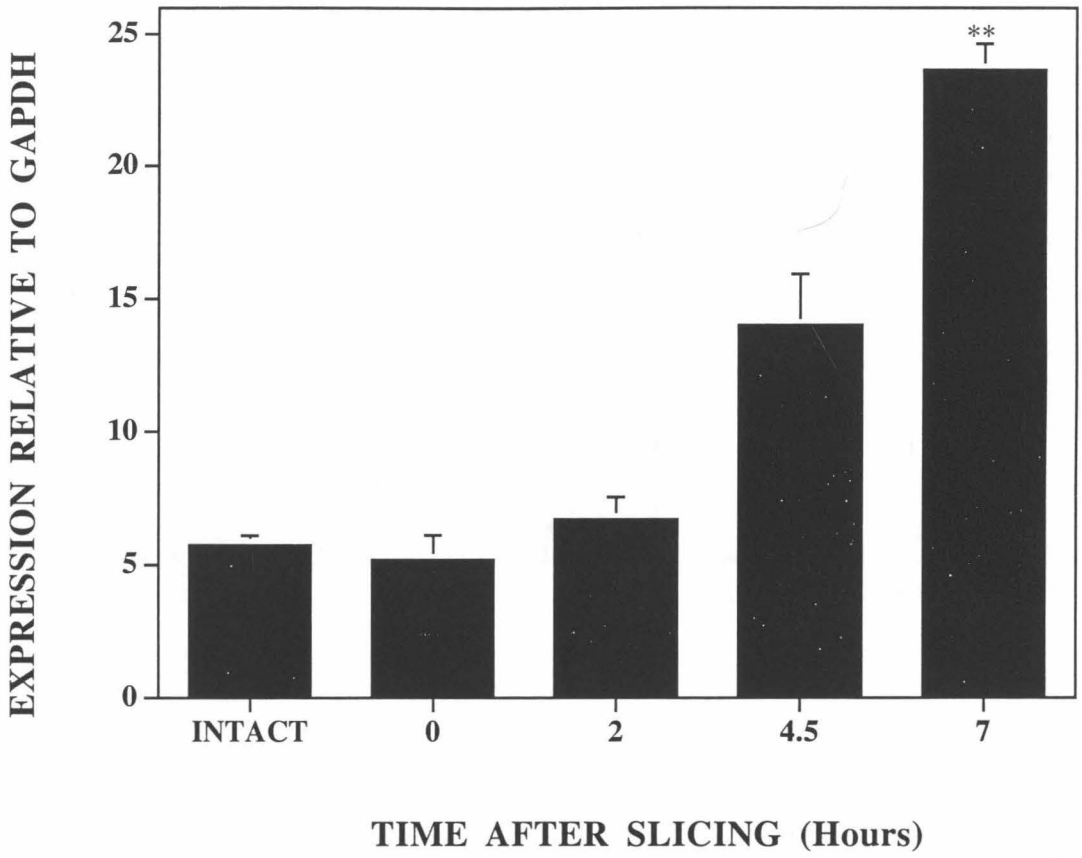


Figure 2. Expression of IL-1β mRNA in hippocampal slices at various times after slice preparation. Hippocampal slices were prepared using a mechanical tissue chopper, followed by recovery in an air-interface chamber perfused with oxygen, and were harvested without being used for electrophysiology. Levels of IL-1β assessed immediately after slice preparation (*0 hours*) did not differ from levels observed in the intact hippocampus (*intact*). However, IL-1β expression progressively increased with time after slicing (**: $p<0.01$, Student's t-test).

Figure 3

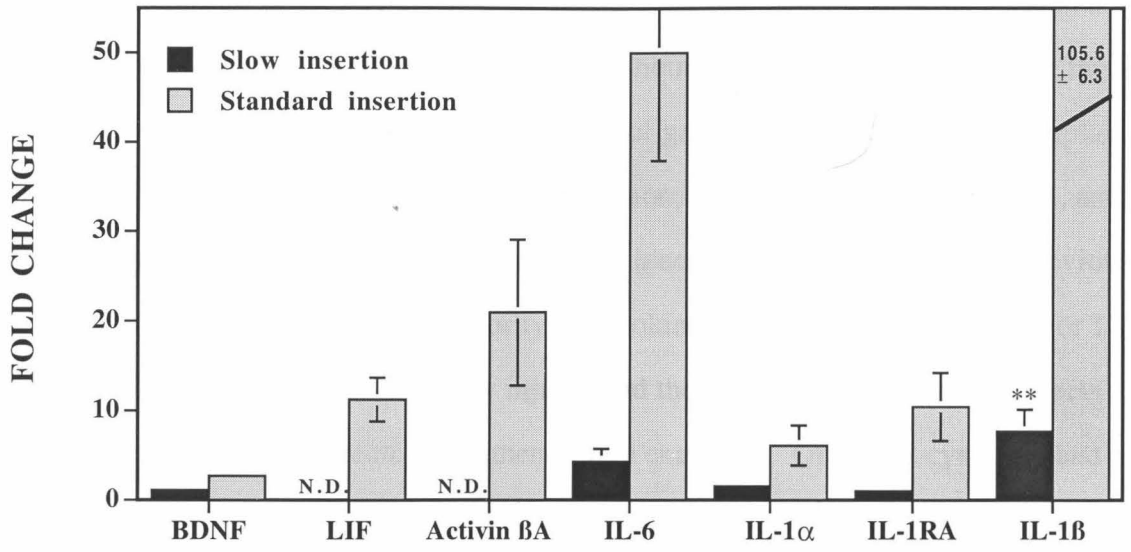


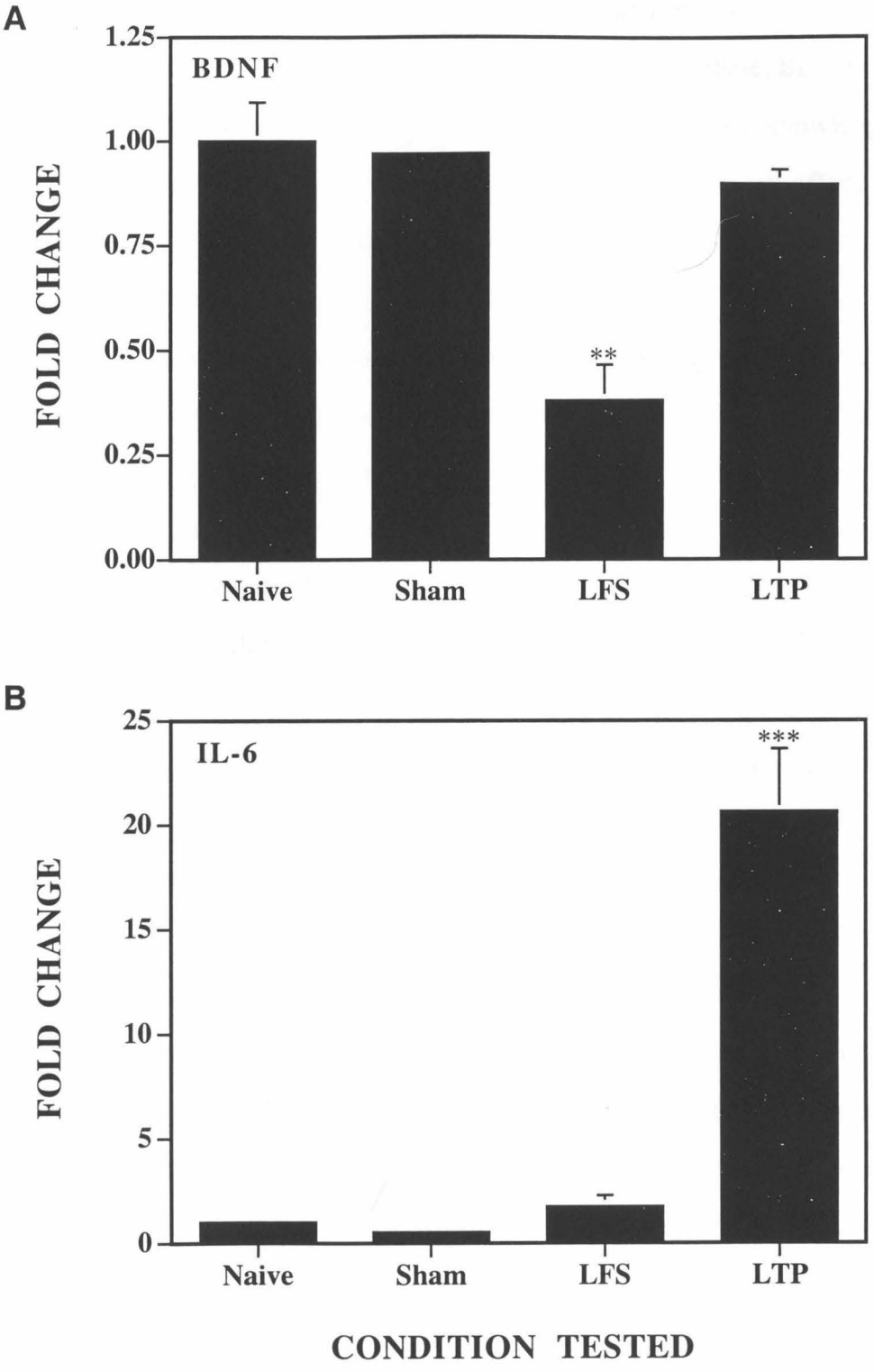
Figure 3. Electrode insertion can cause cytokine induction *in vivo*. The *gray bars* show the up-regulation of several cytokines and growth factors by the tissue damage caused by placing electrodes into the hippocampus for recording. All animals received low-frequency test stimulation, but no tetanic stimulus. Values for all cytokines shown are statistically different from basal expression in the naive, uninjured hippocampus, and are shown as fold-change from basal values (BDNF, LIF, activin βA, IL-6, IL-1α, IL-1RA: $p<0.05$; IL-1β: $p<0.01$). The *black bars* show that, in all cases examined, cytokine up-regulation can be attenuated by slow and careful electrode placement (*N.D.*: LIF and activin βA not determined). Expression of IL-1β is significantly reduced by this technique compared to standard electrode insertion (**: $p<0.01$).

Cytokine expression in the chronic in vivo *preparation.* To further reduce acute effects caused by electrode injury, we utilized a chronic *in vivo* preparation in which the electrophysiology was done three weeks after surgical electrode implantation. This recovery time should separate cytokine induction caused by mechanical injury from cytokine alterations caused by synaptic activity. We examined cytokine expression at the same time point as in the acute preparation, four hours after induction of LTP. In this set of experiments, we included an additional control group: sham animals were implanted with electrodes but received no electrical stimulation, not even the LFS test stimuli, and so were true controls for injury due to electrode insertion. Because we had previously determined that expression of the majority of cytokines was not altered by injury or LTP, only those seven cytokines affected by injury (and therefore possibly masking effects due to synaptic activity) in the acute experiments were examined. Of the six cytokines and one neurotrophin assayed, two were found to be altered by electrophysiological stimulation. We observed a *decrease* in BDNF expression following LFS, but no change from basal (naive) levels after LTP induction (Figure 4*A*). More dramatic was the effect on IL-6 expression; this cytokine increased nearly 20-fold with the induction of LTP (Figure 4*B*). Unlike BDNF, no significant changes in IL-6 were seen in any of the control groups; IL-6 expression was altered solely in the LTP group. One animal was excluded from the calculations of mean and significance shown in Figure 4*B*; this animal was an additional naive control that was added to the experiment several months after the other animals had been sacrificed. The extremely high level of expression seen in this animal was inconsistent with the remaining 9 control animals (naive, sham, and LFS), which may have been due to a difference in age or in housing conditions used after the other animals were removed.

Statistically significant differences between naive and LFS values were also found for IL-1RA and IL-1β ($p<0.05$, Student's t-test); however, only two animals were sampled in the LFS condition for these cytokines (data not shown).

Figure 4. Expression of BDNF and IL-6 is altered by synaptic activity in the chronic *in vivo* preparation. (*A*) Expression of BDNF for two controls, sham-operated animals and animals receiving test-stimuli only (LFS), as well as experimental, LTP induced expression, is normalized to basal values in the naive (intact) hippocampal tissue. Expression of BDNF in the LFS control group is significantly reduced from that in the naive group (p=0.005, Student's t-test, **: p<0.01) and the LTP group (p<0.005). (*B*) Expression of IL-6 is strongly and specifically induced by LTP. Again, expression of IL-6 in sham, LFS, and LTP groups is shown normalized to basal values in the naive (intact) hippocampus. Values were determined four hours after the induction of LTP at the Schaffer-collateral/CA1 synapse, and show significant induction compared to control (naive) expression (***: p<0.005).

Figure 4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DISCUSSION

In this study of the effects of synaptic activity on cytokine expression in the hippocampus, we examined 19 growth factors, cytokines, and receptors, identifying two that are specifically affected by electrical stimulation. While one of these, BDNF, had been previously studied, expression of the cytokine IL-6 has not, to our knowledge, been examined in this context. Because exogenous application of IL-6 can affect synaptic properties (Li et al., 1997), the up-regulation of this cytokine by synaptic activity suggests an interesting way in which synaptic plasticity could be regulated, as discussed in more detail below. We also observed several changes in cytokine expression due to the preparation of tissue for electrophysiological recording *in vitro* and *in vivo*. Cutting slices for *in vitro* experiments and insertion of electrodes *in vivo* both significantly altered cytokine and growth factor mRNA expression. Because several of these intercellular signaling proteins are known to affect synaptic properties, we believe that these results with commonly used methodologies suggest using caution when evaluating prior electrophysiological results.

By comparing mRNA levels in naive, unstimulated hippocampal slices to those in intact hippocampus, we identified three cytokines that are up-regulated and four cytokines that are down-regulated by cutting and incubating slices for *in vitro* recording. Two caveats must be considered, however. First, it is possible that the *decreases* in cytokine expression we observe could be due to loss of blood elements from the ACSF-perfused slice that were present in the intact hippocampus. Indeed, both TGFβ2 and IL-1RA proteins are secreted by leukocytes, although less has been written about the mRNA expression of these factors (Paul, 1993; Malyak et al., 1994). Second, the dentate gyrus was removed from the hippocampal slice tissue prior to RNA extraction, but was included in the intact hippocampal RNA isolation. If the dentate has different levels of cytokine expression than areas CA1-CA3, the inclusion of this subfield may account for some of the differences in expression between slice and intact tissue. To the best of our knowledge,

varied expression across hippocampal subfields has not been demonstrated for any of the cytokines found to be altered by cutting slices.

Potential sources of the three cytokines that were up-regulated by slice preparation are astrocytes or microglia. Leukemia inhibitory expression is up-regulated in astrocytes by surgical injury to the cortex *in vivo* (Banner et al., 1997), and activated microglial cells can produce all three cytokines (Benveniste, 1992; Banner et al., 1997). Although several studies have demonstrated that microglia are activated by organotypic hippocampal slice preparation, the microglial response takes longer than the cytokine up-regulation we observed, as does LIF up-regulation in astrocytes following injury (Coltman and Ide, 1996; Hailer et al., 1996).

Few studies have examined the effects of mechanical damage to the hippocampus caused by making slices for *in vitro* recording (Kirov et al., 1999). While neurons largely recover a normal resting potential and retain the ability to respond to electrical stimulation with an intensity-appropriate output, our results indicate the possibility of artifactual alteration in synaptic properties. For example, IL-1β, which has been shown to inhibit the induction of LTP when applied exogenously (Li et al., 1997), is up-regulated in our hippocampal slices. During the course of this work, Schneider et al. (Schneider et al., 1998) described an immediate up-regulation of IL-1β induced by cutting slices for *in vitro* experiments, which subsided with prolonged recovery time. In our studies, however, IL-1β expression steadily increased with time after slicing, the initial levels being similar to those found in the intact hippocampus (Fig. 2). Four methodological differences between our work and that of Schneider may be relevant. First, we used younger animals than Schneider et al. (6-7 weeks vs. 8-10 weeks). Second, we used a slightly different ACSF formulation. Third, the slices are incubated in different recovery chambers (air-interface vs. immersion). Fourth, the temperature at which the slices were held for recovery was different (22-25^{o} C versus 33-35^{o} C). Such differences may also explain our inability to replicate their finding that IL-1β expression is up-regulated by LTP. Another difference

between our results and prior work concerns BDNF. Patterson et al. (Patterson et al., 1992) described an up-regulation of BNDF following LTP induction *in vitro*, while we found no change in BDNF expression between experimental and control slices. The most obvious difference between these studies is in the time between electrical stimulation and RNA isolation (4 hours versus 1 hour after LTP induction).

Since cytokine induction due to mechanical damage in hippocampal slices might have masked effects of LTP on cytokine expression, we also studied the effects of LTP in the intact hippocampus. We assayed 18 cytokine, growth factor, and receptor mRNAs, comparing expression four hours after LTP induction to that in two controls: naive, unperturbed hippocampus, and hippocampal tissue that had electrodes inserted and received test stimuli (LFS) but no tetanic stimulation. Surprisingly, both the LFS and LTP animals display large increases in the expression of several cytokines. Moreover, although some of the same genes are up-regulated *in vivo* as in the slices, the induction reaches far greater levels in the intact animal. This difference could be due to the infiltration of blood cells with high cytokine expression into the injury site *in vivo*; such cells would not be present in the isolated slice. Alternatively, the dramatic response *in vivo* might be due to signaling through synaptic connections with other areas of the brain that also would not be present in the isolated slice. The naive control used for comparison of basal expression levels in these experiments was the same area of hippocampal tissue removed from the LFS and LTP animals and, like the LFS and LTP tissue, it was also micro-dissected to remove the dentate gyrus. Therefore, the changes in expression seen *in vivo* cannot be explained by differences in cytokine expression across subfields.

Again, few studies have examined the potential injury caused by electrode insertion. The most closely related experiments are studies of cortical stab wounds, usually induced with a scalpel. The tissue damage caused in that paradigm would likely be much more extensive than the damage caused by insertion of fine electrodes. Nonetheless, some of the same cytokines respond to both forms of injury. For example, LIF mRNA is increased

more than 10-fold four hours after electrode insertion in the hippocampus, a response more dramatic than was found at the same time point after a scalpel-induced injury to the cortex (Banner et al., 1997). Importantly, most of the factors we find up-regulated by electrode insertion are able to alter synaptic properties when exogenously applied during LTP experiments (Katsuki et al., 1990; Bellinger et al., 1993; Kang and Schuman, 1995; Cunningham et al., 1996; Li et al., 1997; Schneider et al., 1998).

We find that the chronic *in vivo* preparation, in which a three week recovery period separates the implantation of electrodes from the electrophysiology experiments, eliminates the confounding effects of injury on the study of synaptic activity. Compared to levels in the intact hippocampus, all 7 cytokine mRNAs assayed (each of which was significantly induced by acute electrode insertion) returned to basal levels following the three week recovery. Of these 7, two, BNDF and IL-6, are specifically affected by synaptic activity. Previous work had described BDNF up-regulation *in vivo* following induction of LTP by stimulation of the perforant path (Castren et al., 1993; Dragunow et al., 1993; Bramham et al., 1996). We find a slightly different outcome; BNDF is not up-regulated by LTP of the Schaffer collateral-CA1 synapses, but is *down-regulated* by LFS when compared to either basal expression in the intact hippocampus, or to expression in sham operated control animals. The differences between these results could be due to several factors. Prior studies induced LTP in the perforant path while we examined LTP in the Schaffer collateral pathway. Moreover, earlier studies used awake, behaving animals while we used anaesthetized animals. Our choices were based on the desire to retain the same pathway and stimulation paradigm used in our hippocampal slice and acute *in vivo* experiments. Such methodological differences may also explain why we did not see up-regulation of activin βA or IL-1β, both of which had previously been reported to follow LTP induction in the perforant path of awake behaving animals (Andreasson and Worley, 1995; Schneider et al., 1998). Such differences in results could shed light on divergent mechanisms used in

the two synaptic populations studied or on distinctions between awake and anaesthetized neuronal function.

Our observation of IL-6 up-regulation after the induction of LTP takes on additional interest in light of the ability of exogenous IL-6 to prevent the induction of LTP in the Schaffer collateral pathway when applied for as little as 10 minutes, without affecting previously established LTP (Li et al., 1997). If IL-6 protein is also up-regulated following the induction of LTP, it is possible that release of this cytokine could inhibit subsequent potentiation at nearby synapses, either on the same cell or on neighboring cells. Indeed, studies *in vitro* indicate that certain types of neurons are able to both produce and respond to IL-6 (Marz et al., 1998). Thus, up-regulation of this cytokine by LTP could provide a mechanism for the potentiated synapse to down-regulate its neighbors' ability to respond to subsequent input. This may serve to promote stability in networks with modifiable synapses (Turrigiano, 1999). Normalization of neurons and stabilization of their networks may thus be accomplished by the release of a signal that is correlated with activity, and that can act at many nearby sites in the network. In this context, the increase in IL-6 mRNA, and presumably, IL-6 release after LTP induction would limit the number of synapses changed on a given neuron, or prevent further alterations in synaptic strength in nearby regions that have undergone recent modifications. Future experiments using mice in which the gene for IL-6 has been disrupted may shed more light on the role of this cytokine in hippocampal LTP.

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Chapter 3

Differential regulation of cytokine expression following pilocarpine-induced seizure

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ABSTRACT

While the pathological changes that occur in the brain following seizure have been well characterized, the molecular mechanisms underlying these events are poorly understood. Cell death, reactive gliosis and axonal sprouting are among the best studied alterations in the epileptic brain. Previous work in both the peripheral and central nervous systems suggests that cytokines are capable of affecting each of these processes. To better understand the role of cytokines in seizures and their sequelae, we have characterized cytokine expression in an animal model of epilepsy. Using pilocarpine to chemically induce seizures, and RNAse protection assays to assess mRNA levels, we have quantified changes in expression of several members of the neuropoietic cytokine family following a single, prolonged seizure. Levels of oncostatin M (OSM), leukemia inhibitory factor (LIF), cardiotrophin-1 and ciliary neurotrophic factor were all increased in the hippocampus after seizure, though to differing extents and with markedly different time courses. Cells expressing the most dramatically up-regulated cytokines, LIF and OSM, were identified by combined *in situ* hybridization and immunohistochemistry. The majority of LIF^+ cells in the hippocampus were glial fibrillary acidic $protein^+$ astrocytes, while the majority of OSM^+ cells had the morphology of interneurons and were occasionally co-labeled with neurofilament markers. Both the time course and the localization of cytokine up-regulation following seizure suggest possible roles for these intercellular signaling molecules in epilepsy.

INTRODUCTION

Prolonged or repeated seizures characteristic of epilepsy can have severe consequences on the architecture of the brain. In the hippocampus, increased neuronal cell death and birth, axonal sprouting and reactive gliosis occur in distinct but overlapping sequences, beginning within a day of seizure and causing changes that can last throughout life (McNamara, 1994; Beach et al., 1995; Glass and Dragunow, 1995; Lynch et al., 1996;

Mathern et al., 1996; Parent and Lowenstein, 1997). One of the most common lesions seen in human temporal lobe epilepsies is hippocampal sclerosis, in which neuronal cell loss is characteristically observed in the dentate hilus and area CA3 (McNamara, 1994; Mathern et al., 1996). Several animal models of epilepsy yield similar patterns of cell death, and indicate that selective neuronal loss is one of the earliest consequences of prolonged seizure activity, likely mediated by glutamate-induced excitotoxicity (Schwob et al., 1980; Nadler, 1981; Ben-Ari, 1985; Mello et al., 1993; Lynch et al., 1996). Reactive gliosis, the activation and proliferation of astrocytes and resident microglia, occurs after a delay of several days, and is another feature of sclerosis common to both human epilepsy patients and animal seizure models (Meldrum and Bruton, 1992; Jorgensen et al., 1993; Niquet et al., 1994; Represa et al., 1995; Khurgel and Ivy, 1996). Starting at about the same time as glial proliferation, increased neuronal cell birth has recently been described in the dentate gyrus of two rodent seizure models (Bengzon et al., 1997; Parent et al., 1997; Parent et al., 1998; Scott et al., 1998). These new neurons were initially found to generate abnormal axonal projections that may contribute to epileptogenic changes in the neuronal network, however, it was later shown that the aberrant connections persist even in the absence of newborn neurons (Parent et al., 1998). While generation of new neurons in human patients is controversial, the abnormal projections observed in animal models are also seen in human epilepsies (Parent and Lowenstein, 1997). Known as mossy fiber sprouting, these aberrant granule cell projections into their own dendritic fields may have a role in propagation and/or recurrence of hippocampal seizures (Parent and Lowenstein, 1997).

The molecular mechanisms underlying seizure-induced neuropathological changes in the hippocampus are largely unknown. Because of their ability to alter cell survival, proliferation, and differentiation, cytokines may play a role in the pathophysiology of seizures. Initially described as proteins that mediate signaling between hematopoietic cells, many cytokines and their receptors have also been found in the nervous system. One

group of cytokines that could affect many of the changes occurring in the hippocampus after seizure are the neuropoietic cytokines, which includes LIF, IL-6, ciliary neurotrophic factor (CNTF), oncostatin M (OSM), cardiotrophin-1 (CT-1), interleukin-11 (IL-11) and growth promoting activity (GPA). Several of these cytokines can promote neuronal survival *in vitro*. *In vivo*, LIF and CNTF have been shown to be required for normal motor neuron survival in adult mice and can enhance neuronal survival and axonal sprouting and regeneration following injury (Murphy et al., 1997). Moreover, all members of this family can regulate neuronal gene expression in culture, and LIF mediates specific changes in gene expression that are characteristic of the response to injury in sympathetic and sensory neurons *in vivo* (Patterson, 1994; Murphy et al., 1997). Neuropoietic cytokines also regulate various aspects of glial development and function. In culture, LIF and CNTF can promote the differentiation of glial progenitor cells into mature oligodendrocytes or astrocytes (Murphy et al., 1997). Furthermore, mice lacking a functional LIF gene display alterations in markers for both oligodendrocytes and astrocytes in the adult brain (Bugga et al., 1998; Koblar et al., 1998).

The ability of the neuropoietic cytokines to affect neuronal survival, glial activation and axonal sprouting, together with their presence in the brain and up-regulation following injury and kainic acid injection (Minami et al., 1991; Banner et al., 1997; Murphy et al., 1997), suggested that this family of cytokines has a role in changes that occur in the hippocampus after seizure. To begin testing this hypothesis, we first asked if hippocampal expression of several neuropoietic cytokines can be altered by a single, prolonged seizure. We used the pilocarpine model of status epilepticus (Turski et al., 1983) because it avoids the mechanical injury to the brain caused by electrode placement required for kindling-induced seizures, while reproducing many of the pathological and behavioral features of human temporal lobe epilepsy. After peripheral pilocarpine injection, animals undergo an initial, prolonged seizure followed by a variable silent period, before spontaneous, recurrent seizures develop. Concurrent with the silent period, neuronal cell death, mossy

fiber sprouting and increased neurogenesis have been documented (Leite et al., 1990; Parent et al., 1997). This is the time during which we examined cytokine mRNA changes in the hippocampus. Using a semi-quantitative RNAse protection assay, we characterized the regulation of LIF, CNTF, OSM and CT-1 mRNAs from 2 hours to 7 days after pilocarpine-induced seizure. Cells up-regulating expression of the two most dramatically altered cytokines, LIF and OSM, were then identified by combined *in situ* hybridization and immunohistochemistry.

MATERIALS AND METHODS

Seizure Induction. Adult male Sprague-Dawley rats (200-250g; Simonsen Laboratories, Gilroy, CA) were pretreated by injection of atropine sulfate (1 mg/kg, i.p., Phoenix Pharmaceutical, St. Joseph, MO) 20 minutes prior to induction of status epilepticus by injection of pilocarpine hydrochloride (310-320 mg/kg, i.p., Sigma)(Turski et al., 1983). The time of pilocarpine injection was used as the reference point. Seizures were monitored behaviorally; most animals developed seizures characterized by salivation, forelimb clonus, and rearing before entering a state of continuous stage III/IV seizures according to the classification of Racine (Racine, 1972) that characterize status epilepticus. Seizures were terminated with diazepam (10-12 mg/kg, i.p., Steris Laboratories, Phoenix, AZ) 4 hours after the injection of pilocarpine, and dosing was repeated as necessary to prevent recurrence of seizure behavior. Following termination of seizures, all animals were hydrated with lactated Ringer's solution (Abbott Laboratories, North Chicago, IL) for the remainder of the day (5-10 ml/kg/hr, s.c.). Only animals that displayed continuous convulsive seizure activity were used in the experimental "seizure" group.

Despite treatment with pilocarpine, some animals failed to display any overt signs of seizure activity. These animals were found to be very similar to controls in their mRNA profiles, and so were merged into the control group for statistical analysis. In comparison to saline injected rats, the p value for all cytokines tested at each time point examined in

non-seizing animals was $p > 0.18$ (CNTF: p_{ave} for all time points = 0.617 ± 0.085, LIF: $p_{ave} = 0.356 \pm 0.057$, OSM: $p_{ave} = 0.325 \pm 0.137$, CT-1: $p_{ave} = 0.433 \pm 0.047$). Two exceptions to this generalization were the values for OSM in non-seizing animals at 2 hours ($p<0.05$) and 6 hours ($p<0.1$). Even at both of these times, however, the level of OSM in each group of non-seizure animals remains significantly different from the levels measured in animals in which pilocarpine injection produced seizure. Thus, the changes we find in cytokine expression are due to seizure and not to other effects of the various drugs employed. Additional control animals received atropine, valium and Ringer's injections, but were given saline instead of pilocarpine. Cytokine values in these rats displayed no significant difference from uninjected controls either. Animals maintained for more than 24 hours after seizure were given oral fluids and food as needed until they began feeding on their own.

RNAse Protection Assay. At time points ranging from 2 hours to 7 days (2, 6, 12, and 24 hours, 3 and 7 days) after pilocarpine injection, animals (n = 3-6 per time point for each group) were anaesthetized with Halothane and killed by decapitation. Additional uninjected, naive control animals (n=4) were used to determine basal expression levels. Bilateral hippocapmi were quickly dissected and immediately frozen on dry ice. Total RNA was extracted by a modification of the acid-phenol method (Chomczynski and Sacchi, 1987). Plasmids containing portions of rat CNTF, CT-1, and OSM were cloned by PCR from sciatic nerve (CNTF), superior cervical ganglia (CT-1) or P1 testis (OSM) cDNA. PCR fragments were ligated into pCRII (Invitrogen, San Diego, CA) and sequenced to confirm their identity. The CT-1 clone was a gift of Dr. Jr-Gang Cheng, and the LIF clone was previously described in this laboratory (Yamamori et al., 1989). The housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control and was a gift from Dr. Lisa Banner (Banner and Patterson, 1994). The RNAse protection analysis (RPA) was performed as described (Patterson and Fann, 1992), using 10 μg

(CNTF) or 20 μg (CT-1, LIF, OSM) total RNA per reaction. Briefly, plasmids were linearized and ^{32}P-labeled antisense probes generated by *in vitro* transcription were hybridized to total hippocampal RNA. After overnight hybridization at 55° C, reactions were digested with RNAse A and RNAse T1. Digestion was stopped with proteinase K and RNA was extracted with phenol-chloroform.

Reaction products were separated on denaturing 6% polyacrylamide gels to yield protected fragments of 266 nucleotides for CNTF, 198 nucleotides for CT-1, 388 nucleotides for LIF, 430 nucleotides for OSM and 133 nucleotides for GAPDH. Radioactivity was measured by scanning the protected fragments on a Phosphorimager 445SI (Molecular Dynamics, Sunnyvale, CA) and quantitated with ImageQuant software. The intensities of the protected fragments for LIF, CNTF, OSM, and CT-1 were compared to that of GAPDH for each reaction, and the ratio expressed in arbitrary units. Quantitation of GAPDH levels before and at various times after seizure reveal that the GAPDH mRNA levels do not change significantly with seizure. This is consistent with the finding that GAPDH mRNA levels do not change with neural injury (Banner, Maoyeri, and Patterson 1997), and support the validity of using this gene as an internal control for normalizing mRNA levels across samples. One animal was excluded from RPA calculations for OSM: at 24 hours after pilocarpine injection, the value obtained from this animal for OSM expression was >3-fold that of other animals at this time point. The trend of expression taken from time points surrounding 24 hours after seizure strongly suggested that this single value was an anomaly. All statistical analyses were performed using Microsoft Excel to determine significance by the two sample Student's t-test, assuming equal variances. Significance of the two-tailed p value was set at $p < 0.05$.

In Situ Hybridization. Antisense and sense digoxigenin-labeled RNA for LIF (0.6kb) were transcribed from the full length rat LIF plasmid used in the RNAse protection assay above. For OSM, a cDNA longer than that used for the RPA was needed to improve

sensitivity of the signal for *in situ* hybridization, and this was cloned by nested PCR from hippocampal cDNA obtained from an animal that had been subjected to pilocarpine seizure. Both the inner and outer primer PCR products were ligated into pCRII and sequenced to confirm their identity. This longer OSM cDNA (1.5 kb) was used for *in vitro* transcription. Both the LIF and the OSM digoxigenin-labeled RNA *in situ* probes were generated using the Megascript *in vitro* transcription kit (Ambion, Austin, TX) by adding digoxigenin-UTP (Boehringer Mannheim) to the reaction. The LIF probe was used at full length, while the OSM probe was hydrolyzed to an average length of 200 nucleotides.

Animals (n = 2-3 per time point) were anaesthetized with Halothane and killed by decapitation at 2 hours (OSM), 12 hours (LIF), or 7 days (OSM) after injection of pilocarpine. Uninjected control animals (n=3) were used for comparison to basal expression levels. Brains were removed and immediately frozen in pre-chilled isopentane. After embedding in Cryo-M-Bed (Bright, Huntingdon, UK), 20 μm frozen coronal sections were collected on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) through the septotemporal extent of the hippocampus, and slides were dried for one hour before being stored at -80° C. After air drying, sections were fixed with 4% paraformaldehyde for 20 minutes before digestion with 50 μg/ml Proteinase K in Tris-EDTA buffer (50 mM Tris pH 7.5, 5 mM EDTA) for 10 minutes at room temperature. Sections were then acetylated with acetic anhydride, and prehybridized at 60° C for several hours in hybridization buffer (50% formamide, 5x SSC, 50 μg/ml tRNA, 100 μg/ml heparin, 1x Denhardt's solution, 0.1% Tween-20, 0.1% CHAPS, 5 mM EDTA). This solution was replaced with hybridization buffer containing 1 μg/ml of digoxigenin-labeled RNA probe, and hybridization continued for an additional 12-16 hours at 60° C. At this point, the protocols for LIF and OSM differ slightly because the two probes displayed different characteristics of sensitivity and background. The LIF sections were washed several times in 0.2x SSC at 60° C, followed by two changes of PBS with 0.1% Triton-X and 2 mg/ml BSA (PBT) at room temperature; the OSM sections were washed with 1x,

1.5x, and 2x SSC before a post-hybridization RNAse treatment, followed by several washes in 0.2x SSC at 60° C. All sections (LIF and OSM) were next blocked with 20% sheep serum and 2% Blocking Reagent (Boehringer Mannheim) for several hours at room temperature. The blocking solution was replaced with anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:2000 in blocking solution and the sections were incubated overnight at 4° C. After several 30 minute washes with PBT, staining was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim).

Immunohistochemistry. Following *in situ* hybridization, sections were rinsed several times with PBS and refixed in 4% paraformaldehyde for 20 minutes at room temperature before endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in PBS for 30 minutes. Slides were blocked with several changes of 5% goat serum in PBS with 0.1% Triton-X, followed by incubation with primary antibody diluted in blocking solution overnight at 4° C. The antibodies used were against GFAP (rabbit anti-cow polyclonal 1:500, Accurate Chemical and Scientific Corp., Westbury, NY), neurofilament 160 (NF160)(mouse anti-pig monoclonal 1:100, Sigma), and mixed neurofilament SMI311 (mouse mixed monoclonal, 1:750, Sternberger Monoclonals Inc., Baltimore, MD). Sections were washed several times with blocking solution , followed by incubation for one hour with secondary antibody conjugated to horseradish peroxidase diluted 1:200 in blocking solution; GFAP (goat anti-rabbit polyclonal, Boehringer Mannheim), NF160 and SMI311 (goat anti-mouse polyclonal, Chemicon, Temecula, CA). Following several washes with Tris-Imidazole buffer (TI: 50 mM Tris, pH 7.5, 20 mM Imidazole, 0.1% Triton-X), the peroxidase reaction product was developed with 0.04% diaminobenzidene (Sigma) in TI buffer.

RESULTS

Time course of cytokine expression following pilocarpine seizure. To examine the effect of prolonged seizure activity on the expression of neuropoietic cytokines in the hippocampus, pilocarpine was used to induce status epilepticus in adult rats, and seizures were terminated four hours later with valium. Animals were sacrificed at time points ranging from 2 hours (during seizure activity) to 7 days (during the silent period) after seizure, and cytokine mRNA expression in the hippocampus was determined by RNAse protection assay.

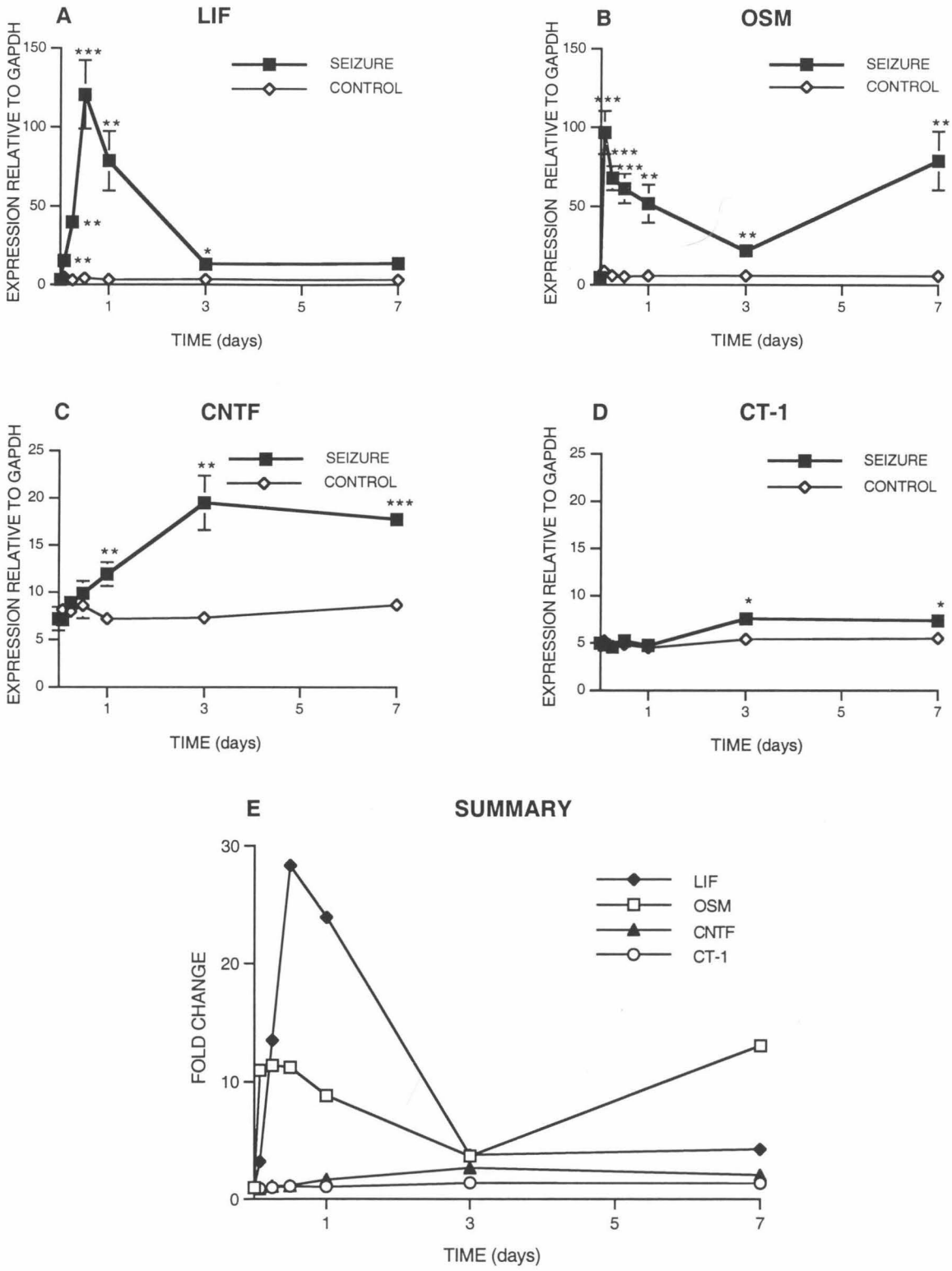
Each of the cytokines examined, LIF, OSM, CNTF, and CT-1 displays a distinct time course and relative increase in expression following seizure. The first cytokine to be induced following pilocarpine injection is OSM. Expression in the hippocampus increases nearly 11-fold by the first time point examined, 2 hours after pilocarpine injection (Fig. 1*B*). The level of OSM mRNA decreases steadily to a low of 3.7-fold over control at 3 days, before a second phase of OSM induction raises levels at 7 days to 13-fold over the control value. Basal OSM expression is relatively low, similar to that of LIF and CT-1, and the response to seizure is significant at all time points tested ($p<0.01$, Students t-Test, 2 sided). One animal was excluded from these calculations (see Materials and Methods). Calculations of significance at 24 hours, and the data presented in Figure 1 were therefore determined after exclusion of this animal.

The largest change in neuropoietic cytokine expression following pilocarpine seizure is with LIF mRNA. While LIF expression peaks later than OSM, LIF mRNA rises to 30-fold higher than control values at 12 hours after pilocarpine injection ($p<0.001$)(Fig. 1*A*). In addition, unlike OSM, LIF displays only a single peak in expression, and returns nearly to basal levels by 3 days after seizure. The difference between seizure and control animals is statistically indistinguishable at one week post-seizure.

The changes in CNTF and CT-1 mRNA levels following seizure are much less dramatic than for OSM or LIF. The first significant rise in CNTF mRNA is not detected

Figure 1. Up-regulation of cytokine expression in the hippocampus following status epilepticus. Graph shows level of expression for pilocarpine-treated status epilepticus (*seizure*) animals and combined pilocarpine-treated non-seizure and saline-treated (*control*) animals at six time points after drug administration. Levels of LIF (*A*), OSM (*B*), CNTF (*C*), and CT-1 (*D*) mRNA were assayed by semi-quantitative RNAse protection assay, and are expressed in arbitrary units as a fraction of the level of GAPDH used in each reaction as an internal control. Each time point represents the mean cytokine:GAPDH expression ratio of 3-6 animals, ± SEM. *Asterisks* denote statistically significant differences from controls at each time point (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; two-tailed Student's t test). Note the change in scale of expression ratio between *A, B* and *C, D*. *E.* Summary of changes in cytokine expression after status epilepticus. Values for each cytokine are displayed as fold change from control values for that cytokine at each time point. Error bars have been omitted for clarity. Of the four cytokines examined, levels of LIF mRNA are changed most dramatically, while OSM increased most quickly.

Figure 1


A
LIF
SEIZURE
CONTROL
EXPRESSION RELATIVE TO GAPDH
TIME (days)
B
OSM
C
CNTF
D
CT-1
E
SUMMARY
FOLD CHANGE

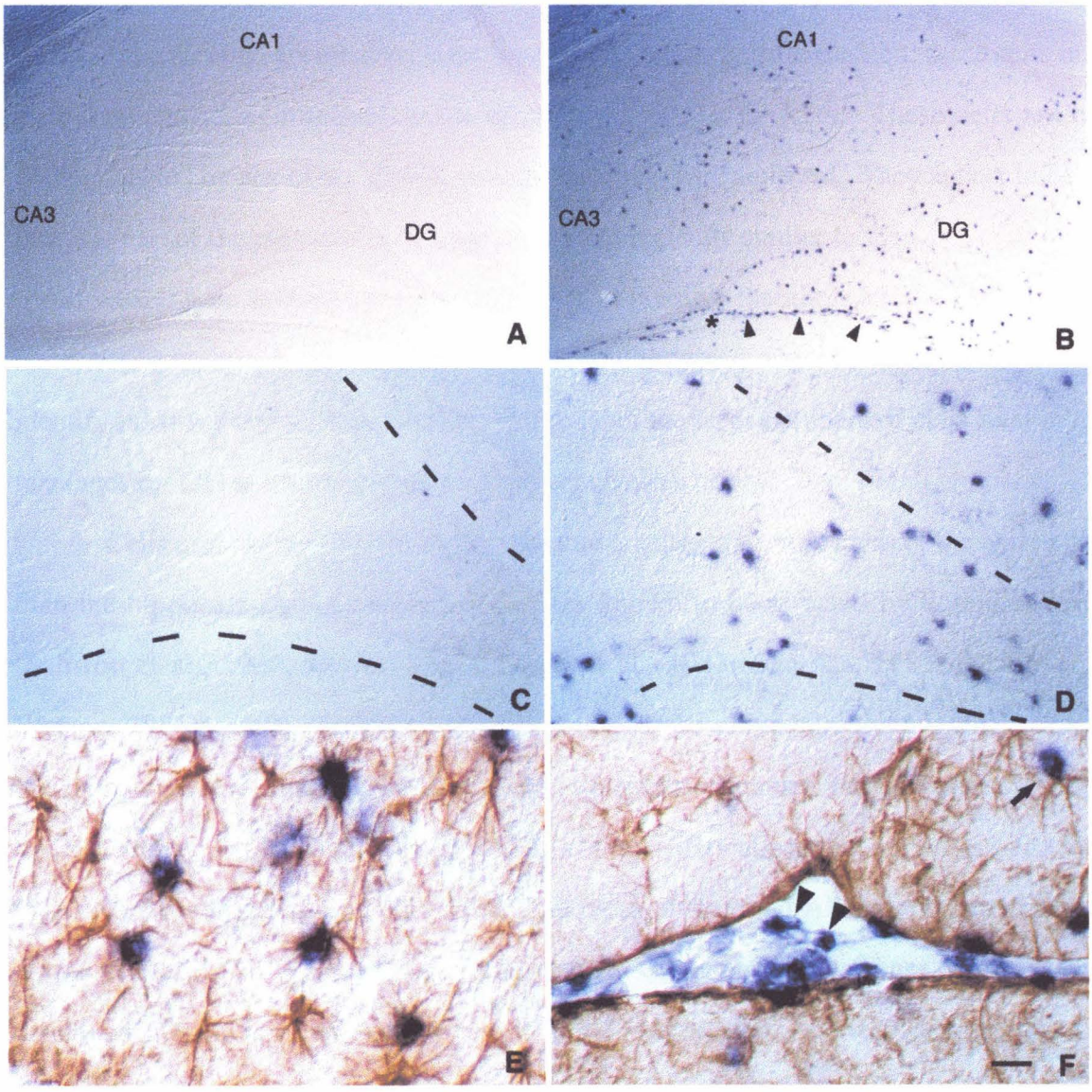
until 24 hours after pilocarpine injection ($p<0.01$)(Fig. 1*C*). Expression is maximal at 3 days, when it reaches values 2.5-fold over baseline ($p<0.01$). The most statistically significant difference in CNTF expression between seizure and control occurs at 7 days ($p<0.001$), with values more than twice baseline. Seizure increases CT-1 mRNA expression in the hippocampus very little. At 3 days and 7 days, experimental levels are only 40% ($p<0.05$) and 35% ($p<0.05$) greater than those for controls (Fig. 1*D*). The major differences in the relative cytokine mRNA levels and their time course are better appreciated when these data are plotted on the same graph (Fig. 1*E*).

Localization of LIF mRNA in the hippocampus following seizure. Given the large changes in LIF and OSM expression evoked by seizure, it is important to identify the cells responsible for these changes. Animals were subjected to pilocarpine-induced status epilepticus and sacrificed at the times of peak cytokine mRNA expression previously determined by RNAse protection analysis. Following *in situ* hybridization, identification of cell types was accomplished by double staining with immunohistochemical markers.

In situ hybridization for LIF mRNA at 12 hours after pilocarpine injection reveals three areas of elevated expression within the hippocampus: the dentate gyrus, the subiculum, and the meninges at the ventral border of the hippocampus (Fig. 2*B* and *D*). In coronal sections, the distribution of LIF^+ cells in the hippocampus appear to cluster around the dorsal ventricle: cells in both the subiculum and the dentate gyrus are most dense medially. Within the hippocampus, fewer, less intensely stained cells are noted in area CA1, while almost no LIF^+ cells are seen in area CA3. In the dentate gyrus, LIF^+ cells are present in both the polymorph (hilus) and molecular layers (Fig. 2*D*), while in CA1, LIF^+ cells are present in both the stratum oriens and stratum radiatum. The LIF^+ cells in all subfields were identified as astrocytes by double-labeling with a GFAP antibody (Fig. 2*E*). While there is evidence that LIF mRNA is expressed at basal levels in neurons of the hippocampus (Lemke et al., 1996), the high stringency conditions used for *in situ*

Figure 2. Localization of LIF mRNA expression in the hippocampus following status epilepticus. *In situ* hybridization for LIF mRNA in untreated animals (*A, C*) and in animals sacrificed 12 hours after pilocarpine-induced seizure (*B, D, E, F*). *A, B.* Low power photomicrographs of coronal sections through the hippocampus show distribution of digoxigenin-labeled LIF cRNA (*blue*). Conditions used for hybridization do not reveal basal LIF expression present in the unstimulated brain that can be detected by RPA (*A, C*). *C, D.* Higher power photomicrographs display staining of individual cells in the dentate gyrus of different sections than pictured in *A, B*. *Dashed lines* indicate the granule cell layer in each section. LIF^+ cells are found in all layers of the dentate gyrus. *E , F.* Immunohistochemistry with GFAP (*brown*) after LIF *in situ* hybridization reveals LIF^+ cells within the parenchyma are $GFAP^+$ astrocytes (*E*), while LIF^+ cells in the pia mater ventral to the dentate gyrus are $GFAP^-$ (*arrowhead* in *F).* Location of cells shown in *F* is indicated by an *asterisk* in *B*; *arrowheads* in *B* indicate extent of meningeal LIF staining (shown enlarged in *F*) in a typical coronal section. Scale bars: *A, B* = 200 μm; *C, D* = 48 μm; *E* = 17 μm; *F* = 21 μm.

Figure 2



hybridization in this study did not permit visualization of LIF expression in control animals (Fig. 2*A* and *C*).

One of the more striking and unexpected areas of LIF up-regulation is in the meninges ventral to the dentate gyrus (Fig. 2*B* and *F*). In some sections, $LIF^+/GFAP^-$ cells in the meninges are seen along the entire extent of the hippocampus (Fig. 2*F*). Because the meninges were left on the hippocampus during its dissection, these cells may have contributed significantly to the quantitation of LIF by RPA. These cells are not double-labeled by any of the glial or neuronal markers we employed. They appear to be an intrinsic part of the pia mater, and they are morphologically similar to the LIF^+ cells of the blood vessels seen in the adjacent thalamus. It is possible that these LIF^+ cells in the pia mater could be invading mast cells, however antibody and histochemical stains used to identify mast cells in adjacent sections fail to label the large numbers of cells seen in the meninges by LIF *in situ* hybridization (data not shown).

Cells expressing LIF mRNA after seizure are also present in areas of the brain other than the hippocampus, and include structures known to be affected by limbic seizures (Schwob et al., 1980; Nadler, 1981; Turski et al., 1983; Ben-Ari, 1985; Paxinos and Watson, 1986). Cells expressing LIF are found in the amygdala, the amygdalostriatal transition area, several dorsal thalamic nuclei, the caudate putamen and the cortex (Fig 4*A*). In areas where GFAP is highly expressed, almost all LIF^+ cells are identified as astrocytes (Fig. 4*B*). The thalamus and the cortex, both areas of lower GFAP staining, are the only places where $LIF^+/GFAP^-$ cells are found in the brain parenchyma. These two areas also display prominent LIF^+ mRNA staining in blood vessels; in the cortex, radial vessels are lined with LIF^+ cells (Fig. 4*D*). In the thalamus, these cells can be clearly identified as part of the blood vessel wall and are $GFAP^-$ (Fig. 4*E*); in the cortex, the presence of GFAP in the end feet of astrocytes surrounding the blood vessels often makes this distinction difficult. The ventral cortex also displays LIF^+ cells along the pial surface; both $GFAP^+$ cells of the glia limitans and meningeal $GFAP^-$ cells are common (Fig. 4*C*).

Localization of OSM mRNA in the hippocampus following seizure. *In situ* hybridization for OSM mRNA at the peak expression times, two hours and 7 days after seizure, yields very different results. At two hours after pilocarpine injection, a small but strongly stained population of cells (approximately <5% of the cells) is positive for OSM, while at 7 days, few if any cells are stained above background. The RPA data, however, indicate that the total amount of hippocampal OSM mRNA is similar at these two time points. Therefore, our *in situ* hybridization results are consistent with the hypothesis that a small population of cells is strongly induced at 2 hours while many more cells are induced at 7 days, but to a much lower level. At two hours, outside of the neuronal cell body layers, OSM^+ cells are almost evenly distributed across the hippocampus (Fig. 3*B* and *D*). A slightly greater number of positive cells are in the dentate gyrus than in areas CA1 or CA3, along with a slight preference for stratum oriens in CA1 and the polymorph layer of the dentate (hilus) (Fig. 3*D*). Unlike LIF expression, no bias towards the ventricle and little staining in the meninges is observed with OSM. Also strikingly different from LIF, almost no OSM^+ cells are $GFAP^+$ astrocytes (Fig. 3*E*). A few of the OSM^+ cells could be positively identified as neurons by double-labeling with intermediate (NF160) or mixed (SMI311) neurofilament antibodies (Fig. 3*F*). The OSM^+ cells have large cell bodies, are localized outside the projection neuron layers, and lack GFAP staining, suggesting that they are hippocampal interneurons. We have attempted to double-label these OSM^+ cells with a variety of interneuron markers, but have not yet succeeded in finding a suitable marker that survives the *in situ* hybridization procedure.

As within the hippocampus, the OSM^+ cells outside of the hippocampus were evenly though sparsely distributed. At two hours after pilocarpine injection, the cortex, corpus callosum, amygdala, hypothalamus and some parts of the thalamus contain a small subpopulation of OSM^+ cells (Fig. 5*A*). As in the hippocampus, almost none of the OSM^+ cells are $GFAP^+$ astrocytes. Especially within the cortex, these $OSM^+/GFAP^-$ cells

Figure 3. Localization of OSM mRNA expression in the hippocampus during status epilepticus. *In situ* hybridization for OSM mRNA in untreated animals (*A, C*) and in animals sacrificed 2 hours after pilocarpine injection, during seizure (*B, D, E, F*). *A, B.* Low power photomicrographs of coronal sections through the hippocampus show distribution of digoxigenin-labeled OSM cRNA (*blue*). *Arrowheads* indicate representative OSM^+ cells. Conditions used for hybridization do not reveal basal OSM expression present in the unstimulated brain that can be detected by RPA (*A, C*). *C, D.* Higher power photomicrographs display staining of individual cells in the dentate gyrus of different sections than pictured in *A, B*. *Dashed lines* indicate the granule cell layer in each section. OSM^+ cells are preferentially located in the polymorphic layer of the dentate gyrus. *E.* Immunohistochemistry for GFAP (*brown*) after *in situ* hybridization for OSM reveals that most (estimated >90%) OSM^+ cells are not $GFAP^+$ astrocytes, although occasional $OSM^+/GFAP^+$ cells can be found. *F.* Immunohistochemistry for mixed neurofilaments (SMI311, *brown*) after OSM *in situ* hybridization demonstrates that some OSM^+ cells in the hilus can be identified as $SMI311^+$ neurons (*arrowhead, inset*). Scale bars: *A, B* = 200 μm; *C, D* = 54 μm; *E* = 23 μm; *F* = 21 μm.

Figure 3

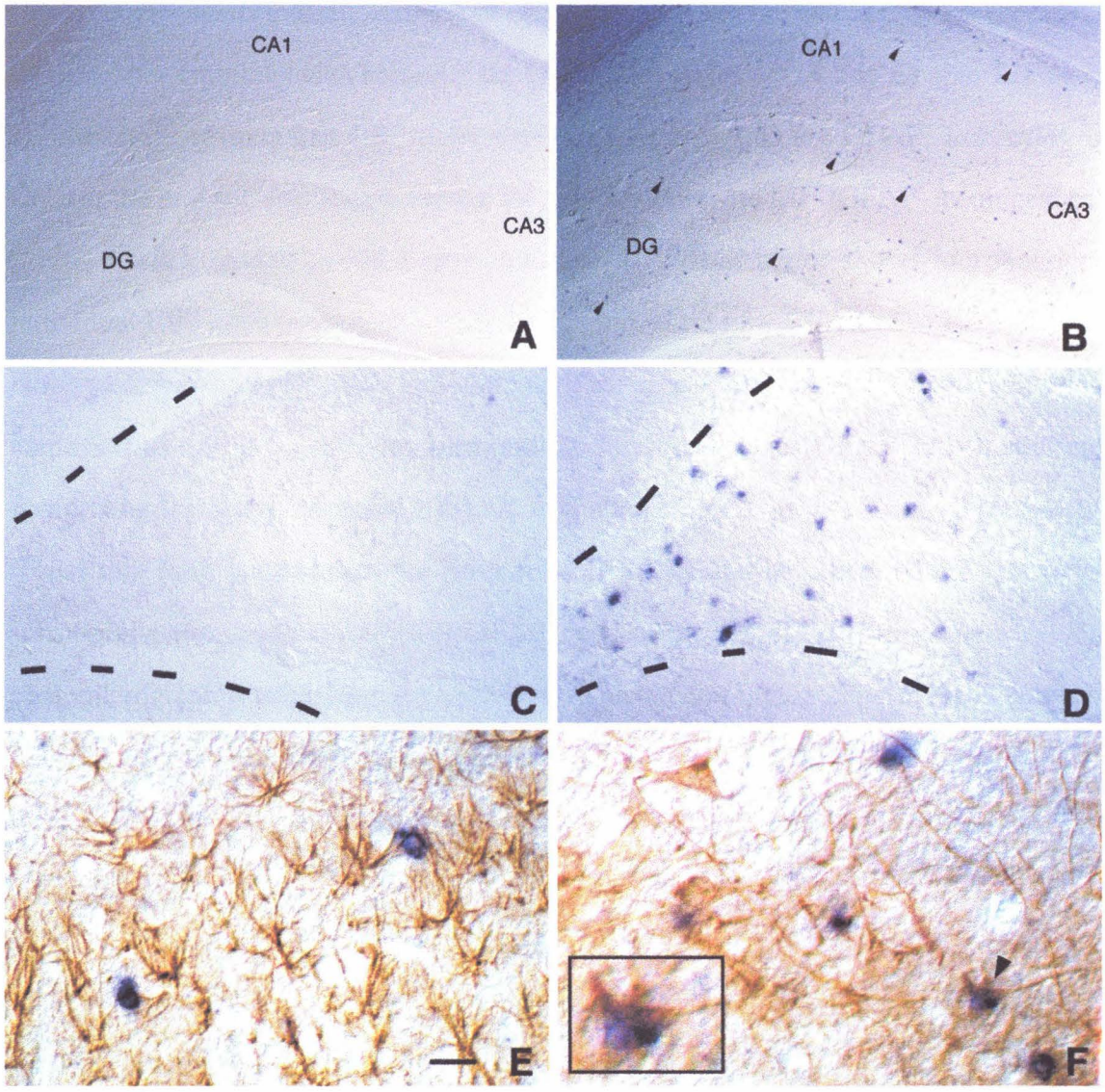
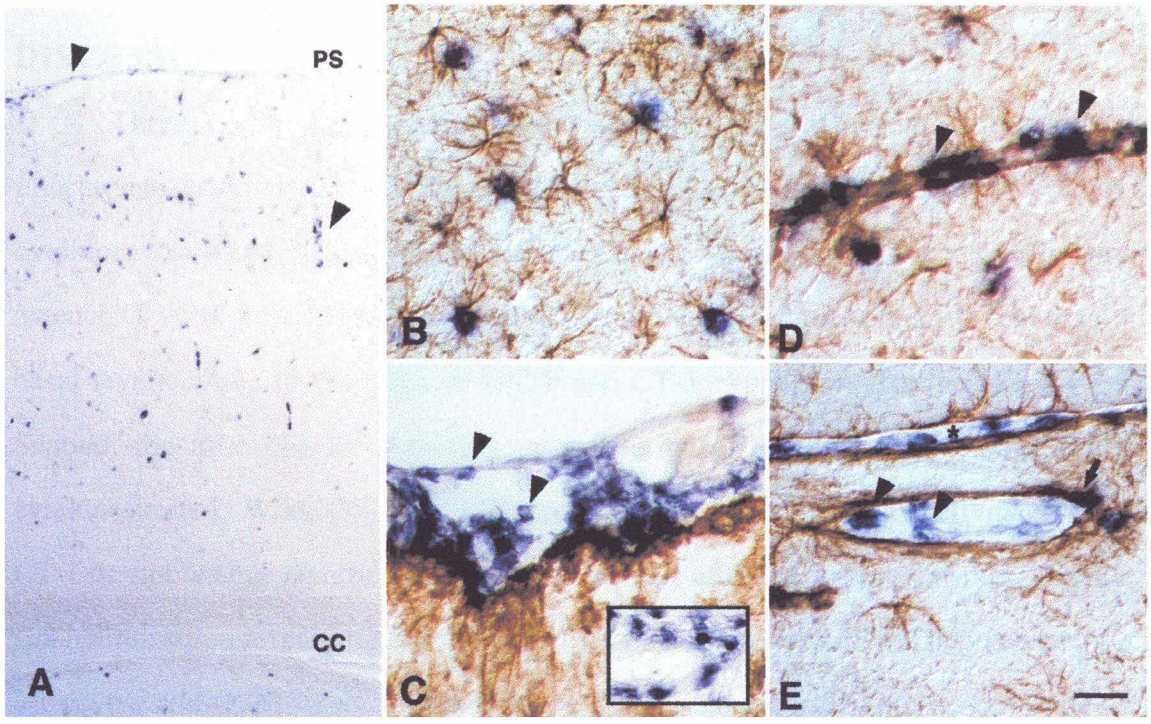


Figure 4. Localization of LIF mRNA expression in areas outside of the hippocampus after status epilepticus. *In situ* hybridization for LIF mRNA in animals sacrificed 12 hours after pilocarpine-induced seizure. *A.* Low power photomicrograph of a coronal section through the somatosensory cortex shows distribution of digoxigenin-labeled LIF cRNA (*blue*). *Arrowheads* indicate staining of cells at the pial surface and in radial cortical blood vessels. *B.* Immunohistochemistry for GFAP (*brown, B, C, D,* and *E*) after LIF *in situ* hybridization reveals that LIF^+ cells within the parenchyma are $GFAP^+$ astrocytes. *C.* Cells at the pial surface, shown here at the rhinal fissure, are $LIF^+/GFAP^-$ meningeal cells (*arrowheads*), similar to those seen in Figure 2F. *Inset* better shows morphology of individual LIF^+ cells at the pial surface before processing for GFAP. *D.* Radial cortical blood vessels also contain LIF^+ cells, although when seen in a transverse plane, can also be identified as $GFAP^+$ astrocytes surrounding the vessel, or as $GFAP^-$ endothelial cells comprising the vessel (*arrowheads*). *E.* Thalamic blood vessels seen in cross section reveal that both possibilities are present: LIF^+ cells can be either $GFAP^+$ astrocytes surrounding the vessel (*arrow*), or $GFAP^-$ cells of the vessel wall (*arrowheads*). This photomicrograph is taken from the same section pictured in Figure 2F; *asterisk* indicates the $LIF^+/GFAP^-$ cells of the pia mater shown in 2F. Scale bars: *A* = 200 μm, *B, C, E* = 32 μm; D = 28 μm . *ps*, pial surface, *cc*, corpus callosum.

Figure 4



have large cell bodies, are localized in all cortical layers, and can occasionally be co-stained with neurofilament markers (Fig. 5*B* and *C*). Combined with the relatively fast up-regulation of OSM, these characteristics are suggestive of cortical interneurons. Overall, both within and outside of the hippocampus, the pattern of OSM expression is quite distinct from that of LIF; most OSM^+ cells are likely to be neurons, while LIF^+ cells are either astrocytic or other non-neuronal cells. In addition, the distribution of OSM^+ cells is generally diffuse across the brain while LIF^+ cells are concentrated in particular areas.

DISCUSSION

Three key findings emerge from our study of cytokine expression following pilocarpine-induced status epilepticus. First, after a single prolonged seizure each of the cytokines examined, LIF, OSM, CNTF, and CT-1, is up-regulated, but to greatly varying extents. Two of the cytokines, LIF and OSM, are increased more than 10-fold over their basal levels, while the changes in CNTF and CT-1 expression after seizure are small. Second, the time course of mRNA up-regulation differs markedly among the four cytokines tested. While OSM and LIF respond within hours to seizure activity, CNTF and CT-1 do not change significantly until several days after the seizure episode. Finally, *in situ* hybridization reveals that LIF and OSM are induced in separate cell populations; LIF in astrocytes and OSM likely in interneurons. Although they may converge on the same signal transducing pathways, each of these four cytokines is differentially regulated by seizure. Thus, the functional redundancy that these cytokines display *in vitro* may not be true for the response to seizure *in vivo.*

The pilocarpine model of status epilepticus has several advantages over other seizure paradigms, particularly for studying cytokines and inflammatory mediators.
Most importantly, seizure initiation is non-invasive, as pilocarpine is administered peripherally, without the need for mechanical injury to the brain produced by intracranial kindling or lesion electrodes. We have found in related studies that the insertion of

Figure 5

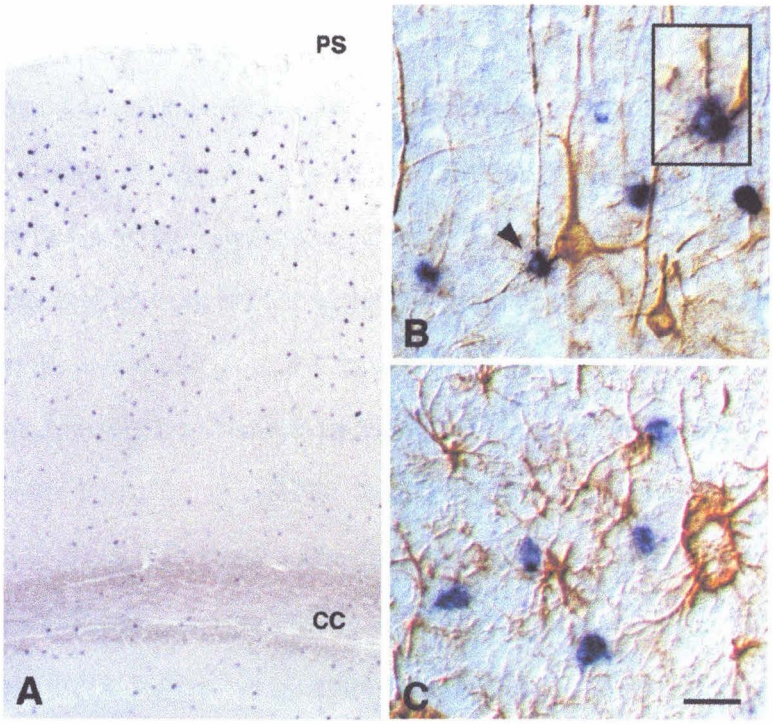


Figure 5. Localization of OSM mRNA expression in the cortex after status epilepticus. *In situ* hybridization for OSM mRNA in animals sacrificed 2 hours after pilocarpine-injection, during seizure. *A.* Low power photomicrograph of a coronal section through the parietal cortex shows widespread distribution of digoxigenin-labeled OSM cRNA (*blue*), with strongest staining in layers II/III. *B.* Immunohistochemistry for mixed neurofilaments (SMI311, *brown*) after OSM *in situ* hybridization demonstrates that some OSM^+ cells in the cortex can be identified as $SMI311^+$ neurons (*arrowhead, inset*). *C.* Immunohistochemistry for GFAP (*brown*) after OSM *in situ* hybridization is consistent with OSM up-regulation in neurons, as most OSM^+ cells are not $GFAP^+$ astrocytes. Scale bars: *A* = 200 μm, *B* = 28 μm, *C* = 24 μm. *ps*, pial surface, *cc*, corpus callosum.

electrodes required to perform electrophysiology can significantly induce certain pro-inflammatory cytokines (J.L. Jankowsky, B.E. Derrick, and P.H. Patterson, in preparation). Seizure-induced neuronal damage in the pilocarpine model is, however, more widespread than in kindling models. Nonetheless, pilocarpine-induced seizures do give rise to sequelae that are similar in many respects to human temporal lobe epilepsy. Pilocarpine-treated animals develop spontaneous recurrent seizures indicative of the human syndromes that often do not develop in other animal models of epilepsy (Mello et al., 1993). Prior to the onset of spontaneous seizures, several pathological alterations occur in the hippocampus that may contribute to the development of seizure recurrence: cell death, cell birth, granule cell dispersion, and mossy fiber sprouting have all been characterized following pilocarpine-induced status epilepticus (Turski et al., 1989; Mello et al., 1992; Mello et al., 1993; Parent et al., 1997). Reactive gliosis, a consequence of seizure often seen in human epilepsy, has been better documented in the kainate or kindling models of epilepsy than in the pilocarpine model (Andersson et al., 1991; Marty et al., 1991; Jorgensen et al., 1993; Niquet et al., 1994; Represa et al., 1995; Khurgel and Ivy, 1996). However, the present results demonstrating elevated LIF expression within the hippocampus exclusively in astrocytes indicates that glia are indeed affected following pilocarpine-induced seizure.

Our results demonstrating the up-regulation of inflammatory cytokines during and after seizure highlights possible similarities between the consequences of seizure and other types of insult such as ischemia and cortical lesion, a comparison that has been made previously (Lindvall et al., 1992; Bengzon et al., 1993; Merlio et al., 1993). Clearly, some aspects of injury and repair are common to all such insults, including cell death, reactive gliosis, and synaptic reorganization. Thus, comparison of prior work on LIF and CNTF in brain trauma to our current findings with seizure may be quite relevant for understanding pathological events in both forms of brain injury. Interestingly, up-regulation of LIF following pilocarpine-induced status epilepticus peaks by 12 hours after

seizure before returning nearly to control levels by 3 days, a time course very similar to that observed following a cortical stab wound (Banner et al., 1997). In both paradigms LIF expression peaks within hours of insult, and within the parenchyma, it is localized almost exclusively to GFAP$^+$ astrocytes. Up-regulation of LIF mRNA in cells of the pia mater outside of the brain is a novel observation. It is noteworthy that LIF expression is increased in these cells outside of the brain with the same time course as in the glial cells of the parenchyma. The delayed response of CNTF after pilocarpine-induced seizure reported here was also observed after cortical aspiration lesion (Ip et al., 1993). In both seizure and lesion injury models, CNTF expression does not peak until 3 days after insult, and remains elevated for the duration of each study. While not localized after seizure, after lesion CNTF-expressing cells, like LIF-expressing cells, are GFAP$^+$ astrocytes.

Since the chronology of pathological changes in the hippocampus after seizure has been carefully characterized, and the pattern of cytokine up-regulation in at least two cases is similar after seizure and cortical injury, correlating the timing of cytokine up-regulation with the occurrence of events after seizure may help elucidate the role of these factors in both seizure-induced changes in the hippocampus and in more general instances of brain injury. With its maximum expression at 12 hours after seizure, it is possible that LIF could be affecting events that occur within the first few days of insult, such as cell death and reactive gliosis. Under different conditions in culture, LIF can enhance neuronal survival or increase neuronal death (Kessler et al., 1993; Kotzbauer et al., 1994; Murphy et al., 1997). There is considerable evidence that LIF also regulates astrocytes in culture. *In vitro*, LIF is expressed by astrocytes and their precursors, and it affects gene expression in these cells, particularly GFAP (Murphy et al., 1997). Study of the brains of mice in which the gene for LIF has been disrupted reveals that GFAP immunoreactivity is also altered *in vivo* (Bugga et al., 1998; Koblar et al., 1998).

The delay of CNTF up-regulation, peaking at 3 days after seizure, makes it an unlikely initiator of astrogliosis, but it could be a downstream effector (Levinson et al.,

1996). Alternatively, since astrocytes are thought to normally express CNTF at basal levels, the observed increase in CNTF mRNA after seizure may simply reflect an increase in the total number of astrocytes expressing this basal level of mRNA, and not an increase in CNTF expression in any one cell or cell type. There is evidence suggesting that astrocytes are induced to proliferate following kindling-induced seizures (Khurgel and Ivy, 1996). This hypothesis might also explain why we observed only a 2.5-fold increase in CNTF after seizure, while Ip et al. (Ip et al., 1993) saw a more than 7-fold increase after cortical lesion: a traumatic injury such as aspirative lesion may induce greater glial proliferation than prolonged seizure. The continued expression of CNTF after its peak at 3 days also correlates with the onset of mossy fiber sprouting. Previous work has shown that exogenous CNTF can induce sprouting of CNS axons *in vivo*(Murphy et al., 1997). Candidate factors that might affect axonal sprouting are rare at this point: only a handful of growth factors and cytokines have been examined more than one week after seizure; of those, even fewer are found to be expressed at elevated levels at that time (Gall et al., 1991; Gall et al., 1994; Riva et al., 1994; Garcia et al., 1997; Elmer et al., 1998).

While LIF and CNTF had previously been shown to be present at low levels and up-regulated in response to injury in the brain, neither OSM nor CT-1 expression had been well characterized in the CNS. Our work provides the first evidence of OSM expression in the brain, its regulation by synaptic activity, and its *in situ* localization after seizure. We find that OSM is expressed at low levels in the adult hippocampus under normal conditions, and is rapidly up-regulated by seizure activity to nearly 10-fold above control levels. *In situ* hybridization for OSM at this time, 2 hours after pilocarpine injection, reveals its expression across all fields of the hippocampus, as well as in the cortex and amygdala. The OSM^+ cells of the hippocampus are predominantly $GFAP^-$, localized outside of the neuronal cell body layers, and can occasionally be co-labeled with markers for neurofilament, suggesting they are hippocampal interneurons.

Although nothing is known about the function of OSM in the CNS, our findings suggest at least two possible roles for this cytokine. First, based on its rapid up-regulation and the fact that other members of the neuropoietic cytokine family can alter synaptic plasticity (Li et al., 1997), it is possible that OSM alters the extent of seizure by affecting neuronal physiology. This cytokine could either exacerbate or suppress seizure activity in the hippocampus, depending on whether it acts in an autocrine manner on GABA-ergic interneurons or in a paracrine manner on nearby glutamatergic granule and pyramidal neurons. This hypothesis assumes that OSM decreases neuronal excitability; there is no *a priori* reason to suppose it could not increase excitability. A second possible function of OSM at this very early time point after seizure onset would be the regulation of other cytokines and growth factors. For instance, OSM can induce IL-6 *in vivo* and *in vitro* (Brown et al., 1991; Richards and Agro, 1994; Wallace et al., 1995), as well as vasoactive intestinal protein and tissue inhibitor of metalloproteinase-1 *in vitro* (Rao et al., 1992; Fann and Patterson, 1994b; Richards et al., 1997). Importantly, all of these potential downstream mediators are also affected by seizure (Minami et al., 1991; Greenwood et al., 1994; de Lanerolle et al., 1995; Rivera et al., 1997). Oncostatin M expression after seizure is biphasic; its mRNA increases to a second peak at 7 days, the latest time point examined. The second expression peak correlates with the beginning of mossy fiber sprouting after seizure, so that would be a point of departure for further investigation.

A variety of intercellular signaling molecules, including growth factors and neurotrophins, have been examined in models of epilepsy ranging from kindling to kainic acid. Expression of most, including NGF, BDNF, and bFGF, is transiently increased after seizure (NT-3 is an exception), and in cases where the experiment tested later time points, expression returns towards basal levels within several days (Gall et al., 1991; Bugra et al., 1994; Gall et al., 1994; Riva et al., 1994; Schmidt-Kastner et al., 1996; Schwarzer et al., 1996). The role of these factors in seizure *in vivo* is not clear, however. Mice heterozygous for the BDNF-gene deletion have a higher threshold for kindling

development (Kokaia et al., 1995). While this neurotrophin can stimulate mossy fiber sprouting in collagen explant cultures, such mossy fiber sprouting is not impaired in slice cultures from homozygous BDNF-deficient mice (Lowenstein and Arsenault, 1996; Bender et al., 1998). The strongest evidence that neurotrophic factors have a role in post-seizure changes in the hippocampus has been suggested by experimentally altering NGF levels in the hippocampus during kindling. When NGF was artificially elevated during the kindling period, seizure development and mossy fiber sprouting were enhanced, and the number of cells lost from the hilar area after seizure was decreased (Adams et al., 1997). Conversely, when anti-NGF antibody was injected locally during kindling, seizure development was delayed and mossy fiber sprouting was reduced (Holtzman and Lowenstein, 1995; Van der Zee et al., 1995).

The identification and characterization of transcripts up-regulated in response to seizure is an important step in understanding how the brain responds to and recovers from traumatic insult. Correlation of the time course of induction with other events evoked by seizure can provide important clues as to possible roles for these factors. Perturbation experiments can now be envisioned with such clues in mind. That evidence could, in turn, provide further understanding of the molecular basis for the pathology and recovery from seizures that is essential for the design of future therapeutic interventions.

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Chapter 4

Leukemia inhibitory factor is required for the astroglial activation that follows ECS-induced seizure

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ABSTRACT

Many forms of neural injury provoke a strong reaction in nearby astrocytes, which is characterized in part by the up-regulation of glial fibrillary acidic protein (GFAP). The factors that mediate this response, and the role of reactive astrocytes in neural injury have not been well characterized, however. The electroconvulsive shock (ECS) model of seizure provides a way to study the up-regulation of GFAP in the absence of cell death. Previous work using ECS had found that GFAP up-regulation is sensitive to protein synthesis inhibitors for a period of 12 hours after the seizure. The timing of this sensitivity is consistent with the hypothesis that a seizure-induced protein mediator is responsible for increased GFAP expression. The cytokine leukemia inhibitory factor (LIF) is a good candidate for mediating the activation of astrocytes because we find that LIF mRNA is up-regulated by ECS-induced seizure and others have found that LIF regulates astrocyte GFAP expression in culture. We tested this hypothesis by evoking ECS in LIF knockout and littermate wild type mice. We find that the GFAP response to ECS is significantly reduced in LIF null mutant mice compared to controls. Thus, LIF plays a key role in the glial response to seizure.

INTRODUCTION

Although the astrocytic reaction to seizure activity has been well characterized, the molecular mechanisms responsible for many of the changes in morphology and gene expression are poorly understood. One of the hallmarks of reactive astrocytes is up-regulation of the cytoskeletal component glial fibrillary acidic protein (GFAP). While this response is most often seen in association with local tissue damage and neuronal death, one seizure model, electroconvulsive shock (ECS), can be used to dissociate these events. Single electroconvulsive seizures result in GFAP mRNA up-regulation in the absence of neuronal loss, while multiple seizures result in both GFAP mRNA and protein up-regulation, again in the absence of neuronal loss (Orzi et al., 1990; Steward, 1994). The

ECS-induced up-regulation of GFAP mRNA is sensitive to cycloheximide treatment for up to 12 hours after the seizure event, implicating *de novo* protein synthesis in the GFAP induction. This suggests that a newly synthesized protein factor is responsible for mediating GFAP expression.

A candidate for the GFAP-regulating protein is leukemia inhibitory factor (LIF). Expression for LIF is strongly up-regulated in the hippocampus by pilocarpine-induced seizure, peaking during the cycloheximide-sensitive time frame of GFAP induction (Jankowsky and Patterson, 1999). Moreover, exogenous LIF can stimulate the GFAP expression in astrocyte progenitor cells *in vitro* (Murphy et al., 1997). In addition, endogenous LIF is required for normal GFAP immunostaining in the female mouse hippocampus, both during development and in adulthood (Bugga et al., 1998; Koblar et al., 1998).

To test the hypothesis that LIF mediates GFAP mRNA induction after ECS, we first established the timing and extent of LIF mRNA up-regulation by ECS in wild type (WT) mice. Finding that LIF is strongly and rapidly up-regulated, we then asked whether GFAP expression after ECS is altered in the absence of LIF. Using LIF KO mice, we find that the induction of GFAP mRNA following ECS requires LIF.

MATERIALS AND METHODS

Electroconvulsive shock. We have maintained LIF KO mice (Stewart et al., 1992) within a colony, with occasional back-crossing to the C57BL/6 parental strain for increased viability and fertility. A PCR-based method was used to genotype the mice (Banner et al., 1998). Electroconvulsive shock seizure (ECS) was induced in adult male LIF null and LIF wild type (WT) mice (6-8 months of age, about 20-30 g) by delivering a 9 mA pulse for 0.5 seconds via ear clip electrodes. The resulting seizure was characterized by immediate tonic extension lasting for 10-20 seconds, followed by a brief period of tonic-clonic movements. Although most animals recovered spontaneously, some animals ceased

breathing and required resuscitation. Mice typically exhibited a post-ictal behavioral depression for 5-10 minutes, and thereafter appeared behaviorally alert.

Non-radioactive in situ *hybridization.* Antisense and sense digoxigenin-labeled LIF cRNAs (0.4 kb) were transcribed from a partial mouse LIF cDNA plasmid. Digoxigenin-labeled RNA *in situ* probes were generated using the Megascript *in vitro* transcription kit (Ambion, Austin, TX) by adding digoxigenin-UTP (Boehringer Mannheim) to the reaction. The LIF probes were used at full length for hybridization.

An initial series of WT mice were sacrificed at various times (6, 12, 24, and 48 hours, n=1 per time point) after a single ECS to determine the time course of LIF mRNA induction. Animals were killed by anesthetic overdose and transcardially perfused with 4% paraformaldehyde in PBS. Brains were removed and post-fixed overnight in 4% paraformaldehyde, and then cryoprotected in 15% sucrose for an additional 24 hours.

Additional WT mice were anaesthetized with Halothane and killed by decapitation (n=1) twelve hours after a single ECS. Unstimulated control animals (n=1) were used to determine basal expression levels. Brains were removed and immediately frozen in pre-chilled isopentane. After embedding in Cryo-M-Bed (Bright, Huntingdon, UK), 20 μm frozen coronal sections were collected through the septotemporal extent of the hippocampus from both paraformaldehyde-fixed and fresh frozen tissue onto polylysine-coated (fixed sections) or Superfrost Plus (fresh-frozen sections) slides (Fisher Scientific, Pittsburgh, PA), and sections were dried for one hour before being stored at -80° C. After air drying, sections were fixed with 4% paraformaldehyde for 20 minutes before digestion with 50 μg/ml Proteinase K in Tris-EDTA buffer (50 mM Tris pH 7.5, 5 mM EDTA) for 10 minutes at room temperature. Sections were then acetylated with acetic anhydride, and prehybridized at 60° C for several hours in hybridization buffer (50% formamide, 5x SSC, 50 μg/ml tRNA, 100 μg/ml heparin, 1x Denhardt's solution, 0.1% Tween-20, 0.1% CHAPS, 5 mM EDTA). This solution was replaced with hybridization buffer containing 1

μg/ml of digoxigenin-labeled RNA probe, and hybridization continued for an additional 12-16 hours at 60° C. Sections were washed several times in 0.2x SSC at 60° C, followed by two changes of PBS with 0.1% Triton-X and 2 mg/ml BSA (PBT) at room temperature. Sections were next blocked with 20% sheep serum and 2% Blocking Reagent (Boehringer Mannheim) for several hours at room temperature. Sections were then incubated overnight at 4° C with anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:2000 in blocking solution. After several 30 minute washes with PBT, staining was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim).

Immunohistochemistry. Following *in situ* hybridization, sections were rinsed several times with PBS and refixed in 4% paraformaldehyde for 20 minutes at room temperature before endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in PBS for 30 minutes. Slides were blocked with several changes of 5% goat serum in PBS with 0.1% Triton-X, followed by overnight incubation (at 4° C) with primary antibody against GFAP (rabbit anti-cow polyclonal, Accurate Chemical and Scientific Corp., Westbury, NY) diluted 1:500 in blocking solution. Sections were washed several times with blocking solution, followed by incubation for one hour with secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit polyclonal, Boehringer Mannheim) diluted 1:200 in blocking solution. Following several washes with Tris-Imidazole buffer (TI: 50 mM Tris, pH 7.5, 20 mM imidazole, 0.1% Triton-X), the peroxidase reaction product was developed with 0.04% diaminobenzidene (Sigma) in TI buffer.

Radioactive in situ *hybridization.* A radiolabeled antisense probe for GFAP was transcribed from a plasmid containing a 1.26 kb cDNA fragment using the T3 promoter to generate a ^{35}S-labeled probe of about 1000 bases in length.

LIF null (n=2) and WT (n=3) mice were killed by anesthetic overdose and transcardially perfused with 4% paraformaldehyde in PBS (pH 7.4) 36 hours after ECS,

the time of peak GFAP expression after electroconvulsive seizure (Steward et al., 1997). Additional null (n=1) and WT (n=1) animals were sacrificed and perfused without prior ECS treatment for use as naive controls. Brains were removed and placed in 4% paraformaldehyde for several days and then were cryoprotected by immersion in 15% sucrose for an additional 24 hours. Coronal sections were cut at 20 μm with a cryostat, and were thaw-mounted onto polylysine-coated slides and stored at -80° C.

In situ hybridization was performed as previously described (Steward et al., 1990; Steward et al., 1997). Sections on slides were post-fixed in 4% paraformaldehyde, treated with proteinase K (1 μg/ml in RNase buffer for 30 minutes), washed in 0.5xSSC, and dried. The slides with sections were placed in humidified petri dishes, covered with 120 μl of hybridization buffer containing 1 μl of probe (0.5-1 x 10^6 cpm) and 2 μg of tRNA. Sections were hybridized overnight at 55° C, treated with RNase, and washed for 2 hours at 55° C in a stringency buffer (0.1x SSC, 10 mM BME, 1 mM EDTA). Sections were exposed to photographic film to obtain film autoradiographs and then dipped in photographic emulsion (Kodak NTB2). The emulsion-coated slides were exposed for 1 week, developed in D19, and stained with cresyl violet.

Quantitative analysis was carried out using the film autoradiograms by measuring the optical density (OD) of the film in three locations - the molecular layer of the dentate gyrus, in which the increases in GFAP mRNA levels are most dramatic, and in two control sites in the thalamus and the cerebral cortex. The data are expressed as the ratio of the OD in the molecular layer of the dentate gyrus to the OD in the thalamus to provide an internal control for variability in hybridization.

RESULTS

LIF expression following ECS in WT mice. An initial series of mice were sacrificed at times ranging from 6 hours to 2 days after a single ECS seizure to determine the time course of LIF mRNA induction following seizure in wild-type mice. *In situ* hybridization

revealed that LIF is strongly but transiently up-regulated at 12 hours after ECS seizure. Additional animals were examined at the time of peak LIF expression to further characterize the location and types of cells responsible for LIF induction. *In situ* hybridization on coronal brain sections revealed that LIF mRNA is induced only in the dentate gyrus (Fig. 1*B*). Increased LIF expression was not seen in any other area of the brain examined, including all other subfields of the hippocampus. Within the dentate gyrus, LIF mRNA is induced in a subpopulation of cells that border the granule cell layer (Fig. 1*D*).

Immunohistochemistry for the astrocyte marker GFAP combined with *in situ* hybridization for LIF showed that GFAP-positive astrocytes are responsible for the majority of mRNA up-regulation (Fig. 2). The double-labeled astrocytes do not display a typical stellate morphology, however. Instead, these LIF-positive cells appear to have either very short, thickened processes, or in those cells that directly abut the granule cell layer, just a single projection, sent radially through the neuronal cell body layer. Previous work has documented the presence of radial glia in the rodent dentate gyrus, and it is possible that these LIF-positive astrocytes are residual radial glia (Kosaka and Hama, 1986).

GFAP expression following ECS. To determine whether GFAP induction following ECS requires LIF, we exposed both wild-type and LIF null mice to a single ECS seizure, and sacrificed them 36 hours later, at the time of peak GFAP expression (Steward et al., 1997). Radioactive *in situ* hybridization was used to determine the location and extent of GFAP mRNA expression. Because it had been previously established that GFAP immunostaining is altered in LIF mutant mice (Bugga et al., 1998; Koblar et al., 1998), we also determined the level of basal GFAP mRNA expression in null mice (without seizure), and compared it with basal levels expressed in WT mice. No differences in basal GFAP mRNA expression (without ECS) were seen between LIF KO and WT mice (Figs. 3 and 4).

Figure 1

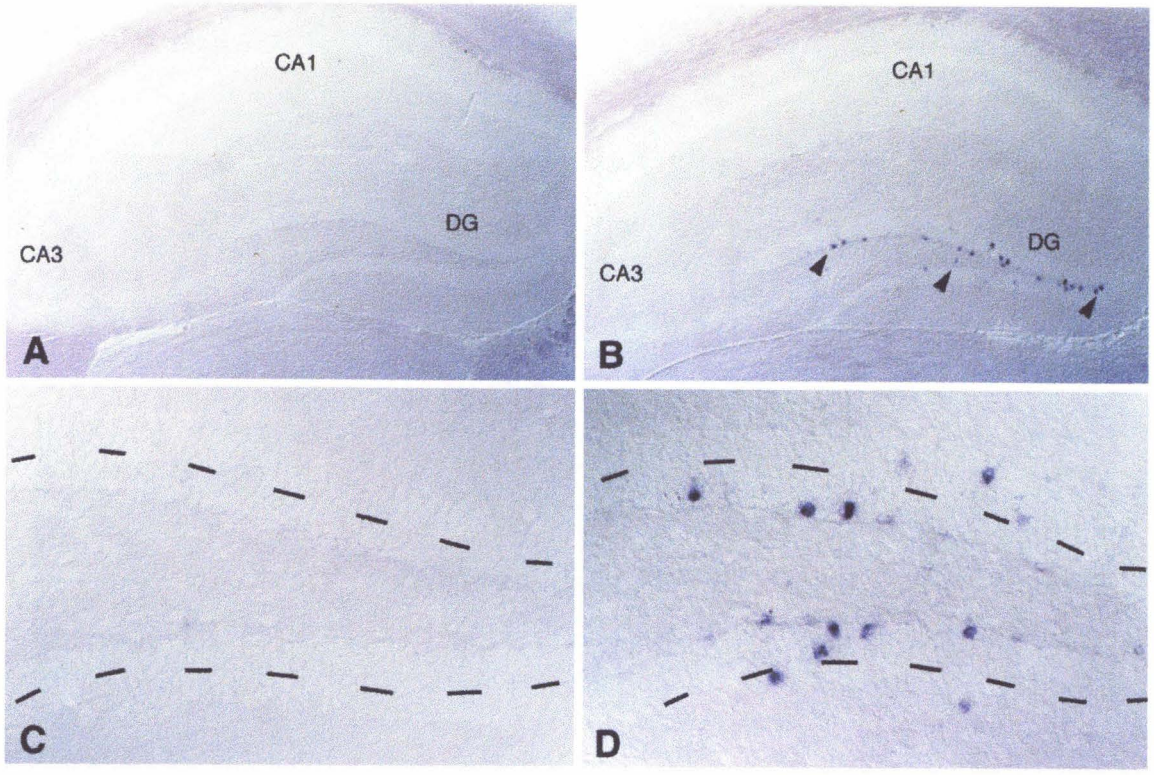

Figure 1. Localization of LIF mRNA expression in the hippocampus following electroconvulsive shock (ECS) seizure. In situ hybridization for LIF mRNA in control animals (*A* and *C*) and in animals sacrificed 12 hours after ECS-induced seizure (*B* and *D*). *A, B.* Low power photomicrographs of coronal sections through the hippocampus show distribution of digoxigenin-labeled LIF cRNA (*blue*). Conditions used for hybridization do not reveal basal LIF expression present in the unstimulated brain that can be detected by RT-PCR. *C, D.* Higher power photomicrographs display staining of individual cells in the dentate gyrus of different sections than pictured in *A* and *B*. *Dashed lines* indicate the granule cell layer in each section. LIF+ cells are found primarily along the granule cell layer of the dentate gyrus (*arrowheads* in *B*).

Figure 2

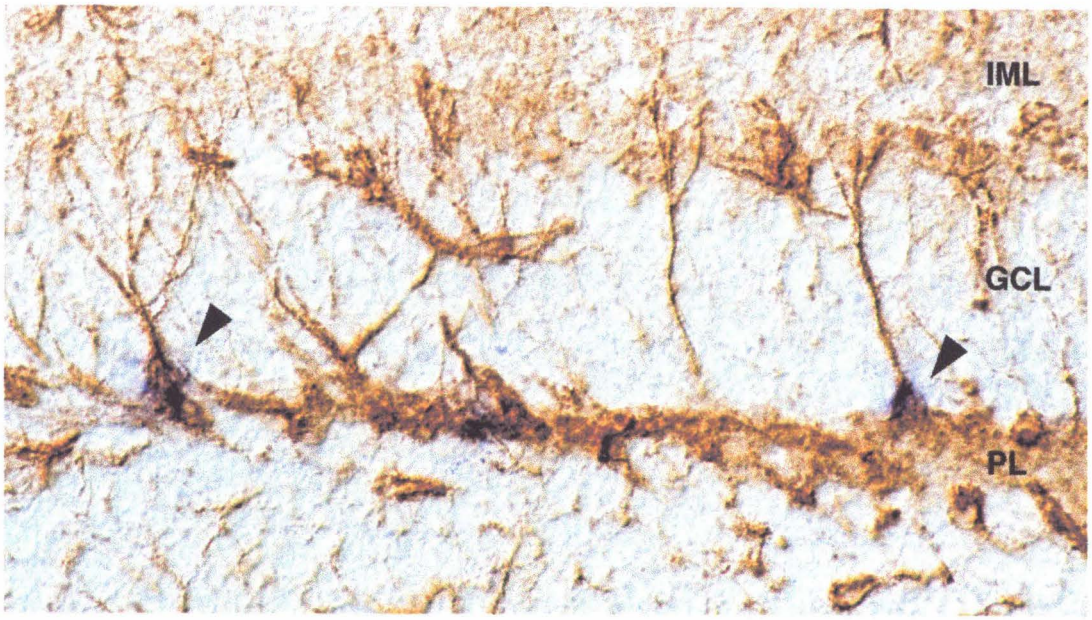

Figure 2. LIF-positive cells in the dentate gyrus are predominantly GFAP-positive astrocytes with the morphology of radial glia. *In situ* hybridization for LIF (*blue*) was followed by immunohistochemistry for GFAP (*brown*) in animals sacrificed 12 hours after a single ECS seizure. LIF+ cells in the subgranule zone generally did not have the stellate morphology of astrocytes in other layers of the hippocampus, but instead appeared to have only a single or branched GFAP+ process sent directly through the neuronal cell body layer (*arrowheads*). *IML*, inner molecular layer; *GCL*, granule cell layer; *PL*, polymorph layer.

The seizure-induced up-regulation of GFAP mRNA is significantly less in the LIF KO mice compared to WT animals (Fig. 3). Quantitation of bound radioactivity in the most dramatically affected area, the molecular layer of the dentate gyrus, revealed that GFAP induction in the absence of LIF is reduced by more than 90% compared to WT mice, when calculated after normalizing the post-ECS GFAP levels to those found in control animals for each genotype (Fig. 4).

DISCUSSION

In the present experiments, we sought to determine if the upregulation of GFAP mRNA following ECS is mediated by LIF. Although we had previously shown that LIF mRNA is strongly induced in the hippocampus following pilocarpine-induced seizure, it was necessary to determine if this also occurs in the much more brief seizure induced by ECS. We show here the timing and localization of LIF mRNA induction after ECS is consistent with a role for this cytokine in regulating GFAP expression. The hypothesis was directly tested by quantifying the induction of GFAP mRNA after ECS in the absence of LIF using KO mice.

The strict temporal and spatial restriction of LIF mRNA induction following ECS seizure is striking. Peak LIF expression is seen at 12 hours after seizure. This time course is similar to that seen in another model of seizure, pilocarpine-induced status epilepticus (Jankowsky and Patterson, 1999). Unlike pilocarpine, however, ECS-induced LIF expression is spatially restricted to the hippocampus, specifically in the dentate gyrus. This is surprising because like pilocarpine, ECS causes generalized seizures that involve widespread areas of the brain. Although little is known about the electrographic nature of ECS in rodents, it is likely that the dentate is one of the most active areas in this form of seizure because of the large number of inputs that converge there. Another possibility is that LIF is induced throughout the hippocampus and in other brain areas, but to levels that are not above the detection threshold required in our *in situ* hybridization conditions. In

Figure 3

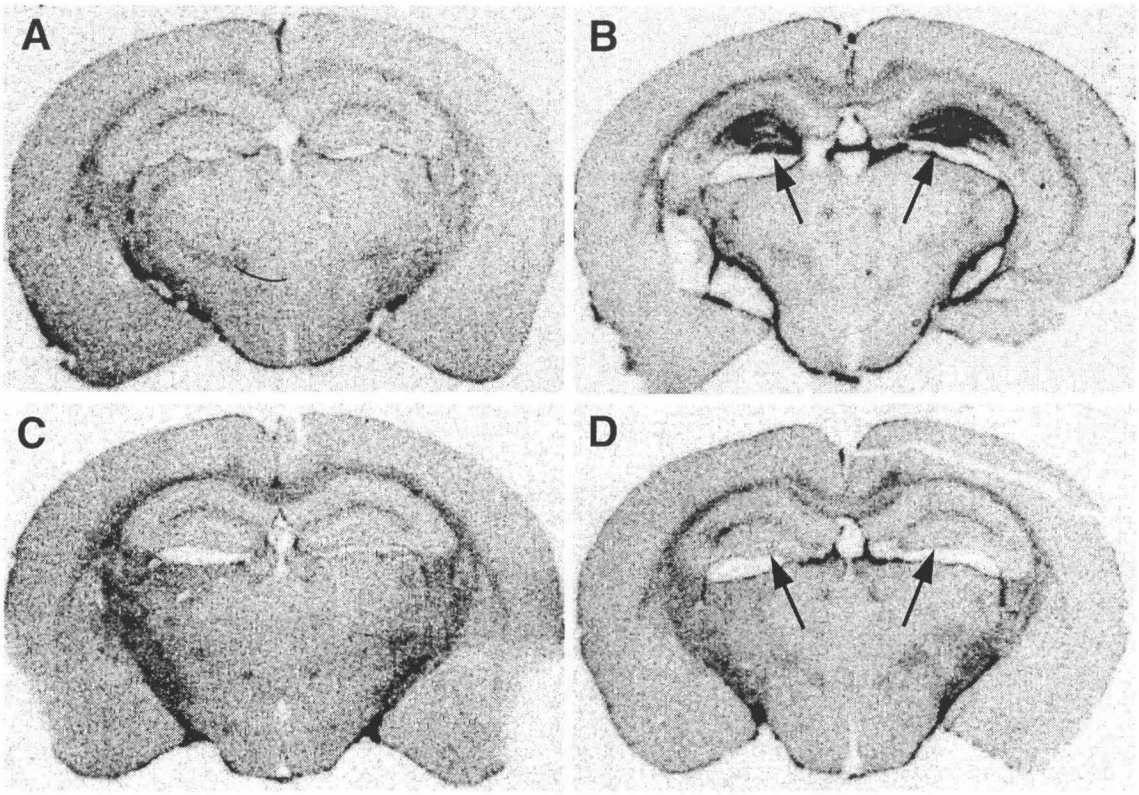

Figure 3. Localization of GFAP mRNA expression following ECS seizure (film autoradiographs). Radioactive *in situ* hybridization for GFAP in control animals (*A* and *C*) and in animals sacrificed 36 hours after a single ECS seizure (*B* and *D*). GFAP is expressed at similar basal levels in both wild-type (*A*) and LIF KO mice (*C*). ECS seizure induces strong GFAP upregulation in the dentate gyrus of wild type animals (*solid arrows*; *B*); this response is substantially attenuated in LIF-mutant mice (*solid arrows*; *D*).

Figure 4

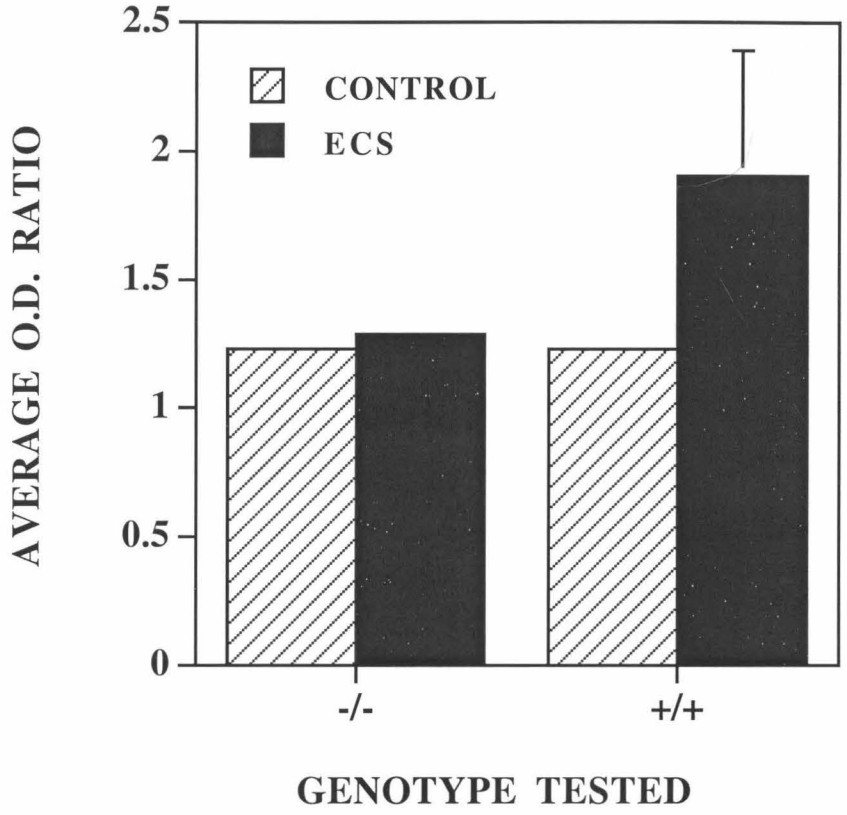

Figure 4. Quantitation of GFAP upregulation in the dentate gyrus following ECS seizure. Using film autoradiographs, measurements were made of the optical density in the molecular layer of the dentate gyrus and in the thalamus in a series of five sections from each animal. The average OD ratio of the two structures was calculated to provide a within-section control for variations in labeling. Average OD ratios for control animals of each genotype are almost identical (*hatched bars*), indicating that KO animals express normal levels of GFAP mRNA under basal conditions. GFAP upregulation in animals sacrificed 36 hours after ECS seizure, however, is substantially reduced in the KO mice compared to WT animals (*black bars*).

either case, the timing and the location of maximal LIF expression are consistent with a role in mediating GFAP induction after seizure: the up-regulation is maximal within the period during which GFAP induction is sensitive to cycloheximide, and is restricted to precisely the same area of the hippocampus in which GFAP is most strongly up-regulated (Steward et al., 1997).

Direct support for the hypothesis that LIF mediates ECS-induced increases in GFAP expression comes from examination of GFAP mRNA levels in LIF KO mice following ECS. The ECS-induced increase in GFAP mRNA in these mice is much less than in the WT mice. Expression of GFAP is not completely eliminated in the null mice, however, indicating that LIF is not the sole factor responsible for GFAP induction. The latter finding is not surprising given that many proteins are able to affect GFAP expression in cultured astrocytes. For example, ciliary neurotrophic factor (CNTF), another member of the neuropoietic family to which LIF belongs, is also a potent inducer of GFA protein *in vitro* (Murphy et al., 1997), and mRNA for CNTF is also up-regulated by seizure activity (Jankowsky and Patterson, 1999). The increase in CNTF mRNA occurs much more slowly than LIF up-regulation after seizure, however, suggesting that it may not be part of the cycloheximide-sensitive mechanism responsible for GFAP induction *in vivo*.

Understanding the regulation of GFAP expression is an important step in understanding how glial cells respond to and recover from seizure activity. Human temporal lobe epilepsy is often accompanied by glial scarring in the hippocampus, but the cause and function of this pathology are not known (Glass and Dragunow, 1995; Lynch et al., 1996; Mathern et al., 1996). In the normal brain, astrocytes are generally believed to play a supportive role, clearing neurotransmitters from extracellular space and buffering ion fluctuations, both of which would be important in seizure-induced neuronal hyperactivity. On the other hand, the astrocyte proliferation that occurs in response to insults such as excitotoxicity may prevent compensatory reorganization of the neuronal network by providing a hostile environment for axonal outgrowth (Niquet et al., 1994; Steward,

1994). Alternatively, astrocytes within the dentate gyrus have been suggested to promote axonal outgrowth, which can result in the formation of inappropriate neuronal connections, thus enhancing the generation of recurrent seizures (Niquet et al., 1994; Represa et al., 1995; Lynch et al., 1996). Activated astrocytes are also known to produce toxic molecules as well as growth factors, which could be involved in neuronal death following seizure (Chao et al., 1995). Clearly, a better understanding of the astrocytic response to episodes of extreme neuronal activity is needed, and insight into the regulatory factors involved in this reaction may help provide tools for manipulating the astrocyte response.

Although the LIF produced by astrocytes is acting in an autocrine/paracrine manner to cause their activation, it is also possible that this cytokine is acting on other cell types following seizure. This is particularly likely given that seizure can involve an inflammatory reaction (Perry et al., 1995), and LIF is a key regulator of inflammation (Gadient and Patterson, in press). Endogenous LIF is required for the normal levels of early infiltration of inflammatory cells following sciatic nerve injury, and for the normal activation of astrocytes and microglia following surgical injury to the cortex (Patterson et al., 1997; Patterson, in press). In addition, application of exogenous LIF is known to affect axonal sprouting and neuronal survival (Murphy et al., 1997; Kurek et al., 1998; Blesch et al., 1999). Finally, endogenous LIF can regulate neuronal gene expression, neuronal death, and neurogenesis in response to axotomy (Rao et al., 1993; Corness et al., 1996; Sun and Zigmond, 1996; Rasika et al., 1999). Since all of these phenomena are part of the response to seizure, LIF must be considered as a candidate for mediating these events as well as astrocyte activation.

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Chapter 5

Summary and future directions

Joanna L. Jankowsky

Using a variety of model systems, I have explored the regulation of cytokine expression within the rodent hippocampus following synaptic activity of both physiological and pathological intensity. In the first set of experiments, we describe the upregulation of IL-6 after LTP induction *in vivo*, as well as the downregulation of BDNF by the low frequency test stimuli used to assess synaptic responses. In turning to models of peripherally-induced seizure, we have identified additional factors, LIF, CNTF, and OSM, that are affected by epileptiform activity within the hippocampus. Finally, we have explored the role of one factor, LIF, in hippocampal seizure pathology using gene-specific knock-out mice. We find that GFAP upregulation after seizure is significantly impaired in the LIF-mutant animals, suggesting that LIF is required for the astroglial response.

One of the most striking and consistent observations across these models of synaptic activity and plasticity is the exquisite regulation of cytokine expression. This control occurs on several levels, the most fundamental being the timing and extent of upregulation. Even following the severe and prolonged seizures induced by pilocarpine, we find that each factor is induced to greatly different extents over very distinct timeframes. In two cases, LIF and CNTF, for which regulation has been explored in other models of neural injury, we find mRNA induction following seizure closely mimics that observed following other forms of CNS injury, such as cortical lesion (Ip et al., 1993; Banner et al., 1997). This supports the idea that cytokine upregulation after seizure does not simply reflect neuronal dysregulation in which many non-essential genes are induced. Rather, the specific factors affected likely play important roles in the response to various forms of injury. In addition, not all factors studied were affected by pilocarpine treatment. One cytokine, CT-1, of the four assayed, is not substantially induced either during or after seizure, providing further support for the relevance of those genes that are specifically upregulated.

Another facet of the careful regulation of cytokine expression following synaptic activity is their distinct localization within different brain structures and cell types.

Following pilocarpine-induced seizure, both LIF and OSM are upregulated throughout the brain. Indeed, this is a model of generalized epilepsy, in which all brain structures are involved to greater or lesser extents. The cellular localization of the two factors, however, is clearly distinct. LIF mRNA is located in GFAP+ astrocytes and additional cells outside of the blood-brain barrier; OSM is located in presumptive GABA-ergic interneurons. More interesting is the change in localization of one particular factor, LIF, in the two models of seizure used in my studies. As stated, following pilocarpine-induced seizure, LIF is upregulated throughout the brain. Following the much shorter seizures of ECS, however, LIF expression is restricted to the subgranular zone of the dentate gyrus. The different expression patterns suggest that LIF regulation is indeed related to both the duration and form of neuronal activity induced. In both types of seizure, as after cortical stab wound (Banner et al., 1997), LIF is found predominantly in GFAP+ astrocytes, again indicating that it may play a similar role in many forms of insult, but that its action is appropriately restricted in each.

A third aspect of cytokine regulation is the number of factors "recruited" by increasingly intense forms of neuronal activity. Following the induction of LTP, a relatively physiological form of synaptic activity, we observed alterations in only two factors, BDNF and IL-6, from more than 19 initially screened. After the pathological activity of prolonged seizure, we found that three out of four factors examined show altered expression. By extrapolating these percentages, it would appear that the number of cytokines "called to action" in the hippocampus is correlated to the intensity, form, or duration of neuronal activity. Indeed, the literature in this area documents only a handful of factors that are affected by hippocampal LTP, but more than 15 factors that are induced by seizure activity (Introduction, Sections 4 and 5). A superficial explanation for this bias in the literature is the relative ease of performing seizure experiments compared to LTP studies which require costly equipment and learned expertise which may influence the models studied in many labs. In our hands, however, the greater number of cytokines affected by

seizure reflects the level of synaptic activity (and subsequent injury) rather than the number of studies performed with each method. One significant caveat to this correlation between activity levels and the number of factors recruited is the presence of tissue damage after severe or repeated seizures that is not observed after the less intense activity of LTP. Cytokines have been well-characterized for their involvement in neural insult, and may be upregulated after seizure not primarily as a result of activity *per se*, but rather in response to subsequent injury.

Distinctions between primary roles in activity or injury will be best parsed out through well-designed functional studies. The final section of my thesis begins to address the role of one seizure-regulated factor, LIF, in astrocytic activation using KO mice. These experiments provided a good starting point for dissecting LIF function in seizure activity and pathology for two reasons. First, both LIF and seizure had previously been well-characterized for their ability to affect GFAP expression (Murphy et al., 1997), and second, the LIF mutant (male) animals survive to adulthood without a significant neural phenotype (Stewart et al., 1992; Murphy et al., 1997). LIF, however, represents just one of the several factors upregulated in our study of pilocarpine-seizure. As KO mice and specific antagonists become available, it will also be interesting to explore the role of OSM and CNTF in seizure and its aftermath.

Like LIF, CNTF also affects many aspects of the astrocytic response, including astroglial gene expression, morphology, and proliferation. In fact, exogenous application of CNTF to the neocortex of adult rodents alone can induce many aspects of the glial reaction (Levison et al., 1996). Because the CNTF mutant mice, like the LIF KO's, survive to adulthood and display no substantial brain aberrations (Masu et al., 1993; DeChiara et al., 1995), they would provide a good model in which to examine the role of this factor in seizure-related gliosis.

The fast, neuronal upregulation of OSM, and the ability of other neuropoietic cytokines to affect neuronal physiology (Introduction, Sections 2, 3.4, and 4.3), suggested

that OSM may alter neuronal activity during the seizure itself. An obvious experiment to test this hypothesis is to examine seizure susceptibility in the KO animals. While we cannot *a priori* know in which direction the KO animals will be shifted, one would predict that the OSM mutant animals will display an altered timecourse of seizure initiation or duration. The second wave of OSM expression observed following pilocarpine seizure suggests that OSM may have yet another role in later seizure-related sequelae. Again, based on the timing of OSM expression, and the ability of other neuropoietics to affect similar changes *in vitro* (Introduction, Sections 2 and 3.2), one might also reasonably look for differences in axonal reorganization after seizure in the WT and KO animals.

Overall, the precise timing, extent, and localization of cytokine expression, combined with initial functional studies, suggest that these changes after synaptic activity or plasticity are not irrelevant side-effects of neuronal dysregulation. The shear number of factors affected, however, brings their individual importance into question. Could some of them simply be the unstopped consequence of factor induction "chains", one leading unchecked to the upregulation of the next? There is limited evidence for both possibilities. In the first case, there are so many roles available in the regulation of neuronal activity and related pathology that the large number of factors induced does not provide an adequate argument against their relevance. In addition, where examined, functional perturbation of cytokine activity during LTP and seizure provides evidence that they do play important roles in both activity and pathology (Introduction, Sections 4.3 and 5.3). The seizure literature, however, also provides examples of mRNA induction in the absence of protein upregulation, revealing dissociation between the control of mRNA expression and the expected functional consequences. Specifically, the upregulation of GFAP mRNA after a single ECS occurs in the absence of later changes in GFAP protein levels (Orzi et al., 1990; Steward, 1994). Instead, several electrically-induced seizures are required to increase both GFAP mRNA and protein (Orzi et al., 1990), indicating that the isolated mRNA upregulation after a single seizure may reflect an aborted or vestigial astrocytic response.

Resolution of the actual roles played by individual factors in synaptic activity, plasticity, and pathology will largely await functional studies either in knockout animals or using specific antagonists to determine how and when they act.

The functional relevance of mRNA changes in terms of the connection to subsequent protein expression and release is especially pertinent when considering the model we propose in Chapter 1, by which IL-6 upregulation after LTP may modulate local synaptic plasticity. Given the limits of our assay system, we do not know if IL-6 protein expression follows its mRNA upregulation, nor under what conditions it is released. Further, if IL-6 protein is produced, what is its range of influence? How far does it diffuse, and what concentration threshold is needed to affect synaptic physiology? Answers to these questions minimally require additional experiments using immunohistochemical and ELISA techniques, provided appropriate species-specific antibodies become available that can bind IL-6 in its native form.

Additionally, our model currently predicts that IL-6 is upregulated within neurons, based on studies in which its basal expression is found primarily in these cells (Introduction, Section 2.6). Of course, other cell types such as astrocytes or invading immune cells may be responsible for IL-6 upregulation after LTP. Glial IL-6 expression would have interesting implications for our model. Astrocytic upregulation would indicate that non-neuronal cells are indeed involved in the brain's response to normal synaptic activity by suggesting that glia secrete activity-regulated factors that can act back on nearby neurons. Upregulation within invading immune cells, however, might pose a significant impediment to our model. In this case, IL-6 release from blood cells might not occur within an activity-restricted location. This could result in the dissociation of the site of synaptic plasticity from the site of IL-6 release and subsequent inhibition of later-occurring LTP. These issues will be addressed shortly using *in situ* hybridization to identify the cell types responsible for IL-6 upregulation *in vivo*.

Review of the literature on IL-6 indicates that it has many actions on neuronal and non-neuronal cells that may be germane to the biology of LTP and synaptic plasticity. Among these, effects of IL-6 on neuronal physiology, morphology, and survival have all been described (Introduction, Sections 2 and 3). Here again, however, knowledge of the timing of IL-6 upregulation after LTP suggests and limits the probable roles it may play at the synapse. For example, given the relatively fast (though controversial; Sorra and Harris, 1998) changes in synaptic morphology after LTP or spatial learning (Trommald et al., 1990; Moser et al., 1994; Trommald et al., 1995), it is unlikely that IL-6, which requires several days to significantly affect neurite outgrowth *in vitro*, contributes substantively to LTP-induced morphological changes. More reasonable would be a role in which IL-6 acts quickly to affect changes observed shortly after LTP induction, such as alterations in neuronal physiology or responsiveness. In this manner, we return to the theme of enlightened suggestion, by which understanding of the timing and localization of cytokine regulation can inform the speculation of their function in synaptic activity, plasticity, and pathology.

As indicated for other factors upregulated after seizure activity, determining the actual role of IL-6 in synaptic plasticity will await carefully-controlled functional experiments. One of the most obvious is how the absence of IL-6 protein affects the induction and maintenance of LTP. This could easily be performed using IL-6 KO mice, which survive to adulthood without any significant CNS phenotype (Fattori et al., 1994; Kopf et al., 1994), or by application of soluble IL-6 receptor to hippocampal slices taken from WT animals. Though somewhat more technically challenging, it would also be of interest to directly test the hypothesis that IL-6 release following LTP acts to prevent potentiation by subsequent tetanic stimulation. This would require information discussed above about the range of IL-6 influence in order to appropriately space several electrode sets within the hippocampus, but from the hypothesis, one would expect that after LTP induction in one set of synapses, potentiation in the second pathway would be

compromised. Further, this inhibition should be absent in IL-6 KO animals, and reversible by IL-6 antagonists in WT animals. A peripherally related experiment that might provide additional insight into the contribution of IL-6 inhibition to neuronal activity examines the susceptibility of IL-6 KO mice to seizure induction. For example, if IL-6 plays a more general role in downregulating neuronal activity in the hippocampus, one would predict that seizure induction would be easier in mice lacking IL-6. Using the kindling model of epilepsy, in which consecutive subthreshold stimulations eventually lead to seizure activity, fewer stimulations would be expected to produce the same seizure milestones (behavioral signs of epileptiform discharge) in IL-6 KOs than in WT mice. In the particular case of IL-6, experimental design is greatly facilitated by the availability of specific antagonists and the apparent normalcy of the KO animals, but in any case, indicate the sorts of experiments that are now required to take cytokine mRNA upregulation from informed suggestion to proven function.

The lack of thoughtful functional experiments is currently the most significant shortcoming in the study of cytokines in models of synaptic activity and plasticity. With so many cytokines to examine, often the literature offers survey information on a great number of factors, but provides a deeper understanding of relatively few. In this respect, study of the neurotrophins in the CNS far surpasses that of cytokines, although their potential to affect neuronal physiology, morphology, differentiation, and survival is similar (Introduction, Sections 2 and 3). Currently, the ease and necessity of basic localization and perturbation experiments dominate the field, and completion of this catalog may be required before more difficult functional experiments are compulsory. Because the number of cytokines increases seemingly exponentially, descriptive experiments may never be exhausted.

Two additional, significant hindrances to cytokine functional experiments must also be acknowledged. The first of these is simply that adult neurobiology is complicated. Presumably many soluble proteins are involved in any given aspect of neuronal activity.

Though this sounds naive, it is, in this instance, intrinsically confounded by the second impediment, that many cytokine factors can act in a redundant fashion (Murphy et al., 1997, Introduction Section 2). *In vitro*, this is evidenced in the nearly identical effects observed after exogenous application of related factors (Introduction Sections 2 and 3.3). Such redundancy, in the form of compensation, is also observed *in vivo*. A good example of this comes from comparison of the single CNTF and LIF KO's to the double KO, in which much a much more severe phenotype is found (Sendtner et al., 1996). Thus even the most carefully designed functional experiments may be thwarted by the complexity of cytokine action *in vivo*.

Occasional examples combining luck and good experimental design provide hope that even these obstacles in understanding cytokine neurobiology can be overcome. One such experiment demonstrated that the microglial response to seizure is completely absent in TNFα KO's (Bruce et al., 1996). This KO has thus provided not only a function for TNF in the response to seizure, but will also allow us to bootstrap our way to addressing more fundamental questions about the nature and significance of the microglial response to pathological neuronal activity. As more functional experiments are approached, additional examples of specific cytokine function as well as important contributions to experimental systems for the exploration of basic neurobiological questions will emerge. A substantial amount of work needs yet to be done, with an emphasis on function in addition to description, but their multifaceted potential to affect aspects of neurobiology as diverse as morphology, gene expression, cell survival and differentiation, combined with their presence in the adult CNS and precise regulation by neuronal activity and injury, suggest that cytokines have much to reveal.

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Appendix

Regulation of LIF, CNTF, and BDNF in the mesolimbic dopamine system by chronic cocaine exposure

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ABSTRACT

The long-term biochemical changes in the mesolimbic dopamine system that characterize persistent exposure to addictive drugs can be altered by administration of exogenous neurotrophic factors or cytokines. We sought to determine if the changes resulting from chronic drug treatment are mediated by alterations in the endogenous levels of such factors. Using an RNase protection and RT-PCR assays, we measured the expression of ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and leukemia inhibitory factor (LIF) in several regions of the mesolimbic dopamine system following chronic cocaine exposure. We report no significant changes in expression of these factors in any brain area examined. Several potential reasons for our negative findings are proposed.

INTRODUCTION

Chronic exposure to drugs of abuse induces long-term biochemical changes within the mesolimbic dopamine system, which may ultimately be responsible for some aspects of drug addiction (Nestler et al., 1996). Several neurotrophic factors and cytokines have recently been linked to key aspects of this drug-induced cellular plasticity (Nestler et al., 1996). Specifically, intracranial infusion of CNTF can mimic the effects of chronic drug exposure. One of the hallmarks of chronic drug exposure is up-regulation of the dopamine synthetic enzyme tyrosine hydroxylase (TH), and this can be reproduced by intracranial infusion of CNTF (Berhow et al., 1995). Subsequent exposure to chronic cocaine or morphine does not further increase TH levels in CNTF-treated animals, indicating that the effects of CNTF are not additive with the effects of morphine or cocaine. This finding led to the hypothesis that CNTF mediates cellular changes caused by chronic drug exposure. Further support for this hypothesis comes from the finding that chronic cocaine administration leads to increased immunoreactivity for Janus kinase 2 (JAK2) specifically within one area of the mesolimbic dopamine system (Berhow et al., 1996). This enzyme is

a key component of the CNTF signaling pathway. Thus, it was reasonable to ask if chronic cocaine administration increases TH levels by up-regulating CNTF expression.

The actions of BDNF are quite distinct from the effects of CNTF. Chronic infusion of BDNF decreases TH levels in specific areas of the mesolimbic dopamine system (Berhow et al., 1995). Moreover, BDNF infusion can prevent TH up-regulation following drug exposure, and can reverse increases in TH when administered after chronic drug treatment. In addition, drug administration itself affects intracellular signaling events. Chronic exposure to morphine or cocaine increases levels of extracellular signal-regulated protein kinase (ERK), an important part of the signaling pathway activated by BNDF (Ortiz et al., 1995; Berhow et al., 1996). Therefore, it is possible that BDNF may also mediate effects of chronic drug exposure, acting in a protective manner to retain or restore the cells' original biochemical state.

MATERIALS AND METHODS

Chronic cocaine treatment. Adult, male Sprague-Dawley (CAMM, Wayne, NJ) were used for this study. Experimental animals were injected i.p. once daily with 0.2 ml of saline containing 20 mg/kg cocaine-HCl for 12 days. Control animals received 12 daily injections of saline. Twenty-four hours after the final injection, animals were sacrificed by decapitation, and the brains removed for dissection. Two sets of animals were used; in the first, tissue from the ventral tegmental area (VTA) and nucleus accumbens (NAc) were obtained as 12-15 gauge punches from coronal cross-sections of brain, along with additional tissue from the frontal cortex (n=6 each, cocaine and control). In the second set of animals, the VTA and the substantia nigra (SN) were harvested (n=6 each, cocaine and control). All tissue was quickly frozen on dry ice and stored at -80^{o}.

RNA isolation. In the first set of animals, total RNA was extracted separately from the frontal cortex of three animals per condition. Because so little tissue was collected from the

other brain areas, the NAc tissue was pooled into two samples per condition, and the VTA tissue was pooled into one sample per condition. In the second set of animals, the VTA and the SN tissue were each pooled into three samples per condition, cocaine and control. Total RNA was extracted from all tissue by a modification of the acid-phenol method (Chomczynski and Sacchi, 1987), and stored at -80° C until use.

RNAse protection assay (RPA). A plasmid containing a fragment of CNTF was cloned by PCR from sciatic nerve cDNA, ligated into pCRII (Invitrogen, San Diego, CA), and sequenced to confirm its identity. The housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control for each CNTF reaction, and was a gift from Dr. Lisa Banner (Banner and Patterson, 1994).

The RPA was performed as described (Patterson and Fann, 1992), using approximately 5 μg total RNA per reaction. Briefly, plasmids were linearized and ^{32}P-labeled antisense probes generated by *in vitro* transcription were hybridized to hippocampal total RNA. After overnight hybridization at 55° C, reactions were digested with RNAse A and RNAse T1. Digestion was stopped with proteinase K and RNA extracted with phenol-chloroform. Reaction products were separated on denaturing 6% polyacrylamide gels to yield protected fragments of 266 nucleotides for CNTF and 133 nucleotides for GAPDH. Radioactivity was measured by scanning the protected fragments on a Phosphoimager 445SI (Molecular Dynamics, Sunnyvale, CA) and quantitated with ImageQuant software. The intensity of the protected fragment for each cytokine was compared to the intensity of the protected fragment of GAPDH in that reaction and the ratio was expressed in arbitrary units.

Reverse-transcription polymerase chain reaction (RT-PCR). The basal levels of LIF and BDNF mRNAs were too low to detect by RPA, requiring the more sensitive RT-PCR assay to measure changes induced by experimental manipulation. Prior to reverse

transcription, RNA was treated with 1 unit of DNAse (Gibco BRL) in 1x DNAse buffer for 15 minutes at room temperature. The DNAse was inactivated by addition of EDTA and heated at 65° C for 10 minutes, then quickly chilled on ice. Random hexamers (0.5 μg, Gibco BRL) were used to prime the RT reaction, and were added just before denaturing the RNA at 70° C for 8 minutes. The final RT reaction mixture consisted of 1x reverse transcription buffer, 1 mM each dNTP (Boehringer Mannheim), 10 mM DTT, 20 units RNAsin (Boehringer Mannheim), and 200 units Superscript II reverse transcriptase (Gibco BRL). Reactions were incubated for 10 minutes at room temperature, followed by 2 hours at 42° C. After synthesis of cDNA was complete, reactions were diluted to a final volume of 150 μl, and separated into 10 μl aliquots placed into thin walled PCR tubes (USA Scientific, Ocala, FL) for storage at -20° C. The following primer sets, listed 5' to 3', were used to amplify specific gene products: LIF: 5' CAA TGC CCT CTT TAT TTC CTA TTA CAC AGC, 3' GGG GAC ACA GGG CAC ATC CAC ATG GCC CAC; BDNF: 5' ATG ACC ATC CTT TTC CTT ACT ATG GT, 3' TCT TCC CCT TTT AAT GGT CAG TGT AC (Zaheer et al., 1995); GAPDH: 5' ACC ACC ATG GAG AAG GCT GG, 3' CTC AGT GTA GCC CAG GAT GC (Brown et al., 1994). Amplification reactions were performed in a final volume of 100 μl, consisting of 10 μl of diluted cDNA, 1 unit of Taq polymerase (Promega, Madison, WI), 1x PCR buffer (Promega), 2.5 mM $MgCl_2$ (except IL-6: 1.5 mM), 0.2 mM of each dNTP, and one set of primers (200 ng each). The cycle programs used to amplify each gene were: LIF and BDNF: 94° C for 5 min., 58° C for 85 sec., 72° C for 1 min. (75 sec. for BDNF) 1x, followed by 39 cycles of 94° C for 30 sec., 58° C for 85 sec., and 72° C for 30 sec. (75 sec. for BDNF); GAPDH: 94° C for 5 min., 60° C for 85 sec., and 72° C for 1 min. 1x, followed by 27 cycles of 94° C for 30 sec., 60° C for 85 sec., and 72° C for 45 sec. To follow the amplification rate of each reaction and identify the linear range, PCR reactions were stopped every 4 cycles starting at 24 cycles (LIF and BDNF) or 12 cycles (GAPDH), and 5 μl aliquots were removed.

cDNA hybridization. Reaction products from each PCR cycle sampled were blotted onto Nytran 0.2 μm nylon membrane (Schleicher & Schuell, Keene, NH), and fixed to the membrane by UV cross-linking (Stratagene Stratalinker) followed by baking at 80° C for 10 min. Prior to hybridization with radiolabeled probes, membranes were prehybridized for 2 hours at 42° C in a solution of 6x SSPE, 1% SDS, 10x Denhardt's solution, 20 μg/ml tRNA (Boehringer Mannheim), and 50 μg/ml herring sperm DNA. During prehybridization, oligonucleotide probes designed to bind the PCR products were end-labeled with ^{32}P using polynucleotide kinase (Boehringer Mannheim), and purified over G25TE spin columns (Boehringer Mannheim). Oligonucleotide probes were tested for specificity by hybridization against end-stage PCR reactions run out on agarose gels and transferred to nylon membrane. Internal oligonucleotide sequences, listed 5' to 3', were: GAPDH: ATC GTG GAA GGG CTC ATG ACC ACA GTC CAT; BDNF: TGG GTC ACA GCG GCA GAT AAA AAG ACT GCA; LIF: AAG TTG TGC GAG CTG TAT CGG ATG GTC ACG TA. After 2 hours, prehybridization buffer was removed, and replaced with 6x SSPE/1% SDS containing 500 kcpm/ml of labeled probe. Hybridization was continued for an additional 14-16 hours at 65° C. Membranes were then washed three times for 10 minutes each in 6x SSPE/ 1% SDS at room temperature, followed by a final wash in 1x SSPE/ 1% SDS for 3 minutes at 65° C.

Quantitation of RT-PCR reactions. Bound radioactivity was quantitated with a Phosphorimager 445SI using Imagequant software. Using samples taken from regular intervals in the PCR amplification, a linear range was determined for each reaction. Over many trials, we found that the amount of PCR product generated by 28 cycles fell in the linear range for the LIF and BNDF reactions, while GAPDH reached linear values by 16 cycles. In order to control for variations in cDNA synthesis between reactions, values for LIF and BDNF expression, taken as the intensity of hybridization of each reaction at 28

cycles, were expressed as a ratio to GAPDH expression amplified 16 cycles from the same initial cDNA reaction. Values for cytokine expression based on this ratio are expressed in arbitrary units.

RESULTS AND DISCUSSION

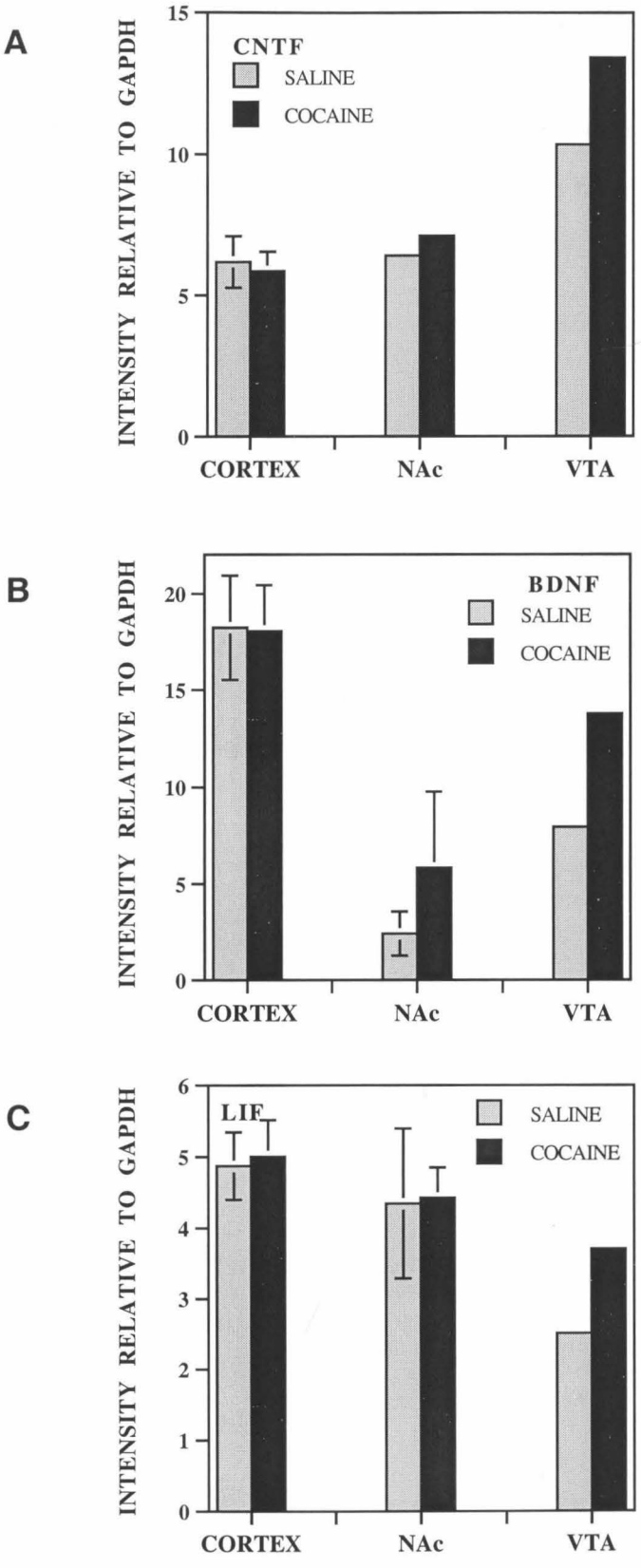
In the present study we sought to determine whether levels of endogenous CNTF, BDNF, and LIF were altered by chronic exposure to cocaine. In the first set of experiments, we measured expression of CNTF, BDNF, and LIF mRNAs in the VTA, and two of its projection areas, the NAc and the frontal cortex. No differences between control and cocaine-treated animals is seen for any of these factors in the cortex or the NAc (Fig. 1). Of note is the finding of almost no BDNF expression in the NAc, in agreement with concurrent work by Conner et al. (1997). With only one sample tested for each condition, the modest increase in expression of all three factors in the VTA of cocaine-treated animals warrants further study (Fig. 1).

By pooling only two animals (rather than all 6) for each RNA isolation in the second set of experiments, we increased the number of VTA samples tested for expression of CNTF, BDNF, and LIF. We also measured mRNA levels for these factors in a fourth brain area, the SN, which is anatomically related to the VTA. With the larger number of VTA samples, we found that the earlier result (showing slightly higher expression in the cocaine-treated animals) was within the standard deviation among samples. When a larger number of VTA samples was analyzed, no significant differences between conditions was observed for any factor tested (Fig. 2). In addition, no substantial changes after cocaine treatment were noted in the SN for any mRNA assayed.

There are several possible explanations for our failure to detect substantial changes in endogenous mRNA expression for any factor or brain region tested following chronic cocaine exposure. Numan et al. (1998) recently described the regulation of neurotrophin and trk receptor mRNAs in catecholaminergic nuclei during a chronic morphine paradigm.

Figure 1. Expression of CNTF (*A*), BDNF (*B*), and LIF (*C*) mRNAs following chronic cocaine exposure (*black bars*) or control saline injection (*grey bars*). Cytokine and neurotrophic factor expression in the frontal cortex (*cortex*), nucleus accumbens (*NAc*), and ventral tegmental area (*VTA*), was determined by RPA or RT-PCR, and is expressed as a ratio to levels of the internal control GAPDH. Note that the BDNF:GAPDH ratio in the NAc is actually 10X lower than shown. No significant differences were found between experimental conditions for any mRNA tested (cortex: $n=3$, NAc: $n=2$, VTA: $n=1$).

Figure 1


A
CNTF
SALINE
COCAINE
INTENSITY RELATIVE TO GAPDH
15
10
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CORTEX
NAc
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BDNF
SALINE
COCAINE
INTENSITY RELATIVE TO GAPDH
20
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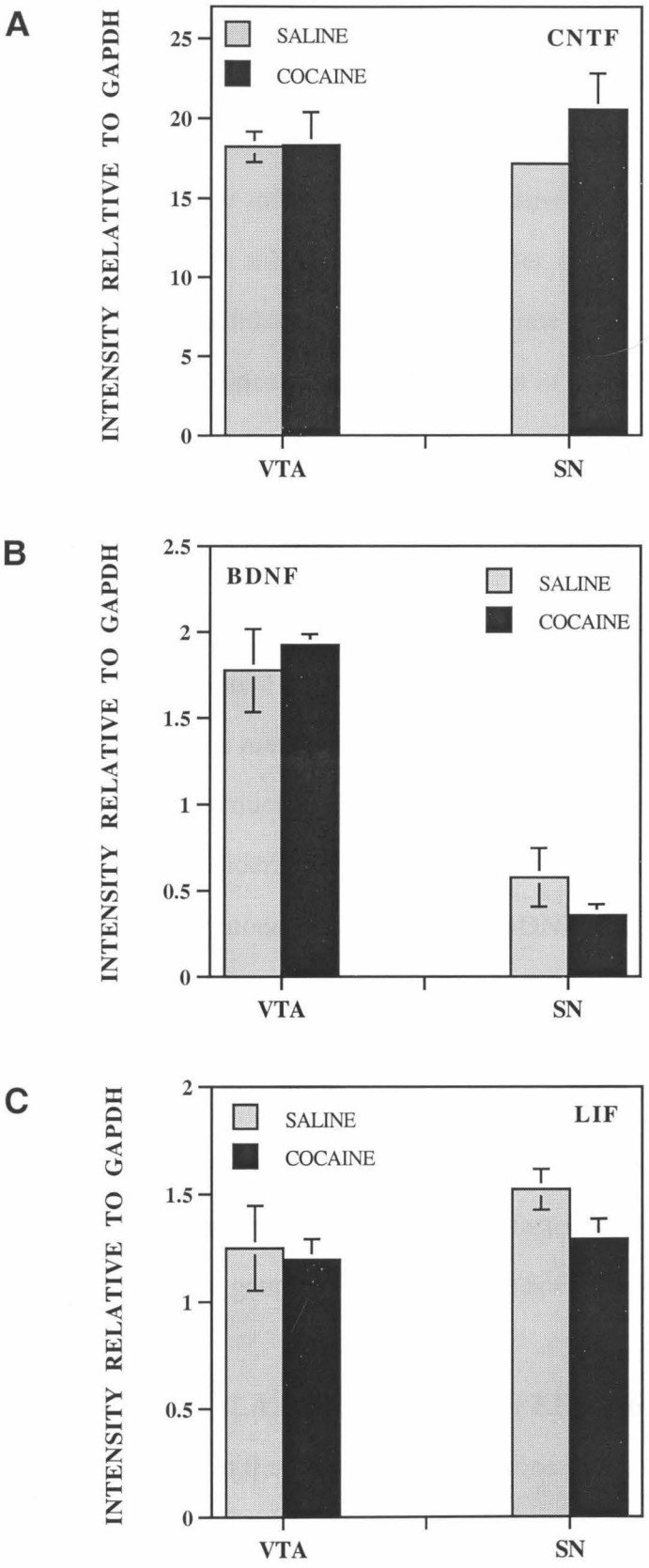
CORTEX
NAc
VTA
C
LIF
SALINE
COCAINE
INTENSITY RELATIVE TO GAPDH
6
5
4
3
2
1
0
CORTEX
NAc
VTA

Figure 2. Expression of CNTF (*A*), BDNF (*B*), and LIF (*C*) mRNAs following chronic cocaine exposure (*black bars*) or control saline injection (*grey bars*). Cytokine and neurotrophic factor expression in the ventral tegmental area (*VTA*) and the substantia nigra (*SN*), was determined by RPA or RT-PCR, and is expressed as a ratio to levels of the internal control GAPDH. No significant differences were found between experimental conditions for any mRNA tested (VTA: $n=3$, SN: $n=3$).

Figure 2


A
INTENSITY RELATIVE TO GAPDH
SALINE
COCAINE
CNTF
25
20
15
10
5
0
VTA
SN
B
INTENSITY RELATIVE TO GAPDH
BDNF
SALINE
COCAINE
2.5
2
1.5
1
0.5
0
VTA
SN
C
INTENSITY RELATIVE TO GAPDH
SALINE
COCAINE
LIF
2
1.5
1
0.5
0
VTA
SN

The most dramatic changes in mRNA levels were found in the locus coeruleus (LC), an area that we did not examine. Consistent with our results, they found no significant changes in any mRNA examined in the VTA. It is therefore possible that we did not assay the appropriate brain area. Alternatively, our drug-treatment paradigm may not have been appropriate to produce significant differences in our cocaine-exposed animals. Numan et al. found only slight changes in mRNA expression after chronic morphine treatment. Instead, it was precipitated withdrawal (using the opiate antagonist naltrexone) that produced several-fold changes of neurotrophin expression in the LC. We are not aware of any such precipitated withdrawal paradigm for chronic cocaine treatment that could have been used to enhance mRNA alterations in our study. Finally, the assays we used to measure mRNA changes may not have been as precise as necessary to detect potentially small differences in expression. Indeed, Numan et al. successfully measured mRNA levels in these brain areas using radioactive *in situ* hybridization, in which very small alterations could be detected in the LC. This method has the advantage of specific localization within the section, by which individual nuclei can be identified and quantified. In conclusion, any or all of these technical considerations may have been responsible for our inability to document changes in the endogenous levels of CNTF, BDNF and LIF in the mesolimbic dopamine system following chronic cocaine exposure.

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