

Tumor necrosis factor-alpha in normal and diseased brain: Conflicting effects via intraneuronal receptor crosstalk?

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Tumor necrosis factor-alpha (TNF- α) is pleiotropic mediator of a diverse array of physiological and neurological functions, including both normal regulatory functions and immune responses to infectious agents. Its role in the nervous system is prominent but paradoxical. Studies on uninflamed or “normal” brain have generally attributed TNF- α a neuromodulatory effect. In contrast, in inflamed or diseased brain, the abundance of evidence suggests that TNF- α has an overall neurotoxic effect, which may be particularly pronounced for virally mediated neurological disease. Still others have found TNF- α to be protective under some conditions of neurological insult. It is still uncertain exactly how TNF- α is able to induce these opposing effects through receptor activation of only a limited set of cell signaling pathways. In this paper, we provide support from the literature to advance our hypothesis that one mechanism by which TNF- α can exert its paradoxical effects in the brain is via crosstalk with signaling pathways of growth factors or other cytokines. *Journal of NeuroVirology* (2002) **8**, 611–624.

Keywords: cytokine; glutamate; human immunodeficiency virus type 1 (HIV-1); inflammation; neurodegenerative; neuron; tumor necrosis factor-alpha

Introduction

Tumor necrosis factor-alpha (TNF- α) is a prototypical inflammatory cytokine, originally characterized as an endotoxin-induced serum factor that caused necrosis of certain tumor cell types (Carswell *et al*, 1975). Since that time, TNF- α has been found to be a key regulator of the immune response, capable of diverse cellular effects, including apoptosis, necrosis, inflammatory effects, proliferative or

growth-promoting effects, and hematopoietic effects (Pan *et al*, 1997). In the central nervous system (CNS), TNF- α is an important modifier of thermoregulation and the hypothalamic-pituitary-adrenal (HPA) axis. More recently, TNF- α has also garnered attention as a significant player in the regulation of cell fate within the nervous system, both in normal brain development and in pathological brain conditions.

Up-regulated in most if not all immune and inflammatory responses in the brain, TNF- α likely has important roles in a broad range of neurodegenerative disease. However, these roles are widely variable and often uncertain: in different settings, TNF- α can be causative, beneficial, or secondary to the disease. There are a multitude of factors that might determine whether TNF- α will have a protective or toxic effect on neurons under pathological brain conditions, and many of those will be discussed here. Which of the effects of TNF- α predominate may be in part dictated by the involvement of viral infection in the disease. TNF- α has been implicated in a number of definitive

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These studies were funded in part by NIH grants T32 AI49815 (SWP and SD); R01 MH56838 (SD and HAG); and P01 MH64570 (SD and HAG). MJB is a trainee in the Medical Scientist Training Program, NIH T32 GM07356.

Received 27 June 2002; revised 27 August 2002; accepted 13 September 2002.

Table 1 TNF- α in viral diseases of the nervous system

<i>Virus</i>	<i>Classification (Family [Genus*])</i>	<i>Host</i>	<i>TNF-α/TNFR</i>	<i>Reference</i>
HIV-1	<i>Retroviridae [Lentivirus]</i>	Human	TNFR-2	Ryan <i>et al</i> , 2001; Poli <i>et al</i> , 1999;
FIV		Cat	TNF- α	Craig <i>et al</i> , 1997
Ovine IV		Sheep	TNF- α	
HTLV-1	<i>Retroviridae [BLV-HTLV retroviruses]</i>	Human	TNF- α	Hoffman <i>et al</i> , 1992
Cytomegalovirus	<i>Herpesviridae [Betaherpesvirinae]</i>	Human	TNF- α	Tong <i>et al</i> , 2001; Cheeran <i>et al</i> ,
		Rodent		2001; Shinmura <i>et al</i> , 1999
Epstein-barr virus	<i>Herpesviridae [Gammaherpesvirinae]</i>	Human	sTNFR-1, sTNFR-2	Feng <i>et al</i> , 1999
Polytropic murine virus (Fr98)	<i>Retroviridae [Mammalian type C retroviruses]</i>	Rodent	TNF- α	Peterson <i>et al</i> , 2001
Chimeric oncornavirus (FrCas(E))	<i>Retroviridae [Mammalian type C retroviruses]</i>	Rodent	TNF- α	Askovic <i>et al</i> , 2001
Theiler's murine encephalomyelitis virus	<i>Picornaviridae [Cardiovirus]</i>	Rodent	TNF- α	Koh <i>et al</i> , 2000; Molina-Helgado <i>et al</i> , 1999
Sindbis virus	<i>Togaviridae [Alphavirus]</i>	Rodent	TNF- α	Trgovcich <i>et al</i> , 1999
Murine hepatitis virus (JHMV)	<i>Coronaviridae [Coronavirus]</i>	Rodent	TNF- α	Lin <i>et al</i> , 1998; Sun <i>et al</i> , 1995
Murine leukemia virus (LP BM5 MuLV)	<i>Retroviridae [Mammalian type C retroviruses]</i>	Rodent	TNF- α	Sei <i>et al</i> , 1997
Canine distemper virus	<i>Paramyxoviridae [Morbillivirus]</i>	Rodent	TNF- α	Bencsik <i>et al</i> , 1996

Note. TNF- α or its receptors, as indicated in column 4, may play a role in the pathology of these viral infections of the nervous system. This is not an exhaustive list.

*Subfamily given for Herpesviridae.

viral disease affecting the nervous system (Table 1), and its role in some of these will be discussed in more detail later. In general, for some neurological diseases that result directly from known and active viral infections, such as human immunodeficiency virus (HIV) infection of the nervous system, the neural effects of TNF- α are almost decidedly toxic. For neurodegenerative diseases not known to have a direct and active viral cause, the role of TNF- α is often more ambiguous, with different investigators reporting toxic, nontoxic, and even protective effects.

There is also a growing list of neurodegenerative diseases, including Alzheimer's disease (AD), schizophrenia, and multiple sclerosis (MS), that are not themselves defined by an active viral infection, but for which prior or latent viral infections may confer increased susceptibility to, or even induce, the disease. In AD (Itzhaki and Dobson, 2002; Perry *et al*, 2001; Verreault *et al*, 2001), schizophrenia (Boin *et al*, 2001; Yolken *et al*, 2000), and MS (see Hemmer *et al*, 2002 and Cermelli and Jacobson, 2000, for review), TNF- α has been ascribed a possible role in either conferring susceptibility to the disease and/or contributing to the pathophysiology of the disease itself. Although demonstrating a causative role is difficult, particularly in clinical studies, evidence that TNF- α plays a significant part in the disease is substantial and convincing. Evidence for the role of TNF- α in the development or progression of schizophrenia is still more equivocal, although the hypothesis has attracted considerable current research attention. Evidence for viral links—particularly causative links as opposed to consequential links—to these diseases is also contested, but the burgeoning literature on the subject in recent years makes the possibility difficult to ignore.

Given the multifaceted functions of TNF- α , it is difficult, yet exceedingly important, to understand the molecular mechanisms by which it may achieve these numerous effects through receptor activation of only a limited set of cell signaling pathways. Here, we provide evidence for our assertion that modulating the signaling pathways of growth factors or other cytokines, either at the receptor level or at the level of downstream signal transduction, may be one mechanism by which TNF- α is able to achieve these paradoxical effects. We propose that much as peripherally derived cytokines may determine the fate of a target immune cell, the complex balance of proinflammatory and anti-inflammatory cytokines in the CNS, along with other trophic factors, may determine a neuron's fate. Thus, CNS cytokines may serve as common mediators for a number of neuropathological conditions that were previously thought unrelated. We will generally be referring to neuron-to-neuron interactions in the brain unless other cell types or regions are specifically mentioned.

Origin of TNF- α signals in the brain

TNF- α originating from a variety of cell types in either the periphery or the brain itself can act on the brain to regulate neural-immune interactions as well as other brain functions. TNF- α derived from peripheral immune cells can act in a hormone-like fashion through the HPA axis and the vagus nerve to stimulate and affect several CNS responses, including neuroendocrine responses, sickness, and other behavioral patterns, as well as sleep and fever (Sternberg, 1997). In turn, feedback from the HPA axis, the sympathetic nervous system, and the peripheral nervous

system act to regulate the immune system. Brain vasculature can also convey cytokine-induced signals to the brain via second messengers, such as nitric oxide (NO) or prostanoids (Licinio and Wong, 1997). Finally, peripherally derived TNF- α can enter the CNS and thus act directly on brain parenchyma by crossing the blood-brain barrier (BBB), either by active transport mechanisms or passive diffusion in the circumventricular organs, including areas of the hypothalamus, pituitary, and pineal gland (Sternberg, 1997). Although much of this action occurs in response to immune activation, there is growing evidence to suggest that peripheral TNF- α and other peripheral cytokines may exert neuromodulatory actions in healthy individuals under noninflammatory (i.e., immune “normal”) conditions—for example, regulation of sleep and feeding (Vitkovic *et al*, 2000).

TNF- α can also be generated *within* the CNS (Sternberg, 1997); all neural cell types are thought to contain TNF- α receptors and be capable of synthesizing TNF- α under some conditions. TNF- α of central origin may be secreted from the brain to modulate peripheral immune functions (Licinio and Wong, 1997), and brain-derived cytokines, such as those from the periphery, are increasingly thought to play substantial roles in modulating normal brain physiology (Vitkovic *et al*, 2000).

TNF- α in the normal CNS

There is considerable evidence implicating roles for TNF- α during normal brain development. TNF- α has been shown to modulate the entire range of neuronal function, from electrophysiology to cell signaling to gene transcription. Its pleiotropic effects in the CNS include regulation of growth factors and other cytokines; moderation of the synthesis and release of the monoamines and of glutamate; modulation of the responsive state of α_2 adrenergic receptors; effects on calcium and potassium ion currents, calcium homeostasis, membrane potentials, and long-term potentiation; and finally, effects on a wide range of second-messenger signaling pathways in neurons (Vitkovic *et al*, 2000; Grazia De Simoni and Imeri, 1998; Munoz-Fernandez and Fresno, 1998; Jarskog *et al*, 1997; Pan *et al*, 1997). Finally, many downstream modulators of TNF receptor activation, such as nuclear factor kappa B (NF- κ B) and p38 mitogen-activated protein kinase (MAPK), have also been implicated in roles that govern cell fate (Yang *et al*, 2002).

Even with this evidence, there is still some debate as to whether the roles of TNF- α in these various processes occur under normal physiological (i.e., homeostatic) conditions and concentrations, or whether these actions only occur at abnormal levels of TNF- α (i.e., at levels associated with significant inflammation in the periphery or in the CNS). Several key observations argue for an important homeostatic role for TNF- α in normal brain: (1) TNF- α and its receptors

are constitutively expressed in discrete brain regions in a manner consistent with a role in endogenous brain function; (2) expression is developmentally regulated; and (3) spatial and temporal variations in expression are consistent with a likely role in regulating sleep and other neuroendocrine and autonomic functions. The following studies will support the hypothesis that TNF- α is both *present* and *functionally active* at the levels found in uninflamed, healthy brain.

In “normal” or control rodent brain, TNF- α protein and/or TNF- α mRNA are expressed by neurons in multiple brain regions, including cortex, striatum, thalamus, hypothalamus, hippocampus, cerebellum, brainstem, and pons (Vitkovic *et al*, 2000). TNF- α mRNA has been detected in subcortical white matter of the normal human CNS (Wesselingh *et al*, 1993); but no other brain regions were included in this study. Although these results do not have a widespread consensus in the literature (Vitkovic *et al*, 2000), variations in data between different laboratories may have been due to differing assay sensitivities, species differences, relative health, or immune status of the subjects or diurnal variations in TNF- α expression (discussed below).

Likewise, TNF- α receptors (TNFRs) have been found in all of the areas where TNF- α protein or mRNA has been found, as well as substantia nigra (in humans) and mesencephalon and basal ganglia (in mouse) (Boka *et al*, 1994), and it has been suggested that the receptor distribution may be even broader (Vitkovic *et al*, 2000). Moreover, unlike TNF- α protein or mRNA production, which is confined to neurons under physiological conditions, both TNFR variants (TNFR-1 and TNFR-2) are constitutively expressed by all neural cell types, with the exception of astrocytes and oligodendrocytes, which predominantly express TNFR-1 (Munoz-Fernandez and Fresno, 1998; Dopp *et al*, 1997). The neuron-specific constitutive TNF- α expression, in conjunction with the broader cell-type and regional expression of TNFRs, suggests a functional, regulatory role for TNF- α .

In developing rodent brain, TNF- α is transiently expressed at high levels in immature embryonic neurons and astrocytes (Munoz-Fernandez and Fresno, 1998). This high, transient expression ceases once the cells mature, except for continued low-level expression in neurons of certain brain regions. This finding would suggest a role for TNF- α in regulating normal brain development.

Neuronal TNF- α expression often exhibits diurnal variation and is highest during peak sleep periods (Bredow *et al*, 1997). This might explain why some studies did not find significant TNF- α expression in neurons, but more importantly, it suggests a role for TNF- α in regulating sleep. This concept is further supported by findings that TNF- α increases non-rapid eye movement (NREM) sleep, and that TNFR-null mice sleep less than control animals (Krueger *et al*, 1998). These data, together with findings of

constitutive TNF- α expression in the pathways innervating autonomic and neuroendocrine-related regions (Pan *et al*, 1997), and TNF- α involvement in feeding regulation (Plata-Salaman *et al*, 1988), suggest roles for TNF- α as a neuromodulator regulating neuroendocrine and other autonomic functions.

TNF- α in CNS disease

TNF- α may play a key role in a wide range of neurodegenerative diseases including ischemia, Parkinson's disease (PD), HIV-1-associated dementia (HAD), MS, epilepsy, traumatic brain injury (TBI), and AD (Grazia De Simoni and Imeri, 1998). The modes by which TNF- α production and activity increase are likely to be similar across this range of diseases, and many of the cellular responses to TNF- α that produce neurotoxicity, will be discussed in the next section.

TNF- α is intimately involved in the CNS immune response to any neural injury, infection, or damage, and therefore levels of TNF- α (and other cytokines) have been shown to increase in the brain in response to many, if not all, neurological insults or diseases (Munoz-Fernandez and Fresno, 1998; Pan *et al*, 1997). Moreover, this may be particularly true of viral infections eliciting either an acute or chronic neuroimmune response (see below and Table 1). In this fashion, TNF- α may be a common mediator or effector of the pathologies seen in many seemingly disparate neurological diseases. Furthermore, there has been sufficient evidence to suggest that (1) brain TNF- α (and other cytokine) levels are *acutely* increased even with peripheral illnesses, or illnesses of *any* nature, and that this increased activity is what moderates fever and other sickness behavior (decreased appetite, somnolence, attention to illness, etc.) (Dantzer, 2001); and (2) in similar fashion, increased cytokine (including TNF- α) expression may alter production, function, and/or turnover of a variety of neurotransmitters, and thus is likely to play a role in many behavioral and psychiatric illnesses (Kronfol and Remick, 2000). In other words, TNF- α may contribute to pathological brain states even in the absence of acute neuronal damage or lasting sequelae. Thus, it is plausible that it may have some impact on the brain with virtually any pathological condition.

Increased TNF- α expression in chronic inflammatory CNS disease appears to be a result of both increased production of TNF- α by immune activated CNS cells, as well as increased entry of TNF- α from the periphery. Brain insult, injury, or disease will trigger a local immune response, resulting in activation of astrocytes and microglia, with a subsequent increase in TNF- α production by these cells and perhaps neurons. In turn, many cytokines, including TNF- α , stimulate production of themselves or other cytokines, which may result in a positive feedback cycle (Munoz-Fernandez and Fresno, 1998; Pan *et al*, 1997).

TNF- α entering from the periphery further enhances this feedback amplification loop of TNF- α production in the brain. In many neurological diseases, the amount of peripheral TNF- α entering the brain increases relative to the normal state (Pan *et al*, 1997). This might occur for several reasons. First, the local CNS immune response may recruit activated (i.e., TNF- α -producing) peripheral leukocytes and macrophages to the brain. Permeability of the BBB also increases with brain inflammation (Pan *et al*, 1997), which may increase access of these peripheral immune cells to the brain parenchyma, as well as increase passage of peripheral TNF- α . Significantly, TNF- α itself may play a key role in increasing BBB permeability and recruiting activated immune cells (Pan *et al*, 1997). This has been most well documented and determined to be of particular significance in MS and experimental autoimmune encephalomyelitis (EAE), and HAD (Pan *et al*, 1997; Epstein and Gelbard, 1995). Finally, any peripheral immune activation will increase the amount of peripheral TNF- α available to enter the brain by the active or passive transport mechanisms mentioned earlier. In illnesses that do not directly affect the nervous system, this phenomenon may simply serve to regulate sickness behavior. However, in illnesses with a direct neurological component, the association of other factors may make it such that this increased peripheral TNF- α actually contributes to the pathology of the neurological illness.

Thus, levels of CNS and/or peripherally derived TNF- α that exceed an as yet undetermined threshold for cellular homeostasis are thought to set forth a cascade reaction such that previously unaffected astrocytes, microglia, or even neurons are stimulated in turn to produce abnormally high concentrations of TNF- α . Although no generalization can be made regarding the persistence of elevated TNF- α levels in the CNS during the course of neurodegenerative diseases, in some cases glia or neurons alone are likely to become the primary producers of TNF in the CNS (Munoz-Fernandez and Fresno, 1998).

In HIV-1 encephalitis, the amounts of both CNS-produced and peripheral TNF- α in the brain are markedly elevated, due to heightened virus-induced activation of both the CNS immune response and the peripheral immune system; direct invasion of the brain by virus-infected macrophages; and other virus-related effects that may further amplify TNF- α production, including recruitment of activated immune cells, increases in BBB permeability, and neurological damage. Not surprisingly, then, TNF- α has been ascribed a significant role in the pathology of this disease (Griffin, 1997). Perhaps the most telling evidence to implicate TNF- α in inflammatory damage to the CNS from virally mediated neurodegeneration comes from work using the LP-BM5 Moloney leukemia virus model for murine acquired immunodeficiency syndrome (MAIDS). Despite being an (onco)retroviral infection, this model mimics many of the key

immunological, biochemical, and neurological features of lentiviral infection in the CNS (Kustova *et al*, 1998). Using LP-BM5–infected mice with a targeted deletion of TNF- α , Iida *et al* (2000) demonstrated that CNS neuropathology and biochemical and neurocognitive effects were substantially ameliorated despite unchanged systemic signs of immunodeficiency compared to wild-type mice infected with LP-BM5. As a variety of viral agents likely cause similar dramatic increases in CNS TNF- α levels via the mechanisms described above, TNF- α or its receptors are also implicated in the pathogenesis of other virally induced neurodegenerative diseases that target the brain and/or spinal cord. Many of these are listed in Table 1.

Other specific examples of roles for TNF- α in mediating the destructive effects of a variety of neurological diseases are easily found. In AD, elevated levels of TNF- α are expressed by microglia associated with amyloid plaques, and beta-amyloid has been shown to stimulate neurotoxic levels of TNF- α from microglia, suggesting a possible role for TNF- α in AD pathogenesis (Munoz-Fernandez and Fresno, 1998). Similarly, TNF- α immunoreactive glial cells have been observed in close proximity to degenerating dopaminergic neurons in PD, and thus TNF- α may also contribute to PD pathogenesis by inducing death of mesencephalic dopaminergic neurons via a ceramide-dependent pathway (Brugg *et al*, 1996). In MS, or the related animal model EAE, levels of TNF- α may correlate with disease progression, and blocking TNF- α ameliorates disease severity (Munoz-Fernandez and Fresno, 1998). One mechanism by which TNF- α is thought to be neurodestructive in MS is by inducing damage to oligodendrocytes (Munoz-Fernandez and Fresno, 1998), although other more direct effects on neurons may also exist. In stroke (ischemia), TBI, and intracerebral hemorrhage, TNF- α production is up-regulated after injury, and is directly correlated with extent of cerebral damage and neurological impairment (Mayne *et al*, 2001; Knoblach *et al*, 1999; Barone *et al*, 1997). Finally, neuronal damage in bacterial meningitis and cerebral malaria may also be triggered by TNF- α (Munoz-Fernandez and Fresno, 1998). Polymorphisms of the TNF- α promoter region have been linked to the development of cerebral malaria (Ubalee *et al*, 2001).

In some of these diseases—most notably MS and EAE, nerve injury, TBI, stroke, AD, and PD—TNF- α has also been attributed a neuroprotective role (Fontaine *et al*, 2002; Kassiotis and Kollias, 2001; Stoll *et al*, 2000; Shinpo *et al*, 1999; Gary *et al*, 1998; Mattson *et al*, 1997; Bruce *et al*, 1996). Perhaps the most dramatic example of this occurs in mice with targeted deletion of TNFRs (Bruce *et al*, 1996). These mice have augmented damage after focal cerebral ischemia or seizures compared to their wild-type controls, leading the authors to conclude that TNF- α plays a neuroprotective role. Later studies from this group (Gary *et al*, 1998) established the dependence

of neuroprotection on the TNFR-1 receptor, following ischemic insult to the hippocampus, whereas other groups using models of MS (Kassiotis and Kollias, 2001) and retinal ischemia (Fontaine *et al*, 2002) have demonstrated that TNFR-2 is involved in neuroprotection while TNFR-1 is involved in neurotoxicity *in vivo*. Perhaps some of the conflicting results observed between *in vitro* and *in vivo* studies may be due to the possibility that not all of the neurotoxic effect of TNF- α seen in these diseases is mediated exclusively by neuronal TNFR activation. For example, TNF- α likely also participates in secondary injury mechanisms such as glial recruitment and activation of glial TNFRs, thus resulting in glial-induced production of reactive oxygen species (ROS), inflammatory factors, and other potentially neurotoxic products (Knoblach *et al*, 1999).

Furthermore, TNF- α is *neuroprotective* to hippocampal organotypic slices when applied *prior* to ischemic stress, but *neurotoxic* when applied *after* the same ischemic insult (Wilde *et al*, 2000). When TNF- α is present after the ischemic insult, CNS damage appears to be mediated by the production of free radicals and the failure of neurons to defend against this type of damage. Ischemic preconditioning (i.e., exposure of neural tissue to low levels of free radicals) may activate signal transduction pathways involved in neuroprotection (Dawson, 2002; Digicaylioglu and Lipton, 2001; Rauca *et al*, 2000).

TNF- α signaling pathways and intraneuronal receptor crosstalk

Thus far, we have seen that TNF- α may have a neuromodulatory role in normal brain function, yet become neurotoxic under some conditions of brain inflammation and disease. Therefore the question becomes how does this change of function occur? Or, in evolutionary terms, why would it? Although the “why” and “how” are difficult to answer, the biological precedents are numerous. Many cytokines and growth factors, including nerve growth factor (NGF), can mediate either neuronal maintenance functions, protection, or death depending on the precise set of circumstances (see Casaccia-Bonofil *et al*, 1999; Loddick and Rothwell, 1999; and below). Therefore, it should be of no surprise to see TNF- α exhibit similar behavior. There are numerous mitigating factors that may impact what role TNF- α has under any given set of circumstances. These include type and location of neuron affected (e.g., inhibitory or excitatory, neurotransmitter synthesized, neuroanatomical location, etc.); precise timing and neural environment under which TNF- α is expressed; circulating and local levels of TNF- α ; whether or not the effects of TNF- α on neurons are primary (i.e., direct) or secondary (i.e., mediated by glia); type of neuronal toxicity involved (i.e., excitatory or nonexcitatory, necrotic or apoptotic); presence or absence of receptor

presensitization (e.g., pretreatment effects); and, finally, receptor state, location, and levels.

Although these factors can provide ideas into *what* might contribute to the paradoxical effects of TNF- α under different brain environments, precisely *how* the varying effects are orchestrated remains elusive. Although much progress has been made towards elucidating the cell signaling pathways induced by TNFR activation, it still remains very much a mystery how TNF- α can modulate such diverse functions through activation of the same limited set of second messenger systems. Therefore, more recent research has turned towards a less reductionist approach, including the interactive effects that TNFRs may have with other colocalized receptors. However, a basic understanding of the TNFR signaling pathways is necessary before embarking on a discussion of this receptor crosstalk.

TNF- α signaling pathways

TNF- α is formed as a 26-kDa transmembrane precursor protein (pTNF- α), from which a 17-kDa soluble form (sTNF- α) is released by proteolytic cleavage (Pelagi *et al*, 2000). Both the membrane-anchored and soluble forms are homotrimerically active ligands that specifically bind and crosslink two members of the TNFR superfamily: the 55-kDa TNFR-1 (a.k.a. CD120a) and the 75-kDa TNFR-2 (a.k.a. CD120b), both of which are constitutively expressed in the brain (Vitkovic *et al*, 2000). Soluble TNF- α has similar affinities for TNFR-1 and TNFR-2, but pTNF- α has a higher affinity for the TNFR-2 (Ledgerwood *et al*, 1999). Some nonspecific binding of TNF- α to the more closely homologous members of the TNFR superfamily, such as the p75NGF receptor, may also occur (Ameloot *et al*, 2001). In most cases, receptor activation is thought to result in internalization and perhaps translocation of the receptor-ligand complex to subcellular localizations, although the significance of the role of ligand or receptor internalization is unclear, because many aspects of TNF- α signaling can still occur when ligand or receptor internalization is inhibited (Jones *et al*, 1999).

Although both TNF- α receptors have traditionally been considered cell surface (i.e., plasma membrane-bound) receptors, they often also exist in subcellularly localized and cleaved, soluble forms (Ledgerwood *et al*, 1999). In fact, at least one group has reported (in endothelial cells) that although most TNFR-2 molecules are located on the cell surface, the majority of steady-state TNFR-1 are localized to the perinuclear golgi complex (Jones *et al*, 1999). However, current understanding is that only the plasma membrane-bound TNFRs associate with the known effector molecules to activate the usual characterized array of downstream cell signals (Ledgerwood *et al*, 1999). Soluble TNFRs, on the other hand, may serve as a means for regulating both TNFR surface expression and, because they still retain ligand-binding capacity, TNF- α bioavailability (Ledgerwood

et al, 1999). The function of subcellularly localized TNFR-1 and TNFR-2 receptors is less clear, because it is possible, but not certain, that TNF- α binds these receptors, and even if ligand binding occurs, it does not induce association with the usual adapter proteins (Jones *et al*, 1999). In the same fashion as soluble TNFRs, this subcellular pool of receptors may also serve to regulate surface receptor density and perhaps TNF- α bioavailability. Nonetheless, more direct roles for these intracellular receptors are suggested by evidence that TNF- α activates signaling mechanisms at multiple subcellular locations, and an as yet unidentified TNF- α -binding protein has been localized to the inner mitochondrial membrane (Ledgerwood *et al*, 1999).

It has often been assumed that TNFR-1 is the primary effector of TNF- α -induced biological outcomes in neurons, but this concept has blurred in recent years. Although the signaling functions of TNFR-2 remain less well understood, it has been shown that TNFR-2 can perform a "ligand passing" role for TNFR-1, or otherwise act in concert with TNFR-1 to supplement, enhance, or moderate its effects (Ledgerwood *et al*, 1999). Moreover, a growing body of evidence suggests that TNFR-2 may also subserve some effects of TNF- α entirely independently of TNFR-1 activation. Based on several knockout studies showing decreased neuronal damage with TNFR-1 knockouts, and increased damage with TNFR-2 knockouts (Fontaine *et al*, 2002; Yang *et al*, 2002), it might also be tempting to conclude that—at least in neurons—TNFR-1 activation induces neuronal cell death, and TNFR-2 activation induces neuronal protection. However, once again, this view may be too simplistic, because TNFR-2 activation alone can promote necrosis in both neuronal cells (Sipe *et al*, 1998) and non-neuronal cells (Pelagi *et al*, 2000). Thus, the role of TNFR-2 in neurons may be not only significant, but also more complicated than the knockout studies might suggest. In short, both TNFR-1 and TNFR-2 can probably transduce either neurotoxic or neuroprotective signals depending on their precise signaling environment.

Because TNFRs lack intrinsic enzymatic activity, ligand-induced receptor cross-linking induces intracellular signaling events by causing a conformational shift in the cytoplasmic tails of the receptors in proximity to adapter proteins (Ledgerwood *et al*, 1999). The TNFR-1 utilizes an ever-growing list of adapter molecules to initiate three primary signaling pathways in response to activation: the caspase (i.e., apoptosis), c-Jun, and (NF- κ B) pathways (Figure 1). Although the cellular effects of the latter two pathways can vary with cell type, in neurons, NF- κ B activation appears to be an important neuroprotective response to inflammation, whereas activation of the c-Jun pathway usually promotes neuronal death (Maggirwar *et al*, 2000; Ham *et al*, 1995; Estus *et al*, 1994). Other TNFR-1-activated second messengers thought to be of particular significance to neuronal

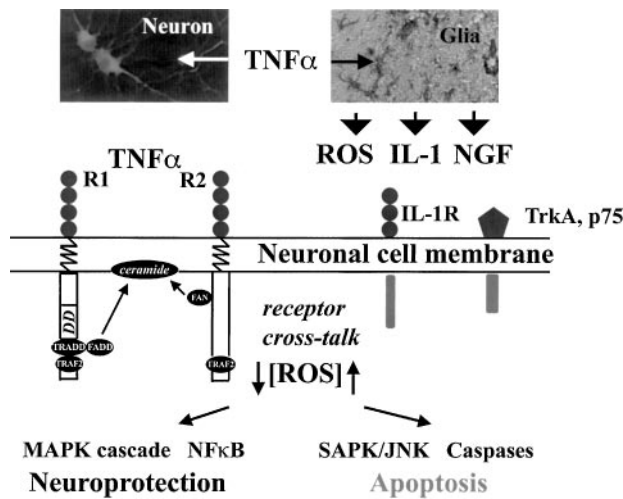


Figure 1 Factors affecting neuronal fate in response to TNFR activation. Some of the concepts discussed in the text that affect cell outcome after activation of neuronal TNF- α receptors are depicted, including presence of glia, other cytokines, ROS production, and crosstalk with other colocalized receptors. See text for details.

function(s) include the extracellularly regulated kinase (ERK) MAPK cascade, which is usually neuroprotective (Bahr *et al*, 2002), and ceramide production, which is generally considered neurotoxic (Marchetti *et al*, 2002), although it may be neuroprotective under some circumstances (Ariga *et al*, 1998). A final common mechanism for TNF- α -mediated neuronal cell death may be generation of ROS, although the precise means by which this occurs is unknown (Rath and Aggarwal, 1999).

TNFR-1 activation first results in the release of silencer of death domain (SODD), which allows for binding of TNFR-associated death domain (TRADD) to the receptor's death domain, a prerequisite step for activation of all downstream signaling pathways (Rath and Aggarwal, 1999). Although TRADD binding to TNFR-1 is sufficient to promote the rest of the apoptotic cascade (i.e., successive activation of Fas-associated death domain (FADD), Fadd-like interleukin-1-converting enzyme (FLICE; aka caspase-8, caspases, etc.), subsequent binding of other effector molecules is necessary for activation of the c-Jun and NF- κ B pathways. By way of illustration, TNFR-associated factor-2 (TRAF-2) binding to TRADD enables activation of the c-Jun pathway, but further binding of receptor-interacting protein (RIP) to TRAF-2 is necessary for NF- κ B induction. In addition, ceramide production can be triggered by both FADD and by a separate receptor domain *outside* of the death domain, which may account for its sometimes opposing effects (Ariga *et al*, 1998). Because TNF- α binding to TNFR-1 can simultaneously induce all of these pathways, the relative availability of certain downstream effector molecules may control which cellular response pathway takes precedence. Also significant are inhibitory molecules or functions that promote one TNFR-activated cascade while inhibiting another (Rath and Aggarwal, 1999).

The TNFR-2 has been shown to activate many of the same pathways, except the TRADD/FADD/FLICE/caspase apoptosis cascade. The reason for this discrepancy is related to the fact that the TNFR-2 does not have a death domain, and therefore does not bind TRADD. Instead, TNFR-2 signaling is initiated by TRAF-2 dimers (either homodimers or heterodimers with TRAF-1) associating directly with the activated receptor (Baker and Reddy, 1998), thus enabling the TNFR-2 to activate many of the same pathways as TNFR-1 with the exception of the caspase cascade. Nonetheless, TNFR-2 itself is still capable of initiating neuronal cell death, even without a known death domain (Ledgerwood *et al*, 1999; Rath and Aggarwal, 1999). Exactly how this occurs is unclear, but could involve c-Jun activation.

Intraneuronal receptor crosstalk

The rapid growth in understanding of TNF- α signaling mechanisms has, unfortunately, not been accompanied by an equally increased understanding of how TNF- α exerts its pleiotropic effects in the brain. This discrepant state of knowledge suggests that the complex effects of TNF- α cannot be explained simply by receptor location or density, or receptor activation of a rote, predetermined set of cellular signaling cascades. A plausible alternative could involve TNFR crosstalk, which could occur either in paracrine fashion, between receptors on interacting cells, or in autocrine fashion, between receptors on the same cell. We will focus on the latter situation.

Conceivably, this could occur on three levels. First, the TNFR-1 and TNFR-2 receptors might interact to control differing effects of TNF- α . Second, a wide variety of cytokines and growth factors activate the same downstream signaling mechanisms, including activation of other cytokines and growth factors, and thus crosstalk may occur among these common signaling pathways. For example, in neuronal cells, FGF has been shown to induce apoptosis via activation of TNFRs (Eves *et al*, 2001). Third, perhaps the most novel and yet unexplored means by which intraneuronal receptor crosstalk might occur is by direct (i.e., receptor-level) inhibition or stimulation of receptor activity by a coacting factor.

One might envision a crosstalk mechanism by which TNFR-2 modulates TNFR-1 function by competing for TRAF-2, thus down-regulating the TRAF-2-dependent TNFR-1 pathways and increasing the likelihood of TNFR-1-induced cell death (which is not TRAF-2 dependent). In this fashion, TNFR-2 signaling may be able to convert a TNFR-1 response from neuromodulatory to neurotoxic. Unfortunately, there is little direct evidence in support of this concept in neurons. However, in separate experiments, competitive inhibition of TRAF-2 binding reduced NF- κ B activation, and reduced TNFR-1-mediated NF- κ B activation enhanced the TNFR-1 apoptotic signal (Brink and Lodish, 1998), thus supporting this as a plausible mechanism.

The second possible mechanism for intraneuronal TNFR crosstalk is interference by, or competition for, similar or identical second messenger molecules activated by non-TNFRs. A wide variety of cytokines and neurotrophic factors use a fairly limited set of cell signaling pathways and transcription factors (e.g., ERK and other MAPK cascades, c-Jun/c-Fos regulation of activating protein-1 [AP-1] transcription, NF- κ B, ceramide, etc.) to achieve their effects. Many of them also use TRAFs. Therefore, competition for a limited pool of kinases or adapter molecules, as influenced by relative effector molecule binding affinities and densities of the competing receptors, could easily influence which receptor signal takes precedence. Moreover, receptor-activated inhibitory molecules might act on the signaling pathways of colocalized receptors, or nonspecific kinase activity might occur by kinases in high abundance.

Two of the more prominent examples of this kind of crosstalk by second messenger systems include TNF- α signal interaction with interleukin-1 (IL-1) and NGF. TNF- α and IL-1 are proinflammatory cytokines that activate nearly identical signaling pathways and transcription factors, and achieve remarkably similar effects, despite structurally unrelated protein and receptor content (Eder, 1997). Moreover, they have an equally remarkable congruence of effects on brain. In healthy brain tissue, like TNF- α , IL-1 is also nontoxic and thought to regulate many of the same neuromodulatory activities, but becomes toxic under conditions of brain inflammation or disease (Rothwell *et al*, 1997). In achieving these effects, their analogous signaling pathways increase the likelihood of synergistic interactions between these two molecules. Both cytokines use TRAFs (TRAF-2 for TNFR, TRAF-6 for IL-1 receptor [IL-1R]) to activate the NF- κ B, c-Jun, and p38 MAP kinase cascades (Eder, 1997). Although NF- κ B activation is usually neuroprotective, activation of c-Jun and the p38 MAPK cascades is usually neurotoxic. Therefore, in an inflamed brain, when both TNF- α and IL-1 levels rise, increased coactivation of both TNFRs and IL-1Rs might be sufficient to shift the balance from neuroprotective to neurotoxic by increasing activation of the neurotoxic pathways (i.e., c-Jun and p38 MAPK) relative to the neuroprotective one (i.e., NF- κ B). There are at least two hypothetical mechanisms by which this might occur in the CNS. First, IL-1R-mediated NF- κ B activation may be weaker than TNFR-mediated NF- κ B activation, whereas both receptors may activate c-Jun and p38 MAPK equally (Eder, 1997), thus increasing the likelihood that IL-1/TNFR interaction will exert neurotoxic synergism. Second, the fact that the TNFRs and IL-1Rs utilize only one common neuroprotective pathway (i.e., NF- κ B), but at least two *different* neurotoxic pathways (i.e., c-Jun and p38 MAPK), might result in (1) increased competition for intermediaries in neuroprotective pathways, or perhaps more likely, (2) increased production of the number of *unique* neurotoxins (with potentially

additive or synergistic effects) relative to the number of *unique* neuroprotectants—either of which would shift the overall net intraneuronal balance towards a neurotoxic cascade.

This next contrasting example of TNFR crosstalk involves interaction with NGF, a neurotrophic factor that binds the p75 and TrkA receptors and is capable of inhibiting TNF- α -induced neuronal cell death (Haviv and Stein, 1999). Which NGF signaling pathways mediate this inhibition? One might expect that most of the protective effects of NGF would be mediated by TrkA, as p75 usually modulates cell death through activation of the JNK pathway and selected caspases (Casaccia-Bonnet *et al*, 1999). Indeed, TrkA does activate the Akt pathway, a serine/threonine kinase that inhibits the apoptotic cascade by phosphorylating and thus inactivating many downstream apoptosis effectors such as Bad and caspase-9 (Wang *et al*, 1999). TrkA activation also activates the protective MAPK pathway. Either or both of these actions may enhance neuronal survival in the face of increased TNF- α signaling, although at least for oligodendrocytes, it has been shown that the Akt pathway probably plays a greater role than the MAPK pathway in NGF rescue of TNF- α -treated cells (Takano *et al*, 2000). In addition, coexpression of TrkA with p75 abrogates the latter's death-inducing effect while still allowing it to maintain NF- κ B activation (Casaccia-Bonnet *et al*, 1999), which likely further contributes to the reduction of TNF- α neurotoxicity. Adding to the complexity of these interactions is the possibility that beta-amyloid can stimulate p75 receptors and, together with TNF- α , exert a synergistic neurotoxic response when TNFRs are colocalized on the same neuron (Perini *et al*, 2002).

Additionally, ceramide and sphingolipid signaling pathways could also play multiple roles in TNFR/NGFR crosstalk. TNF- α activation induces ceramide production, which has been implicated in a large number of pathways contributing to neuronal cell death, including activation of C-Jun N-terminal kinase (JNK); involvement in the neurotoxic phospholipase A2 (PLA2)/arachidonic acid (AA)/reactive oxygen intermediates (ROI) signaling pathway; caspase activation, perhaps in part through decreased activity of Akt; and inhibition of some protective protein kinase C (PKC) variants (Goswamia and Dawson, 2000; Macdonald *et al*, 1999; Ariga *et al*, 1998; Spiegel *et al*, 1998). NGF, in contrast, stimulates ceramidase, which catabolizes ceramide, thus reducing the ceramide-induced activation of any or all of these pathways. Further, NGF also increases sphingosine-1-phosphate (SPP) levels, which not only increases neuroprotective MAPK/ERK activation, but also prevents JNK activation and consequent apoptosis by TNF- α and ceramide (Casaccia-Bonnet *et al*, 1999; Spiegel *et al*, 1998). Moreover, SPP also has a stimulatory effect on phospholipase D, leading to increased diacylglycerol (DAG) activity, PKC stimulation, and subsequent activation of ERK and NF- κ B cascades,

whereas ceramide typically has inhibitory effects on phospholipases and thus reduces DAG generation. However, this last scenario is greatly complicated by the facts that ceramide and SPP can each have opposing effects on different PKC variants, and that PKCs in turn regulate SPP and ceramide levels (Spiegel *et al*, 1998). Nonetheless, the more direct effects of NGF-induced SPP activity are likely to play prominent roles in alleviating neurotoxic effects of TNF- α -induced ceramide production.

Finally, unlike the work of Haviv and Stein (1999) discussed above where NGF completely reversed TNF- α toxicity in PC-12 cells, it is also quite possible that in some instances TNF- α might inhibit survival of neurons dependent on NGF. Indeed, function-blocking antibodies to either TNF- α or TNFR-1 prevented apoptosis after withdrawal of NGF in sympathetic or sensory neurons (Barker *et al*, 2001), suggesting that TNF- α facilitates the death of NGF-deprived neurons. Moreover, MacDonald *et al* (1999) show that AA, a downstream mediator of TNF- α , causes apoptosis in PC-12 cells by inhibiting NF- κ B and PKC-zeta, two components of the NGF signaling pathway. This TNF- α -induced apoptosis cannot be blocked by NGF. These data argue for inhibition of NGF survival signals by TNF- α as another mechanism of intraneuronal receptor crosstalk.

The third and final type of intraneuronal receptor crosstalk to be discussed—direct inhibition (or stimulation) of a colocalized receptor—is perhaps the most novel. In one example of this model, low doses of TNF- α can inhibit the survival promoting effects of insulin-like growth factor-1 (IGF-1) on mouse cerebellar granule neurons by impairing the ability of the IGF-1 receptor (IGF-1R) to phosphorylate tyrosine residues on insulin receptor substrate 2 (IRS2) (the major IGF-1R-docking molecule in primary granule neurons), which in turn inhibits subsequent IRS2-induced activation of the survival enzyme phosphatidylinositol 3' kinase (PI3 kinase) (Venters *et al*, 1999). In this fashion, TNF- α may contribute to neurotoxicity without necessarily having a directly neurotoxic effect itself.

Although this finding is interesting, it also begs one obvious question: If even low doses of TNF- α can inhibit survival promoting effects, then why is low constitutive TNF- α activity in normal brain not neurotoxic? There are several potential answers to this question. First, perhaps TNF- α , even at low levels of activity in normal brain, is always capable of inhibiting some survival signals, but this survival inhibition only begins to have a detrimental or neurotoxic effect on neurons when they are otherwise stressed. For example, brain inflammation or disease unleashes numerous cascades contributing to neuronal insult, many of which have been mentioned here. Therefore, under these conditions, neurons become ever-more dependent on opposing neuroprotective mechanisms designed to ensure their survival, and thus also much more sensitive to the effects of inhibition of any of

these survival promoting effects. In contrast, under nonstressed or “normal” conditions, neurons may be able to tolerate loss of some survival signals without loss of viability. Moreover, there may also be a hypothetical dose threshold at work here, so that only exposure to TNF- α above a certain level—perhaps above the level that is seen in normal brain—will inhibit survival signals. Similarly, concentrations or relative receptor activity of the survival signaling molecule may have to reach a certain level before inhibition from TNF- α or other molecules can occur.

An alternative but related answer is that this mechanism of TNF- α inhibition of survival signals only occurs in brain subjected to inflammation and oxidative stress, but not normal brain, regardless of TNF- α levels. In this scenario, when the redox potential of vulnerable neurons is shifted toward oxidation by increased net production of free radicals or decreased production of free radical-scavenging enzymes, a concentration of TNF- α that is not normally toxic might induce neuronal apoptosis. Support for this concept comes from Castegne *et al* (2001), who demonstrated that administration of an NF- κ B inhibitor *alone* following axotomy of retinal ganglion cells was *neuroprotective* compared to vehicle control. However, coadministration of a glutathione synthesis inhibitor *with* the NF- κ B inhibitor following axotomy caused neuronal apoptosis in these cells. This example demonstrates that either activation or inhibition of the same molecule may have opposing effects depending on the redox status of the cell. This theory may also explain why levels of both TNF- α and IL-1 alone that are nontoxic in normal brain, then become toxic in inflamed brain (Vitkovic *et al*, 2000).

Other studies that have bearing on these issues were conducted by Venters *et al* (1999), who sought to determine whether TNF- α inhibited the ability of IGF-1 to rescue cerebellar granule cells from serum withdrawal. Cell survival in the no-serum control condition was only about 13% to start with, and increasing doses of TNF- α (.001 to 10 ng/ml) had little additional toxic effect. However, TNF- α caused a dose-dependent decrease in the ability of 100 ng/ml IGF-1 to rescue the neurons from serum withdrawal; survival decreased from 80% in the no-serum/no TNF- α /+ IGF-1 condition to about 20% in the no serum/+ TNF- α /+ IGF-1 condition. Although TNF- α itself showed little toxicity in this paradigm, the cells were already at such a low survival level that this does not by any means indicate that TNF- α at some doses would not show toxicity under more normal culture conditions. More importantly, this serum withdrawal paradigm models a very stressed and therefore unnatural state for the neurons. Thus, it is probably a closer representation of inflamed or diseased brain than it is of “normal” brain. Therefore, the findings that TNF- α inhibited IGF-1 survival signals in this experimental model may be in agreement with the idea that inhibition of IGF-1 (or other survival signals) by TNF- α (or other molecules) might

only occur, and/or might only reduce neuronal viability, under conditions of high neuronal stress such as brain inflammation or disease.

In any of these crosstalk examples, glia are likely to play a prominent role. Glia activated in response to inflammation are thought to mediate many of the neurotoxic and neuroprotective signals affecting neurons after brain injury. Thus, glial involvement may have much to do with whether TNF- α or any other molecule is neuroprotective or neurotoxic under any given set of circumstances. For example, both TNF- α and IL-1 are often nontoxic in pure neuronal cultures, but toxic in neurons cultured with glia (Loddick and Rothwell, 1999; Rothwell *et al*, 1997). Further, as we have seen, these effects are often synergistic. In addition, the regional distribution of microglia has a profound influence on the extent of neuronal injury after inflammation-inducing lipopolysaccharide (LPS) injection (Kim *et al*, 2000). There may be several reasons for these critical effects of glia. Glia are most likely responsible for initiating most cytoprotective or cytotoxic cascades, and producing the majority of the neurotrophic and neurotoxic factors. Furthermore, cytokines or neurotrophic factors may affect a neuron very differently depending on the origin of the signaling molecule, and whether the molecule is acting directly on neuronal receptors, or acting via secondary pathways stimulated by ligand activation of glial receptors. Finally, glia can indirectly affect neurons by altering concentrations of a variety of molecules in the synaptic cleft. For example, reduced glutamate uptake by activated glia has often been attributed to excitotoxic neuronal death in a variety of neurological diseases (Hu *et al*, 2000). An additional novel pathological mechanism involves chemokine signaling of stromal-derived factor 1 (SDF-1 α) through its cognate receptor CXCR4 on astrocytes to rapidly release TNF- α into the extracellular space. This process is further amplified by activated microglia (Bezzi *et al*, 2001).

To summarize, intraneuronal receptor crosstalk represents a significant mechanism by which TNF- α and other neuronal signals may have opposing roles under different brain environments. Changing interactions between signaling pathways could explain the shifting roles of TNF- α at different times in the course of a disease, at differing levels of cellular stress, and perhaps between different classes of disease. Many of these concepts are illustrated in Figure 1. For example, patterns of cytokine signaling typical of a neuroimmune response to viral infection may interact with TNF- α signaling in a way that leads to the predominance of toxic effects typically seen during virus-induced neurodegeneration. This may differ from the effects of TNF- α signaling during neuroimmune responses to other types of agents, which may differ yet again from neuroinflammatory responses in the absence of infectious agents. Moreover, the concept of intraneuronal receptor crosstalk itself implies two key prerequisites that, in many in-

stances, are still under-investigated: (1) that receptors for the molecule in question are found colocalized on the same neuron, and (2) that this colocalization occurs in the regions where pathology is found. Published receptor expression studies indicate that this is probable, especially for more ubiquitously expressed molecules like the ones discussed above (Vitkovic *et al*, 2000), but nonetheless, more studies specifically addressing receptor colocalization within particular brain regions and in specific neuronal populations need to be performed to fully elucidate this unfolding mystery.

Other considerations and future directions

Thus far the focus has been primarily on TNF- α as an important benevolent mediator in normal brain development and maintenance, and a toxic mediator in a wide range of inflammatory conditions. Of course, in reality, the picture is not nearly so simple. As we discussed above, in many of the diseases for which we described a (neuro)pathological role for TNF- α , TNF- α has also been shown to have protective effects. Conversely, TNF- α may exert a toxic effect under "normal" neurological conditions. For example, it has been proposed that TNF- α might have a death-inducing role during developmental pruning of variety of neuronal cell types, including sympathetic and sensory (Barker *et al*, 2001) and monoamine (Jarskog *et al*, 1997) neurons.

In some situations, these pleiotropic effects of TNF- α might be explained in light of the hypotheses we present above. For example, primary cell cultures may very well represent more of an inflamed state than a "normal" neuronal state, due to physical stress that is inherent in establishing such cell cultures from primary brain tissue. Moreover, even "pure" neuronal cultures have varying glial content, which can affect cytokine or neurotrophic factor action. Likewise, the use of immortalized neuronal lines is complicated because the differentiating agents used to create "neuronal" phenotypes can activate innumerable complex intracellular signaling pathways regulating cell fate. Furthermore, such cell lines are mitotic, often tumor-derived, and therefore of questionable relevance to normal neuronal physiology. Finally, because cytokine action is closely interrelated with trophic factors, even medium compositions containing differing concentrations of growth factors or other cell factors may be enough to reverse the effects of TNF- α under otherwise identical experimental conditions.

In other situations, we likely have to look beyond the hypotheses presented here for additional explanations. It has been suggested that for some disease states, like nerve regeneration, early TNF- α presence may be beneficial to neurons, but become destructive with continued chronic exposure in the later disease state (Stoll *et al*, 2000). Diametrically opposed,

other investigators have suggested that TNF- α is toxic in the early postinjury phase, but neuroprotective at later time points (Shohami *et al*, 1999). This observation is based on the work of Scherbel *et al* (1997) in a model of TBI in TNF- α knockout mice. Following cortical contusion, these mice recovered faster than the wild-type controls, but had greater neurological deficits between 1 and 4 weeks after the injury, suggesting that lack of TNF- α was implicated in the chronic phase of recovery from TBI. Other work has suggested that pretreatment with TNF- α confers neuroprotective activity against excitotoxic cell death resulting from brain injury (Gary *et al*, 1998; Mattson *et al*, 1997). Exactly how this pretreatment effect works is unclear. However, it has been shown that cytokines and neurotrophic factors can moderate the levels of other cytokines or neurotrophins and their receptors (Munoz-Fernandez and Fresno, 1998), and that p75 receptor expression alone confers a relative dependence on neurotrophins (Casaccia-Bonofil *et al*, 1999). It could be posited that TNF- α pretreatment affects receptor levels of other trophins, or that it reduces neuronal dependence on trophic factors, thus making it protective. Taking all of these results and concepts together, it would be interesting to make a comparison of the effects of TNF- α in these models when applied pre-, peri-, or postinjury. For example, as we mentioned earlier, TNF- α is *neuroprotective* to hippocampal organotypic slices when applied *prior* to ischemic stress, but *neurotoxic* when applied *after* the same ischemic insult (Wilde *et al*, 2000). This and many other examples cited above indicate that *timing* and *duration* of TNF- α exposure relative to injury may well determine whether its effects are neuroprotective or neurotoxic, and elucidation of these critical details is therefore imperative to developing safe and effective therapies for brain injury.

A critical, unresolved conundrum relates to the evolutionary basis for the ability of TNF- α to simultaneously signal both protective and toxic pathways *within the same inflamed environment*. On evolutionary grounds, why this occurs may be difficult to answer, but it further elucidation of exactly how TNF- α might exert its many effects will likely come from progress in several areas, including greater understanding of glial involvement in these processes; subcellular localization and function of TNF- α and

other cytokine/neurotrophic factor receptors, especially at the nuclear and mitochondrial levels; regulation of cleavage activity of both TNF- α and its receptors, and how that affects signaling; regulation of endocytosis of both cleaved and uncleaved receptors and TNF- α ; other TNF- α -induced signaling pathways such as Jak/STAT, as well as continued search for additional molecules that inhibit signaling; and finally, how structural analysis of TNF- α and TNFRs relates to their function.

It will also help to understand how and why the effects of TNF- α differ in neurons (nonmitotic) versus non-neuronal (mitotic) cells. To our knowledge, the signaling pathways are the same, yet TNF- α often has completely contradictory effects in neurons versus other cell types. Thus, as suggested herein, the differentiation state of a neuron may greatly dictate its response to TNF- α , whether it be in response to a virus, or some other inflammatory response. Lastly, to better understand TNF- α function in the brain, we also need to further our understanding of the role of TNF- β in the brain. TNF- β [a.k.a. lymphotoxin (LT)- α] is highly active in the immune system but generally considered to play a lesser role in the brain than TNF- α (Munoz-Fernandez and Fresno, 1998), and therefore we made no mention of it here. However, because TNF- β signals through the exact same receptors as TNF- α , if it is present in the brain, it could mediate any of the same effects, and participate in the same crosstalk mechanisms, as TNF- α . For example, in a magnetic resonance imaging (MRI) study of MS patients, Kraus *et al* (2002) recently found that TNF- β serum levels were significantly elevated in patient subgroups with (1) higher numbers of lesions, and (2) greater disease burden (total lesion area), respectively.

In closing, this work has endeavored to present evidence for contrasting roles of TNF- α in normal versus diseased brain, and advance the concept that intraneuronal receptor crosstalk might be one mechanism responsible for some of the opposing roles of TNF- α . It is only by thinking in these interactive terms, and continuing to focus on the kinds of areas mentioned above where our knowledge is still deficient, that we can hope to gain more insight into the complexity of interactions of TNF- α and other cytokines in the brain.

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