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EFFECTS OF TUMOR NECROSIS FACTOR ALPHA ON NITRIC OXIDE SYNTHASE IN NERVE GROWTH FACTOR-RESPONDING CELLS

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by
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Increased cytokine levels have been observed in post mortem brains of Alzheimer Disease (AD) patients accompanied by a decrease in nerve growth factor (NGF)-responsive cholinergic neurons. I report a synergistic effect of the cytokine tumor necrosis factor alpha (TNFα) and NGF on expression of nitric oxide synthase (NOS), in rat pheochromocytoma PC12 cells. NGF/TNFα-promoted iNOS can be toxic in PC12 cells. Using PC12 cell mutants lacking the low affinity p75NTR receptor, we also demonstrate that this receptor is required to mediate iNOS expression, likely acting through the transcription factor nuclear factor kappa B (NF-κB). To determine if specific regions of the brain display a selective susceptibility to TNFα enhanced iNOS expression, I investigated the effects of *in vivo* stereotaxic injections of recombinant rat TNFα into the fourth ventricle of adult male C57BL/6 mice. There was a robust induction of iNOS expression following the injection of TNFα, which was restricted to the basal forebrain and hippocampus. No iNOS expression was detected in the striatum, or cerebellum. Determining whether the role of TNFα is one key to the selective

neurodegeneration of NGF-responsive neurons in may allow for novel therapeutic strategies.

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AMP, adenosine monophosphate; AraC, cytosine β-D-arabinofuranoside; AD, Alzheimers disease; BCA, bicinchoninic acid assay; BDNF, brain derived neurotrophic factor; BFA, basal forebrain area; BFCN, basal forebrain cholinergic neurons; CBLM, cerebellum; CNS, central nervous system; CRE, cyclic-AMP response element; DMEM, Dulbecco's minimal essential medium; GDNF, glial derived neurotrophic factor; IGF, insulin-like growth factor; IL-1β, interleukin-1beta; iNOS, inducible nitric oxide synthase; HIPP, hippocampus; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor kappa B; NGF, nerve growth factor; NFT, neurofibrillary tangles; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; NT, neurotrophin; NTR, neurotrophin receptor; PC12, pheochromocytoma; PCN, penicillin; PDTC, pyrrolidinedithyocarbamate; PKA, protein kinase A; PNS, peripheral nervous system; PSI, proteosome inhibitor; SDS, sodium dodecylsulfate; SEAP, secreted alkaline phosphatase; S.E.M, standard error of the mean; SOD, super oxide dismutase; Strep, streptomycin; SP, senile plaques; STR, striatum; TNFα, tumor necrosis factor alpha; TNFR1, tumor necrosis factor alpha receptor 1; TrkA, troponin-like receptor kinase A; TTBS, tris-buffered saline with tween 20

BACKGROUND AND SIGNIFICANCE

Neurodegeneration and Alzheimers Disease

Alzheimer's disease (AD) is characterized as an age-associated dementia in which there is a progressive decline in mental capabilities; specifically in language and visuospatial functions, ultimately resulting in motor system dysfunction and personality changes. Perhaps even more unfortunate than the individual symptoms are a reliance on a "diagnosis by exclusion" and an utter lack of preventive therapeutics. AD is the major cause of dementia and affects about 11% of the population over 65 years of age and 50% over the age of 85 (Hof et al. 1995). Postmortem neuropathological criteria, originally described by Alois Alzheimer in 1906, remain the only definitive hallmarks of the disease to this day. These include histopathological alterations in the cerebral cortex, specifically the presence of both neurofibrillary tangles (NFTs) and extracellular depositions of the β-amyloid protein (Aβ). The distribution and density of these alterations in the brain are used to confirm the clinical diagnosis of AD.

NFT's are cytoskeletal alterations consisting of abnormal tau protein, a protein that normally stabilizes microtubules in healthy neurons, which when heavily phosphorylated can disrupt axonal transport resulting in altered protein metabolism, synaptic malfunction and impaired retrograde transport of neurotrophic factors and appear as dystrophic neurites. Additionally, masses of fine (7-10 nm) filaments, comprised of the 4kDa peptide Aβ that is derived from the larger β–amyloid precursor

protein (APP), form in the extracellular space. These depositions can occur diffusely or as more compact spherical structures known as senile plaques (SP). When these plaques are spatially localized with dystrophic neurites they are called neuritic plaques.

AD often presents after a lengthy preclinical phase and although profound cerebral atrophy occurs, only a highly specific subset of nerve cells shows vulnerability to degeneration in the malignant phase. Specifically, while excitatory pyramidal cells in layers II, III and V demonstrate this vulnerability, GABAergic inhibitory interneurons are unaffected (Hof et al. 1991) (Hof et al. 1993); (Sampson et al. 1997). Additionally, association neocortical areas are particularly susceptible, but primary motor and sensory areas are not (Terry et al. 1981); (Morrison and Hof 1997). This specificity underlies the clinical difficulties in making a definitive diagnosis as the affected areas have more subtle roles in cognition and are not as easily observed as motor or sensory deficits might be. Finally, different areas of the brain manifest pathological changes at different timepoints in the progression of the disease. For example, medial temporal regions are affected earlier and then the pathology spreads to other neocortical areas (Braak and Braak 1985), thus the characteristic memory loss appears first and subsequent cognitive declines as the disease course progresses.

Cholinergic Deficits in AD

Neurons that are particularly affected in AD include the basal forebrain cholinergic neurons (BFCNs; Davies and Maloney 1976; Perry et al. 1977; Whitehouse et al. 1982). BFCNs provide the major subcortical source of cholinergic innervation to the

neocortex (Calarco and Robertson 1995; Eckenstein et al. 1988; Lysakowski et al. 1989; Perry et al. 1978; Whitehouse et al. 1982) and a decline in the number, size and/or function of the cholinergic cells in the basal forebrain may be responsible for some of those cognitive impairments that characterize AD (McGeer et al. 1984; Rinne et al. 1987; Swaab et al. 1994; Terry et al. 1991; Vogels et al. 1990). BFCNs are known to be dependent on a constant supply of target-derived neurotrophic factors such as nerve growth factor (NGF; Hartikka and Hefti 1988; Hefti 1986). It is interesting to note that the NGF-responsive cholinergic neurons of the basal forebrain are affected in AD while the cholinergic pontomesencephalotegmental neurons, which do not express the NGF receptors, are not affected (Woolf et al. 1989a; Woolf et al. 1989b); perhaps suggesting that is the cholinergic NGF-dependent phenotype which is more at risk in AD. While it is not clear whether there are significant changes in the levels of NGF in AD (Hellweg et al. 1998) the treatment of AD patients with NGF may not be effective and, more significantly, may be impractical because of severe side effects (Jonhagen 2000).

Neurotrophins and AD

Trophic factors support cellular proliferation and promote cell survival and are synthesized by a variety of cells including neurons and glia. Neurotrophins (NT) are a class of trophic factors characterized as small, highly basic proteins that dimerize to function, for example, in embryonic development by coordinating complex patterns of neuronal connectivity. Their role is briefly described as a process by which only neurons whose axons have innervated appropriate targets compete for limited NT support thus

eliminating inappropriate or less than optimal connections by means of programmed cell death. This developmentally regulated cell death by deprivation of trophic support has been termed "neuronal pruning" (Oppenheim 1991). NT signaling works through two cell surface receptors, one being described as a high affinity tyrosine kinase receptor (Trk), specific to each NT, and the other a common low affinity p75 receptor ($p75^{NTR}$; for a review see (Teng and Hempstead 2004)). The Trk receptors consist of a single transmembrane stretch comprised of a NT-specific extracellular domain and a conserved intracellular kinase domain (for a review see (Huang and Reichardt 2003)). The $p75^{NTR}$ receptor is part of a superfamily of receptors characterized by cysteine-rich repeats in the extracellular region (Mallett and Barclay 1991)and contains a intracellular Type II death domain similar to the Type I death domains of the Fas and tumor necrosis factor alpha receptor I (TNFR1; for a review see (Barker 1998)).

Nerve Growth Factor

Nerve growth factor (NGF) is the prototypical NT, first described by Levi-Montalcini in 1951. NGF exists as a 26-kDa homodimer that though not covalent is relatively stable, even at low concentrations. Other NTs include NT-3 (Hohn et al. 1990;Maisonpierre et al. 1990), NT-4/5 (Hallbook et al. 1991) and brain-derived growth factor (BDNF; (Leibrock et al. 1989). NGF is important the differentiation and survival of developing BFCNs and is also implicated in the maintenance of mature and survival of lesioned BFCNs. In 1981 Appel suggested that deficiency in NTs, such as NGF, could account for the selective vulnerability seen in various forms of neurodegeneration (Appel

1981). However, subsequent studies failed to show changes in NGF protein or message levels in AD brain or any evidence that failed NGF synthesis could account for the degeneration of BFCNs (Mufson et al. 1996;Mufson et al. 1999;Crutcher et al. 1993;Scott et al. 1995). However this still leaves open a possibility for failed NGF signaling (Mufson et al. 1994).

The NGF dimer binds with high affinity to the 140-kDa TrkA receptor, the most prominent CNS population of NGF-responsive neurons expressing TrkA being the BFCN, however other populations of peripheral neurons such as the nociceptive sensory neurons are wholly dependent upon NGF for their survival (Hamanoue et al. 1999b;Maggirwar et al. 1998). Upon binding there is receptor dimerization, autophosphorylation and receptor internalization by endocytosis. The signaling cascade begins upon autophosphorylation and continues as the endosome is retrogradely transported back to the perikarya, where it triggers signal transduction pathways regulating expression for neuronal survival (for a review see (Kalb 2005). For example, NGF is produced in hippocampal neurons and following release binds to TrkA receptors on presynaptic axon terminals from BFCNs consequently forming extended protein signaling complexes which are internalized possibly in part by clathrin-coated pits (for a review see (Howe and Mobley 2005). These signaling complexes are then transported along microtubules, by which TrkA is linked directly to dynein, until it reaches the cell body where the signal is communicated to the nucleus triggering gene expression for survival and cholinergic function (Tirassa et al. 2003; Yuhara et al. 2003; Auld et al. 2001). The common low-affinity $p75^{NTR}$ also binds NGF, and all known NTs with

similar affinity (Squinto et al. 1990), with signaling that is less well understood. The low affinity receptor is thought to act by increasing the affinity of Trk receptor for its ligand as well as having direct signaling pathways (Bibel et al. 1999). The pathways associated with $p75^{NTR}$ receptor stimulation have been found to both promote and inhibit apoptosis depending on the model system and the presence or absence of Trk receptors (for a review see (Bredesen and Rabizadeh 1997;Rabizadeh and Bredesen 2003).

Neuroinflammation and Nitrosative Stress in AD

Although the brain has been regarded as "immunologically privileged", it is vulnerable to constitutive host defenses such as inflammation. Neuroinflammation is thought to play a prominent role in neurodegeneration and is associated with a variety of acute and chronic insults in both the central (CNS) and peripheral nervous system (PNS; (Floyd 1999; Mcgeer and Mcgeer 2001a). Examples of neurotraumatic or neurodegenerative conditions where the occurrence or role of neuroinflammation has been documented include peripheral nerve injury (Creange et al. 1998;Chandross 1998;Sung et al. 2004) acute and chronic spinal cord injury (Popovich and Jones 2003;Hausmann et al. 2003a;Hausmann et al. 2003b;Bareyre and Schwab 2003), traumatic brain injury (Bayir et al. 2002); (Morganti-Kossmann et al. 2002); (Lenzlinger et al. 2001), stroke (Sundararajan and Landreth 2004; Dirnagl 2004; Danton and Dietrich 2003), amyotrophic lateral sclerosis (ALS, Consilvio et al. 2004; Pompl et al. 2003; Mcgeer and Mcgeer 2002) and Alzheimer Disease (Cacquevel et al. 2004;Gupta and Pansari 2003; Mcgeer and Mcgeer 2001b; Mcgeer and Mcgeer 2003).

An inflammatory hypothesis for the onset of sporadic AD has been supported by independent retrospective clinical studies that have shown treatment with nonsteroidal anti-inflammatory drugs may delay the onset of symptomatic progression of AD (in 't Veld et al. 1998; Mackenzie and Munoz 1998). Amyloid-based genetically modified mouse models of AD demonstrate significant inflammatory markers and astrogliosis (Frautschy et al. 1998;Stalder et al. 1999;Wegiel et al. 2001) associated with SP that can be ameliorated with chronic administration of ibuprofen (Lim et al. 2000;Yan et al. 2003). Specifically increased levels of cytokines associated with a proinflammatory state have been found in the brains of AD patients, as well as mouse models (Mehlhorn et al. 2000;Apelt and Schliebs 2001;Abbas et al. 2002;Patel et al. 2005), and proinflammatory cytokine polymorphisms that increase expression of such cytokines as interleukin 1-beta (Nicoll et al. 2000;Mrak and Griffin 2001), interleukin 10 (Ma et al. 2005) and tumor necrosis factor alpha (McCusker et al. 2001;Ma et al. 2004) are thought to represent risk factors for developing AD.

Tumor Necrosis Factor alpha

Inflammatory markers in AD have included high CNS levels of tumor necrosis factor alpha (TNFα) elevated in AD cerebral spinal fluid (CSF) and brain (Tarkowski et al. 1999; Tarkowski et al. 2003) but not in either serum or blood cells (Maes et al. 1999;Lombardi et al. 1999). TNFα is a 17-kDa cytokine, originally identified by its antitumor activity but is actually a mediator for a variety of various inflammatory responses by Carswell et al. in 1973 (for a review see Baud and Karin 2001). TNFα, like

NGF, elicits its effects through two distinct receptors, TNFR1 and TNFR2 (Vandenabeele et al. 1995). Trimeric TNFα binds and induces trimerization and subsequent recruitment of signaling complexes to the cytoplasmic domains of the receptors. TNFR1 is ubiquitously expressed in nucleated cells and is thought to mediate inflammatory and apoptotic stimuli through its Fas-associated death domain (FADD; for a review see (Thorburn 2004) while on the other hand, TNFR2 expression is more limited to specific cell types and is associated with anti-apoptosis through the TNF-receptor-associated factor 1 (TRAF1; for a review see (Carpentier et al. 2004). TNFα is one of many stimuli, including other proinflammatory cytokines, bacterial lipopolysaccharide, UV irradiation, phorbol esters and reactive oxygen intermediates, which are potent stimulators of the transcription factor nuclear factor kappa B (NF-κB) (for reviews see (Baeuerle and Baltimore 1996; Grilli and Memo 1999)).

Nuclear Factor Kappa B

NF-κB was first identified as a transcription factor that binds specifically to the DNA sequence (5'-GGGACTTTCC-3') in the intronic enhancer of the immunoglobulin κ light chain gene (Sen and Baltimore 1986). It has been extensively implicated as a modulator of multiple genes involved in inflammation and cell survival (Clarkson and Watson 1999;DeLuca et al. 1999;Mercurio and Manning 1999;Taglialatela et al. 1997;Taglialatela et al. 1998). Currently there are five identified mammalian NF-κB proteins; p50 (NFκB1), p52 (NFκB2), p65 (RelA), RelB and c-Rel important in CNS function and pathology (for a review see (Meffert and Baltimore 2005). NF-κB family

proteins are identified by a shared homology within a 300 amino acid domain called the Rel homology domain, which contains sequences necessary for nuclear localization, DNA binding and dimerization with other Rel family members with both homo- and heterodimers being possible. Three members of this family have transcription activation domains (RelA or p65, RelB and c-Rel) with the other members being identified as inhibitors of transcriptional activation. The most prototypic subunit combination is the p50/p65 heterodimer found in the cytoplasm bound to an inhibitor in the absence of stimulation. NF-κB activity is controlled by members of the so-called IκB family, a family of inhibitor proteins that are identified by clusters of 30-33 amino acid ankyrin repeats, which mediate their interactions with the Rel homology domains of NF-κB proteins. This association masks the nuclear localization sequence of the NF-κB proteins and sequesters them in the cytosol. NF-κB is typically sequestered in the cytoplasm, but when dissociated from a phosphorylated IκB, allow NF-κB dimers to translocate to the nucleus and bind to promoter sequence to initiate *de novo* gene transcription (for a review see (Karin et al. 2004)). Activation of NF-κB proteins depends on activation of IκB kinases (IKKs) for phosphorylation of IκBα through one of two possible pathways (for a review see (Bonizzi and Karin 2004). The classical pathway involves serine (residues 32 and 36) phosphorylation by the serine/threonine kinases named IKKα or β and requires IKKγ. This phosphorylation marks the inhibitor protein for ubiquination and subsequent proteosomal degradation. An alternate pathway associated with hypoxia, reperfusion and growth factor activation of NF-κB uses tyrosine (residue 42) phosphorylation, which

does not result in ubiquination or degradation, but that prevents association with NF-κB proteins through an unknown mechanism (Bui et al. 2001) and does not require IKKγ. This alternate pathway leads to p52/RelB heterodimerization (for a review see (Delhalle et al. 2004).

NF-κB is also a redox-sensitive transcription factor (Marshall et al. 2000). Overexpression of SOD inhibits NF-κB (Li et al. 1998)and hydrogen peroxide has been shown to induce phosphorylation and degradation of IκB (Schoonbroodt and Piette 2000). The presence of a free sulfhydryl group necessary to bind DNA also makes NF-κB sensitive to oxidizing conditions in the nucleus. This may explain why NF-κB can be activated by some free radicals (Park et al. 1997) and inhibited by others (Peng et al. 1998) as both translocation and DNA binding are necessary for transcriptional activity.

NF-κB in Aging and AD

Models of senescence have also demonstrated an altered NF-κB status (Gosselin and Abbadie 2003). Increased activation and levels of nuclear NF-κB have been found in aged rat brain (Toliver-Kinsky et al. 2002;Bernard et al. 2004) without accompanying changes in cytoplasmic (inactive) protein levels (Korhonen et al. 1997). This increased NF-κB activation is likely not mediated by changes in the expression of IκB inhibitor proteins (Helenius et al. 2001) but rather perhaps through age-associated decreased proteosomal activity (Keller et al. 2000). As noted above, it is thought that activation of the classical pathway of NF-κB is associated with proteosomal degradation of the

inhibitor proteins perhaps implicating a shift towards the proteosome-independent alternate pathway in aged tissue.

Aβ peptide is thought to activate NF-κB (Behl et al. 1994) and studies of postmortem brain tissue from AD patients reveal increased NF-κB activity in cells that were involved in the neurodegenerative process (Terai et al. 1996; Kaltschmidt et al. 1997; Kaltschmidt et al. 1999; Mattson and Camandola 2001). In addition, immunohistochemical studies suggest that levels of NF-κB activity are increased in BFCNs of AD patients (Boissiere et al. 1998). Inhibiting NF-κB , both in vitro (Taglialatela et al. 1997) and in vivo (Taglialatela et al. 1998), results in neuronal cell apoptosis that is resistant to NGF rescue (Taglialatela et al. 1997;Macdonald and Taglialatela 2000;Macdonald et al. 1999). Thus, perturbations of NF-κB signaling are likely to play a role in the onset/progression of neurodegeneration perhaps as a failure to successfully respond to stressors in senescence (Helenius et al. 1999) and neurodegeneration or more directly through the chronic induction of inflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6) or inducible nitric oxide synthase (iNOS).

Other transcription factors mediating gene transcription in the brain

The family of cyclic AMP (cAMP) response element binding (CREB) proteins are mainly involved in the initiation of gene transcription downstream of signal-transduction pathways that lead to an increase in cytosolic levels of cAMP. cAMP activates protein kinase A (PKA) by promoting the dissociation of catalytic subunits from

the cytosolic holo-protein complex thus allowing their nuclear localization and subsequent phosphorylation of CREB proteins. Although the exact mechanism is not yet known, phosphorylated CREB increases gene transcription of genes containing the cis-acting palindromic CRE-consensus sequence (TGACGTCA) in the promoter region. The time course for CREB stimulation peaks approximately 30 minutes followed by a gradual decrease to basal levels over several hours through the actions of various phosphatases. Activator protein complex-1 (AP-1) transcription factors, like CREB, is also not a single protein but rather a mixture of dimers of the Jun family (c-Jun, JunB and JunD) of basic leucine-zipper (bZIP) associated with related proteins of the Fos (c-Fos, FosB and Fra1) giving subtly different functions depending upon complex mixtures of AP-1 dimers. Activated AP-1 increases gene transcription of genes containing a palindromic DNA motif (TGAG/CTCA) in response to a large number of extracellular stimuli, including NGF (Kamata et al. 2005).

Nitric Oxide Synthase

Free radicals are molecules with unpaired electrons making them highly reactive. Oxidative phosphorylation reactions convert molecular oxygen to water by successively adding electrons and protons. Intermediate products include reactive oxygen species (ROS) such as superoxide (O_2^-), hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2). Although H_2O_2 is not a free radical it is converted to hydroxyl radicals by reduced iron. The brain is particularly susceptible to damage by ROS for a number of reasons. First, the brain relies almost entirely on oxidative phosphorylation reactions to meet it

relatively high metabolic needs. The neuronal membranes, which are high in polyunsaturated fatty acids, are potential substrates for peroxidative damage via the hydroxyl radicals. Finally, the levels of catalytically available iron in the brain are relatively high while the relative levels of antioxidant enzymes are low as compared to other tissues in the body (Gutteridge and Halliwell 1989). An increased production of free radicals either induced by an overdrive of endogenous ROS generating species or by exogenous oxidative insults challenges the cellular redox homeostasis maintained by the levels of antioxidant enzymes leading to chronic oxidative stress and subsequent impairment of function.

Inducible nitric oxide synthase (iNOS or NOS-II) is one of three isoforms of the enzyme responsible for nitric oxide (NO) production in the body (McCann et al. 1996; McDonald and Murad 1996). Like the other two isoforms, neuronal (nNOS or NOS-I) and endothelial (eNOS or NOS-III), iNOS converts arginine and oxygen, along with cofactors, into NO and citrulline. NO is a highly diffusible gas and the under basal conditions is produced in small amounts by calcium-dependent nNOS and eNOS. NO activates soluble guanylate cyclase, leading to the production of cGMP, a second messenger implicated in a number of physiological functions. However, high amounts can be produced by calcium-independent iNOS. This increased production, especially under conditions of oxidative stress, can form peroxynitrite by competing with super-oxide dismutase (SOD) for the super oxide free radical (OH). Peroxynitrite is a highly reactive charged molecule with cytotoxic properties. iNOS is transcriptionally regulated by cytokines (IL-1, IL-2, IL-6 and TNFα) and the bacterial endotoxin,

lipopolysaccharide (LPS) and requires NF-κB. Promoter analysis has shown the human iNOS gene contains 4 cooperative NF-κB binding motifs that are bound by cytokine-induced NF-κB activation (Taylor et al. 1998).

Although iNOS message or protein has not been reported in healthy brain, it is reportedly increased in AD brain (Lee et al. 1999) while levels of nNOS in areas susceptible to neurodegeneration such as entorhinal cortex and hippocampus demonstrate decreased levels of nNOS-positive neurons in patients with AD (Thorns et al. 1998). High levels of NO are known to induce cell damage by overcoming cellular defenses against oxidative stress, another known component of AD-associated neurodegenerative events. Peroxynitrite can modify proteins by nitrosylating tyrosine residues and nitrosylated residues may be used as an index for the presence of peroxynitrite, thought to be an inhibitor or acetylcholine synthesis (Guermonprez et al. 2001). The end products or "footprints" of peroxynitrite reactions are observed in AD tissues as increased amounts of nitrotyrosine-modified proteins in addition to other oxidative modifications such as malondialdehyde, advanced glycation end products (AGEs), free carbonyls, SOD and heme oxygenase (Balazs and Leon 1994; Colton and Chernyshev 1996; Hensley et al. 1995; McCann 1997; Pappolla et al. 1992; Smith et al. 1994; Smith et al. 2000). Despite this evidence, the source of nitrogen and oxygen radicals *in* vivo is not completely understood and its precise source of production in AD remains undetermined.
nNOS is usually expressed constitutively expressed in neurons and is thought to be very different in terms of its role in the progression of neurodegenerative events. As neurons strongly expressing nNOS demonstrate resistance to neurodegeneration while low nNOS

expressions is associated with signs of neurodegeneration (Thorns et al. 1998;Tao et al. 1999;Simic et al. 2000) or even directly protective against excitotoxic insults to cholinergic nuclei (Guevara et al. 2002). Additionally, In AD brain the levels of nNOS mRNA are decreased in frontal cortex and hippocampus (Norris et al. 1996). However, the exact mechanism for this "protective effect" remains elusive and is confounded by the appearance of expression of nNOS in reactive astrocytes in AD brain (Simic et al. 2000) and its possible involvement in nitric oxide-stimulated guanine nucleotide exchange in the regulation of proliferation and differentiation promoting signaling pathways such as p21ras (Luth et al. 2000). However, nNOS is thought to be responsible for production of NO in normal human brain (Blum-Degen et al. 1999) and the down regulation of this isoform has been associated with other age-related deficiencies in the CNS (Yu et al. 2000). Taken together the observations could suggest that while the role of the common NOS end product NO may have pleiotropic effects, the loss of nNOS expression and gain of iNOS expression could be relevant to the progression of degenerative and age-associated decreased in neuronal function and survival.

Overall Significance

Although the pathogenesis of AD is far more complicated than cholinergic dysfunction, understanding the mechanisms of selective impairment in BFCNs may help to understand the etiology of the reduced cholinergic function, which is associated with many of the cognitive deficits seen in AD. This mechanism should be based on conditions of neuroinflammation, specifically increased CNS cytokine levels, impinging

on NT-responsive neurons leading to conditions of oxidative stress and ultimately impaired function/phenotype. By understanding this sequence of events, it may be possible to look for markers indicating patients who may be at risk, before clinical presentations of memory loss. As all of the current therapies are based on symptomatic replacement of acetylcholine, this knowledge will be instrumental to devise treatments that protect those neurons that are selectively damaged in AD, thus preventing the onset/progression of those cognitive deficits that most severely disable AD patients.

MATERIALS AND METHODS

Materials

All common reagents and chemicals were obtained from Sigma-Aldrich, St Louis, MO, USA. Recombinant rat TNFα, and IGF were obtained from R&D Systems, Minneapolis, MN, USA, purified mouse NGF from Harlan Bioproducts, Indianapolis, IN, USA, and pyrrolidine dithiocarmbamate (PDTC), the octapeptide proteasome inhibitor (PSI), PD98059, K252a and 1400W from Calbiochem, San Diego, CA, USA.

Clonal Cell Lines

Stock cultures of rat pheochromocytoma cells (PC12; kind gift from Dr Lloyd Greene, Columbia University, New York, NY, USA) and PC12 mutant cell lines, i.e. NNR and $PC12^{p75-}$ (kind gifts from Dr. Uri Saragovi, McGill University, Montreal, QC, Canada) were maintained in 10 mL RPMI-1640 culture medium supplemented with 5% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich) and 5% (v/v) heat-inactivated horse serum (Sigma-Aldrich), in a humidified cell incubator (Cedco, Portland, OR, USA) at 37°C and 5% CO_2 atmosphere. All procedures were performed under sterile conditions in a laminar flow cell culture hood (Labconco, Kansas City, MS,) for routine cell maintenance, half of the medium was replaced every other day and at confluency, cells were split into two flasks.

Primary Cell Culture

Primary forebrain cultures were prepared from d0 Sprague-Dawley rats (Harlan, Minneapolis, MN, USA) using the method of Kiss (Kiss et al. 1994) with minor modifications. Briefly, following procedures within Institutional Animal Care and Use Committee (IACUC) guidelines under protocol #00-03-016, animals were decapitated and forebrains dissected from brains under a "dead air" plexiglass hood with the aid of a dissecting microscope. Tissue was triturated using a sterile fire-polished glass pipette in ice-cold Hanks Buffered Saline Solution (HBSS) without Mg2+ or Ca2+(Sigma-Aldrich). Dissociated cells were seeded at 5X105 cells/cm2 on polylysine-coated coverslips in Dulbecco's minimal essential medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (PCN/Strep; Invitrogen). Glial proliferation was prevented by treatment with 4 mM of the mitotic inhibitor, cytosine -D-arabinofuranoside (AraC; Sigma-Aldrich) beginning on the third day of culture. On day 6, the cultures were washed 3 times with 10 mL of serum-free medium and serum medium was replaced with DMEM containing the N2 medium supplement (Invitrogen).

Organotypic Brain Slice Culture

Organotypic slice cultures were obtained using the method developed by Stoppini (Stoppini et al. 1991). Briefly, 20 day old Sprague-Dawley rat pups (Harlan Biosciences, Indianapolis, IN, USA) were decapitated and 400 micron thick sections were obtained by using a 51425 Tissue Slicer (Stoelting Co. Wood Dale, Il, USA) in ice-cold Hanks

medium. Individual slices were cultured for one week in F12/DMEM (Sigma-Aldrich) supplemented with 1% PCN/Strep and AraC with 25% fetal bovine serum on AnoporeTM membranes (25 mm diameter, 0.02 μm pore size) of cell and tissue culture inserts (Nunc, Rochester, NY, USA). After a week in culture, slices were washed 3 times with 10 mL of serum-free medium and serum medium was replaced with F12/DMEM containing N2 medium supplement before treatment.

Lactate Dehydrogenase Release Cytotoxicity Assay

LDH activity was assayed in 25 μL of cell culture medium using a colorimetric kit (Roche, Indianapolis, IN, USA), according to manufacturer's instructions.

Reporter Gene Assay

The secreted alkaline phosphatase assay (SEAP) expression vector, containing the SEAP gene under nuclear factor kappa B (NF-κB), AP1 or cyclic AMP-response element (CRE) enhancer control, was purchased from Clontech (Palo Alto, CA, USA). Transfection of cells was performed using a liposomal packaging system. Briefly, 1.2 pmol of expression vector were mixed with DMRIE-C (Life Technologies, Carlsbad, CA, USA) in a 1:3 DNA to liposome ratio and allowed to equilibriate at room temperature for 30 min. The DNA/liposomes were diluted in 400 μl serum free transfection medium (Optimem; Invitrogen) and then added to approx 100,000 cells in a 12 well cell culture plate (Corning Inc, New York, NY, USA). The cells were allowed to take up the liposomal DNA for 3 hours before being washed with 10 mL RPMI-1640 culture

medium supplemented with 5% (v/v) heat-inactivated fetal bovine serum and 5% (v/v) heat-inactivated horse serum and returned to cell culture medium. Cells were allowed to recover for 24 hours before any treatments. 100 μL aliquots of medium from cells transfected with the SEAP reporter vectors was assayed at various time points after treatment for alkaline phosphatase activity by using the chemiluminescent Great EscAPe SEAP assay (Clontech, Palo Alto, CA, USA), according to manufacturer's instructions.

Western Blot Analysis

Protein Extraction

Cells and tissue samples were lysed using an SDS-based lysis buffer (2% SDS, 5mM EDTA, 50 mM Tris, 1 mM each of dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF) and 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich). Following an ice-cold phosphate buffered saline (PBS; Invitrogen) wash, cells were lysed with the SDS-lysis buffer and the sonicated with a model W-10 Cell Disruptor (Heat Systems Ultrasonics, Plainview, NY, USA). Samples were then centrifugated at 20,000 x g for 20 min at 4°C to remove cell tissue and debris. After centrifugation the supernatant was collected and stored at –80°C. Protein content was measured using the standard bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Electrophoresis and Electroblotting

Protein extracts (40 μg) were diluted in 4μL 6X SDS loading buffer (each 10 mL of buffer contains: 7 mL 4X Tris/Cl (1.5 M Tris/Cl and 0.4% SDS), 3 mL glycerol, 1g SDS, 0.93g DTT and 1.2 mg bromophenol blue), boiled for 5 min and loaded into a 6% SDS-polyacrylamide gel. Gels were run for approximately one hour at 100V in a Tris-Glycine buffer (20 mM Tris, 150 mM glycine, 0.1% SDS pH 8.3) so that the appropriate high molecular weight standards were sufficiently separated. Sample separation was followed by electrophoretic transfer to nylon-backed nitrocellulose membranes (BioRad, Hercules, CA, USA) overnight at 25 V and 4°C.

Immunoblotting

Following transfer, all incubations were at room temperature in 0.5% Tween in Tris buffered saline (TTBS; 1.21g Tris, 5.84g NaCl, 1 mL Tween 20 with 999 mL dd H_2O pH 7.5). The membranes were blocked for one hour in 5% milk in TTBS. Primary monoclonal anti-iNOS and anti-nNOS (purchased from Signal Transduction Laboratories, San Diego, CA, USA) or the polyclonal antibody anti-TNFR1 (purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:1000 (v/v) in 2.5% milk in TTBS at 1:1000 and membranes were incubated with the antibody for one hour at room temperature. Membranes were washed 3 times for 10 min each in TTBS before incubating for one hour with a horseradish-peroxidase conjugated secondary antibody (BioRad, Hercules, CA, USA) at 1:7500 (v/v) in 2.5% milk in TTBS. Finally, membranes were washed again in TTBS 3 times for 10 min each. Immunoreactive bands

were visualized by a chemiluminescent (ECL) western blot detection kit (Amersham Biosciences, Piscatay, NJ, USA) according to manufacturer's instructions. Images were captured employing a computer-aided 12 bit monochrome camera (UVP, Upland, CA, USA).

Animals

Animals were housed singly (pregnant dams/dams with pups), doubly (rats), or 5 to a cage (mice) in Animal Resource Center housing in cages with food/water *ad libitum* and a 12-hour light dark cycle. Cages were changed twice per week by animal husbandry personnel and all animals were closely monitored for signs of distress or poor health.

Animal Treatment

Each young (about 3 months, 20-25 g) male C57BL/6 mouse was anesthetized with isoflurane gas (Abbot Laboratories, North Chicago, IL, USA) and placed in a stereotaxic instrument (Kopf Model 900 with mouse adaptor) equipped with a mouse anesthesia mask (Kopf Model 907) to deliver 2-3% isoflurane and oxygen during the procedure. An isothermal pad (Model 39; Braintree Scientific, Braintree, MA, USA) was used to maintain body temperature. The scalp was shaved, incised and retracted so that the bregma and lambda sutures on the skull were fully exposed. A hole was drilled at the appropriate location using a drill (Dremel Model 395). A 10 microliter syringe (Hamilton, Reno, NV) was secured in a microinjection unit (Kopf Model 5000) and stereotaxically positioned into the fourth ventricle, at the following coordinates; AP: -6.0 mm from bregma on the midline and DV: -4.0 mm from the top of the skull, was used to

inject 5 microliters of TNFα /vehicle over 5 minutes. 5 minutes after injection the needle was removed, the scalp was sutured close and animals closely monitored until they fully recovered from anesthetic. Groups of animals included TNFα injected and vehicle (artificial cerebrospinal fluid, aCSF: 140 mM NaCl; 3 mM KCl; 2.5 CaCl2; 1 MgCl2; 1.2 Na2HPO4; pH 7.4) injected. All animals were monitored closely for discomfort and were humanely euthanized 24 hr after treatment by CO_2 asphyxiation according to procedures reviewed and approved by the Institutional Animal Care and Use Committee protocol 02-07-047.

Tissue Dissection for Biochemistry

Upon sacrifice, the cerebellum, hippocampus, basal forebrain area (including septum) and striatum were rapidly dissected, snap-frozen in liquid nitrogen and stored at -80°C for protein extractions and western blot analysis.

Tissue Fixation for Immunohistochemistry

Animals were overdosed with 50 mg/kg pentobarbital and transcardially perfused with ice-cold PBS until perfusion was visually assessed as completely penetrating hepatic tissues. Each animal was then perfused with 100 mL 4% phosphate-buffered paraformaldehyde. The brains were excised and post-fixed in 4% paraformaldehyde overnight at 4C. The brains were then cryoprotected by immersion in 30% sucrose in PBS for 72 hrs at 4C. Each brain was then placed in a cryotray, covered in Optimal Cutting Temperature compound (OCT; Tissue-Tek, Torrence, CA, USA) and snap frozen in 2-methylbutane (Sigma-Aldrich) cooled to –70C with liquid nitrogen. Brains were

then stored at –80C until sectioned on a Leica CM1900 cryostat at a thickness of 16 μm on gelatin subbed slides (Fisher Scientific, Vernon Hills, IL, USA) and stored at –80 until use.

Immunohistochemical Staining

Sections were hydrated for 15 min in TBS and then blocked and permeabilized for 1 hr with 0.025% Triton X-100 (Fisher Scientific) and 5% normal goat serum (Sigma-Aldrich). Primary antisera were diluted at 1:100 in TBS with Triton X-100 and followed with TBS washes for 15 min, repeated three times. Secondary fluorescent antibodies were diluted 1:400 in TBS with Triton X-100 and followed with TBS washes for 15 min, repeated three times. Sections were then mounted with Fluromount G (Southern Biotechnology Associates Inc., Birmingham, AL, USA) and coverslips for viewing.

Confocal Microscopy

Images were obtained using a Zeiss LSM510 META laser scanning confocal microscope with laser excitations at 633nm (Texas Red), 351 nm (DAPI) and 488 nm (FITC). All image analysis was performed using Meta-morph imaging software v6.0 (Universal Imaging Corporation, Downingtown, PA, USA).

RT-PCR

Total RNA was extracted with Trizol Extraction Kit (Gibco BRL, San Diego, CA, USA) according to manufacturer's instructions. One μg of total RNA from each sample was applied to Ready-to-go RT-PCR Beads (Amersham Biosciences, Piscatay, NJ, USA) and

used to complete the amplification protocol according to manufacturer's instructions. Primer sequences for rat iNOS were as follows; forward 5'-CAC GGA GAA CAG AGT TGG –3' and reverse 5'-GGA ACA CAG TAA TGG CCG ACC- 3'. Amplified samples were run on agarose gels and stained with ethidium bromide. Images were captured using a 12 bit monochrome camera (UVP, Upland, CA, USA).

Flow Cytometry

One μg of antibody (either αTrkA or $\alpha p75^{NTR}$ purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA) was labeled with Zenon Rabbit IgG labeling kit from Molecular Probes (Eugene, OR) according to manufacturer's instructions and incubated for 1 hr with the cells in suspension. After incubation, labeled cells were visualized and quantified using a Becton Dickinson FACS Vantage Flow Cytometer set at appropriate instrument parameters.

Statistical Analysis

Where appropriate, data were expressed as mean +/- standard error of the mean (S.E.M.), and analyzed by student unpaired two-tailed *t* test with significance set at $p<0.05$.

LIGAND REQUIREMENTS FOR NERVE GROWTH FACTOR/TUMOR NECROSIS FACTOR ALPHA-PROMOTED ALTERATIONS OF NITRIC OXIDE SYNTHASE EXPRESSION

Abstract

Inflammation and oxidative stress play a critical role in neurodegeneration associated with acute and chronic insults of the nervous system. Notably, affected neurons are often responsive to and dependent on trophic factors such as NGF. Based on our laboratory's previous work showing that in NGF-responsive PC12 cells the cytokine TNFα and NGF synergistically induce the expression of the free radical-producing enzyme iNOS, I proposed that NGF-responsive neurons might be selectively impaired as a consequence of elevated TNFα levels. With the aim of identifying possible therapeutic targets, in the present study I determined the pharmacological requirements of NGF/TNFα-promoted responses including the induction of iNOS and inhibition of nNOS. Using defined medium I found that the NGF/TNFα-promoted iNOS expression did not require any additional factors present in normal cell culture medium. Notably, TNFα was unable to promote iNOS expression in concert with Insulin-like Growth Factor (IGF), which uses a tyrosine kinase receptor that promotes signaling pathways similar, albeit not identical to the high-affinity NGF receptor TrkA. Although the NGF/TNFα-promoted iNOS induction was saturable with regards to NGF concentrations, it was dose-dependent with respect to TNFα. Continued signaling from

both TNFα and NGF were required to sustain NGF/TNFα-promoted effects with iNOS and nNOS protein expression levels returning to baseline within 24 hours of withdrawal of either ligand. Here I also report that TNFα treatment in PC12 cells results in the reversible NGF-dependent induction of iNOS and inhibition of nNOS expression and is independent of NO. Lastly, *in vitro* results from primary neuronal and organotypic brain slice cultures demonstrate that NGF/TNFα-promoted iNOS expression could be elicited in neuronal systems other than the PC12 cells.

Introduction

There seems to be an intimate relationship between pro-inflammatory cytokines, oxidative stress and trophic factors that underscores the neuropathological consequences of extrinsic (e.g. traumatic) or intrinsic (e.g. disease-related) injury to the nervous system. Relevant to this observations, our laboratory's previous work showed that in the NGF-responsive rat pheochromocytoma (PC12) cells, a neuroblast-like cell line that has been extensively used in neurobiological studies (Levi et al. 1988;Durham et al. 1995), TNFα induces expression of the free radical nitric oxide (NO) synthesizing enzyme NOS II (iNOS), only when in the presence of NGF acting through its high affinity receptor TrkA (Macdonald and Taglialatela 2000). These results illustrated a possible mechanism through which TNFα could selectively target NGF-responsive cells and there induce oxidative stress. Indeed, perturbed levels of NOS and NO-derived oxidative damage have been reported in both acute and chronic neurodegenerative conditions (Emerit et al.

2004), including spinal cord injury (Isaksson et al. 2005;Urushitani et al. 1998;Diaz-Ruiz et al. 2002), stroke (Parmentier-Batteur et al. 2001;Sarchielli et al. 2003) and AD (Luth et al. 2002;Luth et al. 2001;Law et al. 2001;de la Monte et al. 2000;Lee et al. 1999). However, not all NO production leads to oxidative damage; for example, nNOS expression is crucial to enteric neuron function (for a review see (Barrachina et al. 2001) and nNOS has been shown to negatively regulate proliferation and promote survival and differentiation of neuronal cell precursors (Ciani et al. 2004).

Here I report that in PC12 cells TNFα treatment specifically results in a reversible NGF-dependent induction of iNOS and inhibition of nNOS expression. My results show that such induction of iNOS and inhibition of nNOS occurs at the mRNA level and is also reversible upon withdrawal of TNFα/NGF treatment. While neither iNOS induction nor nNOS inhibition can be sustained by either TNFα or NGF alone after initial treatment with both ligands, TNFα failed to promote iNOS expression in the presence of a growth factor other than NGF (IGF). Furthermore, NGF/TNFα-promoted iNOS expression could be observed even when cells where maintained in serum-free, defined cell culture medium. Lastly, here I report that a rapid expression of iNOS protein produced by the simultaneous exposure to NGF and TNFα can also be obtained in primary and organotypic neuronal cultures containing NGF-responsive neurons.

Materials

All routine reagents and chemicals were obtained as in Chapter 2. Primary monoclonal anti-iNOS and anti-nNOS were purchased from Signal Transduction Laboratories.

Methods

General methods for cell culture, western blotting, RT-PCR, primary and organotypic culture were used as outlined previously in Chapter Two, Materials and Methods.

Results

Treatment with NGF and TNFα results in a TrkA-dependent induction of iNOS expression in PC12 cells, primary and organotypic cultures

The upper panel of Figure 3.1A shows a western blot detecting iNOS in PC12 cells treated simultaneously with 10 ng/ml NGF and 10 ng/ml TNFα in the presence or absence of 50 nM K252a, an inhibitor of phosphorylative events associated with tyrosine kinase receptor activation that has been shown to block the function of the high affinity NGF receptor TrkA (Ohmichi et al. 1992). There was a marked induction of iNOS expression only in cells simultaneously treated with NGF and TNFα, while neither treatment alone elicited any effect. Furthermore, K252a completely abolished NGF/TNFα-promoted iNOS induction, suggesting that TrkA function is essential to

mediate it. As shown in Figure 3.1B, there was an induction of iNOS mRNA in PC12 cells treated with NGF and TNFα but not in cells treated with either factor alone. I also found that the synergistic induction of iNOS by NGF and TNFα is not a phenomenon limited to the PC12 cell system but can be elicited also in primary forebrain mixed rat neuronal cultures (Figure 3.1C) and in long-term organotypic rat brain slice cultures (Figure 3.1D). In either case, TNFα alone produced a weak (Figure 3.1C) or no induction of iNOS (Figure 3.1D), while NGF was ineffective. However, treating either culture simultaneously with TNFα and NGF elicited a more robust induction of iNOS expression.

NGF/TNFα-promoted iNOS expression is not mediated by elements in serum or another growth factor, IGF

To exclude the involvement of unknown serum factors, NGF/TNFα-promoted induction of iNOS was determined in cells cultured for 24 hr in serum free or in defined medium N2 (Figure 3.2A). There was a detectable iNOS induction in both serum free- and defined medium-cultured cells, although much reduced in serum free conditions, which is predictable as the cells cannot survive in the absence of serum. Since insulin is present in both serum and the N2 supplement, and can activate the insulin-like growth factor (IGF) receptor, I asked whether TNFα may synergize with IGF, which is also present in serum, to induce iNOS expression. The results shown in Figure 3.2B indicate that this is not the case.

NGF/TNFα-promoted iNOS expression is dose-dependent with respect to both NGF and TNFα and reversible with the removal of either ligand from the culture medium

Figure 3.3A shows western blots detecting iNOS in cells treated with increasing concentrations of NGF (top panel) or TNFα (bottom panel), in the presence or absence of a fixed amount of TNFα or NGF, respectively. Either factor was ineffective when added alone at any of the concentrations tested. However, there was a marked dose-response increase in iNOS expression when increasing concentrations of NGF or TNFα were added in the presence of a fixed amount of TNFα or NGF, respectively. Figure 3.3B shows a representative western blot detecting iNOS expression in cells continuously treated with NGF and TNFα as compared to cells in which the combined treatment was withdrawn after 24 hr. The expression of iNOS returned to basal, undetectable, levels between 24 and 48 hr after withdrawal of both TNFα and NGF. Furthermore, as shown in Figure 3.3C, withdrawal of either NGF or TNFα alone was sufficient to abolish iNOS expression induced by the combined treatment, both at the protein (upper panel) and mRNA level (lower panel).

Prolonged treatment with NGF and TNFα results in a reversible inhibition of nNOS message and protein in PC12 Cells

I then asked whether a similar TNFα/NGF combined treatment in PC12 cells may affect nNOS expression as well. I treated naïve PC12 cells for 72 hrs with 10 ng/mL of

recombinant rat TNFα and/or 10 ng/mL purified mouse NGF. While there was no effect of either TNFα or NGF treatment alone, simultaneous treatment with both TNFα and NGF (Both) results in an abolishment of mRNA levels (Figure 3.3B) and a corresponding decrease in nNOS protein levels (Figure 3.3A). Figure 3.3C shows results from PC12 cells that were either cultured in normal medium (Cont) or treated for 72 hrs with both TNFα and NGF (Both). Treatments were then washed out and then the cells were cultured for an additional 6 or 24 hours in either normal medium (Cont) or in the presence of both TNFα and NGF (Both). Removal of TNFα and NGF for 6 hours (Wash) was sufficient to allow for a significant reemergence of nNOS protein levels as compared to cells washed but then reexposed to TNFα and NGF (Both). This may be more fully appreciated by comparing cells 24 hours after removing TNFα and NGF (Wash) and comparing them to cells cultured remaining in the presence of TNFα and NGF (Both). 24 hrs after the removal of TNFα and NGF nNOS levels have almost returned to basal levels, suggesting that the loss of nNOS expression represents a reversible change in phenotype rather than a selection for PC12 cell subcultures expressing low levels of nNOS.

NGF/TNFα-promoted nNOS inhibition is dose dependent with respect to both NGF and TNFα

Figure 3.4 shows western blots from experiments testing the effect of varying concentrations of either TNFα (3.4A) or NGF (3.4B). The aim of this set of experiments

was to address whether the phenomenon was dose-dependent with respect to either agent. In figure 3.4A, PC12 cells were treated with varying concentrations of TNFα (0.1-100 ng/mL) in the presence or absence of a constant concentration of NGF (10 ng/mL) and. It may be appreciated that after 72 hours of treatment. No dose of TNFα was sufficient to alter nNOS protein levels in the absence of NGF. However, increasing TNFα concentrations were able to act in concert with 10 ng/mL NGF to decrease nNOS levels at all concentrations tested and in a dose-dependent manner. In figure 3.4B, cells were cultured for 72 hours with varying concentrations of NGF (0.1- 100 ng/mL) in the presence or absence of 10 ng/mL TNFα. Again I observed that nNOS protein levels were less affected in cells treated with NGF alone as compared to cells receiving NGF in the presence of TNFα and there was a greater decrease in nNOS protein at higher doses of NGF.

NGF/TNFα-promoted iNOS expression and nNOS inhibition is independent of the end-product NO

Next we sought to determine whether NOS activity, which we had previously reported to be strongly induced following TNFα/NGF treatment in PC12 cells (Macdonald and Taglialatela 2000), could play a role in the delayed decrease of nNOS expression. Therefore we assessed the effect a generic NOS inhibitor (LNAME) and a specific iNOS inhibitor (1400W) on TNFα/NGF-induced iNOS expression and nNOS decreases in PC12 cells. The western blot shown at the top of figure 3.6 illustrates that

the combined TNFα/NGF treatment (Both) induced iNOS (figure 3.6A upper) and inhibited nNOS (figure 3.6A lower) even in the presence of the generic NOS inhibitor LNAME. Similar results were obtained when a more specific iNOS inhibitor (1400W) was used instead. The western blot in figure 3.6B shows that, as expected, there was a robust induction of iNOS expression under these treatment conditions. As shown in figure 3.6B (top), LNAME did not affect TNFα/NGF-induced iNOS expression, nor did it affect the associated down-regulation of nNOS expression (bottom). In addition, treating PC12 cells with the NO donor SIN-1 did not sustain iNOS expression after NGF and TNFα were withdrawn (Fig. 3.6C, top panel), thereby further suggesting that sustained iNOS expression in response of the combined NGF/TNFα treatment is independent of NO. The bottom panel of Figure 3.6C shows a western blot detecting nitrotyrosine in the same protein samples shown in the top panel of Figure 4C and demonstrate that SIN-1 was active. Note that in this experiment the relatively low level of nitrotyrosine in cells simultaneously treated with NGF and TNFα is to be expected since L-arginine availability in PC12 cells is an obvious limiting factor for the resulting iNOS activity (see also figure 4.3). Furthermore, consistent with the results reported in Figure 5.3A, inhibition of NOS activity by L-NAME did not affect NFκB activation by NGF/TNFα combined treatment (Fig. 5.3D).

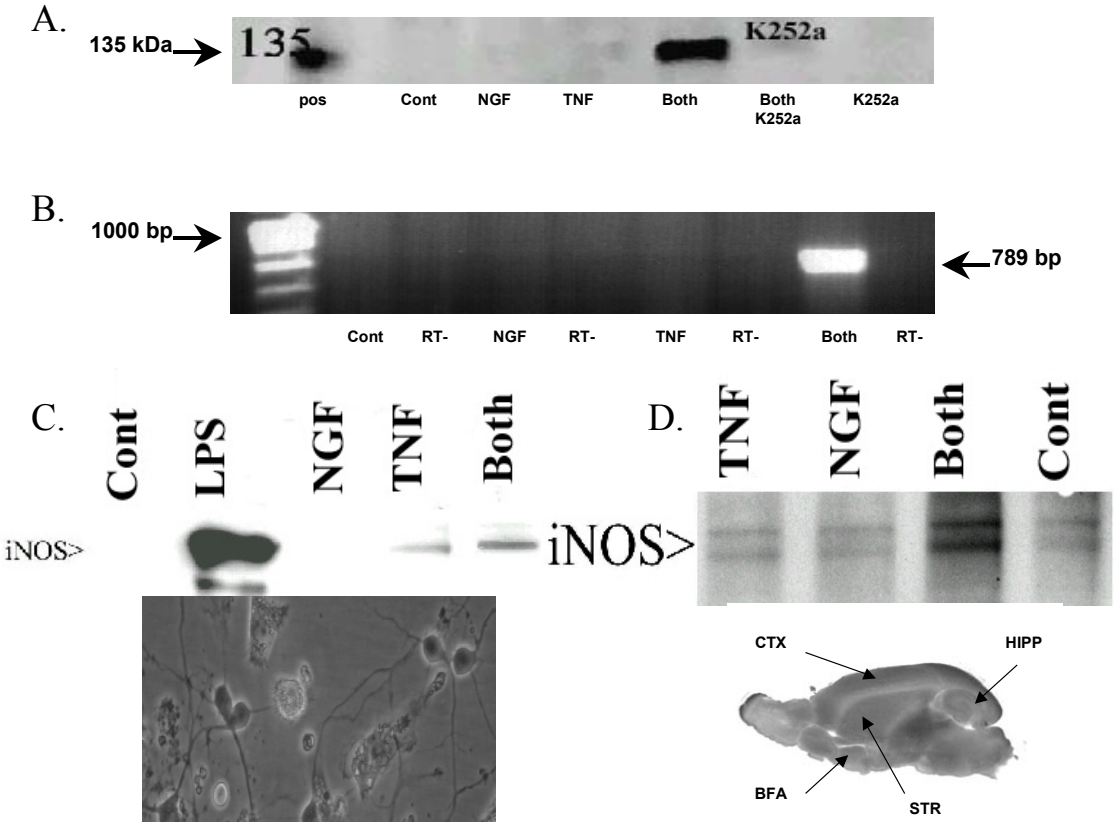

A.
135 kDa
135
K252a
pos
Cont
NGF
TNF
Both
Both K252a
K252a
B.
1000 bp
789 bp
Cont
RT-
NGF
RT-
TNF
RT-
Both
RT-
C.
Cont
LPS
NGF
TNF
Both
iNOS>
D.
TNF
NGF
Both
Cont
iNOS>
CTX
HIPP
BFA
STR

Figure 3.1: A. Western blot analysis detecting the presence of iNOS in 40 μg total protein extracts from PC12 cells treated for 24 hr with 10 ng/ml NGF and 10 ng/ml TNFα , individually or combined (Both), in the presence of 50 nM of the receptor tyrosine kinase inhibitor K252a. Positive control (Pos) is 4 μg of total protein extracts from mouse macrophages. **B.** RT-PCR detecting iNOS mRNA in PC12 cells treated for 24 hr with 10 ng/ml NGF and 10 ng/ml TNFα , individually or combined (Both) compared to untreated cells (Cont). Internal PCR controls lacking reverse transcriptase (RT-) were performed on each sample as shown. **C.** Western blot analysis detecting the presence of iNOS in total protein extracts from rat primary mixed forebrain cultures treated for 24 hr with 10 ng/ml NGF and 10 ng/ml TNFα , individually or combined (Both). Protein extracts from cultures treated with 10 nM LPS were used as positive control. The insert at bottom shows a representative photomicrograph of primary cultures used in this experiment. **D.** Western blot analysis detecting the presence of iNOS in total protein extracts from rat organotypic brain slice cultures treated for 24 hr with 10 ng/ml NGF and 10 ng/ml TNFα , individually or combined (Both). The insert at bottom shows a representative photomicrograph of a brain slice used in this experiment. CTX: cortex; HIPP: Hippocampus; STR: Striatum; BFA: Basal Forebrain Area.

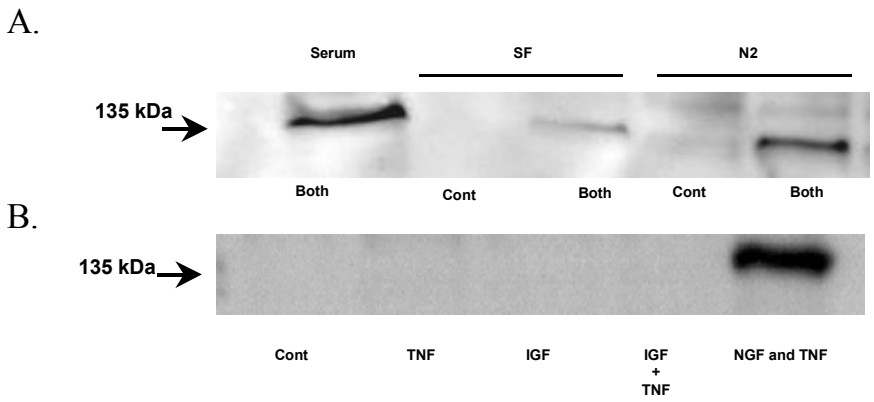


Figure 3.2: A. Western blot detecting iNOS in total protein extracts from PC12 cells simultaneously treated for 24 hr with 10 ng/ml NGF and 10 ng/ml TNFα in medium containing serum, in serum free medium (SF) or in defined medium (N2). **B.** Western blot analysis detecting the presence of iNOS in total protein extracts from PC12 cells treated for 72 hr with 100 ng/ml IGF and 10 ng/ml TNFα , individually or combined, as compared to cells simultaneously treated with 10 ng/ml NGF and 10 ng/ml TNFα or untreated controls (Cont).

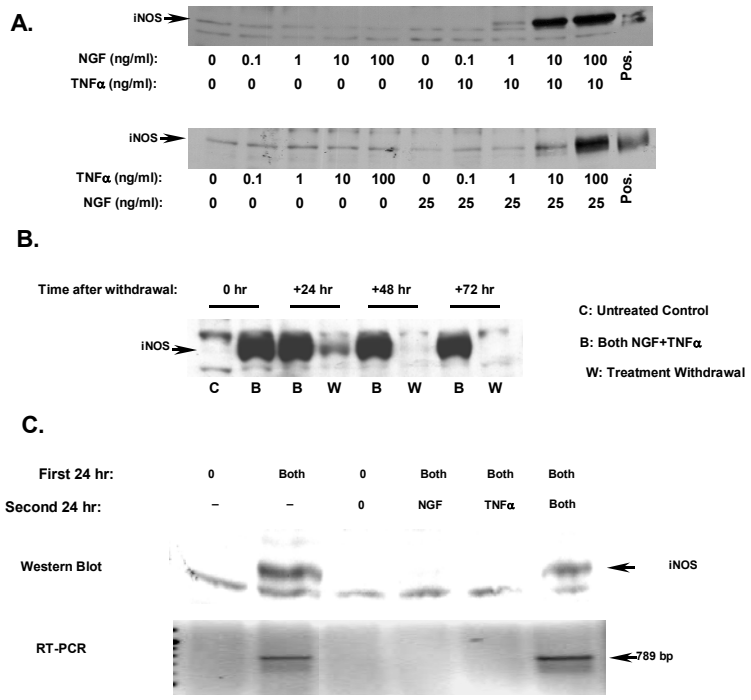


Figure 3.3: A. Western blots detecting iNOS in total protein extracts from PC12 cells treated for 24 hr with increasing concentrations of NGF in the presence or absence of 10 ng/ml TNFα **(Top)** or treated with increasing concentrations of TNFα in the presence or absence of 25 ng/ml NGF **(Bottom)**. Positive control (Pos) is 4 μg of total protein extracts from mouse macrophages. **B.** Western blot analysis detecting iNOS in total protein extracts from PC12 cells simultaneously pre-treated with 10 ng/ml NGF and 10 ng/ml TNFα. At 24 hr treatment was withdrawn and the presence of iNOS was determined 24, 48, and 72 hr thereafter. **C.** Western blot analysis **(Top)** and RT-PCR **(Bottom)** detecting iNOS protein and mRNA in total protein extracts and total RNA from PC12 cells simultaneously pre-treated for 24 hr with 10 ng/ml NGF and 10 ng/ml TNFα (Both). After 24 hr, treatment was withdrawn and replaced with either NGF or TNFα alone or with both and iNOS expression determined 24 hr thereafter.

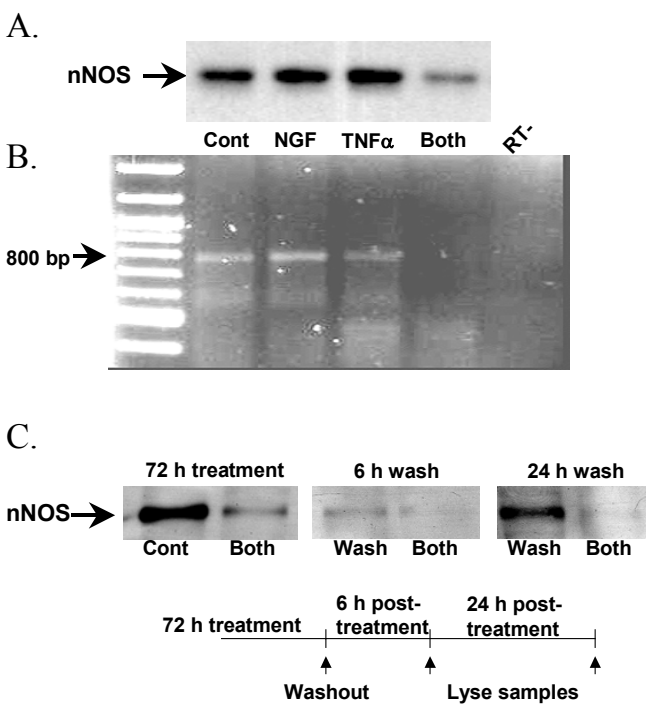
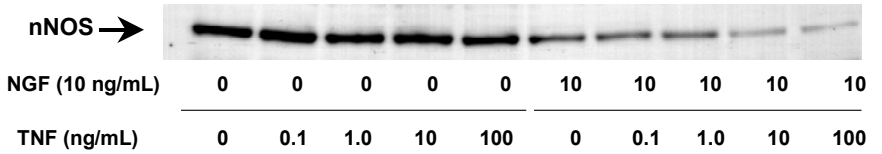


Figure 3.4: A. nNOS expression in PC12 cells after 72 hours of treatment with 10 ng/mL each of NGF and TNFα as determined by immunoblot and **B.** RT-PCR **C**. PC12 cells were washed and serum medium was replaced with either normal RPMI-1640 culture medium or with culture medium supplemented with 10 ng/mL each of NGF and TNFα and then cultured for an additional 6 or 24 hrs and nNOS expression determined by immunoblot.

A.



B.

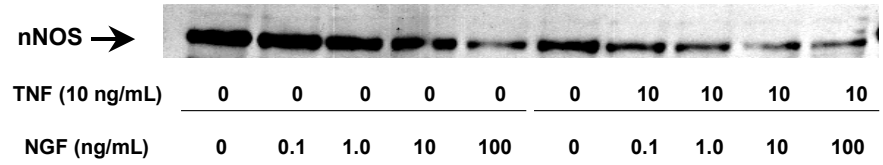


Figure 3.5: A. nNOS expression as determined by immunoblot in PC12 cells after 72 hours of treatment with either 10 ng/mL of NGF and varying concentrations of TNFα or 10 ng/mL of TNFα and **B.** varying concentrations of NGF.

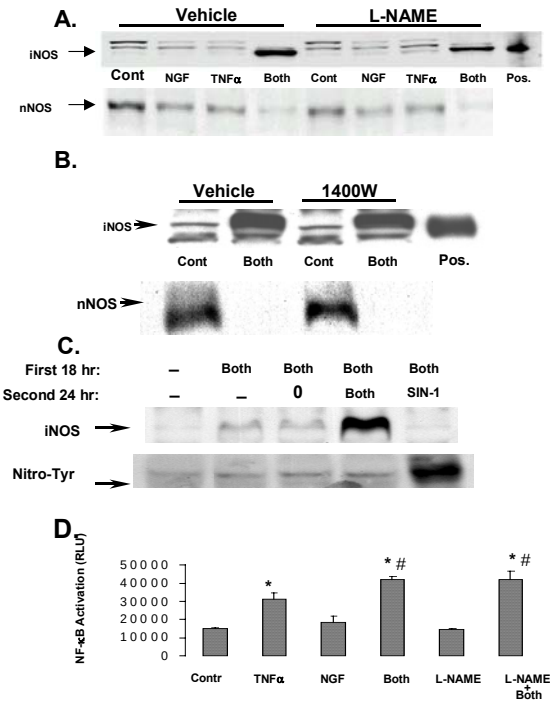


Figure 3.6: **A**. Western blot detecting iNOS (upper) and nNOS (lower) expression in total protein extracts from PC12 cells treated for 24 hr with 10 ng/ml NGF and 10 ng/ml TNFα, either individually or simultaneously (Both). Cells were further treated with vehicle or 0.5 mM of the generic NOS inhibitor L-NAME. Positive control (Pos) is 4 mg of total protein extracts from mouse macrophages. **B**. Western blot detecting iNOS (upper) and nNOS (lower) expression in total protein extracts from PC12 cells simultaneously treated with 10 ng/ml NGF and 10 ng/ml TNFα (Both), in the presence or absence of 10 nM of the iNOS-specific inhibitor 1400W. Cells were further treated with vehicle or 0.5 mM L-NAME. Positive control (Pos) is 4 mg of total protein extracts from mouse macrophages). **C**. Western blot analysis detecting iNOS (upper) and nitrotyrosine (lower) in total protein extracts from PC12 cells simultaneously treated for 18 hr with 10 ng/ml NGF and 10 ng/ml TNFα (Both). At 18 hr, treatment was withdrawn and replaced with vehicle (0), NGF plus TNFα (Both), or 1 mM of the NO donor SIN-1 and expression of iNOS or presence of nitrotyrosine was determined 24 hr thereafter. **D**. NFκB transcriptional activity (as measured by a transiently transfected SEAP reporter vector) in PC12 cells treated for 24 hr with 10 ng/ml NGF, 10 ng/ml TNFα or NGF plus TNFα (Both) in the presence of 0.5 mM L-NAME. Data are shown as mean ± S.E.M. from 3 independent replicates. * and #: $p<0.05$ vs. control or TNFα -alone cells, respectively (two-tailed unpaired Student's t-test).

Discussion

The work that I presented here stems from original observations made by our laboratory showing that iNOS expression and subsequent NO production can be synergistically induced by NGF and TNFα in a TrkA-dependent manner in PC12 cells (Macdonald and Taglialatela 2000). My present results extend that observation to two neuronal culture systems, primary neonatal rat forebrain mixed cultures and organotypic rat brain slice cultures. Both culture systems contain NGF-responsive cholinergic neurons, which are primarily located in the basal forebrain/septum area (Hefti 1986). Since I consistently observed a higher iNOS expression if NGF is added simultaneously to TNFα, I propose that iNOS expression was induced selectively in NGF-responsive cells. These results do not allow us to rule out the possibility that intermediate factors induced by TNFα or NGF may play a role in sensitizing indirectly neurons to NGF or TNFα, respectively. However, the results in PC12 cells shown in Figure 3.2 seem to exclude such a possibility. Indeed, while increasing concentrations of either NGF or TNFα can induce iNOS only when applied in the presence of fixed amounts of TNFα or NGF, respectively, withdrawal of NGF and TNFα allows for a prompt ablations of iNOS expression (Figure 3.2B). However, neither NGF nor TNFα alone is sufficient to sustain iNOS expression following withdrawal of TNFα or NGF (Figure 3.2C). These observations suggest that the simultaneous and continuous presence of both factors is

required to sustain iNOS induction/expression and that cell sensitization through a priming mechanism seems unlikely.

To exclude the involvement of unknown serum factors, NGF/TNFα-promoted induction of iNOS was determined in cells cultured for 24 hr in serum free or in defined medium N2 (Figure 3.2A). There was a detectable iNOS induction in both serum free- and defined medium-cultured cells, although much reduced in serum free conditions, which is predictable as the cells cannot survive in the absence of serum or N2 supplements. Since one of the major component of the N2 supplement is insulin, which is also present in serum and can activate the insulin-like growth factor (IGF) receptor, we asked whether TNFα may synergize with IGF, which is also present in serum, to induce iNOS expression. The results shown in figure 3.2B, however, indicate that this is not the case.

I report here the effects on nNOS expression in signaling paradigm resulting from sustained (>72 hours) exposure to NGF and TNFα in PC12 cells, a model for NGF responsive neurons. This NGF/ TNFα–induced inhibition of nNOS is independent of NO production and thus could take place in inflamed tissues irrespective of NO contributions from other NOS isoforms. Finally, I report here that this change of phenotype, reflected by the loss of nNOS expression in response to signaling cascades elicited with the combined NGF and TNFα may be reversible as the enzyme expression returned to basal levels within 24 hours of washing out the treatment as seen in Figure 3.4C. These results also suggest that rather than a selective TNFα toxicity in a population of nNOS

expressing cells, it is a reversible global loss of nNOS expression. Others have reported that NGF alone can inhibit nNOS expression in dissociated cultures of rat dorsal root ganglia (DRG; (Thippeswamy and Morris 1997), however, the authors note the presence of non-neuronal cells in the preparations leaving a possibility that TNFα could have also been present (not measured). More recently this group has reported that increased NGF expression in target tissues contributes to the loss of nNOS expression in developing DRG (Thippeswamy et al. 2005). While seemingly in contrast to my assertion that both NGF and TNFα are required for suppression of nNOS expression, it could also be hypothesized that simultaneous developmental increases represent a physiological role for the combined NGF/ TNFα–induced nNOS expression. Under conditions of chronic inflammation this developmental response could then lead to the loss of essential enzymatic activity in adult tissues.

We have previously reported that TNFα and NGF can synergistically promote iNOS expression in PC12 cells (Macdonald and Taglialatela 2000), leaving the possibility that the decrease of nNOS observed here could be due to negative feedback acted by iNOS-derived NO. However, our results seem to exclude this possibility since the combined TNFα/NGF treatment effectively reduced nNOS expression even in the presence of a selective iNOS inhibitor or a generic NOS inhibitor (figure 3.6A). This inhibition of nNOS is independent of NO production and thus could take place in inflamed tissues irrespective of NO contributions from other NOS isoforms. We also show that this phenotypic change is reversible in PC12 cells (figure 3.3C) and may have

therapeutic implications for the treatment of disease conditions that include increased TNFα production and decreased nNOS expression, including IBD. This observation also suggests that scavenging of NO, which has been shown to be increased in neurodegenerative diseases including IBD tissues (Iwashita et al. 1995), may not be an efficient therapeutic approach to correct the decrease in nNOS expression that may accompany these diseases, even though colonic injury models in transgenic animals have suggested a protective role for nNOS and pathological role for iNOS (Beck et al. 2004). Rather, our results would suggest that targeting of the yet unclear signaling pathways mediating selective TNFα-promoted nNOS down-regulation in NGF-responsive neurons should be considered for the development of future therapies.

Overall, my data indicate the possibility that a convergence between NGF-promoted trophic signaling and TNFα could selectively endanger NGF-responsive neurons under conditions of neuroinflammation because of a synergistic action between TNFα and NGF to induce iNOS and inhibit nNOS expression. Given that the product produced by both of these enzymes is NO, there is the possibility that induction of one isoform results in the inhibition ot the other isoform's expression, however, in chapter five I will present evidence that this mechanism is independent of NO production and likely a specific result of NGF/TNFα-promoted signaling pathways. Therefore, my results suggest the attractive idea that one mechanism through which pathologically high levels of TNFα affect certain trophic factor-responsive neurons may involve changes in nitrinergic function and a switch from activity dependent nNOS-mediated NO production

to constitutively active iNOS-mediated NO overproduction. My results also suggested that understanding the pharmacological mechanisms mediating the synergistic NGF/TNFα-promoted changes may provide novel therapeutic targets for the prevention of certain neurodegenerative events associated with acute or chronic injury of the nervous system.

RECEPTOR INVOLVEMENT IN MEDIATING NERVE GROWTH FACTOR/TUMOR NECROSIS FACTOR ALPHA SIGNALING IN PC12 CELLS

Abstract

Our laboratory has previously reported a synergistic effect of the cytokine, TNFα, and NGF on expression of iNOS, in rat pheochromocytoma PC12 cells. Here I show that NGF/TNFα-promoted iNOS can be toxic in PC12 cells. Furthermore, using PC12 cell mutants lacking the low affinity $p75^{NTR}$ receptor and wild type PC12 cells transfected with chimeric receptors having the extracellular domain of TNFRI and the intracellular domain of either p75NTR or TrkA NGF receptors, I also demonstrate that the simultaneous presence of both types of NGF receptors is necessary to mediate the induction of iNOS expression promoted by the combined TNFα/NGF treatment. These results suggest that that selective neurodegeneration as a result of Neuroinflammation-associated free radical production may occur in neurons bearing both $p75^{NTR}$ and TrkA, such as BFCN, may allow for novel therapeutic strategies.

Introduction

Arguments for inflammatory involvement in AD pathogenesis have been proposed based on evidence of increased proinflammatory cytokines such as TNFα and

IL-1 in CSF and cortex of AD brain (Tarkowski et al. 2003;Tarkowski et al. 1999). Risk factors for AD such as head injury are also known to be associated with increased circulating concentrations of inflammatory cytokines and reduced numbers of basal forebrain cholinergic neurons (Murdoch et al. 1998). TNFα has also been show to contribute to the death of NGF-dependent neurons *in vitro* (Barker et al. 2001) as well as in TNFα-overexpressing transgenic mice (Lombardi et al. 1999). Using pheochromocytoma cells (PC12) as a well-established *in vitro* model of NGF-responsive cholinergic neurons, our and other laboratories have demonstrated an increased susceptibility to TNFα administered in the presence of NGF (Mielke and Herdegen 2002;Macdonald and Taglialatela 2000). The molecular mechanisms mediating such apparent increased susceptibility to TNFα have not yet been fully elucidated. One possible candidate is the inducible nitric oxide synthetase (iNOS), one of three isoforms of an enzyme responsible for nitric oxide (NO) production. Levels of iNOS message or protein have not been reported in healthy brain but are reportedly increased in AD brain (Lee et al. 1999), presumably leading to the production of high levels of nitric oxide (NO). Such abnormal levels of NO are thought to induce cell damage through reactive nitrogen species (RNS)-mediated oxidative stress, another known component of AD-associated neurodegenerative events as revealed by both carbonyl and nitrotyrosine formations in the AD cortex (Smith et al. 1997).

Perturbed levels of NOS and NO-derived oxidative damage have been reported in both acute and chronic neurodegenerative conditions (Emerit et al. 2004), including spinal cord injury (Isaksson et al. 2005;Urushitani et al. 1998;Diaz-Ruiz et al. 2002),

stroke (Parmentier-Batteur et al. 2001;Sarchielli et al. 2003) and AD (Luth et al. 2002;Luth et al. 2001;Law et al. 2001;de la Monte et al. 2000;Lee et al. 1999). However, TNFα alone has not been shown to be an effective inducer of human iNOS promoter activity (Darville and Eizirik 1998) or of rat cortical iNOS expression when administered intracerebroventricularly (Kong et al. 2000). Nonetheless, TNFα has been shown to contribute to the death of NGF-dependent neurons *in vitro* (Barker et al. 2001), in TNFα-overexpressing transgenic mice (Aloe et al. 1999), and following direct administration into the NGF-dependent mouse cholinergic basal forebrain (Wenk et al. 2003a). Therefore, my previous results suggest the attractive idea that one mechanism through which increased levels of TNFα affect certain trophic factor-responsive neurons may involve NO-derived oxidative damage brought about by a synergistic induction of iNOS. Understanding the molecular mechanisms mediating the synergistic NGF/TNFα-promoted induction of iNOS may thus provide novel therapeutic targets for the prevention of certain neurodegenerative events associated with acute or chronic injury of the nervous system.

Through the use of $p75^{NTR}$-deficient PC12 cell mutants and chimeric TNF/NGF receptor constructs I report that NGF/TNFα-promoted iNOS in PC12 cells requires the simultaneous presence of both the low-affinity p75NTR and the high-affinity TrkA NGF receptors. D3, a commercially available peptidomimetic compound reported to be a selective TrkA agonist exhibits partial agonist properties in my model. I also report that TrkA-deficient PC12 "NNR" mutants lack TNFR1 making them unsuitable for this study.

Materials

All routine reagents and chemicals were obtained as described in Chapter 2. Primary monoclonal anti-iNOS antibodies were purchased from Signal Transduction Laboratories. Primary polyclonal anti-TNFR1 antibody was purchased from Santa Cruz Biotechnologies.

Methods

General methods for cell culture, western blotting, flow-cytometry, and LDH assay were used as outlined previously in Chapter Two, Materials and Methods.

Results

Pharmacological elucidation of NGFR involvement fails with a commercially available ligand

Previously, K252a, a receptor tyrosine kinase inhibitor, was used by our laboratory to demonstrate the requirement for the TrkA receptor in mediating NGF/TNFα-promoted iNOS induction. Here, I sought to determine whether TrkA activation alone would be sufficient to mediate this event in conjunction with TNFα using a commercially available peptidomimetic ligand, D3. However, initial studies to demonstrate TrkA activation with D3 using a serum deprivation model in PC12 cells failed to provide any evidence for TrkA activation by D3. Indeed, when administered 24 hrs after serum withdrawal, at concentrations sufficient to bind TrkA (1 ng/mL), NGF

was almost completely effective a rescuing serum-deprived PC12 cells. However, when D3 and NGF were administered together, D3 appeared to compete with NGF for binding to TrkA and made NGF less effective at rescuing serum deprived PC12 cells at 20 and 500 pg/mL (Figure 4.1).

NGF/TNFα-promoted iNOS expression can be toxic in PC12 cells

TNFα alone can be toxic to PC12 cells at high concentrations (>50 ng/ml) and relatively high concentrations of NGF have been shown to protect cells from this stress (Pappas et al. 2003). Paradoxically, the additional treatment of PC12 cells with a low dose of NGF (10 ng/ml) exacerbated TNFα toxicity (Figure 4. 2 lower). This increased toxicity was paralleled by induction of iNOS elicited by the combined NGF/ TNFα treatment (Figure 4.2, Above), thus implicating iNOS-derived NO in cell death. To explore this possibility, PC12 cells were treated with 10 ng/ml of TNFα alone or in combination with 10 ng/ml NGF, in the presence or absence of L-arginine (the substrate for NOS which is present in limiting amounts under normal culture conditions) and L-NAME (a generic NOS inhibitor). As figure 4.3 shows, L-arginine greatly increased PC12 cell toxicity produced by the NGF/ TNFα combined treatment but was ineffective in cells treated with TNFα alone. Additional treatment with L-NAME completely blocked toxicity induced by NGF/ TNFα combined treatment in the presence or absence of L-arginine, thus supporting the idea that iNOS-derived NO participates in producing PC12 cell toxicity in response to NGF/ TNFα treatment.

Both TrkA and p75NTR required for NGF/TNFα-promoted iNOS expression

Next, I subcloned a PC12 mutant cell line ($PC12^{p75NTR\ (-)}$) that lacks p75NTR expression while retaining TrkA at levels comparable with wild type PC12 cells (Figure 4.4A). $PC12^{p75NTR\ (-)}$ cells exposed to the combined NGF/TNFα treatment did not show any induction of iNOS expression as compared to the parent cell line (Figure 4.4B). It is important to note that the $PC12^{p75NTR\ (-)}$ cells used here express TNFα receptor type 1 (TNFR1) at levels comparable (or even higher) than wild type PC12 cells (Figure 4.4C). Therefore lack of iNOS induction by the NGF/TNFα combined treatment in these cells cannot be ascribed to lack of TNFα responsiveness.

The results obtained in $PC12^{p75NTR(-)}$ would suggest that p75NTR is essential to mediate iNOS induction by the combined TNFα/NGF treatment while the results obtained using K252a (Figure 3.1) would suggest a prominent role for TrkA. In order to ultimately ascertain the relative role of the two NGF receptors in mediating TNFα/NGF-promoted iNOS induction I made use of PC12 cells transiently transfected with expression vectors coding for chimeric TNFα/NGF receptors constructed as described by (Rovelli et al. 1993). As can be appreciated by the colored diagram in the upper panel of figure 4.5, these constructs bear the ligand binding domain from the human TNFR1 and the signal transduction domain from rat NGF receptors, either TrkA or p75NTR. Previously, it has been shown that transfection with these chimeras allows for TNF-promoted NGF signaling (Rovelli et al. 1993). Figure 4.5 (lower panel) shows a western

blot detecting iNOS in PC12 cells individually or simultaneously transfected with chimeric TNFα receptors bearing the intracellular domain of p75NTR (p75) or TrkA. Transfected cells were then treated either with TNFα and NGF alone, or with both TNFα and NGF. As expected, the combined TNFα/NGF treatment induced a robust expression of iNOS in these PC12 cells, regardless of the presence of any transfected expression vector. As also expected, NGF alone did not elicit iNOS expression in any of the transfected cells. Similarly, TNFα alone did not induce iNOS in cells transfected with either TNFR/p75NTR or TNFR/TrkA chimeric receptors. However, TNFα promptly induced iNOS expression in cells transfected with both TNFR/p75NTR and TNFR/TrkA chimeric receptors.

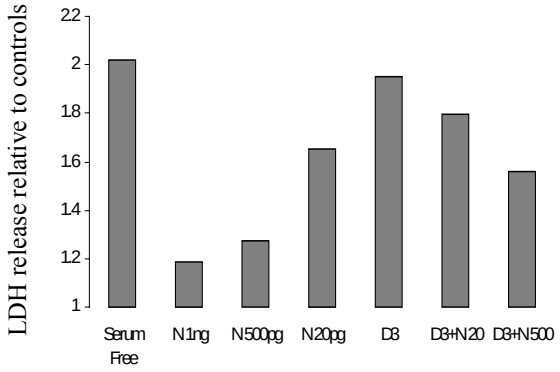


Figure 4.1: Ability to rescue 24 hr serum-deprived PC12 cells (a TrkA-mediated event) was determined by a LDH release into cell culture medium as compared to cells in serum-containing RPMI 1640. 10 μM D3, a selective TrkA agonist, was ineffective at preventing cell death and also inhibited NGF-mediated rescue when given in combination.

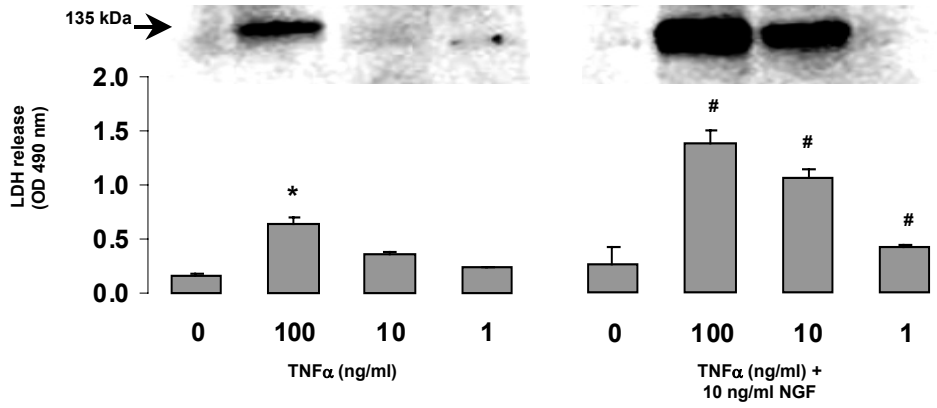


Figure 4.2: (Top) Western blot detecting iNOS in total protein extracts from PC12 cells treated for 48 hr with 1, 10, and 100 ng/ml TNFα in the presence or absence of 10 ng/ml NGF. **(Bottom)** cytotoxicity LDH assay in the culture medium from the same cells shown in the top panel. Data are shown as mean ± S.E.M. from 3 independent replicates. * and #: $p<0.05$ vs. untreated control cells and $p<0.05$ vs. matched TNFα-alone treated cells, respectively (two-tailed unpaired Student's t-test).

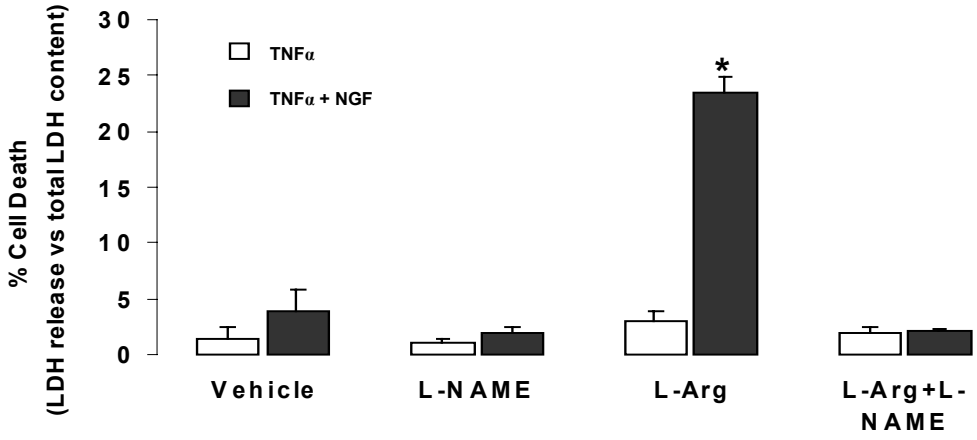


Figure 4.3: LDH release assay in PC12 cells treated for 48 hr with 10 ng/ml TNFα in the absence (open bars) or presence (gray bars) of 10 ng/ml NGF. Cells were additionally pretreated with vehicle, 0.5 mM of the generic NOS inhibitor L-NAME, 1 mM of the NOS substrate L-Arginine or L-Arginine plus L-NAME. Data are shown as mean ± S.E.M. from 3 independent replicates. *: $p<0.001$ vs. matched TNFα-alone treated cells (two-tailed unpaired Student's t-test).

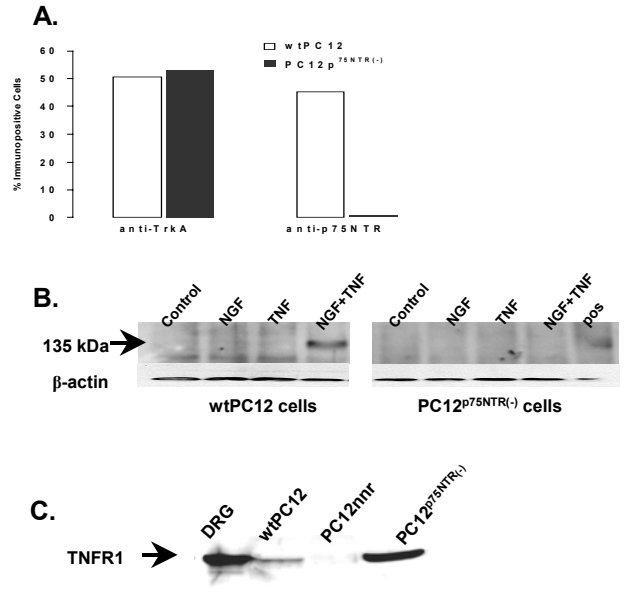


Figure 4.4: **A.** Graph depicting the percentage of TrkA- or p75NTR- immunopositive cells in wild type (wt)PC12 cells and PC12 cell mutants lacking the low affinity NGF receptor ($PC12^{p75NTR(-)}$) from flow cytometry data. **B**. Western blot detecting the presence of iNOS in wtPC12 cells and $PC12^{p75NTR\ (-)}$ cells treated for 24 hr with vehicle (Cont), 10 ng/ml NGF, 10 ng/ml TNFα or NGF plus TNFα (Both). Membrane was re-probed for β-actin (lower panel) to control for equal protein loading. Positive control (Pos) is 4 μg of total protein extracts from mouse macrophages. **C.** Western blot detecting the presence of TNFR-I in total protein extracts from wtPC12 cells and $PC12^{p75NTR\ (-)}$ cells. Twenty μg of total protein extracts from rat dorsal root ganglia (DRG) were used as a positive control.

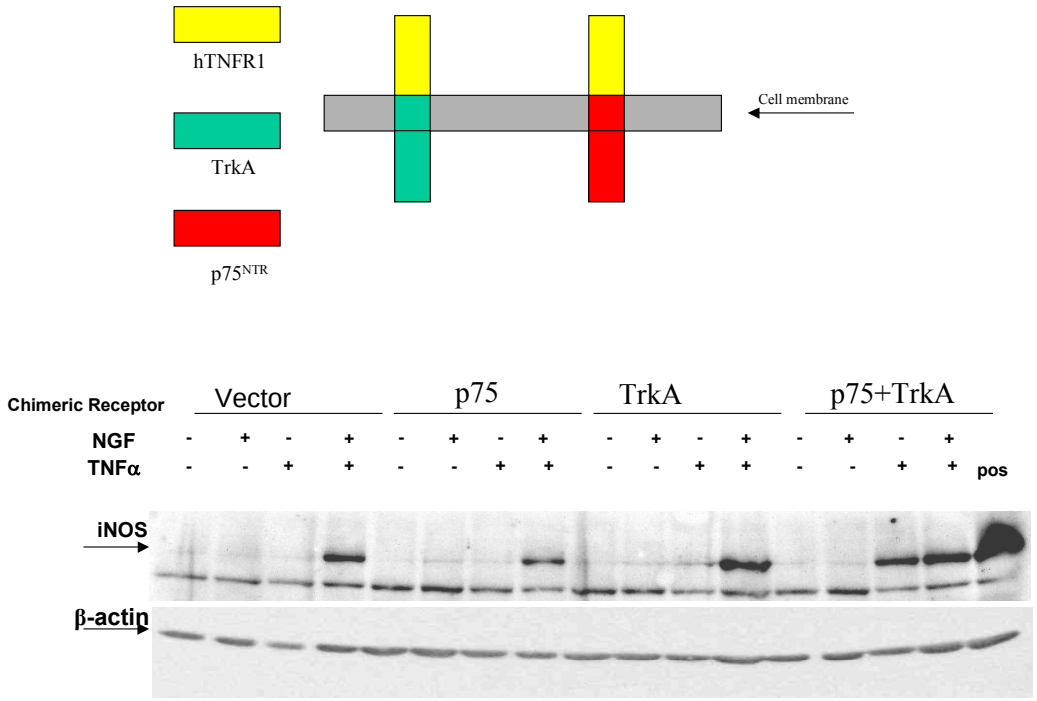


Figure 4.5: The upper diagram illustrates the nature of the chimeric proteins used for this set of experiments. The colored regions denote an extracellular human TNFR1 binding region (yellow) and an intramembrane and signaling domain from either TrkA (green) or $p75^{NTR}$ (red). Below is western blot detecting iNOS in 40 μg total protein extracts from PC12 cells treated for 24 hr with 10 ng/ml human TNFα, 10 ng/ml NGF, or both. Twenty-four hr before treatment, cells were transfected with either an empty vector or expression vectors for chimeric receptor proteins bearing the human TNFR1 ligand binding domains and the intracellular domain of either rat $p75^{NTR}$ or TrkA NGF receptors ($p75^{NTR}$, TrkA or p75NTR+TrkA). Positive control (Pos) is 40 μg of total protein extract from wild type PC12 cells treated with both rat TNFα and NGF. Membrane was re-probed for β-actin (bottom panel) to control for equal protein loading and is representative from three independent transfections and treatments.

Discussion

My results indicate that NGF/TNFα-promoted iNOS induction in PC12 cells can result in cytotoxicity. Paradoxically, my data show that NGF enhanced TNFα toxicity in PC12 cells (Figure 4.2 lower panel). This is seemingly in contrast with our previous work showing that high levels of NGF (>100 ng/ml) protected PC12 cells from TNFα [62]. However, it is important to appreciate that the concentrations of NGF used here are much lower (10 ng/ml) than the ones that were shown to effectively rescue from TNFα toxicity. Thus, the two phenomena may be distinct and have separate physiologic relevance: on the one hand, basal neurotrophic levels of NGF which are normally taken up by NGF-responsive neurons from target sources may endanger such neurons under conditions of acute or chronic elevated TNFα (Wenk et al. 2003b); on the other hand, high NGF levels may protect through stimulation of cell antioxidant mechanisms (Perezpolo et al. 1990). Indeed, NGF/TNFα-promoted PC12 cell death was exacerbated by excess of the NOS substrate L-arginine and prevented by L-NAME (a NOS inhibitor, Figure 4.3), thus indicating that cytotoxicity may be directly related to increased expression of iNOS (Fig. 4.2) and its enzymatic activity and subsequent oxidative stress brought about by its end product NO. L-NAME is a generic NOS inhibitor that cannot discriminate among the different NOS isoforms. Therefore, I cannot exclude the possibility that cytotoxicity may be due to induction of isoforms other than iNOS. However, as I showed in Chapter 3, I have evidence to suggest that while non-stimulated PC12 cells express only the neuronal

isoform of NOS (nNOS), exposing them to the same combined NGF/ TNFα treatment as employed in the present study completely abolishes nNOS protein and messenger RNA (Figure 3.3B). Therefore, it seems unlikely that nNOS activity may contribute significantly to cytotoxicity in response to NGF/ TNFα treatment in PC12 cells.

I found that mutant PC12 cells that lack expression of the p75NTR receptor failed to respond in terms of iNOS expression when simultaneously treated with NGF and TNFα. That PC12 cells bearing only the TrkA receptor failed to respond the combined NGF/TNFα treatment suggests that signaling from p75NTR in combination with TNFα is necessary to induce iNOS expression. On the other hand, our previous work illustrated the importance of TrkA-associated signaling in mediating NGF/TNFα-promoted induction of iNOS [43] (see also figure 3.1). These results are only apparently in contrast. Indeed, in an admittedly artificial system making use of chimeric constructs I observed that only in the presence of both TNFα-responsive NGF receptor signaling can TNFα promote iNOS expression when added alone. Whether this is a consequence of simultaneous but independent signaling of both types of NGF receptors (Woo et al. 2004) or recruitment of intracellular signalling elements uniquely driven by the simultaneous activation of both NGF receptors' signaling domains remains to be investigated. On the other hand, these results exclude the possibility that the combined action of TNFα and NGF may derive from yet undescribed interaction(s) of the extracellular domains of their respective receptors following ligand binding.

Thus, my combined results would indicate that there exists a specific pathway requiring the simultaneous expression or both types of NGF receptors that is synergistically induced by TNFα and NGF to promote expression of iNOS. Indeed, others have also noted signaling pathways that require the simultaneous expression of both TrkA and $p75^{NTR}$ (Lad et al. 2003;Szutowicz et al. 2004) as well as the convergence of TrkA and $p75^{NTR}$-mediated signaling impinging upon specific transcription factors (Foehr et al. 2000). Furthermore, recent reports in neurons of TNF-promoted signaling occurring selectively in the presence of the glutamate agonist NMDA (Floden et al. 2005) illustrate the importance of considering the signaling "context" when studying the effects of cytokine treatment.

Overall, my data indicate the possibility that a convergence between NGF-promoted trophic signaling and TNFα could selectively endanger NGF-responsive neurons under conditions of neuroinflammation because of a synergistic action between TNFα and NGF to induce iNOS expression. For example, TNFα–overexpressing transgenic mice show selective neurodegeneration of NGF-responsive basal forebrain cholinergic neurons (Aloe et al. 1999) and direct TNFα administration in the brain of mice results in an impairment of basal forebrain cholinergic function (Wenk et al. 2003b). However, whether induction of iNOS and subsequent oxidative damage may play a role in these two models remains to be determined.

SIGNALING PATHWAYS MEDIATED BY TUMOR NECROSIS FACTOR ALPHA IN NERVE GROWTH FACTOR-RESPONSIVE CELLS *IN VITRO* AND *IN VIVO*

Abstract

The final aim of this project includes elucidating some of the intracellular signaling requirement mediated by the combined NGF/TNFα treatment in the hopes of identifying therapeutic targets. Using a reporter gene assay I investigated the synergistic effect of the combined NGF/TNFα treatment in PC12 cells and found that it is mediated through NF-κB. Inhibitors of NF-κB and MAPK demonstrate that iNOS induction requires NF-κB and is not a function of differentiaton. Finally I use acute intracerebroventricular (icv) injections of recombinant TNFα in mice to show a region specific expression of iNOS immunoreactivity. I propose that TNFα-promoted induction of cytotoxic iNOS in NGF-responsive neurons may be relevant to the mechanisms underlying BFCN demise in AD.

Introduction

Using PC12 cell mutants lacking the low affinity p75NTR receptor, I demonstrate that this receptor is likely acting through the transcription factor nuclear factor kappa B (NF-κB). Our data also suggest that NGF/TNFα-promoted iNOS induction requires

activation of the transcription factor nuclear factor kappa B promoted by both TNFα and NGF. The activation of NF-κB by NGF and TNFα is independent of NO production as is the induction and inhibition of iNOS and nNOS protein expression respectively (see Chapter 3). To determine if specific regions of the brain display a selective susceptibility to TNFα enhanced iNOS expression, I investigated the effects of *in vivo* stereotaxic injections of recombinant rat TNFα into the fourth ventricle of adult male C57BL/6 mice. There was a robust induction of iNOS expression following the injection of TNFα, which was restricted to the basal forebrain and hippocampus. No iNOS expression was detected in the striatum, or cerebellum. Determining mechanisms for modulation of NFκB-promoted control of selective genes could serve as a potential therapeutic target to prevent neuroinflammation-induced neuronal damage.

Materials

All routine reagents and chemicals were obtained as described in Chapter 2. Primary monoclonal anti-iNOS antibodies were purchased from Signal Transduction Laboratories.

Methods

General methods for cell culture, western blotting, transfection, immunohistochemistry and reporter gene assay were used as outlined previously in Chapter Two, Materials and Methods.

Results

NGF/TNFα-promoted iNOS expression is specifically mediated through and requires NF-κB activation in part by the p75NTR

Figure 5.1 shows results from PC12 cells transiently transfected with a secreted alkaline phosphatase reporter gene construct (SEAP) promoted by enhancer sequences specific for nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), cAMP-responsive element (CRE) or Tal (non-inducible control). Twenty-four hr after transfection cells were treated with 10 ng/ml each of TNFα and NGF (alone or combined) and SEAP released in the culture medium (an index of endogenous transcription factor activation) was assayed 3 hr and 12 hr later. At 3 hr (figure 5.1 upper), cells treated with TNFα showed a significant increase in NF-κB activity but not AP-1 or CRE. Cells treated with NGF alone showed at 3 hr no significant increase in NF-κB, AP1 or CRE activity. When cells were exposed to the combined NGF/ TNFα treatment, there was a robust increase in NF-κB activity that was significantly higher than the response induced by the individual treatment with TNFα. On the other hand, neither AP-1 nor CRE activity were significantly affected by the combined NGF/ TNFα treatment. At 12 hr (figure 5.1 lower), both TNFα and NGF/TNFα combined treatments significantly increased NF-κB activity, but were not statistically significantly different. NGF-treated cells showed a significant increase in AP-1 and CRE activity at 12 hr, while

NF-κB activity was not affected. As a result, there was also a significant increase in AP-1 and CRE activity elicited by the NGF/TNFα combined treatment at 12 hr. Neither NGF nor TNFα (alone or combined) elicited any effect on the control reporter construct Tal, either at 3 or 12 hr.

Involvement of NF-κB was further explored by determining the extent to which pharmacological inhibition of NF-κB would block NGF/TNFα-promoted iNOS induction in PC12 cells. As shown in figure 5.2A, treatment of PC12 cells with either pyrrolidine di-thio-carbamate (PDTC) or the octapeptide proteasome inhibitor PSI (two effective NF-κB inhibitors that have distinct mechanisms of action (La Rosa et al. 2004;Taglialatela et al. 1997;Taglialatela et al. 1998), completely abolished NGF/ TNFα-promoted iNOS induction. In this experiment, PD98059, a selective MAPK inhibitor, was used as a negative control. Both NF-κB inhibitors effectively blocked NF-κB-mediated transcriptional activity as determined by SEAP reporter gene assay (Figure 5.2B), whereas PD98059 had no effect. However, PD98059 completely blocked NGF-promoted neurite outgrowth (Figure 5.2C), an event that in PC12 cells is dependent on MAPK activation (Pang et al. 1995).

Given the reported stimulation of NFkB activity by NGF through the p75NTR, I investigated the importance of this receptor in mediating TNFα/NGF-promoted induction of NFkB activity. In order to achieve this, I made use of PC12 cells that lack p75NTR as I preciously described in Chapter 4. As shown in Figure 5.2D, NF-κB activity was not significantly increased by the NGF/TNFα combined treatment over the levels induced by

TNFα alone in PC12$^{p75NTR\ (-)}$ (Figure 5.2D), which is consistent with the results that I reported in Chapter 4 (Figure 4.4B) as compared to the parent cell line. As I mentioned in Chapter 4, it is important to note that the PC12$^{p75NTR\ (-)}$ cells used here express TNFα receptor type 1 (TNFR1) at levels comparable (or even higher) than wild type PC12 cells (Figure 4.4C). Therefore lack of iNOS induction by the NGF/TNFα combined treatment in these cells cannot be ascribed to lack of TNFα responsiveness (as can also be appreciated by the NFκB response induced by TNFα alone shown in figure 5.2D).

Regioselective iNOS expression can be observed in mice with an intraventricular TNFα administration

The results reported above indicate that iNOS induction may be promoted by TNFα selectively in NGF-responsive neural cells expressing both TrkA and p75NTR receptors, such as the cholinergic neurons in the basal forebrain which innervate the hippocampus and the cortex (Varga et al. 2003)or the olfactory receptor neurons (Carter and Roskams 2002). In order to test this hypothesis, I stereotaxically injected 5 μL of various concentrations of TNFα, or vehicle, in the fourth ventricle of the CNS of C57/BL6 mice and determined iNOS expression in various brain regions 24 hr following the injection. Figure 5.3 (top panel) shows immunohistochemistry demonstrating the presences of cholinergic neurons in the area we dissect out as the BFA. The lower panel is a schematic of the area in which the injections were made. Figure 5.4 shows Western blots detecting iNOS expression in total cell extracts from the basal forebrain (BFA),

hippocampus (HIPP), cerebellum (CBLM) and striatum (STR). At the highest concentrations used (1 and 2 μg/mouse), TNFα elicited iNOS expression in the basal forebrain area and hippocampus, but not in the cerebellum or striatum. At lower doses (0.25 and 0.5 μg/mouse) TNFα did not elicit iNOS expression in any of the brain regions studied. Notably, both the basal forebrain and the hippocampus are known to be associated with NGF-responsive BFCN, with cell bodies being localized in the basal forebrain and terminals innervating the hippocampus (Varga et al. 2003). Because the injections were made through the cerebellum into the fourth ventricle, it is also important to note that iNOS expression was not observed in the cerebellum or in the striatum suggesting that the iNOS induction in response to TNFα is restricted to selected sensitive CNS regions, rather than representing a more ubiquitous glial response.

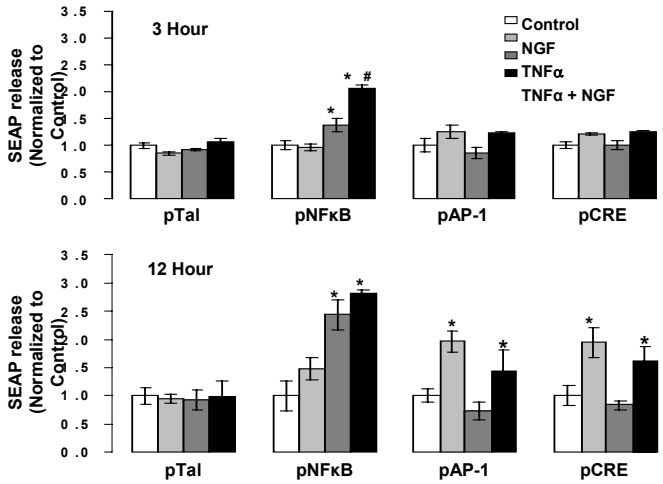
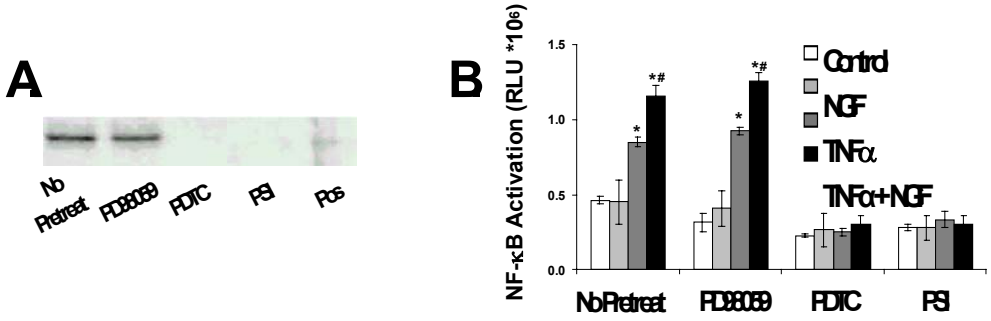
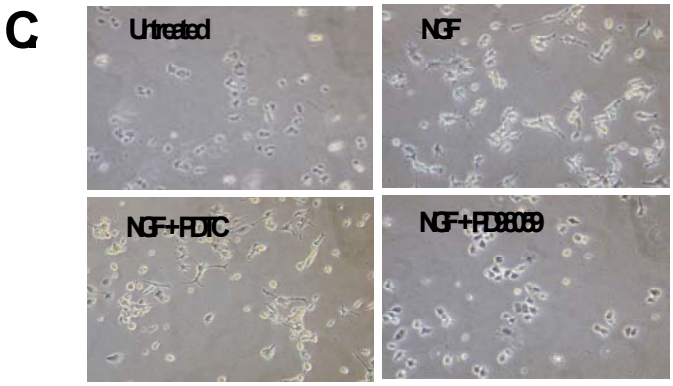


Figure 5.1: Detection of SEAP in the culture medium of PC12 cells transfected with a SEAP reporter gene construct under the transcriptional control of enhancers specific for NF-κB, AP-1 or CRE. pTal is the non-enhanced control SEAP reporter vector. Twenty-four hr after transfection, cells were treated with vehicle (Control), 10 ng/ml NGF, 10 ng/ml TNFα or NGF plus TNFα (Both) and the presence of SEAP in the culture medium assayed 3 hr **(Top)** or 12 hr **(Bottom)** thereafter. Results are expressed as percent of control cells in each transfection group (N=3). * and #: $p<0.05$ vs. control and TNFα - alone, respectively (two-tailed unpaired Student's t-test).


A
No Pretreat
PD98059
PDTC
PSI
Pos
B
NF-κB Activation (RLU *10⁶)
1.5
1.0
0.5
0.0
*
*#
Control
NGF
TNFα
TNFα+NGF
NoPretreat
PD98059
PDTC
PSI


C
Untreated
NGF
NGF+PDTC
NGF+PD98059

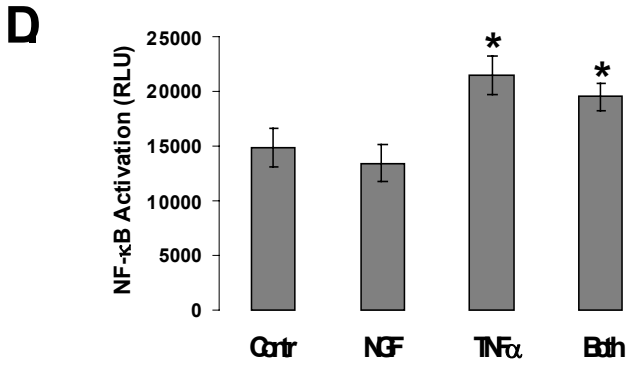

D
NF-κB Activation (RLU)
25000
20000
15000
10000
5000
0
*
*
Contr
NGF
TNFα
Both

Figure 5.2: A. Western blot detecting iNOS in PC12 cells simultaneously treated with 10 ng/ml NGF and 10 ng/ml TNFα for 24 hr. Thirty minutes before NGF/ TNFα treatment cells were pre-treated with 10 μM pyrrolidinedithyocarbamate (PDTC), 2 μM of a oligopeptide proteosome inhibitor (PSI) or 10 μM of a MAPK inhibitor (PD98059). **B.** SEAP release in the culture medium of PC12 cells transfected for 24 hr with an NF-κB-sensitive SEAP reporter gene construct and treated for 12 hr with vehicle (Control), 10 ng/ml NGF, 10 ng/ml TNFα or NGF plus TNFα in the presence of 10 μM PD98059, 10 μM PDTC or 2 μM PSI. Data are shown as mean ± S.E.M. from 3 independent replicates. * and #: $p<0.05$ vs. control or TNFα -alone cells, respectively (two-tailed unpaired Student's t-test). **C.** Representative photomicrographs of PC12 cells treated for 48 hr with 10 ng/ml NGF in the presence or absence of 10 μM PD98059 or 2 μM PDTC. **D.** NFκB transcriptional activity (as measured by a transiently transfected SEAP reporter vector) in PC12 cells treated for 24 hr with 10 ng/ml NGF, 10 ng/ml TNFα or NGF plus TNFα (Both) in the presence of 0.5 μM L-NAME. Data are shown as mean ± S.E.M. from 3 independent replicates. * and #: $p<0.05$ vs. control or TNFα -alone cells, respectively (two-tailed unpaired Student's t-test).

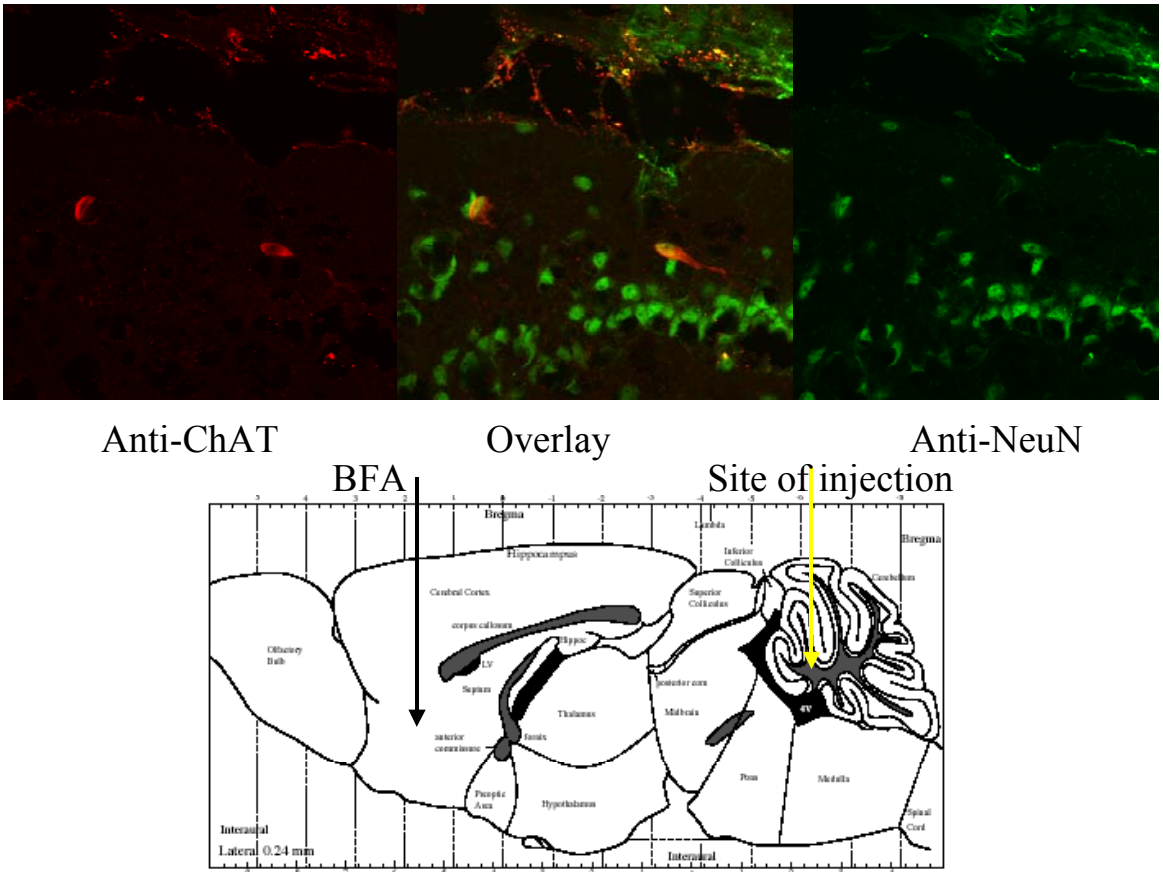


Figure 5.3: Top panels depict representative immunofluorescent micrographs of ChAT neurons the region included the dissected brain areas used for western blot in Figure 5.4. The bottom panel is adapted from Paxinos 2000 and demonstrates where the injection of TNFα was made in the mouse brain.

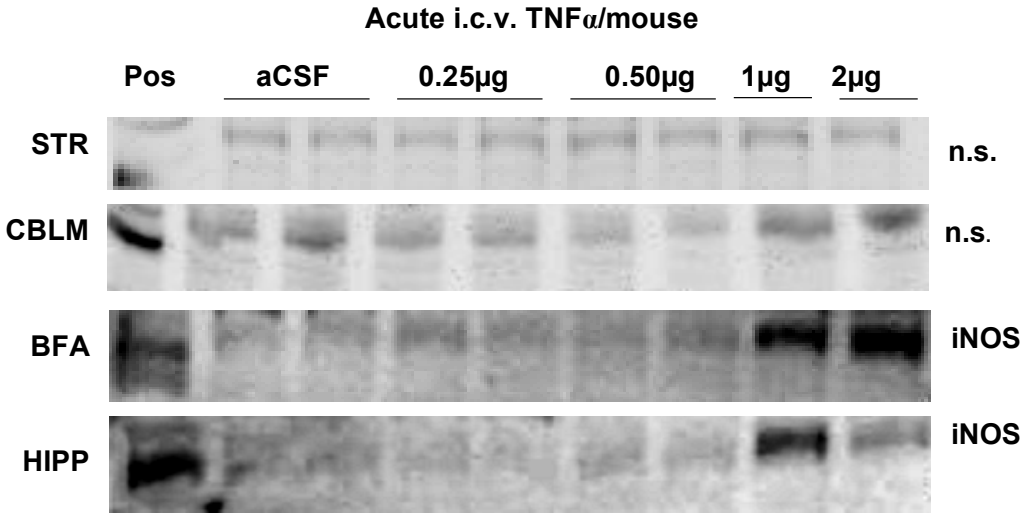


Figure 5.4: Western blot detecting iNOS expression in total cell extracts from the striatum (STR), cerebellum (CBLM), basal forebrain (BFA) and hippocampus (HIPP) of mice 24 hr after receiving a stereotaxic injection in the fourth cerebral ventricle of artificial cerebrospinal fluid (aCSF) or TNFα at the concentrations shown. Pos: iNOS positive control (macrophage total protein extracts).

Discussion

Several studies have indicated that NGF can induce NF-κB through the low affinity p75NTR receptor (Hamanoue et al. 1999a;Burke and Bothwell 2003). Analysis of the transcriptional activity promoted by NF-kB, AP-1 and CRE revealed that, of the three, only NF-kB is likely to mediate synergistic iNOS induction by TNFα and NGF. Consistently, while NF-kB inhibition blocked both transcriptional activity and iNOS induction MAPK inhibition did not affect either (Figs. 5.2A&B). I also observed that NF-κB activity was not induced by the combined NGF/TNFα treatment in PC12 cells mutants lacking p75NTR expression (Figure 5.2D). Therefore, I conclude that NFκB activation is necessary to promote iNOS induction by the TNFα/NGF combined treatment. I also conclude that p75NTR may play a crucial role in such NFκB activation.

Our *in vivo* studies demonstrate that even high levels of TNFα in the CSF do not produce global iNOS expression, suggesting that TNFα *per se* is not a potent inducer of iNOS within the CNS. Rather, our results show that increased iNOS expression in response to i.c.v. injection of TNFα in mice can only be observed in brain regions that contain NGF-responsive cholinergic neurons with presumable intact NGF signaling, like the basal forebrain, or areas that receive extensive innervation from NGF-responsive cholinergic neurons, such as the hippocampus. This suggests the attractive possibility that

a convergence between NGF-promoted trophic signaling and TNFα could selectively target NGF-responsive cholinergic neurons under conditions of chronic inflammation.

Indeed our results *in vitro* indicate that there exists a specific pathway that can result in cytotoxic levels of iNOS-derived NO. PC12 cell death was exacerbated by excess L-arginine and prevented by L-NAME, a non-selective NOS inhibitor. Other researchers have also noted an increased sensitivity to TNFα by PC12 cells differentiated with NGF(Mielke and Herdegen 2002). Our results, however, seem to exclude that differentiation of PC12 cells may have played a role, at least in the experimental paradigm used here. First, in our experimental conditions, iNOS expression occurs as early as three hours after exposure to the combined NGF/TNFα treatment (Macdonald and Taglialatela 2000), earlier than any morphological differentiation induced by NGF. Second, blockade of NGF-induced differentiation by a MAPK inhibitor (Figure 5.2C) had no effect on the NGF/TNFα–promoted iNOS expression (Figure 5.2A). Thus, our results suggest that NGF-responsive cells bearing both the high and low affinity NGF receptors may be most vulnerable to otherwise non-toxic levels of TNFα by virtue of a synergistic induction of iNOS.

Taken together, the results that I presented in this Chapter indicate that iNOS induction resulting from TNFα/NGF combined treatment requires a specific signaling pathway involving NFkB and can result in cell toxicity. These results also demonstrate that TNFα injected in vivo in the brain can result in iNOS expression selectively in those

brain areas where the cells bodies or terminals of NGF-responsive neurons are located, the basal forebrain and hippocampus, thus corroborating my mechanistic in vitro studies.

CONCLUSIONS

This project focused on investigating the effects of a prototypical inflammatory molecule, TNFα, in a model for NGF-responsive neurons, PC12 cells. It has been well established that conditions of chronic inflammation, including increased levels of TNFα, occur in a variety of neurological disorders in both the peripheral and central nervous system. These disorders manifest and are often classified according to which specific neuronal phenotypes are most at risk for impairment. For example, in AD it is the NGF-responsive cholinergic neurons in the basal forebrain that are amongst the earliest affected cells in the progression of the disease and many of the therapeutic mainstays of clinical treatment are meant to correct the resulting cholinergic deficiencies. However, the underlying cause for the selective impairment of these neurons is not known. Given the importance of understanding the etiology of a disease for designing and implementing curative therapies it is critical that the mechanism for this selective neuronal impairment be found.

The basal forebrain cholinergic neurons provide major subcortical cholinergic input to such areas as the cortex and hippocampus and are selectively impaired in AD. Many of the cognitive deficits in AD can be attributed to decreased cholinergic function and indeed AChE inhibitors can temporarily ameliorate these deficits. These neurons require

continued trophic support for survival and maintenance of phenotype in the form of target-derived NGF, which they receive from areas of innervation such as the cortex and hippocampus and retrogradely transport back to their soma in the basal forebrain. There have been no decreases in NGF levels found in early AD nor has NGF administration proven to be efficacious in rescuing these neurons in AD suggesting that decreased trophic signaling is not the immediate cause for loss of cholinergic phenotype. In these neurons the trophic actions of NGF are mediated through two receptors, the TrkA and $p75^{NTR}$, which are abundantly present on the cholinergic terminals these cells project. An alternate hypothesis that still implicates NGF signaling is that by virtue of expressing both types of NGF receptors and continually responding to NGF, these neurons are experiencing a unique stress under conditions of chronic inflammations such as increased levels of TNFα. Increased CSF levels of the pro-inflammatory cytokine,TNFα, precede the clinical diagnosis of AD suggesting a causal role for the molecule in the development of the behavioral deficits that are attributed to BFCN impairment. There are also other reasons to suspect TNFα impingement on NGF-responsive cells such as structural similarities between the TNFR1 receptor and the $p75^{NTR}$ receptor as well as shared signaling paradigms in this superfamily of receptors.

Although the TNFR1 receptor is most closely associated with the so-called death domain signaling pathways, there does not seem to be a frank loss of neurons in the basal forebrain, but rather a loss of phenotype reminiscent of decreased trophic support. So an attractive mechanism for this loss of phenotype would include a signaling pathway resulting in impairment in neurons that bear both NGF receptors as a result of increased

TNFα levels. This mechanism would have the advantage of implicating a molecule thought to be increased early in the manifestation of the disease and would also explain why BFCN lose their phenotypes in the presence of trophic support while neighboring phenotypes are spared. Clues as to what sort of signaling mechanism may be involved can be found in careful postmortem examination of brain samples from AD patients. Studies have shown that alterations in transcription factors such as NF-κB have been found in neurons of both aged and AD brain. These alterations in NF-κB levels and/or activities could negatively impact physiological functions such as learning and memory as well as interfere with normal maintenance of cellular phenotype. Additionally, there also have been numerous studies implicating an excess of free radicals and ROS in aged and AD brain. Causes for increased protein, DNA and lipid modifications arising from ROS and RNS could include both increased production of free radicals as well as a decreased capacity for scavenging these damaging molecules. Evidence in AD for an increased production of the free radical NO, comes from postmortem brain demonstrations of nitrosylated protein modifications as well as an increased expression the iNOS enzyme, which promotes excess NO production. The present study attempts to link TNFα signaling in the context of a NGF-responding cell to alterations in NF-κB signaling as well as altered free radical production of through the induction of iNOS protein.

Given that AD is known to be a multifactorial process with both genetic and environmental influences it seems unlikely that a single mechanism or hypothesis will adequately explain all of the diverse features of the syndrome that we label AD. The

difficulties are compounded by the temporal reality, which is that this disease progresses over the course of years and may present differently in poorly defined "stages". However, using a reductionist approach, the present study focused on a specific neuronal population and factors known to be involved early in the progression of the disease. My hypothesis is that TNFα may synergize with NGF-promoted neurotrophic signaling and present signaling pathways/outcomes that may be absent in other neuronal populations also responding to TNFα.

The bulk of these investigations used PC12 cells that bear both the TrkA and $p75^{NTR}$ NGF receptors. This neuroepithelial-derived cell line is a well-established model for NGF-responsive neurons. Building on work performed previously by Dr Nancy MacDonald, I confirmed that NGF/TNFα-promoted signaling did not require any additional ligands present in cell culture medium. Although the NGF/TNFα-promoted iNOS response was saturable with regards to NGF concentrations, it was dose-dependent with respect to TNFα, suggesting that trophic concentrations of NGF were sufficient to make the cell more responsive to increasing levels of the cytokine. Additionally, I found that continued signaling from both TNFα and NGF were required to sustain NGF/TNFα-promoted iNOS expression with iNOS protein expression levels returning to baseline within 24 hours of withdrawal of either ligand. This important observation meant that the response is reversible and not a permanent phenotypic change or cell selection artifact.

Turning from ligands to receptor requirements was an important step in determining which populations of cells would most likely be at risk based on receptor

expression. I sought to confirm previous work demonstrating a TrkA requirement, based on inhibition experiments, for this signaling phenomenon by using commercially available selective peptidomimetic agonists. The compound tested was determined to be unsuitable for our purposes for reasons of partial agonism. We then turned to mutant cell lines that lacked either NGF receptor. Again we determined that this approach was not ideal as the TrkA-deficient mutant PC12 cells lacked TNFR1 expression. However, the $p75^{NTR}$-deficicient mutant PC12 cells lines surprisingly failed to respond to the combined NGF/TNFα treatment despite the fact that they expressed the TrkA receptor, we knew to be required, as well as marked levels of TNFR1. Our next strategy turned out be most fruitful and explained the seeming contrast. In an admittedly artificial but arguably elegant system, we made use of chimeric protein constructs bearing the ligand binding domain from the human TNFR1 and the intracellular signal transduction domain from one or the other NGF receptor. This tool meant that the cells transfected with the appropriate chimera would respond solely to TNFα in terms of iNOS expression. Only transfection with both chimeric constructs, giving the cell TNFα-promoted NGF signaling domains from both the high affinity TrkA and low affinity $p75^{NTR}$ receptors, produced iNOS expression in TNFα alone treated cells. This was direct evidence that both NGF receptors were required to mediate this synergistic signal with TNFα and explained the results of both the TrkA inhibition and $p75^{NTR}$-deficient mutant cell experiments. Moreover, it suggested that only a very few population of neurons would be selectively at risk from exposure to TNFα promoted signaling. As noted previously,

the BFCN are among these TrkA/ $p75^{NTR}$-expressing populations and would be at risk in conditions of inflammation. Other CNS populations bearing both NGF receptors and affected AD are the olfactory receptor neurons, which demonstrate iNOS expression and early AD patients often present with decreased olfaction.

After investigating the receptor requirements, we turned to possible signaling mechanisms involved in mediating the NGF/TNFα-promoted iNOS expression. First, we ruled out the possibility that NGF-induced differentiation was involved through the use of MAPK inhibition. Second, using transcription factor-specific reporter gene assays we implicated NF-κB activation downstream of both NGF and TNFα independent of NO production. We ruled out synergistic activation of two other transcription factors, CREB and AP-1, both have which have are not heavily implicated in chronic neurodegenerative events such as AD. Finally, using NF-κB inhibitors we observed that NF-κB is necessary for iNOS expression and fittingly, the $p75^{NTR}$-deficient mutants failed to demonstrate the NGF/TNFα-promoted activation of NF-κB as seen in the wild type cells.

As noted previously, a mechanism for the loss of cholinergic phenotype in AD would be preferable to direct cytotoxicity as frank loss of neurons is not demonstrable early in the course of the pathology. Although excess NO production may be cytotoxic, we only observed marked cell loss in culture when the cells were treated with high levels of TNFα, which by itself may promote apoptotic pathways, or with arginine supplementation, a substrate for NO production. We observed a NO-independent inhibition of nNOS expression after prolonged exposure to NGF/TNFα in culture. This

effect is also reversible suggesting a loss of phenotype rather than selection for low-expressing nNOS populations. That this effect is NO-independent suggests that it is not simple product inhibition enzymatics but rather a change in phenotype as seen by the loss of nNOS expression, an enzyme known to promote cholinergic phenotypes *in vivo.* Additionally, that this effect is not due NO production suggests that although free radical scavenging may not be completely efficacious in preventing this loss of cholinergic phenotype, as has been evidenced by the lackluster performance of antioxidants in the treatment and prevention of the disease as compared to anti-inflammatory therapies which have been show to slow the progression and delay onset, which would presumably represent an intervention at the ligand level rather than signal product.

In conclusion, I have presented results from an investigation of the effects of proinflammatory conditions represented by the cytokine, TNFα, may selectively affect cells bearing both the high affinity TrkA and low affinity $p75^{NTR}$ receptor and responding to physiologic NGF. Of course, these results do not tell the entire story and there remain many important determinations to be made. My preliminary experiments in primary cultures and *in vivo* treatments suggested that these observations are not limited to a clonal cell line. However, the PC12 cells remain a useful tool to see if NGF can act similarly with other proinflammatory molecules such as interleukins or interferon-γ. Other cell lines could be used to investigate whether TNFα can act in concert with other neurotrophins, such as BDNF or NT-3, in cell bearing $p75^{NTR}$ and TrkB or TrkC respectively. But perhaps more dramatic evidence could be obtained by observing the BFCN in transgenic animal models. The model used in the present investigation

involved direct TNFα administration in mice and assessing iNOS expression in NGF-responsive areas. Although exogenous administration of TNFα was the most direct model, it perhaps not the most appropriate as it produced systemic effects such as hypothermia and was accompanied with significant mortality. Of perhaps more relevance would be mouse overexpressing transgenic models, especially those with temporal control of TNFα expression. However, recent reports of selective increases in cytokine levels in amyloid expressing transgenic mice represent an exciting opportunity to assess the responses of NGF-responsive cholinergic populations before and during chronic increases in central cytokine levels. It would be critical to include TNFα blocking antibodies as a control in implicating the cytokine in promoting BFCN impairment. Current studies in these animal models are centered on amyloid plaque clearance and scavenging in an attempt to abrogate behavioral deficits. However, our studies suggest that cholinergic impairments could be occurring before the observance of amyloid plaques, as TNFα levels have been shown to increased along with soluble beta-amyloid levels. The strength of my current work is in trying to provide a small piece of an overall puzzle of a deteriorating brain. This piece has the features of known facets in AD such as the NGF-responsive cholinergic involvement, inflammatory contributions and free radical production. In addition to the focus on single components of the disease process such as amyloid metabolism or tau processing as causative agents, perhaps investigations into what makes different populations of cells respond as they do to the same changes in the brains environment. Does the phenotype of BFCN, such as the need

for sustained trophic support and continued expression of $p75^{NTR}$ and TrkA less able to cope with the changes in AD brain? Does this inability to cope result in a change into a more adaptable but less functional phenotype? Unfortunately, with a disease that is presents and progresses over years in an individual patient it may be very difficult to distinguish the "causes" and the compensatory "responses", but perhaps if one looks very closely at the BFCN as a sort of "sentinel" cell population some insight as to original changes in the brain environment may be gained.

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Vita

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