Published in final edited form as:

Brain Res. 2008 January 10; 1188: 189–197.

Interleukin -1 β interferes with signal transduction induced by neurotrophin-3 in cortical neurons

Rungtip Soiampornkul^a, Liqi Tong^b, Wipawan Thangnipon^a, Robert Balazs^b, and Carl W. Cotman^b

^aNeuro-Behavioral Biology Center, Institute of Science and Technology for Development, Mahidol University, Salaya Campus Nakorn Pathom 73170, Thailand ^bUniversity of California Irvine, Institute for Brain Aging and Dementia, Irvine, California, CA 92697-4540

Abstract

It was previously observed that IL-1 β interferes with BDNF-induced TrkB-mediated signal transduction and protection of cortical neurons from apoptosis evoked by deprivation from trophic support (Tong et al 2007). Here we investigated whether the effect of the cytokine on neurotrophin signaling is more general. The influence of IL-1 β on NT-3 signaling was therefore studied under conditions when NT-3 primarily activated the TrkC receptor. The cytokine reduced NT-3-induced activation of MAPK/ERK and Akt, but did not interfere with Trk receptor autophosphorylation. IL-1 β reduced tyrosine phosphorylation of the docking proteins, IRS-1 and Shc, which convey receptor activation to the downstream protein kinase cascades. These are the steps that are also inhibited by IL-1 β in BDNF-induced signal transduction. The functional consequences of the effect of IL-1 β on NT-3 signaling were severe, as NT-3 protection of the trophic support-deprived cortical neurons was abrogated. In view of the role in the maintenance and plasticity of neurons of ERK, Akt and CREB, which are activated by neurotrophins, elevated IL-1 β levels in the brain in Alzheimer's disease and other neurodegenerative diseases might contribute to the decline in cognitive functions before the pathological signs of the disease develop.

Keywords

IL-1 β ; NT-3; signal transduction; cortical neurons

1. Introduction

Neurotrophins are a family of structurally related proteins that regulate the survival, differentiation and maintenance of different populations of peripheral and central neurons and they are also essential for modulating neuronal plasticity (Bibel & Barde 2000, Huang & Reichardt 2001, Leingartner et al 1994, Segal et al 1992). BDNF and NT-3 support the survival and differentiation of select populations of neurons in a partially redundant manner (Minichiello & Klein 1996). The effects are primarily mediated through high affinity Trk receptors: BDNF signals by activating TrkB, whereas NT-3 is more promiscuous (Huang &

*Correspondence: Liqi Tong, Ph.D., Institute for Brain Aging and Dementia, University of California Irvine, Irvine, CA 92697-4540, Tel: (949)-824-6071, Fax: (949)-824-2071, Email: tongl@uci.edu.

Rungtip Soiampornkul and Liqi Tong contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Reichardt 2003), but activates preferentially TrkC (Lamballe et al 1991). Even if neurons expressing both Trk receptors, BDNF and NT-3 may have specific biological effects (Ip et al 1993, Leingartner et al 1994, McAllister et al 1999, Segal et al 1992) and there is convincing evidence that in vivo, at least in the inner ear, NT-3 signals exclusively through the TrkC receptor (Stenqvist et al 2005), indicating that TrkB and TrkC are not functionally redundant even when coexpressed in individual neurons.

We have been studying the influence of factors that may contribute to neuronal dysfunction in early stages of Alzheimer's disease (AD), when pathological changes are not yet manifest (Tong et al 2004, Tong et al 2001). Recently we examined the effect of the proinflammatory cytokine IL-1 β on BDNF signaling and function (Tong et al 2007). IL-1 β levels are elevated in the brain with aging and even more so in neurodegenerative disorders, such as AD. We observed that IL-1 β , under conditions when it does not compromise neuronal viability, interferes with BDNF signaling by reducing activation of the docking proteins that convey the signal from the activated Trk receptors to the Ras/MAPK and PI3-K/Akt pathways, and thus elicits a type of neurotrophin resistant state (Tong et al 2007). The functional consequences were severe, as neuronal vulnerability was increased. The neuroprotective function of IGF-1 that is another important neurotrophic factor is similarly compromised in cerebellar granule cells by another proinflammatory cytokine TNF α that elicits a reduction of the activation of the docking protein IRS-2 (Venters et al 1999).

In order to test how general is the proinflammatory cytokine-induced neurotrophic factor resistance, we have now studied the effect of IL-1 β on NT-3 signaling. Our observations showed that in cerebral cortical neurons, NT3-mediated activation of the Ras/MAPK and PI3-K/Akt pathways is inhibited by IL-1 β exposure at the same steps at which BDNF signaling is affected, at the docking proteins IRS-1 and Shc. The cytokine interference with NT-3 signaling compromised NT-3 function by abrogating the neuroprotective ability of the neurotrophin. Thus proinflammatory cytokines induce a situation that is similar to insulin resistance in the periphery, whereby the functions of critical neurotrophic factors are compromised in nerve cells.

2. Results

2.1. NT-3 mediates signal transduction primarily through TrkC receptor activation

We observed previously that in our cerebral cortical neuron-enriched cultures, exposure to IL-1 β alone for 2h has no effect on the level of phosphorylated MAPK/ERK or Akt, but the cytokine interferes with BDNF signaling and function (Tong et al 2007). Here we examined the influence of IL-1 β on functions of another neurotrophin NT-3 that plays critical roles in the development and maintenance of certain neuronal populations (Bibel & Barde 2000, Huang & Reichardt 2003). These effects are primarily mediated by high affinity TrkC receptors. Only some neurons in the adult cerebral cortex express high affinity neurotrophin receptors (Miller & Pitts 2000). Therefore, we examined the expression of TrkC receptors in the neurons in our culture and compared that with the expression of TrkB receptors (Fig. 1). Immunocytochemistry showed that virtually all neurons (cells labeled with MAP-2 antibodies) expressed both TrkB (Fig. 1A/a, c and e) and TrkC receptors (Fig. 1A/b, d and f), indicating the coexistence of the two receptors in individual neurons. Control studies showed that the respective antibodies specifically recognized TrkC or TrkB receptors (Fig. 1B).

NT-3 can activate not only TrkC, but also TrkA and TrkB receptors (Huang & Reichardt 2003). To evaluate whether or not under our experimental conditions NT3 specifically stimulated TrkC receptors we examined the level of autophosphorylated TrkB after treating cells with BDNF or NT-3 (each 10 ng/ml, 15 min). In comparison with BDNF, the stimulation by NT-3 of TrkB phosphorylation was low ($25 \pm 8\%$, $n = 3$)(Fig. 1C). On the other hand, the

NT-3-induced activation of the downstream effector of the PI3-K pathway was only slightly less ($86 \pm 6\%$, $n = 3$) than that caused by BDNF (Fig. 1D), indicating the efficacy of NT-3 signaling through the TrkC receptor. To evaluate that NT-3 signaling is mediated predominantly via the TrkC receptor, we also examined the neurotrophin-induced phosphorylation of synapsin I at the critical Ser 62 and 67 residues, which has been observed previously to be affected by BDNF and not NT-3 in synaptosomal preparations (Jovanovic et al 2000). Compared with BDNF, NT-3 was less effective in inducing the phosphorylation of synapsin I in synaptic function competent hippocampal slice cultures, when synaptic organization is already well established: the estimates were $35 \pm 7.5\%$ ($n = 3$) of the values obtained with BDNF (not shown). It seems therefore that under our experimental conditions the effects of NT-3 are mediated primarily, but not exclusively through TrkC receptors.

Because compared with BDNF information on signal transduction induced by NT-3 in cortical neurons is limited, we also examined some basic properties of the NT-3-elicited cellular responses in our cultures. NT-3 triggered a time- and concentration-dependent increase in the activated level of both isoforms of MAPK/ERK, p42 and p44. Nearly maximal effect was obtained after exposure to 10 ng/ml NT-3 for 15 min, and unless otherwise mentioned, these were the conditions employed in this study.

2.2. IL-1 β interferes with NT-3-induced signal transduction

Cognitive impairment is a major and early characteristic of AD (Bookheimer et al 2000, Snowden et al 2000, Snowden et al 1996) and IL-1 β levels are known to be elevated in the brain in AD (Mrak & Griffin 2001). We examined, therefore, the influence of this cytokine on NT-3-induced signal transduction processes that are known to be relevant for synaptic plasticity and cognitive functions. The MAPK/ERK pathway is one of the critical signaling systems subserving synaptic plasticity (Sweatt 2001), and IL-1 β treatment resulted in a reduction of NT-3-induced activation of ERK (Fig. 2A). Immunocytochemical detection showed that NT-3 activated ERK in virtually all cells, and IL-1 β treatment reduced the intensity of labeling per cell, rather than causing a reduction in the expression of activated ERK in a subpopulation of cells (Fig. 2B).

The PI3-K kinase cascade mediates the survival promoting effect of BDNF under certain conditions (Brunet et al 2001, Hetman et al 1999, Tong et al 2004). PI3-K generates 3'-phosphorylated phosphoinositides that act through multiple mechanisms to regulate the downstream effector of PI3-K, the protein kinase Akt/PKB (Datta et al 1999). NT-3, like BDNF, induced the activation of Akt, and IL-1 β interfered with the neurotrophin-elicited elevation of the level of phosphorylated Akt [$73 \pm 7.2\%$ ($n = 3$) of the level in NT-3-stimulated cells] (Fig. 2C).

One of the targets of activated MAPK/ERK is via Rsk the transcription factor CREB that can also be activated by Akt. Activated CREB plays a major role in processes of neuronal plasticity (Abel & Kandel 1998, Silva et al 1998). The transcription promoting activity of CREB involves phosphorylation of the Ser133 residue (Montminy 1997). NT-3, like BDNF (Tong et al 2004), induced CREB phosphorylation at Ser133 (Fig. 2D). IL-1 β treatment compromised the activation of this transcription factor [$65 \pm 5.5\%$ ($n = 3$) of the level in NT-3-stimulated cells]. The effect of IL-1 β on NT-3-induced signal transduction was specific, as the IL-1 β receptor antagonist IL-1ra blocked the cytokine-induced suppression of the activation of MAPK/ERK and CREB (Fig. 3).

2.3. IL-1 β does not affect the activation of Trk receptors, but interferes with NT-3 signaling at the level of the docking proteins IRS-1 and Shc

Inhibition of the activation of Trk receptors would provide a simple mechanism for the interference by the cytokine with neurotrophin signaling. However, tyrosine phosphorylation of the Trk receptor was not suppressed by IL-1 β (Fig. 4A and B). In terms of the tyrosine phosphorylated Trk receptor level in the NT-3-exposed cultures, the estimate in the IL-1 β -pretreated cells was $101 \pm 3.5\%$ ($n = 3$).

We have observed previously that the IL-1 β and the amyloid peptide A β_{1-42} reduce BDNF signal transduction in cortical neurons through the Ras/MAPK and PI3-K/Akt pathways by interfering with the activation of the docking proteins Shc and IRS-1 respectively, which link the activated TrkB receptor with the respective downstream signaling cascades (Tong et al 2007, Tong et al 2004). Fig. 4C-F show that IL-1 β also interferes with NT-3 signaling at the docking steps by reducing the activated Trk receptor-induced Tyr phosphorylation of IRS-1 and Shc.

2.4. Effect of NT-3 and IL-1 β on apoptosis induced by deprivation of cells from trophic support

Neuron survival is compromised by deprivation of the trophic support provided by either serum or the B-27 culture additives, but cells can be rescued by BDNF (Hetman et al 1999, Tong et al 2004). NT-3 reproduced the survival promoting effect of BDNF (Fig. 5). The activation of PI3-K/Akt has been shown to be instrumental for the BDNF-evoked protection of cortical neurons from apoptosis elicited by deprivation from trophic support (Hetman et al 1999). As IL-1 β suppressed NT-3-induced activation of Akt (see Fig. 2), we examined the effect of the cytokine on the protection of the B-27-deprived neurons by the neurotrophic factors. IL-1 β alone had no significant effect on the survival of the deprived cells, but the cytokine compromised the NT-3-evoked rescue of neurons (Fig. 5).

3. Discussion

The main finding of this study is that IL-1 β at concentrations at which neuronal viability is not compromised, interferes with NT-3-induced signaling through the Ras/MAPK/ERK and PI3-K/Akt pathways and reduces the activation of the critical transcription factor CREB, which play crucial roles in synaptic plasticity (e.g. Abel & Kandel 1998, Silva et al 1998, Sweatt 2001). Therefore, elevated levels of the cytokine, which occurs even during normal aging e.g. (Gooney et al 2004) and is very much augmented in neurodegenerative disorders, such as AD (Mrak & Griffin 2001), may significantly contribute to cognitive impairment that is characteristic already at the early stages of this disease (Bookheimer et al 2000, Naslund 2000, Snowden et al 2000). The functional consequences of the suppression of neurotrophin signaling were robust. IL-1 β treatment compromised the protective effect of NT-3 in neurons that were deprived from trophic support. This shows that although IL-1 β alone did not reduce cell viability, it rendered neurons vulnerable. Thus proinflammatory cytokines when chronically present in elevated levels may affect neuronal functions adversely by compromising the adaptive potential of the cells and making them vulnerable to insults of various kinds.

Compared with BDNF, information on NT-3-induced signal transduction in central neurons is limited (Liot et al 2004, Marsh & Palfrey 1996). In our cultures, virtually all the live neurons expressed both functional TrkC and TrkB receptors, as judged by the immunocytochemical detection of the receptors as well as the activated signaling intermediates (Fig. 1, Fig. 2B and Fig. 5A in ref 37 respectively). This is consistent with previous observations on neuronal cultures (Lindholm et al 1993). However in vivo even in layer V of the adult somatosensory

cortex, where Trk receptor expressing cells are most abundant, only about half of the cells express these receptors (Miller & Pitts 2000). Although TrkC is the preferred receptor of NT-3, this factor is also able to activate TrkA and TrkB receptors (Huang & Reichardt 2003), but the efficiency and potency of NT-3 for the activation of TrkB is much lower compared with BDNF (Escandon et al 1994, Lamballe et al 1991). Similarly, in our cultures, 10 ng/ml NT-3 activated TrkC receptors to a much greater degree than TrkB receptors (Fig. 1C and D). The different Trk receptors activate the same major signaling pathways (Huang & Reichardt 2003). Nevertheless, cellular responses are often specific (Segal 2003), even when individual neurons express both TrkB and TrkC receptors. It was observed after genetic manipulation of the Trk receptors and neurotrophins that in vivo in the inner ear NT-3 maintains survival of cells and target innervations only through interaction with TrkC (Stenqvist et al 2005). Further, while both BDNF and NT-3 can modulate dendritic arborization, they antagonize each other's action in the developing cerebral cortex (McAllister et al 1999). Moreover, BDNF, but not NT-3 is survival factor for immature cerebellar granule cells, whereas the mature cells require NT-3 (Lindholm et al 1993, Segal et al 1992, Skaper et al 1998). In these cells, BDNF, but not NT-3 enhances neurite outgrowth, and NT-3 promotes neurite fasciculation (Segal et al 1995). BDNF, but not NT-3, upregulates the expression of NT-3 and p75 neurotrophin receptor (Leingartner et al 1994) and protects granule cells from apoptosis induced by cytosine arabinoside (Ara-C) and other insults (Leeds et al 2005). However, in our cerebral cortical cultures, both NT-3 and BDNF were neuroprotective for the trophic support-deprived cells (Tong et al 2004).

A major site of cytokine interference with neurotrophin signaling was localized to the docking steps that convey Trk receptor activation to the downstream signaling cascades. We reported previously that IL-1 β interferes with BDNF/TrkB signaling and the site of interference is also at the docking proteins (Tong et al 2007). The wide-ranging nature of regulation of signal transduction at the level of the scaffolding proteins is indicated by the observation that interference at these sites is not restricted to IL-1 β inhibition of neurotrophin signaling via IRS-1 in cerebral cortical neurons. Venters et al. (1999) have observed that the signaling of another important neurotrophic factor IGF-1 is inhibited at IRS-2 by another proinflammatory cytokine TNF α in another type of neuron the cerebellar granule cells. Further, factors other than proinflammatory cytokines interfere with neurotrophic factor signaling at the docking steps. We reported earlier that A β ₄₂ reduces BDNF signaling by interfering with the activation of IRS-1 and Shc (Tong et al 2004) and the reduction of IGF-1 signal transduction by excitotoxic insults involves the inhibitory Ser phosphorylation of IRS-1 (Garcia-Galloway et al 2003). It seems therefore that the docking step in the signal transduction pathways serves as a major regulatory site both for IGF-1 and for neurotrophins, where insults of various kinds, including proinflammatory cytokines (Venters et al 1999 and present work) and amyloid peptides (Tong et al 2004), can lead to inhibition of signal transduction. There are several potential intracellular signaling mechanisms that may mediate the inhibition by IL-1 β of TrkC receptor activation of the docking proteins. One attractive possibility is that IL-1 β -induced suppression of NT-3 signaling is mediated by ceramide. Ceramide is one of the factors which is implicated in the development of insulin resistance, as it can lead to interference with insulin signaling by affecting the activation of the docking proteins (Strle et al, Zick, 2001). IL-1 β -induced ceramide signaling has also been demonstrated in neurons (Davis et al, 2006). We observed the involvement of ceramide in IL-1 β -mediated suppression of BDNF signaling (Tong et al, 2007). The effect of the cytokine was blocked by inhibition of ceramide generation by both the de novo pathway and neutral sphingomyelinase, but not acidic sphingomyelinase.

The interference with neurotrophin/IGF-1 signaling has a parallel in the inhibition of insulin signaling. It is well known that proinflammatory cytokines, such as IL-1 β can compromise insulin signaling in a variety of peripheral tissues (Strle et al 2004, White 2003, Zick 2001), a condition referred to as "insulin resistance". The mechanism involves a suppression of the

activation of the docking proteins through inhibitory phosphorylation of certain Ser/Thr residues, which is also the likely mechanism underlying the cytokine inhibition of signaling by neurotrophic factors in neurons (Zick 2001). We suggest that like insulin resistance in the periphery, there is a neurotrophic factor resistance in the CNS, which may be involved in cognitive impairment that precede the florid features of diseases such as AD through interference with signaling critical for synaptic plasticity.

4. Experimental procedures

4.1. Cell culture

Primary cultures of dissociated cerebral cortical neurons were prepared from the brains of embryonic day 18 (E 18) rats as described previously (Tong et al 2007). Cells plated at 2.5×10^5 cells/cm² were cultured in poly-L-lysine coated six well plates (for western blot analysis) or on 24 well plates (for immunocytochemistry and assays for cell viability) and maintained in serum-free optimal Dulbecco's modified Eagles medium (DMEM) supplemented with B-27 components (Invitrogen, Carlsbad, CA). Cultures were maintained for 5–7 days before treatments.

4.2. Experimental treatment

Cells were treated with IL- β for 2 h before the addition of NT-3 (Peprotech, Rocky Hill, NJ). Human recombinant IL-1 β (Prepro Tech, Rocky Hill, NJ) was dissolved in DMEM and used after one freeze-thaw cycle. The IL-1 receptor antagonist IL-1ra (R&D Systems, Minneapolis, MN) was used in 100-fold excess, and when applied, cells were preincubated for 30 min before exposure to IL- β . After the indicated incubation time (usually 15 min) in the presence or absence of the neurotrophic factors, cells were processed for either biochemical (electrophoresis or immunoprecipitation) or immunocytochemical analysis.

4.3. Western blot analysis

Cells were processed as described (Tong et al 2007). Cells were lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na₃VO₄, 20 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 0.7 μ g/ml pepstatin, 1 μ g/ml leupeptin. An aliquot of the lysate (20 μ g of protein) diluted in sample buffer [0.125 M Tris-HCl, pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS, and 10% (v/v) β -mercaptoethanol] was boiled for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide gel). Proteins were then electrotransferred to PVDF membranes. Membranes were incubated at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween and 5% nonfat milk for 60 min to block the nonspecific binding. Following incubation with primary antibody for 2 h at room temperature, the membranes were treated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG) (Vector Laboratories, Burlingame, CA) at 1:500 dilution in the blocking solution for 60 min. Blots were then washed four-times with PBS containing 0.1% Tween. Immunolabeling was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia, Piscataway, NJ) according to the recommended conditions. Immunoreactivity was quantified using densitometric analysis.

The following antibodies were used for the biochemical studies: from Upstate (Charlottesville, VA), phosphorylated CREB (P-CREB) (detects CREB phosphorylated at Ser133, 1:2,000), total CREB (T-CREB, 1:2,000), Shc (the antibody recognizes all isoforms; 1:1000), total Trk (T-Trk, 1:1000); from Cell Signaling (Beverly, MA), phosphorylated MAPK/ERK (P-MAPK) (detects p44/42 MAPK phosphorylated at Thr202 and Tyr204, 1:2000), total-MAPK (T-MAPK, 1:2000), phosphorylated Akt (P-Akt) (detects Akt phosphorylated at Ser473, 1:1000), total Akt (T-Akt, 1:1000), phosphorylated Trk (P-Trk) (detects Trk phosphorylated at Tyr490, 1:500). Quantification of the data obtained from Western blots derived from cultures under the

various experimental conditions was done relative to the estimates in the BDNF-treated cultures. Data were analyzed using Student's *t*-test and when appropriate one-way ANOVA using Fisher-PSD for post hoc comparisons.

4.4. Immunoprecipitation

Cells were lysed in 500 μ l of immunoprecipitation buffer (1% Triton- X-100, 150mM NaCl, 50mM Tris pH 8.0, 0.2 mM sodium ortho-vanadate, 0.2mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin, 1 mg/ml leupeptin, and 1 mg/ml antipain). Protein concentration of the centrifugation-clarified lysates was determined using the Micro-BCA protein assay (Pierce, Rockford, IL). Proteins were immunoprecipitated with anti-Shc or IRS-1 antibodies at 4°C overnight and treated with G-Sepharose at 4 °C for 2 h. The immune complexes were pelleted and washed three times with 1 ml immunoprecipitation buffer. Finally the pellets were suspended in 60 μ l SDS-sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromphenol blue) and proteins were separated on a 10% SDS-PAGE gel and blotted with anti-phosphotyrosine 4G10 (Upstate, Charlottesville, VA) and anti-Shc and anti-IRS-1 antibodies.

4.5. Immunocytochemistry

For the detection of TrkB and TrkC receptors, cultures at 5 DIV were fixed with 4% paraformaldehyde at room temperature for 20 min and then rinsed twice with PBS. Cells were then permeabilized by incubation in PBS containing 0.3% Triton for 20 min, followed by exposure to a blocking solution (5% goat serum and 0.3% Triton in PBS) for 30 min. Antibodies recognizing Trk receptors were from Santa Cruz Biotech. (Santa Cruz, CA). Antibodies to TrkB (SC-12) or TrkC (SC-117) were diluted (1:50) in the blocking solution. Cultures were incubated without shaking with the diluted antibodies at 4°C overnight and then washed three times for 10 min before incubation with biotinylated anti-rabbit antibodies for 1 h. After three washes, cultures were incubated in the presence of Streptavidin conjugated Alexa Fluor 568 (1:200) (Molecular Probes) in PBS for 1 h. Cells were then washed three times in PBS before being subjected to further processing. Similar to the procedure described for Trk receptor detection, cultures were incubated with monoclonal MAP-2 antibodies (Sigma, St. Louis, MO) (1:500) overnight, followed by exposure to anti-mouse antibodies conjugated with Alexa Fluor 488. Finally, cells were washed three times in PBS and mounted in Fluoromount G.

The specificity of Trk receptor staining was examined by repeating the procedure using antibodies to TrkB and TrkC preabsorbed at room temperature for 2 h with the peptides used to raise the Trk antibodies, which are specific to the carboxyl terminal cytoplasmic domains of TrkB (SC-12P) and TrkC (SC-117P) (ratio of antibodies to peptides 1:5 by weight).

In some experiments, triple staining was also performed to detect the presence of Trk receptors on viable neurons. In these experiments, after staining for the Trk receptors and MAP-2, cultures were thoroughly washed and exposed to Hoechst 33342 (diluted in water 1:200) for 20 min followed by three rinses with PBS before mounting in Fluoromount-G.

For the detection of activated MAPK/ERK, cells were fixed in 4% paraformaldehyde on ice for 20 min, washed twice with phosphate-buffer saline (PBS), once with cold ethanol, followed by two washes with PBS. Cultures were then incubated in a blocking solution at room temperature for 1 h, rinsed with Tris-buffered saline (TBS, 0.05 M Tris in 0.9%NaCl, pH 7.4), and kept at 4°C overnight in TBS containing 5% bovine serum albumin (BSA) and anti-P-MAPK monoclonal antibody (1:1000). Cultures were then rinsed with buffer and incubated in biotinylated goat anti-mouse IgG. After a further wash, cultures were incubated in the presence of avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) for 90 min. They were then rinsed with 0.1 M Tris-HCl, pH 7.6 and incubated in Tris-HCl

containing 0.05% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide for 5–10 min. The reaction was stopped by washing the cultures with PBS.

4.6. Deprivation from trophic support

Deprivation from B-27, like that from serum (Hetman et al 1999), compromised neuronal survival (Tong et al 2004), thus permitting the testing of the effect of NT-3 and the influence of IL-1 β on the survival of the deprived cells. The B-27-containing medium was removed from the cultures at 5 DIV. Cells were washed twice with DMEM and then incubated in DMEM for 36 h in the absence or presence of NT-3 (10 ng/ml) $\pm$ IL-1 β (50 ng/ml). Control cells were treated the same way, but were incubated in B-27-containing DMEM. Neuronal survival was determined by trypan blue exclusion (Pike et al 1993) or using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Tong et al 2007).

Acknowledgements

We are indebted to Hilda Martínez-Coria for excellent technical assistance and Christina Tu for the immunocytochemical studies on the Trk receptors. This work has been supported by National Institute of Health Grant P01-AG-000538.

References

- Abel T, Kandel E. Positive and negative regulatory mechanisms that mediate long-term memory storage. *Brain Research Brain Research Reviews* 1998;26:360–78. [PubMed: 9651552]
- Bibel M, Barde YA. Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev* 2000;14:2919–37. [PubMed: 11114882]
- Bookheimer SY, Strojwas MH, Cohen MS, Saunders AM, Pericak-Vance MA, et al. Patterns of brain activation in people at risk for Alzheimer's disease. *N Engl J Med* 2000;343:450–6. [PubMed: 10944562]
- Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol* 2001;11:297–305. [PubMed: 11399427]
- Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* 1999;13:2905–27. [PubMed: 10579998]
- Davis CN, Tabarean I, Gaidarova S, Behrens MM, Bartfai T. IL-1 β induces a MyD88-dependent and ceramide-mediated activation of Src in anterior hypothalamic neurons. *J Neurochem* 2006;98:1379–1389. [PubMed: 16771830]
- Escandon E, Soppet D, Rosenthal A, Mendoza-Ramirez JL, Szonyi E, et al. Regulation of neurotrophin receptor expression during embryonic and postnatal development. *J Neurosci* 1994;14:2054–68. [PubMed: 8158256]
- Garcia-Galloway E, Arango C, Pons S, Torres-Aleman I. Glutamate excitotoxicity attenuates insulin-like growth factor-I pro-survival signaling. *Mol Cell Neurosci* 2003;24:1027–37. [PubMed: 14697666]
- Gooney M, Messaoudi E, Maher FO, Bramham CR, Lynch MA. BDNF-induced LTP in dentate gyrus is impaired with age: analysis of changes in cell signaling events. *Neurobiol Aging* 2004;25:1323–31. [PubMed: 15465630]
- Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J Biol Chem* 1999;274:22569–80. [PubMed: 10428835]
- Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* 2001;24:677–736. [PubMed: 11520916]
- Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem* 2003;72:609–42. [PubMed: 12676795]
- Ip NY, Li Y, Yancopoulos GD, Lindsay RM. Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. *J Neurosci* 1993;13:3394–405. [PubMed: 7688038]

- Jovanovic JN, Czernik AJ, Fienberg AA, Greengard P, Sihra TS. Synapsins as mediators of BDNF-enhanced neurotransmitter release. *Nat Neurosci* 2000;3:323–9. [PubMed: 10725920]
- Lamballe F, Klein R, Barbacid M. trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell* 1991;66:967–79. [PubMed: 1653651]
- Leeds P, Leng Y, Chalecka-Franaszek E, Chuang DM. Neurotrophins protect against cytosine arabinoside-induced apoptosis of immature rat cerebellar neurons. *Neurochem Int* 2005;46:61–72. [PubMed: 15567516]
- Leingartner A, Heisenberg CP, Kolbeck R, Thoenen H, Lindholm D. Brain-derived neurotrophic factor increases neurotrophin-3 expression in cerebellar granule neurons. *J Biol Chem* 1994;269:828–30. [PubMed: 8288635]
- Lindholm D, Dechant G, Heisenberg CP, Thoenen H. Brain-derived neurotrophic factor is a survival factor for cultured rat cerebellar granule neurons and protects them against glutamate-induced neurotoxicity. *Eur J Neurosci* 1993;5:1455–64. [PubMed: 7904521]
- Liot G, Gabriel C, Cacquevel M, Ali C, MacKenzie ET, et al. Neurotrophin-3-induced PI-3 kinase/Akt signaling rescues cortical neurons from apoptosis. *Exp Neurol* 2004;187:38–46. [PubMed: 15081586]
- Marsh HN, Palfrey HC. Neurotrophin-3 and brain-derived neurotrophic factor activate multiple signal transduction events but are not survival factors for hippocampal pyramidal neurons. *J Neurochem* 1996;67:952–63. [PubMed: 8752100]
- McAllister AK, Katz LC, Lo DC. Neurotrophins and synaptic plasticity. *Annu Rev Neurosci* 1999;22:295–318. [PubMed: 10202541]
- Miller MW, Pitts FA. Neurotrophin receptors in the somatosensory cortex of the mature rat: co-localization of p75, trk, isoforms and c-neu. *Brain Res* 2000;852:355–66. [PubMed: 10678763]
- Minichiello L, Klein R. TrkB and TrkC neurotrophin receptors cooperate in promoting survival of hippocampal and cerebellar granule neurons. *Genes Dev* 1996;10:2849–58. [PubMed: 8918886]
- Montminy M. Transcriptional regulation by cyclic AMP. *Annu Rev Biochem* 1997;66:807–22. [PubMed: 9242925]
- Mrak RE, Griffin WS. Interleukin-1, neuroinflammation, and Alzheimer's disease. *Neurobiol Aging* 2001;22:903–8. [PubMed: 11754997]
- Naslund, J. Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. *JAMA* 2000;283:1571–77. [PubMed: 10735393]
- Pike CJ, Burdick D, Walencewicz AJ, Glabe CG, Cotman CW. Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. *J Neurosci* 1993;13:1676–87. [PubMed: 8463843]
- Segal RA. Selectivity in neurotrophin signaling: theme and variations. *Annu Rev Neurosci* 2003;26:299–330. [PubMed: 12598680]
- Segal RA, Pomeroy SL, Stiles CD. Axonal growth and fasciculation linked to differential expression of BDNF and NT3 receptors in developing cerebellar granule cells. *J Neurosci* 1995;15:4970–81. [PubMed: 7623126]
- Segal RA, Takahashi H, McKay RD. Changes in neurotrophin responsiveness during the development of cerebellar granule neurons. *Neuron* 1992;9:1041–52. [PubMed: 1463606]
- Silva AJ, Kogan JH, Frankland PW, Kida S. CREB and memory. *Annu Rev Neurosci* 1998;21:127–48. [PubMed: 9530494]
- Skaper SD, Floreani M, Negro A, Facci L, Giusti P. Neurotrophins rescue cerebellar granule neurons from oxidative stress-mediated apoptotic death: selective involvement of phosphatidylinositol 3-kinase and the mitogen-activated protein kinase pathway. *J Neurochem* 1998;70:1859–68. [PubMed: 9572269]
- Snowdon DA, Greiner LH, Markesbery WR. Linguistic ability in early life and the neuropathology of Alzheimer's disease and cerebrovascular disease. Findings from the Nun Study. *Ann N Y Acad Sci* 2000;903:34–8. [PubMed: 10818486]
- Snowdon DA, Kemper SJ, Mortimer JA, Greiner LH, Wekstein DR, Markesbery WR. Linguistic ability in early life and cognitive function and Alzheimer's disease in late life. Findings from the Nun Study. *Jama* 1996;275:528–32. [PubMed: 8606473]

- Stenqvist A, Agerman K, Marmigere F, Minichiello L, Ernfors P. Genetic evidence for selective neurotrophin 3 signalling through TrkC but not TrkB in vivo. *EMBO Rep* 2005;6:973–8. [PubMed: 16142215]
- Strle K, Broussard SR, McCusker RH, Shen WH, Johnson RW, et al. Proinflammatory cytokine impairment of insulin-like growth factor I-induced protein synthesis in skeletal muscle myoblasts requires ceramide. *Endocrinology* 2004;145:4592–602. [PubMed: 15256490]
- Sweatt JD. The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J Neurochem* 2001;76:1–10. [PubMed: 11145972]
- Tong L, Balazs R, Soiampornkul R, Thangnipon W, Cotman CW. Interleukin-1beta impairs brain derived neurotrophic factor-induced signal transduction. *Neurobiol Aging*. 2007
- Tong L, Balazs R, Thornton PL, Cotman CW. Beta-amyloid peptide at sublethal concentrations downregulates brain-derived neurotrophic factor functions in cultured cortical neurons. *J Neurosci* 2004;24:6799–809. [PubMed: 15282285]
- Tong L, Thornton PL, Balazs R, Cotman CW. Beta -amyloid-(1–42) impairs activity-dependent cAMP-response element-binding protein signaling in neurons at concentrations in which cell survival is not compromised. *J Biol Chem* 2001;276:17301–6. [PubMed: 11278679]
- Venters HD, Tang Q, Liu Q, VanHoy RW, Dantzer R, Kelley KW. A new mechanism of neurodegeneration: a proinflammatory cytokine inhibits receptor signaling by a survival peptide. *Proc Natl Acad Sci U S A* 1999;96:9879–84. [PubMed: 10449788]
- White MF. Insulin signaling in health and disease. *Science* 2003;302:1710–1. [PubMed: 14657487]
- Zick Y. Insulin resistance: a phosphorylation-based uncoupling of insulin signaling. *Trends Cell Biol* 2001;11:437–41. [PubMed: 11684411]

Fig. 1. Virtually all cultured cortical neurons express both TrkB and TrkC receptors

A. Immunocytochemical detection of TrkB (a) and TrkC (b) receptors using specific antibodies (red). Neurons were detected using MAP-2 antibodies (c and d) (green). The merged images (orange) show that almost all neurons express TrkB (e) as well TrkC (f) receptors. In independent experiments (not shown), an additional staining with Hoechst 33342 dye showed that virtually all the live nerve cells expressed these receptors. **B.** Specificity of the Trk antibodies. Merged images (orange) are shown after double staining with antibodies recognizing MAP-2 (green) and Trk receptors (red) using antibodies to TrkB (a) and TrkC (c). Double staining with Trk antibodies preabsorbed with peptides used for the antibody production and specific for C-terminal sequences of TrkB (b) and TrkC (d) showed only green images (MAP-2). **C.** Compared with the autophosphorylation of the TrkB receptor induced by BDNF, the effect of NT-3 is low ($25 \pm 8\%$, $n = 3$). Cultures were exposed to 10 ng/ml of BDNF or NT-3 for 15 min. Immunoprecipitates obtained with TrkB antibody were blotted with phosphotyrosine antibodies (anti-pY) and TrkB antibodies. **D.** In spite of the relatively low activation of TrkB receptor by NT-3, the degree of NT-3-induced activation of Akt was only slightly less than that obtained with BDNF (cells were treated with the neurotrophins at 10 ng/ml for 15 min), consistent with NT-3 predominantly activating TrkC-mediated signaling.

Fig. 2. NT-3-induced signal transduction is reduced by IL-1β treatment

Activation of MAPK/ERK: **A**, Cortical neuronal cultures at 5 DIV were pretreated with IL-1β for 2 h. Western blots show the influence of IL-1β on the levels of phosphorylated MAPK/ERK (P-MAPK) upon NT-3 exposure (10 ng/ml, 15 min). The cytokine had no effect on the total level of MAPK and on its own did not increase P-MAPK. IL-1β (50 ng/ml) reduced NT-3 activation of p42 to $70 \pm 2.2\%$ and p44 to $70 \pm 4.5\%$ of that in controls ($n = 3$). **B**, Immunocytochemical detection shows that treatment with IL-1β (50 ng/ml) decreased the labeling of MAPK/ERK in NT-3-stimulated cultures (5 DIV). **C**, IL-1β pretreatment decreased the NT-3-induced increase in the level of activated Akt (P-Akt) to $73 \pm 7.2\%$ ($n = 3$) of control, but had no effect on total Akt (T-Akt) levels. **D**, Western blot shows a concentration dependent reduction by IL-1β (2 h pretreatment) of NT-3-induced increase in the level of Ser133 phosphorylated CREB (P-CREB). Note that the phosphorylated CREB content is not negligible under basal conditions and it is unaffected by 2 h exposure to IL-1β. However, pretreatment with the cytokine reduced NT-3-activated CREB to only slightly above the basal level.

Fig. 3. IL-1 receptor antagonist (IL-1RA) prevents IL-1 β interference with the NT-3-induced activation of MAPK (A, B) and CREB (C, D)

Representative Western blots are shown in A and C, while the quantification is displayed in B and D. Unless otherwise mentioned, here and in the other legends, quantification of Western blots are percentage estimates compared to the NT-3-treated controls (n = 3) and significance (<0.05) is indicated by *.

Fig. 4. IL-1 β interferes with NT-3-induced tyrosine phosphorylation of the docking proteins but not Trk receptor

A, Representative Western blot, using an antibody against a phosphorylated conserved Tyr in the juxtamembrane domain of Trk receptors (see Methods). **B**, Quantification of the effect of IL-1 β (50 ng/ml) on NT-3-activation of the receptor ($101 \pm 3.5\%$, $n = 3$). **C–F**, IL-1 β (50 ng/ml, 2 h) interferes with NT-3-induced phosphorylation of the docking proteins IRS-1 and Shc. **C**, IL-1 β pretreatment decreased NT-3-induced level of Tyr-phosphorylated IRS-1 analyzed by immunoprecipitation with an IRS-1 antibody, followed by Western blotting with anti-phosphotyrosine antibody (top panel) and with anti-IRS-1 antibody (bottom panel). **D**, Quantification of the effect of IL-1 β on NT-3-activation of IRS-1. **E**, Activation of Shc was analyzed by immunoprecipitation using an Shc antibody that recognizes all isoforms, followed by Western blotting with anti-phosphotyrosine antibody (top panel) and with anti-Shc antibody (bottom panel). The isoforms are indicated as a, b and c (approximate molecular weights, kDa:

66, 52 and 46, respectively). *F*, Quantification of the effect of IL-1 β pretreatment on NT-3 activation of Shc.

Fig. 5. IL-1 β abrogates NT-3 protection of cortical neurons from trophic support deprivation-induced cell death

The B-27-containing “trophic” medium was removed from cultures and after a DMEM wash, cultures were incubated for 36 h in DMEM in the presence or absence NT-3 (10 ng/ml) $\pm$ IL-1 β (50 ng/ml). With IL-1 β there was a 1 h preincubation period before the removal of the B-27 medium. In controls, B-27 medium was removed, but replaced after a DMEM wash with new B-27 medium. Cell survival was assayed using the MTT assay; data are mean $\pm$ S.E. ($n = 3$). Significance ($p < 0.05$) determined by ANOVA (with Fisher’s PLSD as the post hoc test) in comparison with either control cells (*) or cultures treated with NT-3 only (**) is indicated.