

Inflammatory cytokines IL-1 β and TNF- α regulate p75^{NTR} expression in CNS neurons and astrocytes by distinct cell-type-specific signalling mechanisms

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ABSTRACT

The p75^{NTR} (where NTR is neurotrophin receptor) can mediate many distinct cellular functions, including cell survival and apoptosis, axonal growth and cell proliferation, depending on the cellular context. This multifunctional receptor is widely expressed in the CNS (central nervous system) during development, but its expression is restricted in the adult brain. However, p75^{NTR} is induced by a variety of pathophysiological insults, including seizures, lesions and degenerative disease. We have demonstrated previously that p75^{NTR} is induced by seizures in neurons, where it induces apoptosis, and in astrocytes, where it may regulate proliferation. In the present study, we have investigated whether the inflammatory cytokines IL (interleukin)-1 β and TNF- α (tumour necrosis factor- α), that are commonly elevated in these pathological conditions, mediate the regulation of p75^{NTR} in neurons and astrocytes. We have further analysed the signal transduction pathways by which these cytokines induce p75^{NTR} expression in the different cell types, specifically investigating the roles of the NF- κ B (nuclear factor κ B) and p38 MAPK (mitogen-activated protein kinase) pathways. We have demonstrated that both cytokines regulate p75^{NTR} expression; however, the mechanisms governing this regulation are cytokine- and cell-type specific. The distinct mechanisms of cytokine-mediated p75^{NTR} regulation that we demonstrate in the present study may facilitate therapeutic intervention in regulation of this receptor in a cell-selective manner.

Key words: neurotrophin receptor, nuclear factor κ B (NF- κ B), p38 mitogen-activated protein kinase (p38 MAPK).

INTRODUCTION

The p75^{NTR} (where NTR is neurotrophin receptor) is known to play multiple roles in regulating neuronal survival, death and axonal growth (Greene and Rukenstein, 1981; Rabizadeh et al., 1993; Frade et al., 1996; Maggirwar et al., 1998; Friedman, 2000). p75^{NTR} is more widely expressed during development than in adults (Yan and Johnson, 1988; Friedman, 2000). However, this receptor is re-expressed in several pathological conditions such as traumatic brain injury, seizure, ischaemia, oxidative stress and axonal injury (Kokaia et al., 1998; Roux et al., 1999; Casha et al., 2001; Ramos et al., 2007). The role of p75^{NTR} in these pathological conditions has been proposed to be involved in neurodegeneration. For example, pilocarpine-induced seizures induces p75^{NTR} up-regulation in the hippocampus (Roux et al., 1999) and mediates neuronal apoptosis by activating the caspase 9,6,3 cascade (Troy et al., 2002). These studies suggest that there is a tight correlation between p75^{NTR} expression after injury and neurodegeneration. Although there are many studies demonstrating that p75^{NTR} is up-regulated in pathological conditions, the mechanisms regulating p75^{NTR} expression after injury are not well defined.

IL-1 β (interleukin-1 β) and TNF- α (tumour necrosis factor- α) are essential pro-inflammatory cytokines released from several cell types, including astrocytes and microglia, after brain injury (Giulian et al., 1986). IL-1 β and TNF- α have several physiological functions in neuroinflammation. In particular, IL-1 β is known to induce production of other cytokines and growth factors (Benveniste, 1992; Merrill and Benveniste, 1996), change blood flow (Monroy et al., 2001; Maher et al., 2003) and affect neuroendocrine responses and

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Abbreviations: ERK, extracellular-signal-regulated kinase; I κ B, inhibitor of nuclear factor κ B; IL-1 β , interleukin-1 β ; IL-1Ra, IL-1 receptor antagonist; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, minimal essential medium; NF- κ B, nuclear factor κ B; NTR, neurotrophin receptor; p-I κ B, phosphorylated inhibitor of nuclear factor κ B; p-p38, phosphorylated p38 MAPK; RT, reverse transcriptase; TNF- α , tumour necrosis factor- α .

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the activity of the HPA (hypothalamic–pituitary–adrenal) axis (Berkenbosch et al., 1987; Sapolsky et al., 1987). In addition, both IL-1 β and TNF- α have direct influences on neurodegeneration (Zhao et al., 2001; Thornton et al., 2006), and inhibition of endogenous IL-1 β or TNF- α protects against neuronal injury that occurs after cerebral ischaemia (Relton and Rothwell, 1992; Meistrell et al., 1997). In addition to effects on neurodegeneration, IL-1 β also affects remyelination by oligodendrocytes after injury (Mason et al., 2001). These cytokines are highly expressed in Alzheimer's disease (Griffin et al., 1989; Fillit et al., 1991; Wang et al., 1997), Parkinson's disease (Mogi et al., 1994, 1996) and spinal cord injury (Wang et al., 1997; Xu et al., 1998), conditions in which p75^{NTR} is induced. IL-1 β and TNF- α are up-regulated within 2 h after several types of injury (Minami et al., 1992; Taupin et al., 1993; Buttini et al., 1994; Fan et al., 1996; Eriksson et al., 2000), but p75^{NTR} induction after seizure takes 24 h (Roux et al., 1999). Therefore cytokine induction precedes p75^{NTR} induction after damage, and may play a role in regulation of p75^{NTR} expression. Indeed, results from microarray analysis on astrocytes after IL-1 β treatment showed increased p75^{NTR} mRNA (John et al., 2005).

In the present study, we demonstrate that the pro-inflammatory cytokines IL-1 β and TNF- α up-regulate p75^{NTR} in hippocampal neurons and astrocytes. However, the underlying signalling mechanisms to induce p75^{NTR} are different with distinct cytokines and cell types, suggesting a diverse mechanism of regulating cell-specific p75^{NTR} expression after injury.

MATERIAL AND METHODS

Materials

IL-1 β was generously provided by Dr Ronald Hart (Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, NJ, U.S.A.). TNF- α was purchased from R&D systems. SB203580 and SN-50 were obtained from Alexis Biochemicals. Eagle's MEM (minimal essential medium), Ham's F12 and penicillin/streptomycin were purchased from Invitrogen. All other materials were obtained from Sigma.

Neuronal cultures

Neuronal cultures were prepared as described previously (Farinelli et al., 1998; Friedman, 2000). Rat hippocampi were dissected from embryonic day 18, dissociated, plated on to poly-D-lysine (0.1 mg/ml)-coated dishes, and maintained in serum-free medium. The medium consisted of a 1:1 mixture of Eagle's MEM and Ham's F12 supplemented with glucose (6 mg/ml), putrescine (60 μ M), progesterone (20 nM), transferrin (100 μ g/ml), selenium (30 nM), penicillin (0.5 units/ml) and streptomycin (0.5 μ g/ml). Cultures were maintained in 5%

CO₂ at 37°C for 5 days before treatment. All animal studies were conducted using the NIH (National Institutes of Health) guidelines for the ethical treatment of animals with approval of the Rutgers Institutional Animal Care and Use Committee.

Astrocyte cultures

Astrocyte cultures were prepared as described previously (Srinivasan et al., 2004). Rat hippocampi were dissected from embryonic day 21, dissociated, plated on to poly-D-lysine (0.1 mg/ml)-coated flasks, and maintained in Eagle's MEM with Earle's salts, 2 mM L-glutamine, 15% heat-inactivated fetal bovine serum, 6 mg/ml glucose, penicillin (0.5 units/ml), and streptomycin (0.5 μ g/ml). After 7–9 days incubation in 5% CO₂ at 37°C, cells were shaken at 450 rev./min for 10 min to remove microglia and neurons, followed by a fresh medium change. Cells were shaken at 225 rev./min overnight to eliminate additional non-astrocytic cells. Cells were replenished with fresh medium containing 0.1 mM cytosine arabinoside and maintained for 3 days. Trypsinized and replated cells were kept in 5% CO₂ at 37°C for 4 days before treatment (McCarthy and de Vellis, 1980).

Immunoprecipitation and Western blot analysis

Cells were lysed in RIPA buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P40, 0.5% deoxycholic acid and 0.5% SDS] supplemented with a protease inhibitor mixture (Roche Products), 1 mM sodium vanadate and 5 mM sodium fluoride. Proteins were quantified using the Bradford assay (Bio-Rad) and equal amounts of protein were run on a 10% polyacrylamide gel and transferred on to a nitrocellulose membrane. Membranes were blocked in 5% (w/v) non-fat dried skimmed milk in TBST [TBS (Tris-buffered saline; 10 mM Tris and 150 mM NaCl, pH 7.4) containing 0.05% Tween 20] and then probed with antibodies against p75^{NTR} (Upstate Biotechnology), actin (Sigma), p-p38 [phosphorylated-p38 MAPK (mitogen-activated protein kinase)], p-I κ B {phosphorylated-I κ B [inhibitor of NF- κ B (nuclear factor κ B)]; Cell Signaling Technology}. p-p38 and p-I κ B blots were stripped and reprobed with anti-p38 and anti-I κ B (Cell Signaling Technology) antibodies respectively. Bands were visualized by X-ray film exposure using ECL (enhanced chemiluminescence; Pierce). For I κ B ubiquitination studies, cells were treated with IL-1 β or TNF- α for the indicated periods, then lysed in buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100 and protease inhibitors], and centrifuged (20817 g for 15 min at 4°C). Supernatants were incubated with anti-I κ B antibody overnight at 4°C and then incubated with Protein A-agarose at 4°C for 2 h. Immunoprecipitates were washed three times with lysis buffer and analysed by Western blot for ubiquitin (Santa Cruz Biotechnology). Blots were scanned and quantified using

Photoshop. Statistically significant differences were analysed by one-way ANOVA with Tukey's post-hoc analysis.

Biotinylation of cell-surface proteins

Cells were treated with IL-1 β or TNF- α for 8 h and washed with pre-chilled PBS once, and with PBS⁺⁺ (PBS containing 1 mM MgCl₂ and 2.5 mM CaCl₂) twice. Cell-surface proteins were biotinylated with sulfo-NHS-SS-Biotin (Pierce) at 4°C for 1 h, quenched with glycine, and washed with PBS⁺⁺ twice. Biotinylated cells were lysed in buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.5% deoxycholate, protease inhibitor mixture, 1 mM sodium vanadate and 5 mM sodium fluoride, and lysates were incubated with streptavidin-agarose (Pierce) overnight at 4°C. After centrifugation (4500 g for 3 min at 4°C), supernatants were saved and pellets were washed with lysis buffer three times. Pellets and supernatants were analysed by Western blot for p75^{NTR} and actin.

Immunostaining

To visualize nuclear translocation of NF- κ B, cells were plated on plastic Lab-Tek slide wells and treated with IL-1 β or TNF- α with or without pre-incubation with SN-50 for 30 min. Cells were fixed in 4% (w/v) paraformaldehyde, washed with PBS, permeabilized with PBS plus 0.3% Triton X-100, blocked in 5% (v/v) goat serum, and then incubated with anti-p65 antibody (Santa Cruz Biotechnology) overnight at 4°C. Slides were washed three times with PBS, incubated with secondary antibodies coupled to the Alexa Fluor[®] 555 fluorophore (Molecular Probes) for 1 h at room temperature (25°C), then washed with PBS three times. Hoechst 33342 (1 μ g/ml; Sigma) was used to visualize the nuclei.

Quantitative real-time RT (reverse transcriptase)-PCR

Primary hippocampal neurons or astrocytes were treated with IL-1 β or TNF- α for 2, 4 or 8 h, and mRNA was isolated using TRIzol[®] reagent (Invitrogen). cDNA was generated using SuperScript[™] II RT with random hexamers (Invitrogen), and SYBR-green-based quantitative real-time PCR was performed using primers specific for p75^{NTR} (rat, forward 5'-CTGATGCTGAATGCGAAGAG-3' and reverse 5'-TCACCA TATCCGCCACTGTA-3') or actin (forward 5'-TCATGAAGTGTGAC GTTGACATCCGT-3' and reverse 5'-CTTAGAAGCATTGCG GTGCACGATG-3') with the comparative C_T method ($\Delta\Delta$ C_T) (ABI).

RESULTS

To investigate whether pro-inflammatory cytokines such as IL-1 β and TNF- α induce p75^{NTR} in different cell types, cultured hippocampal neurons and astrocytes were treated

with IL-1 β or TNF- α for 1, 4, 8, 12 or 24 h. Levels of p75^{NTR} protein were evaluated by Western blot analysis, and mRNA was determined by quantitative PCR. The expression of p75^{NTR} was increased by IL-1 β and TNF- α in both neurons and astrocytes relative to actin (Figure 1). Elevated p75^{NTR} mRNA expression peaked after 4 h of treatment, and the protein increase was maximal after 8 h of treatment with cytokines.

Mechanisms of IL-1 β regulation of p75^{NTR}

In our previous studies, we have demonstrated that IL-1 β activates distinct signalling pathways in neurons and astrocytes. IL-1 β activates the classical NF- κ B pathway in astrocytes but not in neurons, whereas p38 MAPK [but not JNK (c-Jun N-terminal kinase) or ERK (extracellular-signal-regulated kinase) MAPK] is activated by IL-1 β in hippocampal neurons (Srinivasan et al., 2004). Therefore we investigated the underlying signalling mechanisms governing IL-1 β regulation of p75^{NTR} in both cell types.

We first confirmed the activation of specific signalling pathways in the different cell types. IL-1 β induced robust transient phosphorylation of p38 MAPK in hippocampal neurons (Figure 2A). Activation of NF- κ B signalling was monitored by phosphorylation and degradation of I κ B, which is necessary for nuclear translocation of NF- κ B. In neurons, no I κ B phosphorylation was detected (results not shown), consistent with our previous study showing that IL-1 β does not activate NF- κ B in hippocampal neurons (Srinivasan et al., 2004). However, in astrocytes there was robust increase in p-I κ B 5 min after IL-1 β treatment, with a subsequent loss of I κ B protein (Figure 2B). Interestingly, IL-1 β also activated p38 MAPK signalling in astrocytes, p-p38 MAPK increased within 5 min after IL-1 β treatment and peaked at 10 min in astrocytes (Figure 2C). Thus IL-1 β activates both NF- κ B and p38 MAPK signalling in astrocytes, but only p38 MAPK in hippocampal neurons.

We next examined which signalling pathway was responsible for the IL-1 β -mediated p75^{NTR} up-regulation in the different cell types. Neurons or astrocytes were pretreated either with a p38 MAPK inhibitor SB203580 (10 μ M) or an NF- κ B inhibitor SN-50 (10 μ M) for 30 min. IL-1 β was then applied to the cells for 8 h, and p75^{NTR} levels were analysed by Western blot. In hippocampal neurons, the p38 MAPK inhibitor prevented the IL-1 β -mediated p75^{NTR} up-regulation (Figures 3A and 3B). As expected, the NF- κ B inhibitor SN-50 did not prevent the p75^{NTR} expression in neurons. In astrocytes, both the p38 MAPK inhibitor and the NF- κ B inhibitor completely prevented the IL-1 β induction of p75^{NTR} expression (Figures 3C and 3D), indicating that p75^{NTR} induction in astrocytes requires both the p38 MAPK and NF- κ B pathways. Confirmation that the inhibitors blocked the relevant signalling pathways is shown in Supplementary Figure S1 (at <http://www.asnneuro.org/an/001/an001e010add.htm>).

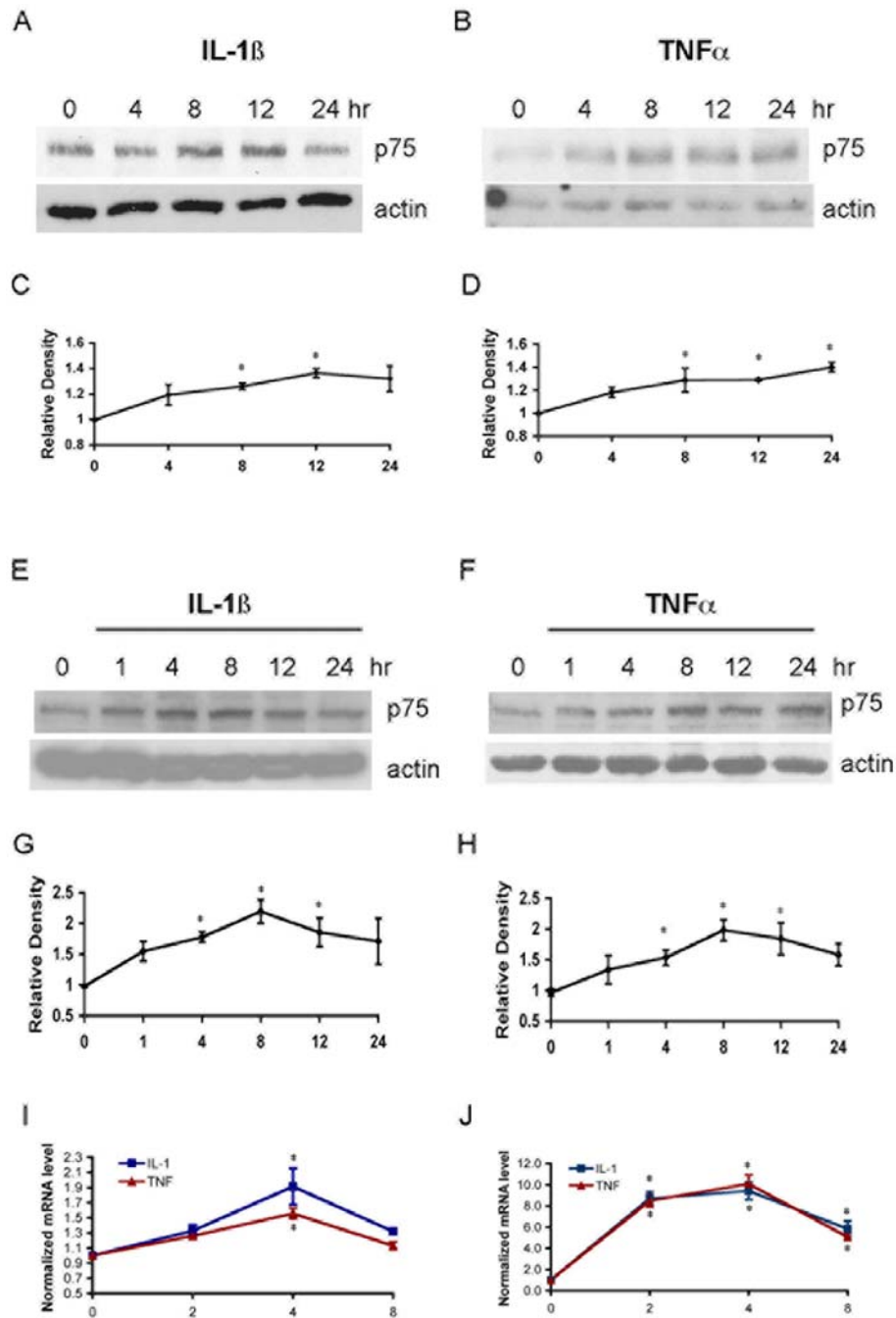


Figure 1 IL-1 β and TNF- α induce p75^{NTR} in neurons and astrocytes (A–D) Cultured hippocampal neurons were treated with either (A) IL-1 β (10 ng/ml) or (B) TNF- α (10 ng/ml) for 4, 8, 12 or 24 h, and were then lysed and analysed by Western blot for p75^{NTR} and actin. (C) and (D) Quantification of blots from three experiments as in (A) and (B) respectively. Densitometric values were normalized to actin and are expressed relative to the untreated cells (time 0). (E–H) Cultured hippocampal astrocytes were treated with either (E) IL-1 β (10 ng/ml) or (F) TNF- α (10 ng/ml) for 1, 4, 8, 12 or 24 h, and were then lysed and analysed by Western blot for p75^{NTR} and actin. (G) and (H) Quantification of blots from three experiments as in (E) and (F) respectively. Densitometric values were normalized to actin and are expressed relative to the untreated cells (time 0). (I and J) Quantitative real-time PCR analysis of p75^{NTR} mRNA in hippocampal neurons (I) or astrocytes (J) treated with IL-1 β or TNF- α expressed relative to untreated control cultures. The significance was determined by ANOVA with Tukey’s post-hoc analysis. * indicates values significantly different from control at $P < 0.05$.

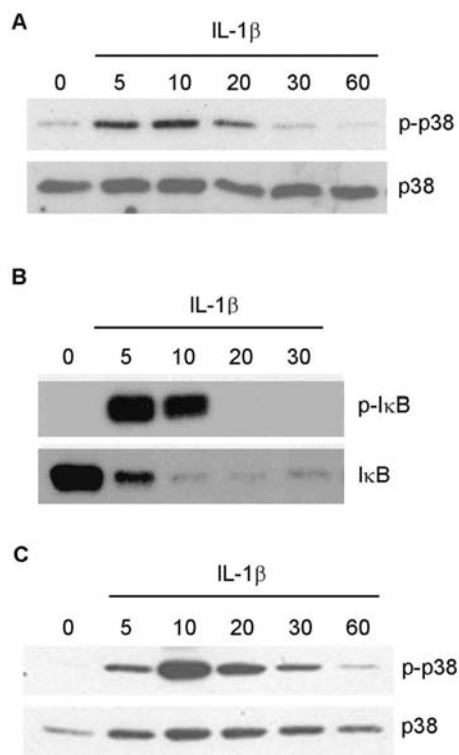


Figure 2 IL-1 activates p38 MAPK in both neurons and astrocytes, and NF- κ B only in astrocytes

(A) Western blot analysis of p-p38 MAPK activation by IL-1 β in neurons. Hippocampal neurons were cultured for 5 days, and treated with IL-1 β for 5, 10, 20, 30 and 60 min and probed for p-p38 MAPK. The blot was stripped and re-probed for total p38 MAPK (bottom panel). (B) Western blot analysis of p-I κ B in astrocytes. Cultured astrocytes were treated with IL-1 β for 5, 10, 20 and 30 min and probed for p-I κ B. The blot was stripped and re-probed for total I κ B, which was degraded after phosphorylation (bottom panel). (C) Hippocampal astrocytes were treated with IL-1 β for 5, 10, 20, 30 and 60 min and probed for p-p38 MAPK. The blot was stripped and re-probed for total p38 MAPK (bottom panel).

Mechanisms of TNF- α regulation of p75^{NTR}

Both IL-1 β and TNF- α elicited increased p75^{NTR} expression in hippocampal neurons; however, the signalling mechanisms activated by these cytokines were distinct. In contrast with the effects of IL-1 β , TNF- α elicited a modest transient phosphorylation of I κ B (Figures 4A and 4C), but failed to induce activation of p38 MAPK (Figures 4B and 4D). Moreover, p65 staining in nuclei was visible with TNF- α -treated neurons, but not with IL-1 β -treated neurons (Figure 4E), confirming activation of the NF- κ B pathway by TNF- α in neurons.

In astrocytes, TNF- α treatment induced phosphorylation of I κ B, which peaked at 10 min (Figure 4F) and decreased afterwards. I κ B protein was degraded after phosphorylation (Figure 4F). TNF- α also activated the p38 MAPK pathway in astrocytes, inducing robust phosphorylation that peaked at 10–20 min after treatment (Figure 4G). Thus TNF- α , like IL-1 β , induced activation of both the NF- κ B and p38 MAPK pathways in hippocampal astrocytes.

Interestingly, although TNF- α induced phosphorylation of I κ B in both hippocampal neurons and astrocytes, loss of I κ B protein was only observed in astrocytes (Figure 4F), not in neurons (Figure 4A). Once I κ B is phosphorylated, it goes through polyubiquitination and proteasome-dependent degradation (Karin and Ben-Neriah, 2000). Therefore we investigated whether the ubiquitination of I κ B occurred in neurons. Astrocytes and neurons were treated with TNF- α for the indicated times and the cell lysates were immunoprecipitated with anti-I κ B antibody, and Western blot analysis was used to detect ubiquitination. There was increased ubiquitinated I κ B in the TNF- α -treated astrocytes; however, no ubiquitination was detected in neurons treated with TNF- α (Figure 4H), consistent with the lack of I κ B degradation.

TNF- α induced phosphorylation of I κ B and nuclear translocation of NF- κ B in hippocampal neurons, suggesting that the increase in p75^{NTR} expression induced by this cytokine might be mediated by NF- κ B. Treatment of the neurons with the NF- κ B inhibitor SN50, but not the p38 MAPK inhibitor SB203580, prevented the TNF- α -evoked increase in p75^{NTR} (Figures 5A and 5B), demonstrating that TNF- α regulates p75^{NTR} expression via NF- κ B signalling, whereas IL-1 β regulates p75^{NTR} via p38 MAPK signalling in hippocampal neurons (Figure 3).

Similar to the effects in neurons, the p38 MAPK inhibitor did not prevent the TNF- α -mediated p75^{NTR} up-regulation in astrocytes (Figures 5C and 5D), whereas NF- κ B inhibition blocked TNF- α -mediated p75^{NTR} expression (Figures 5C and 5D). Confirmation that the inhibitors blocked their respective signalling pathways is shown in Supplementary Figure S1. Thus, in astrocytes, IL-1 β requires both the p38 MAPK and NF- κ B pathways to regulate p75^{NTR} expression; however, TNF- α regulates p75^{NTR} only through the NF- κ B pathway. Moreover, TNF- α signalled via the NF- κ B pathway to regulate p75^{NTR} in both hippocampal neurons and astrocytes, whereas IL-1 β signalled via the p38 MAPK pathway in both cell types and via NF- κ B only in astrocytes.

Cell-surface expression of p75^{NTR}

p75^{NTR} is a cell-surface receptor that can bind a variety of different ligands to mediate distinct cellular functions. To assess whether the elevated p75^{NTR} was localized to the cell surface, biotinylation assays were performed on cytokine-treated neurons and astrocytes. Nearly all of the detectable p75^{NTR} was present at the cell surface after 8 h of treatment with IL-1 β or TNF- α in neurons (Figure 6A) and little p75^{NTR} was detected in the intracellular fraction. Similar to neurons, IL-1 β - and TNF- α -treated astrocytes had elevated p75^{NTR} at the cell surface, but not in cytosolic compartment (Figure 6B), suggesting that p75^{NTR} induced by cytokines is present at the cell surface in both neurons and astrocytes. Specificity of the biotin pulldown is shown in Supplementary Figure S2 (at <http://www.asnneuro.org/an/001/an001e010add.htm>).

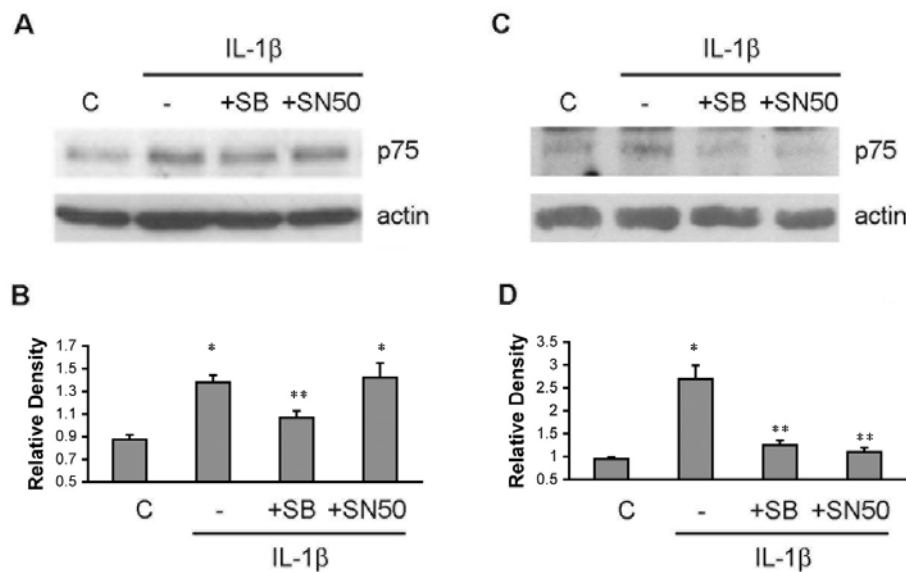


Figure 3 Signalling pathways required for IL-1 β induction of p75^{NTR} in hippocampal neurons and astrocytes (A) Hippocampal neurons were pretreated with SB203580 (10 μ M) or SN-50 (10 μ M) for 30 min and then 10 ng/ml IL-1 β was added to the culture for 8 h. Western blot analysis was used to assess p75^{NTR} and actin levels. (B) Quantification of three blots as shown in (A). * indicates values are significantly different from control, $P < 0.05$; ** indicates IL-1 β +SB203580 is significantly different from IL-1 alone or IL-1 β +SN-50, $P < 0.05$. (C and D) IL-1 induces p75^{NTR} through both the NF- κ B and p38 MAPK pathways in astrocytes. Cultured astrocytes were pretreated for 30 min with the p38 MAPK inhibitor SB203580 (10 μ M) or the NF- κ B inhibitor SN-50 (10 μ M) prior to IL-1 β treatment for 8 h. Western blot analysis was used to detect the p75^{NTR} and actin levels. (D) Quantification of three blots as shown in (C). * indicates values are significantly different from control, $P < 0.05$; ** indicates IL-1 β +SB203580 and IL-1 β +SN-50 are significantly different from IL-1 alone, $P < 0.05$. C, control; SB, SB203580.

DISCUSSION

p75^{NTR} is induced in CNS neurons after many types of injury (Kokaia et al., 1998; Roux et al., 1999; Casha et al., 2001; Ramos et al., 2007), and can mediate many different cellular responses, including neuronal apoptosis or survival, and regulation of axonal growth (Rabizadeh et al., 1993; Frade et al., 1996; Maggirwar et al., 1998; Friedman, 2000). However, the signals that regulate p75^{NTR} induction in neurons are not well characterized. Since many different types of pathophysiological conditions induce expression of p75^{NTR}, it is likely that inflammatory events common to these conditions may regulate this receptor. A previous study has demonstrated that hypo-osmotic stress can regulate p75^{NTR} by increasing cellular levels of Sp1 in primary cortical neurons (Ramos et al., 2007). In the present study, we investigated whether the pro-inflammatory cytokines IL-1 β and TNF- α , which are highly expressed in the brain following injury (Minami et al., 1992; Taupin et al., 1993; Buttini et al., 1994; Fan et al., 1996; Eriksson et al., 2000), might play a role in p75^{NTR} regulation. Not only are these cytokines highly expressed in the brain under these pathophysiological conditions, but IL-1 is also expressed by microglia during neonatal development (Giulian et al., 1988), a time during which p75^{NTR} is widely expressed throughout the brain. Thus this cytokine may regulate expression of p75^{NTR} developmentally as well as after injury.

In addition to the seizure-induced regulation of p75^{NTR} that we (Troy et al., 2002) and others (Roux et al., 1999), have observed in brain neurons, we recently demonstrated that this receptor is also induced in astrocytes following pilocarpine-induced seizures (Cragolini et al., 2009). In astrocytes, we have determined that stimulation of p75^{NTR} with NGF negatively regulates cell proliferation (Cragolini et al., 2009). Thus we investigated the mechanisms that regulate p75^{NTR} induction in both primary hippocampal neurons and astrocytes. We have demonstrated in the present study that the pro-inflammatory cytokines IL-1 β and TNF- α induce p75^{NTR} expression in both hippocampal neurons and astrocytes. However, the underlying signalling pathways leading to p75^{NTR} induction was cytokine- and cell-type-specific. Whereas IL-1 β induced p75^{NTR} via p38 MAPK in neurons, and via both p38 MAPK and NF- κ B in astrocytes, TNF- α induced p75^{NTR} via NF- κ B both in neurons and astrocytes. Thus inflammatory cytokines play a key role in regulating p75^{NTR} expression, and the cellular specificity may provide a possible therapeutic target for CNS diseases.

Mechanisms of IL-1 β regulation of p75^{NTR}

The release of IL-1 β after injury elicits diverse effects including the production of other cytokines and growth factors, thereby promoting inflammatory activity. We have shown in the present study that IL-1 β induced p75^{NTR} expression in primary hippocampal neurons and astrocytes.

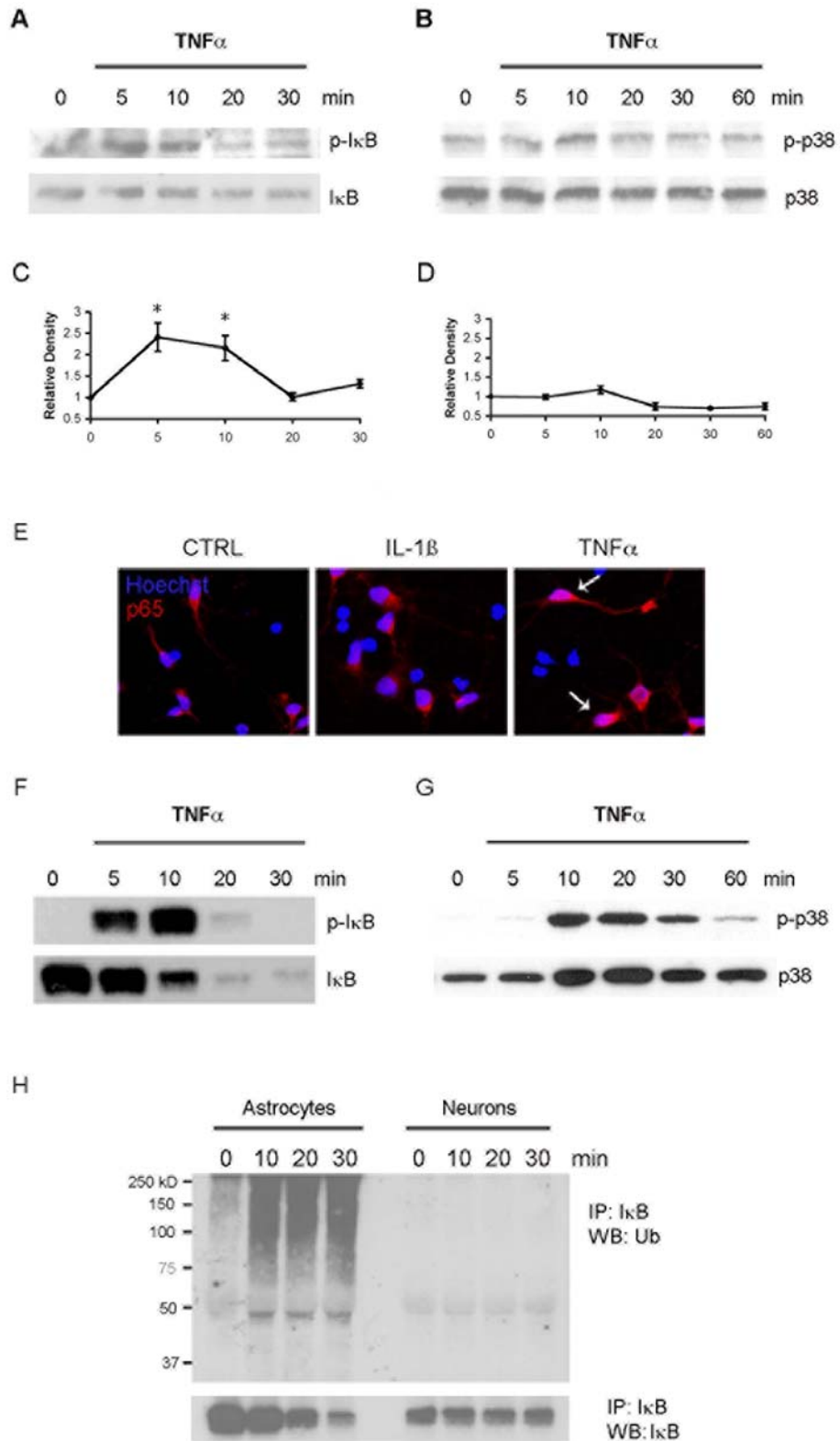


Figure 4 **TNF- α phosphorylates I κ B in neurons, and both I κ B and p38 MAPK in astrocytes**
(A) Hippocampal neurons were cultured for 5 days and treated with TNF- α for the times indicated. Lysates were probed for p-I κ B, stripped and re-probed for total I κ B. **(B)** Neuronal lysates were probed for p-p38 MAPK, stripped and re-probed for total p38 MAPK. **(C)** Quantification of three blots as shown in **(A)**; TNF- α treatment significantly induced p-I κ B at 5 and 10 min, * P <0.05. **(D)** Quantification of three blots as shown in **(B)**; TNF- α treatment did not induce phosphorylation of p38 MAPK in hippocampal neurons. **(E)** Neurons were treated with IL-1 β or TNF- α for 30 min, fixed and immunostained for p65. Nuclei were identified with Hoechst labelling. Note the presence of nuclear p65 in TNF- α -, but not IL-1 β -, treated neurons. CTRL, control. **(F)** Cultured astrocytes were treated with TNF- α for the times indicated. Astrocyte lysates were probed for p-I κ B, stripped and re-probed for total I κ B. **(G)** Astrocyte lysates were probed for p-p38 MAPK, stripped and re-probed for total p38 MAPK. **(H)** Analysis of ubiquitinated I κ B in astrocytes and neurons. Cells were treated with TNF- α as indicated. Cell lysates were immunoprecipitated (IP) with anti-I κ B and probed for ubiquitin (Ub). Blots were stripped and re-probed for I κ B. Note that levels of total I κ B decreased over time in the astrocytes, but not in the neurons. The molecular mass in kDa is indicated on the left-hand side of the blot. WB, Western blot.

The increased p75^{NTR} expression was transient and returned to baseline by 24 h, which may be due to the actions of IL-1Ra, the naturally occurring IL-1 β receptor antagonist (Hannum et al., 1990), providing negative feedback. Many signalling pathways can be activated by IL-1 β in different cell types, including the classic NF- κ B pathway, as well as the JNK, ERK and p38 MAPK pathways (O'Neill, 2002; Dunne and O'Neill, 2003). We have previously established that hippocampal neurons utilize a different signalling pathway compared with astrocytes (Srinivasan et al., 2004). Although IL-1 β activated NF- κ B in astrocytes, it failed to do so in hippocampal neurons, activating only p38 MAPK signalling (Srinivasan et al., 2004). Our present study shows that IL-1 β activation of p38 MAPK was necessary for p75^{NTR} induction by this cytokine in the hippocampal neurons.

In contrast with neurons, IL-1 β activated both the p38 MAPK pathway and the NF- κ B pathway in astrocytes. Pharmacological inhibitors were used to determine which of these pathways regulated the induction of p75^{NTR}

expression in astrocytes. Interestingly, inhibition of either pathway prevented the increase in p75^{NTR} expression, indicating that both p38 MAPK signalling and the NF- κ B pathways are required for p75^{NTR} induction by IL-1 β .

Mechanisms of TNF- α regulation of p75^{NTR}

TNF- α is another major pro-inflammatory cytokine that is produced in the brain after injury (Taupin et al., 1993; Fan et al., 1996) and frequently acts synergistically with IL-1 β . In contrast with the actions of IL-1 β , TNF- α activated the NF- κ B pathway in hippocampal neurons, indicated by phosphorylation of I κ B and nuclear translocation of NF- κ B, demonstrating that these two inflammatory cytokines signal via distinct pathways. Inhibition of NF- κ B nuclear translocation with SN-50 prevented TNF- α -mediated p75^{NTR} induction in the hippocampal neurons, confirming that the distinct pathways activated by the two different cytokines both lead to induction of p75^{NTR}.

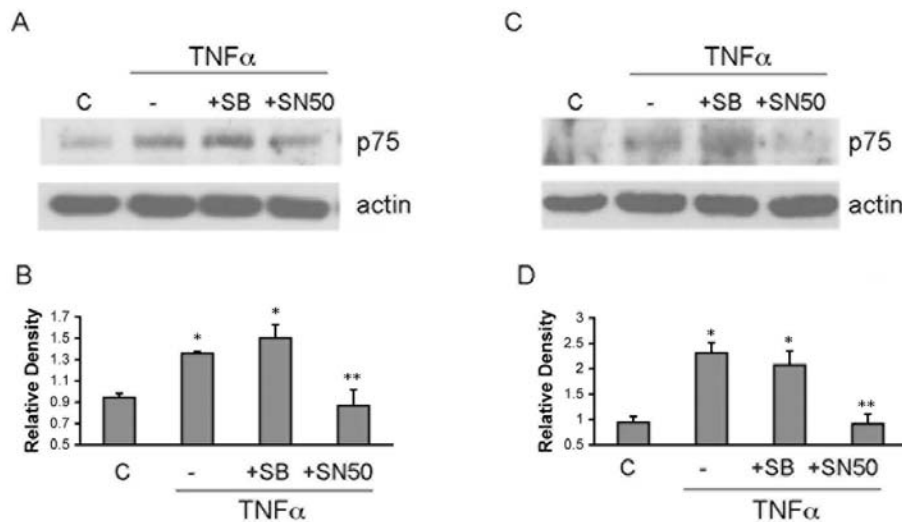


Figure 5 **TNF- α induces p75^{NTR} via NF- κ B in both neurons and astrocytes**
(A) Hippocampal neurons were pretreated with SB203580 (10 μ M) or SN-50 (10 μ M) for 30 min and then 10 ng/ml TNF- α was provided for 8 h. Western blot analysis was used to assess p75^{NTR} and actin levels. **(B)** Quantification of three blots as shown in **(A)**. * indicates levels are significantly different from control, P <0.05; ** indicates TNF- α +SN-50 is significantly different from TNF- α alone, P <0.05. **(C)** Hippocampal astrocytes were pretreated with SB203580 (10 μ M) or SN-50 (10 μ M) for 30 min and then 10 ng/ml TNF- α was added to the culture for 8 h. Western blot analysis was used to assess p75^{NTR} and actin. **(D)** Quantification of three blots as shown in **(C)**. * indicates levels are significantly different from control, P <0.05; ** indicates TNF- α +SN-50 is significantly different from TNF- α alone, P <0.05. SB, SB203580. C, control.

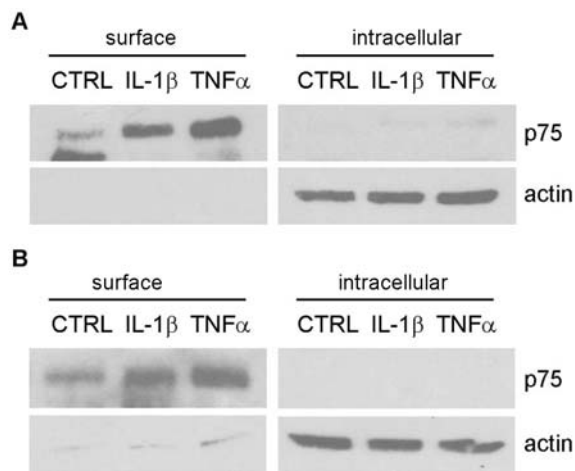


Figure 6 Increased p75^{NTR} expression induced by IL-1 β and TNF- α is present on the cell surface in both neurons and astrocytes. Cultured hippocampal neurons (A) or astrocytes (B) were treated with IL-1 β or TNF- α for 8 h, and incubated with biotin for 1 h. Cell lysates were precipitated with streptavidin. Biotinylated cell-surface protein and non-biotinylated intracellular proteins were analysed by Western blotting for p75^{NTR}. Blots were stripped and re-probed for actin, which was only present in the intracellular fraction. CTRL, control.

Interestingly, although TNF- α treatment of hippocampal neurons lead to I κ B phosphorylation, there was no subsequent ubiquitination and degradation of the protein as normally seen in astrocytes and other cell types (Verstrepen et al., 2008). An alternative pathway for activation of NF- κ B has been demonstrated as a MEKK3 (MAPK/ERK kinase 3)-dependent pathway in which I κ B is phosphorylated and dissociated from NF- κ B, but is not degraded (Yao et al., 2007). Since that is what we have observed in response to TNF- α treatment of neurons, this pathway is likely to be the one involved. In astrocytes, TNF- α activated both the NF- κ B and p38 MAPK pathways, similar to the effects of IL-1 β . However, blocking NF- κ B nuclear translocation with SN-50 prevented TNF- α -mediated induction of p75^{NTR}, whereas the p38 inhibitor had no effect, indicating that the NF- κ B pathway mediates p75^{NTR} induction by TNF- α in both neurons and astrocytes. This is in contrast with the regulation of p75^{NTR} by IL-1 β in astrocytes, which required signalling via both the NF- κ B and p38 MAPK pathways. Thus these two key inflammatory cytokines have a common target in regulation of the p75^{NTR}, but the mechanisms by which they regulate expression of this receptor are distinct both in hippocampal neurons and astrocytes (Figure 7).

Since p75^{NTR} is a cell-surface receptor that binds a variety of ligands to mediate different cellular effects, it was important to determine whether the inflammatory cytokines not only increased expression of this receptor in the neurons and astrocytes, but whether the elevated receptor was on the cell surface where it may be activated by ligand. Biotinylation experiments determined that the increased p75^{NTR} expression was nearly completely present on the cell surface, confirming that the receptor would be accessible for ligand binding.

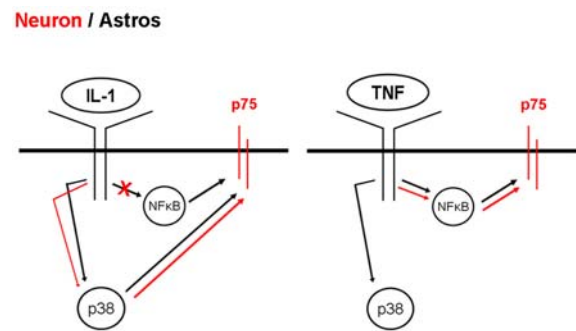


Figure 7 Schematic diagram showing the signalling pathways activated by IL-1 β and TNF- α in neurons (red) and astrocytes (black) leading to induction of p75^{NTR}.

The p75^{NTR} plays an important role in neuronal apoptosis, serving as a receptor for proneurotrophins that are released in the hippocampus following seizures (Volosin et al., 2008). Thus understanding the mechanisms that regulate expression of this receptor may serve as a potential therapeutic target for neuroprotection following seizures or other injuries. There has been contradictory data on the effect of direct administration of IL-1 β to the rat brain. Whereas one group has shown that IL-1 β and TNF- α administration caused neuronal loss in the substantia nigra (Carvey et al., 2005), most studies have shown that IL-1 β alone did not directly affect healthy neurons either *in vitro* or *in vivo* (Chao et al., 1995; Lawrence et al., 1998). However, IL-1 is known to exacerbate neuronal degeneration, and the naturally occurring IL-1Ra has been shown to be neuroprotective in models of ischaemia and excitotoxicity (Simi et al., 2007). The neuronal induction of p75^{NTR} by inflammatory cytokines may contribute to neurodegeneration in these pathological conditions.

Our recent studies have demonstrated that p75^{NTR} is induced in hippocampal astrocytes as well as neurons, and serves to attenuate proliferation of these cells (Cragolini et al., 2009). Since excessive glial proliferation can lead to scarring and inhibition of the potential for regeneration, the induction of p75^{NTR} on astrocytes may be beneficial. Thus it may be therapeutically beneficial to inhibit p75^{NTR} induction in hippocampal neurons to prevent neuronal loss, yet permit p75^{NTR} induction in astrocytes to attenuate glial proliferation. The distinct mechanisms of cytokine-mediated p75^{NTR} regulation that we have identified in the present study may facilitate the cell-type-specific regulation of this receptor and the functional consequences.

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