Interleukin-1β regulates p75 expression and increases vulnerability to proNGF-mediated apoptosis

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Abstract

Many types of injury such as seizure, ischemia, and oxidative stress cause upregulation of $p75^{NTR}$ (neurotrophin receptor) in brain neurons, where it promotes apoptosis, however the mechanism by which $p75^{NTR}$ is regulated under these conditions is not well understood. Proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) are highly produced under these injury conditions and, in particular, are expressed rapidly in the rat hippocampus after seizure. IL-1β is known to increase neuronal vulnerability under many conditions, although it does not directly induce neuronal death. Recently, we have shown that these cytokines regulate $p75^{NTR}$ induction both in neurons and astrocytes *in vitro*. Here, we show that IL-1β infusion into the brain induces $p75^{NTR}$ in neurons of the CA1 area of the hippocampus. While IL-1β induction of $p75^{NTR}$ is not sufficient to induce cell death, we demonstrate that IL-1β primes the neurons by recruiting $p75^{NTR}$ and its coreceptor sortilin to the cell surface, making the neurons more vulnerable to subsequent challenge by proNGF. These results suggest a mechanism by which IL-1β exacerbates neuronal death following injury.

IL-1β is a major proinflammatory cytokine released under conditions of injury, infection, or disease, and is known to be involved in diverse actions in the central nervous system. Although many studies have shown that IL-1β alone does not induce neuronal death either in vitro (Thornton et al., 2006) or in vivo (Lawrence et al., 1998), it has synergistic effects on neuronal damage when provided with other cytokines (Chao et al., 1995; Hu et al., 1997). Moreover, release of IL-1 exacerbates traumatic, ischemic or excitotoxic stimulated neurotoxicity (Yamasaki et al., 1995; Patel et al., 2003), and blocking IL-1 with the receptor antagonist (IL-1ra) attenuates neuronal loss (Allan et al., 2005), suggesting that IL-1β indirectly contributes to neuronal injury.

Our lab has recently reported that proinflammatory cytokines such as IL-1β and TNFα regulate expression of the p75 neurotrophin receptor ($p75^{NTR}$) both in neurons and astrocytes *in vitro* (Choi and Friedman, 2009). The $p75^{NTR}$ has diverse 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). This multifunctional receptor is abundantly expressed in the brain during development, however its expression is limited in the adult brain (Yan and Johnson, 1988). $p75^{NTR}$ is upregulated following many types of

brain injury such as traumatic brain injury, seizure, ischemia, oxidative stress and axonal injury (Kokaia et al., 1998; Roux et al., 1999; Casha et al., 2001; Ramos et al., 2007) as well as in CNS neurodegenerative diseases such as Alzheimer's disease (Hu et al., 2002). The upregulated $p75^{NTR}$ in these pathological conditions has been suggested to be directly involved in neurodegeneration. $p75^{NTR}$ is highly expressed in the hippocampus after pilocarpine induced seizure (Roux et al., 1999) and induces neuronal cell death by activating the intrinsic caspase cascade (Troy et al., 2002). Furthermore, the unprocessed NGF precursor, proNGF, which is a potent ligand for $p75^{NTR}$, is also released after injury and induces neuronal apoptosis (Beattie et al., 2002; Volosin et al., 2008).

IL-1β is also known to regulate NGF mRNA expression (Spranger et al., 1990; Friedman et al., 1991), although the form of the NGF protein that is produced has not been identified. Therefore, IL-1β may be involved in neurodegeneration by regulating the receptor as well as ligands that promote neuronal death.

In this study, IL-1β infusion into brain increased $p75^{NTR}$ expression but did not induce cell death *in vivo*. We therefore investigated the functional role of $p75^{NTR}$ upregulation after IL-1β treatment and show that IL-1β specifically

exacerbated proNGF induced hippocampal neuronal death by recruiting the receptor complex to the cell surface.

Material and methods

Materials. IL-1β was generously provided by Dr. Ron Hart, (Rutgers University, Piscataway, NJ) and NGF was provided by Genentech, Inc. (South San Francisco). Furin-resistant proNGF was generously provided by Dr. Barbara Hempstead. Eagle's MEM, Ham's F12, and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). All other materials were obtained from Sigma (St.Louis, MO).

Stereotaxic cannulation of the hippocampus

Male Sprague Dawley rats (250-275g) were anaesthetized with ketamine (50mg/kg)/xylazine (10mg/kg) and placed in a stereotaxic frame for bilateral implantation of cannula into dorsal hippocampus. The following coordinates were used: anterior-posterior = -3.1mm from bregma, lateral = ±2 mm from midline, dorsoventral = -3mm from skull (Paxinos et al., 1985). Skull holes were made with a dental drill and the guide cannula and support screw were fixed with dental cement. After 7 days, 10μg

(in 0.5μl) of IL-1β was infused unilaterally into the hippocampus via the guide cannula at a rate of 0.5μl/min. Animals found to have an incorrectly placed cannula were excluded.

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 Facilities Committee.

Immunocytochemistry

Two days after IL-1β infusion, rats were anesthetized with ketamine/xylazine and perfused transcardially with saline followed by 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde before being cryoprotected in 30% sucrose for 2 d. Brains were then sectioned on a cryostat (Leica), and mounted onto charged slides for immunostaining. Frozen brain sections (12 μm) were warmed at 37°C for 1 min, washed with PBS, blocked in 10% goat serum with PBS plus 0.3% Triton X-100, and then incubated with anti-$p75^{NTR}$ (Upstate; 1:500) and NeuN (Chemicon; 1:500) overnight at 4°C. The sections were then washed three times with PBS for 15 min, followed by incubation with the secondary antibodies, Fluor 488-conjugated

donkey anti-rabbit and texas red-conjugated goat anti-mouse (Jackson; 1:500) at room temp in the dark for 1 h, washed three times with PBS for 15 min. Hoechst 33342 dye (1 μg/ml; Sigma) was added into PBS during the last wash to label nuclei. Sections were mounted with anti-fading medium (ProLong Gold; Invitrogen) and were examined by fluorescence microscopy (Nikon). Cell death was examined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining following manufacturer's manual (Roche, Mannheim, Germany).

Analysis of Cerebrospinal fluid (CSF)

Rats were anesthetized with ketamine/xylazine and placed in a stereotaxic frame for collecting CSF from cisterna magna using a 25 gauge needle. Only CSF samples that did not contain blood contamination were mixed with protease inhibitors, flash frozen, and stored at -80°C until analysis.

Western blot analysis.

Cells were lysed in RIPA buffer (50mM Tris-HCl, pH7.5 150mM NaCl, 5mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.5% SDS) supplemented with a protease inhibitor mixture (Roche Products, Welwyn Garden City, UK), 1mM sodium vanadate,

and 5mM sodium fluoride. Proteins were quantified by Bradford assay (Bio-Rad, Hercules, CA), equal amounts of proteins were run on 10 % polyacrylamide gel, and transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat milk in TBST and then probed with antibodies to $p75^{NTR}$ (Upstate Biotechnology, Inc., Lake Placid, NY), and actin (Sigma, St. Louis, MO), NGF (Sigma). Bands were visualized by enhanced chemical luminescence (Pierce, Rockford, IL). NRH2 antibody

Quantitative real-time reverse transcription PCR

Dorsal hippocampus was freshly homogenized, mRNA and proteins were isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNA was generated using SuperScript™ II Reverse Transcriptase 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′, reverse: 5′-TCACCATATCCGCCACTGTA-3′), NGF (rat, forward: 5′-CAAGGACGCAGCTTTCTATCCTG-3′, reverse: 5′-CTTCAGGGACAGAGTCTCCCTCT-3′), or actin (forward:5′-TCATGAAGTGTGACGTTGACATCCGT-3′, reverse :5′-

CTTAGAAGCATTTGCGGTG CACGATG-3′) with the comparative C_T method ($\Delta\Delta C_T$)(ABI).

Neuronal cultures

Hippocampal neuronal cultures were prepared as described previously (Farinelli et al., 1998; Friedman, 2000). Rat hippocampi were dissected from embryonic day 18, dissociated, plated on poly-D-lysine (0.1mg/ml)-coated dishes, and maintained in a serum-free environment. The medium consisted of a 1:1 mixture of Eagle's MEM and Ham's F12 supplemented with glucose (6mg/ml), insulin (25μg/ml), putrescine (60μM), progesterone (20nM), transferrin (100μg/ml), selenium (30nM), penicillin (0.5U/ml), and streptomycin (0.5μg/ml). Cultures were maintained in 5% CO_2 at 37°C for 5 days and subjected to IL-1β treatment for the times indicated.

Survival assay.

Survival of cultured hippocampal neurons was assayed by a method we have described previously (Farinelli et al., 1998; Maroney et al., 1999; Friedman, 2000). After removal of the medium, cultured cells were lysed, and intact nuclei were counted using a hemocytometer. Nuclei of dead cells either disintegrate or, if in the process of dying,

appear pyknotic and irregularly shaped. In contrast, nuclei of healthy cells are phase bright and have clearly defined limiting membranes. Cell counts were performed in triplicate wells.

Biotinylation of cell surface proteins

Cells were treated with IL-1β for 6 h and washed with pre-chilled PBS once and with PBS^{++} (PBS containing 1mM $MgCl_2$ and 2.5 mM $CaCl_2$) 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 50mM Tris, 150mM NaCl, 1mM EDTA, 1% Nonidet P40, 0.5% deoxycholate, protease inhibitor mixture, 1mM sodium vanadate and 5mM sodium fluoride, and lysates were incubated with streptavidin-agarose (Pierce) overnight at 4°C. After centrifugation (4500g for 3min at 4°C), supernatants were saved and pellets were washed with lysis buffer three times. Pellets and supernatants were analyzed by Western blot for $p75^{NTR}$ (upstate), sortilin (BD), and transferrin receptor (Invitrogen).

siRNA treatment

siRNAs were designed with a 5' thiol on the sense strand, synthesized, and

HPLC purified (Thermo Fisher Scientific, Dharmacon Products). Sequence for the sense strand was 5'-GCAACAUCAUUCCUGUCUA-3'. siRNA duplexes with a 5' thiol on the sense strand were linked to Penetratin 1 (Q-Biogene, Carlsbad, CA) as described previously (Davidson et al., 2004). The linked products were confirmed by SDS-PAGE using silverSNAP stain Kit II (Pierce, Rockford, IL). Luciferase siRNAs were used as control siRNA; 5'-CGUACGCGGAAUACUUCGA-3'.

In Situ Zymography

One day following IL-1β infusion, rats were anesthetized with ketamine/xylazine, and their brains were flash frozen, sectioned on a cryostat (Leica), and mounted onto charged slides for in situ zymography. Fresh frozen sections (12μm) were covered with a buffer containing 1% low-melting point agarose (BioRad, Hercules, CA), 0.1 M Tris, pH 7.5, 2.5% milk and 20 μg/mL plasminogen. Areas of tPA acivity were detected in black spots on dark field of views, where the endogenous enzyme degraded the substrate.

Results

IL-1β increases $p75^{NTR}$ in vivo

To investigate whether IL-1β induces $p75^{NTR}$ expression *in vivo*, IL-1β (10ng) was directly infused into one hippocampal hemisphere through a cannula. As a control, saline was infused into the contralateral hippocampus. $p75^{NTR}$ mRNA was increased in the hippocampus that had been infused with IL-1β compared to the contralateral side with saline infusion (Fig. 1A). Western blot analysis of tissue samples taken from dorsal hippocampus shows that $p75^{NTR}$ protein was also elevated by IL-1β (Fig. 1B). Since IL-1β can affect multiple cell types, sections were double labeled with anti-$p75^{NTR}$ and a neuronal marker, NeuN to examine where $p75^{NTR}$ was regulated following IL-1β infusion. In addition to non-neuronal cells, $p75^{NTR}$ expression was found in hippocampal neurons (Fig. 1C), consistent with our previous *in vitro* data (Choi and Friedman, 2009). In light of previous work showing cell death mediated by increased $p75^{NTR}$ expression, we examined whether IL-1β induced cell death as a result of $p75^{NTR}$

induction. Neither IL-1β nor saline infusion induced TUNEL positive cells (Fig. 2), which is consistent with many studies demonstrating that IL-1β alone does not induce neuronal cell death (Lawrence et al., 1998). These findings suggest that $p75^{NTR}$ induction alone is not sufficient to mediate cell death.

IL-1β exacerbates proNGF mediated hippocampal neuron death

To investigate the functional consequences of IL-1β-mediated $p75^{NTR}$ upregulation, we examined the effect of NGF and proNGF on IL-1ß-primed neurons in culture. Hippocampal neurons were pretreated with IL-1β for 4-6 hours to allow $p75^{NTR}$ induction, and then subjected to either NGF or proNGF treatment overnight. Both NGF (100ng/mL) and proNGF (1-10ng/mL) caused hippocampal neuronal death (Fig. 3), consistent with previous studies (Friedman, 2000; Volosin et al., 2008). Interestingly, whereas IL-1β did not affect NGF mediated cell death (Fig. 3A), IL-1β pretreatment exacerbated proNGF induced cell death (Fig. 3B), suggesting that IL-1β may make neurons selectively more vulnerable to proNGF.

IL-1β recruits $p75^{NTR}$ and sortilin to the plasma membrane

Since sortilin is a required coreceptor for $p75^{NTR}$ in proNGF-mediated neuronal

cell death (Nykjaer et al., 2004), we examined the level of sortilin and $p75^{NTR}$ at the cell surface following IL-1ß treatment. Biotinylation assays showed increased sortilin and $p75^{NTR}$ on the cell surface in IL-1β primed neurons (Fig. 4), while transferrin receptor levels were unchanged by IL-1β treatment. These results suggest that IL-1β elicits recruitment of sortilin as well as $p75^{NTR}$ to the plasma membrane, which appears to increase vulnerability to proNGF treatment.

IL-1β mediated NRH2 induction increases surface localization of sortilin and $p75^{NTR}$

A recent study demonstrated that NRH2, a mammalian homologue of $p75^{NTR}$, may regulate sortilin trafficking to the cell surface (Kim and Hempstead, 2009). Furthermore, coexpression of NRH2 and $p75^{NTR}$ has been reported in subpopulations of cells where proNGF-mediated cell death was elicited (Kanning et al., 2003; Murray et al., 2004). Thus, we examined whether IL-1β may regulate NRH2 as well as $p75^{NTR}$, to mediate sortilin trafficking. Neurons were treated with either 1ng/mL or 10ng/mL of IL-1β for 6 hours. NRH2 was induced at the same concentration of IL-1β (10ng/mL), which is required to increase $p75^{NTR}$ expression (Fig 5A). To determine whether NRH2 may regulate

the surface expression of sortilin, we used siRNA to knock down NRH2 expression. NRH2 siRNA or a nonspecific control (luciferase) siRNA was linked to penetratin to enhance the cellular delivery of the siRNA into hippocampal neurons (Davidson et al., 2004). Pretreatment of hippocampal neurons with NRH2 siRNA prevented the IL-1β-induced increase in NRH2, but not $p75^{NTR}$, expression (Fig 5B). Moreover, preventing the increase of NRH2 abolished the IL-1β mediated surface translocation of both sortilin and $p75^{NTR}$, suggesting that NRH2 induction is required for IL-1β to direct sortilin and $p75^{NTR}$ to the cell surface (Fig 5C).

IL-1β releases NGF into the CSF

IL-1β is known to regulate NGF expression and secretion in the brain (Spranger et al., 1990; Yasuda et al., 2007). To confirm the NGF regulation by IL-1β, tissue samples were taken from dorsal hippocampus 4 hour after IL-1β or saline infusion and were analyzed by quantitative PCR. IL-1β increased NGF mRNA levels compared to saline (Fig 6A). Since proNGF has been shown to be involved in cell death as a ligand for $p75^{NTR}$ following injury (Beattie et al., 2002; Volosin 2008), we examined which forms of NGF were secreted following

infusion of IL-1β. The cerebrospinal fluid (CSF) was collected from the rats and analyzed by Western blot. The CSF sample taken from IL-1β infused rats showed increased levels of mature NGF compared to the CSF samples from control rats (Fig. 6B). Secreted proNGF was not detected in any of samples, suggesting that IL-1β, in the absence of injury, regulates NGF expression and the subsequent secretion of mature NGF.

We have recently demonstrated that enzymes such as MMP7 and tPA that process proNGF cleavage are reduced following seizures, leading to increased extracellular proNGF (Le and Friedman, 2012). To investigate whether IL-1β may alter proNGF processing enzyme activity to result in increased NGF rather than proNGF, in situ zymography for MMP7 and tPA was performed. The IL-1β infused side showed no change in MMP7 activity (not shown), but increased tPA activity compared to the saline infused side (Figure 6C), suggesting that IL-1β increases NGF possibly by increasing the tPA-mediated cleavage of proNGF to NGF. Taken together, these data suggest that IL-1β induces $p75^{NTR}$ and NGF expression, but does not stabilize proNGF in vivo and therefore by itself is not sufficient to induce cell death.

Discussion

Many changes occur in the brain following injury or in neurodegenerative disease that lead to neuronal loss. We have previously demonstrated that induction of $p75^{NTR}$ in hippocampal neurons and the stabilization of proNGF in the extracellular environment contribute to neuronal loss following seizure-induced injury (Troy et al, 2002; Le and Friedman, 2012). Additional studies have demonstrated that $p75^{NTR}$ induced after many types of injury or in several pathological diseases may play a role in neuronal degeneration (Armstrong et al., 1991; Syroid et al., 2000; Casha et al., 2001; Beattie et al., 2002; Yan and Feng, 2004), however the underlying mechanisms by which $p75^{NTR}$ is regulated have not been extensively studied. Recently, our lab has determined that proinflammatory cytokines, IL-1β and TNFα increase the expression of $p75^{NTR}$ in hippocampal neurons and astrocytes in culture. Since the induction of proinflammatory cytokines, specifically IL-1β, is a key feature of the injury response, we investigated whether this cytokine was sufficient to mediate the changes in the brain that lead to neuronal loss. In the present study, we have shown that IL-1β infusion directly into the hippocampus *in vivo* in the absence

of injury induced $p75^{NTR}$ expression but failed to mediate cell death, leading to an investigation of the functional role of $p75^{NTR}$ after elevation by IL-1β. We found that $p75^{NTR}$ elevated by IL-1β promoted proNGF-mediated neuronal death. IL-1β recruited both $p75^{NTR}$ and sortilin to the plasma membrane, making the neurons more vulnerable to cell death upon proNGF stimulation.

IL-1β increases $p75^{NTR}$ and NGF *in vivo*

The induction of proinflammatory cytokines following injury is responsible for various cellular functions such as inflammation, the production of other cytokines, growth factors, and neurotrophins. The current study has shown increased levels of $p75^{NTR}$ following direct infusion of IL-1β into the hippocampus without injury, suggesting that increased $p75^{NTR}$ in response to seizure or in disease may be due to a direct effect of proinflammatory cytokines that are elevated. We also found that IL-1β itself was not sufficient to mediate cell death *in vivo* even with the elevated $p75^{NTR}$ expression, suggesting that $p75^{NTR}$ induction itself is insufficient to induce cell death. IL-1β has previously been shown to induce NGF mRNA in the brain, but the form of the NGF protein induced had not previously been determined. We confirmed the increase in NGF mRNA, and demonstrated that

mature NGF, but not proNGF, was secreted following IL-1β treatment.

IL-1β exacerbates proNGF-mediated hippocampal neuron death

Since IL-1β can exacerbate neuronal death after injury, we investigated whether the increases in $p75^{NTR}$ expression might contribute to enhanced cell death in IL-1β primed neurons. Interestingly, priming the neurons with IL-1β increased vulnerability to proNGF, but not NGF, in hippocampal neurons.

In order for proNGF to activate $p75^{NTR}$ mediated cell death, sortilin is required as a coreceptor together with $p75^{NTR}$ (Nykjaer et al., 2004). In this study, we showed that IL-1β recruits sortilin to the cell surface as well as $p75^{NTR}$. Sortilin is mostly present in the cytosol (Sarret et al., 2003), and IL-1β induction of sortilin trafficking to plasma membrane may be a crucial factor to cause the $p75^{NTR}$ mediated cell death observed following injury. We also examined the mechanisms by which IL-1β recruited sortilin to cell surface. A recent study demonstrated that NRH2, a homolog of $p75^{NTR}$, can regulate sortilin trafficking to the cell surface (Kim and Hempstead, 2009). Here we demonstrated that IL-1β induced NRH2 as well as $p75^{NTR}$, and the induction of NRH2 was required for sortilin surface translocation. Interestingly, NRH2 knockdown also blocked the

$p75^{NTR}$ surface localization although it did not affect the increased total $p75^{NTR}$ expression observed following IL-1β treatment. Since NRH2 has been reported to associate with both p75 and sortilin (Kim and Hempstead, 2009), NRH2 may direct both receptors to the cell surface. Furthermore, coexpression of NRH2 and $p75^{NTR}$ has been reported in subpopulations of cells where proNGF-mediated cell death was elicited (Kanning et al., 2003; Murray et al., 2004). Thus, it is highly plausible that IL-1β may regulate NRH2 as well as $p75^{NTR}$, to facilitate proNGF mediated cell death after injury or in disease by increasing surface level of these receptors.

Consequences of $p75^{NTR}$ induction following IL-1β treatment

We confirmed that direct infusion of IL-1β into the hippocampus of the rat brain increased NGF mRNA levels, consistent with previous studies (Spranger et al., 1990). Although many studies have shown that IL-1β elicits induction of NGF mRNA, those studies did not clarify the forms of NGF protein that were produced. Here, we have shown that IL-1β caused the release of mature NGF, not proNGF, in contrast to what we have observed after seizures and what has been detected in disease. Our previous studies showed that seizure-induced injury

caused reduced activity of the proNGF processing enzymes, tPA and MMP7 (Le and Friedman, 2012). In contrast, IL-1β increased tPA activity, which activates plasmin, in turn enhancing proNGF cleavage. Since injury induces many changes in the brain in addition to the induction of cytokines, it appears that IL-1β alone is sufficient to induce $p75^{NTR}$ expression, but not the alteration in proNGF processing enzymes required to stabilize proNGF in the extracellular environment. In fact, IL-1β elevated activity of tPA, resulting in the increase of mature NGF, not proNGF, in the extracellular environment. It is likely that the presence of NGF rather than proNGF fails to elicit $p75^{NTR}$ mediated cell death, such as occurs in the hippocampus after injury or in AD. Although this study demonstrated that IL-1β is involved in $p75^{NTR}$ regulation following injury or in disease, the elevated $p75^{NTR}$ by IL-1β was not sufficient to induce cell death in the uninjured brain. These results indicate that additional mechanisms are likely to be involved in regulating proNGF processing enzymes to stabilize proNGF following injury or in disease. Moreover, it has been reported that proNGF isolated from Alzheimer's patients is highly glycosylated and more stable than proNGF from normal individuals (Pedraza et al., 2005), suggesting possible additional modification mechanisms for proNGF.

Whereas the majority of studies have demonstrated that IL-1β alone does not initiate damage in healthy cells, IL-1β is known to exacerbate neuronal death with other cytokines (Chao et al., 1995; Hu et al., 1997) or after injury (Yamasaki et al., 1995; Lawrence et al., 1998). However, the mechanisms by which IL-1β contributes to neuronal degeneration have been unknown. Here, we demonstrate that IL-1β may sensitize neurons and make them more vulnerable to proNGF mediated cell death by increasing the availability of the $p75^{NTR}$/sortilin complex at the plasma membrane. Specifically, recruiting sortilin to cell surface favors proNGF mediated $p75^{NTR}$ activation. Interestingly, this correlates with the environmental changes in AD where increased levels of IL-1β and proNGF have been reported (Griffin et al., 1989; Fahnestock et al., 2001). Thus, IL-1β may serve as a key switch altering the ratio of receptors in the cell surface following injury or in disease; thereby supporting proNGF mediated signaling. Overall, this study suggests that the exposure to inflammatory cytokines following injury or in disease may alter the surface levels of $p75^{NTR}$ and sortilin and may exacerbate neuronal death by creating a cellular environment more vulnerable to proNGF mediated neuronal death following injury or in neurodegenerative disease.

Figure legends

Figure 1. Unilateral IL-1β infusion increases $p75^{NTR}$ expression *in vivo*

Rats were cannulated 7 days before infusion with IL-1β (10ng). **A**. $p75^{NTR}$ mRNA is induced by IL-1β. Tissue was taken after 4 hr treatment with IL-1β (mean±SEM, n = 3). Asterisk denotes difference from saline ($p<0.05$). **B**. 2 days after the infusion, each hippocampus was taken for Western blot assay. $p75^{NTR}$ expression was increased with IL-1β infusion. Quantification of blots from three different experiments and densitometric values were normalized to actin and are expressed relative to the saline control (CTRL). Error bars represent SEM. * $P<0.05$ relative to CTRL, two-tailed t test. **C**. 2 days after the IL-1β infusion, brains were perfused, sectioned through the hippocampus, immunostained with anti-$p75^{NTR}$ (green) and anti-NeuN (red). IL-1β infusion increased $p75^{NTR}$ expression (arrows) in the CA1 region of the hippocampus (right column) compared to saline infusion (left column). Scale bars = 50μm. n=6.

Figure 2. IL-1β is not sufficient to mediate cell death *in vivo*

A. TUNEL staining showed negative labeling with both saline (left column) and

IL-1β (right column) infusion, suggesting that IL-1β itself does not directly elicit cell death. Double staining for TUNEL (green) and Hoechst (blue). Images are representative of two independent experiments. **B**. Positive control TUNEL assay with DNase-I. Scale bars = 50μm.

Figure 3. IL-1β primed neurons are more vulnerable to proNGF than NGF

A. Bars show relative cell number (mean±SEM, n = 4 independent experiments) in hippocampal neuronal cultures. Cells were treated for 4-6 h with IL-1β (10ng/mL), followed by overnight treatment with NGF (100ng/mL). IL-1β did not exacerbate NGF-mediated neuronal death. Asterisks denote difference from untreated control ($p < 0.05$). **B**. Bars indicate relative cell number (mean±SEM, n = 4 independent experiments) in hippocampal neuronal cultures treated with IL-1β (10 ng/mL) for 4-6 hrs, followed by proNGF (1-10ng/mL). ProNGF elicited more cell death in IL-1β primed neurons. * denotes difference from control and # indicates difference from proNGF alone ($p<0.05$; one-way ANOVA and Tukey's post hoc analysis).

Figure 4. IL-1β recruits sortilin and $p75^{NTR}$ receptors to the plasma membrane.

A. Cultured hippocampal neurons were treated with IL-1β for 8h, incubated with biotin for 1h, and lysates were precipitated with streptavidin. Biotinylated cell surface protein and nonbiotinylated intracellular proteins were analyzed by Western blotting for sortilin, $p75^{NTR}$ and transferrin receptor. Blots were stripped and re-probed for actin, which was only present in the intracellular fraction. Quantification of sortilin (**B**) and $p75^{NTR}$ (**C**) from three independent blots. Asterisk denotes difference from control ($p<0.05$; one-way ANOVA and Tukey's post hoc analysis).

Figure 5. IL-1β-mediated NRH2 induction facilitates surface localization of sortilin and p75

A. Cultured hippocampal neurons were treated with IL-1β (1 or 10 ng/ml) for 6h, and were then lysed and analyzed by Western blot for NHR2, $p75^{NTR}$, and actin. 10ng/mL of IL-1β increased NRH2 expression as well as $p75^{NTR}$ expression. Quantification of blots from three experiments and densitometric values were normalized to actin and are expressed relative to the untreated cells (time 0). * indicates values significantly different from control ($p<0.05$; one-way ANOVA and Tukey's post hoc analysis). **B**. Neurons were treated with a

penetratin-linked NRH2 siRNA (NRH2si) or penetratin-linked luciferase siRNA as control (CTsi) with or without IL-1β treatment. NRH2 siRNA specifically inhibited the induction of NRH2, but not $p75^{NTR}$, by IL-1β. Quantification of blots from three experiments and densitometric values were normalized to actin and are expressed relative to the control. * indicates values significantly different from control (CTsi, C) at $p<0.05$. **C**. Neurons were treated with either NRH2 siRNA or control siRNA with or without the addition of IL-1β (10ng/mL) for 6 h, and incubated with biotin for 1h. Cell lysates were precipitated with streptavidin. Biotinylated cell surface protein and nonbiotinylated intracellular proteins were analyzed by Western blotting for sortilin, $p75^{NTR}$ and transferrin receptor.

Figure 6. IL-1β induces increased secretion of mature NGF

A. Bars indicate NGF mRNA level (mean±SEM, n = 3) by qPCR after 4 h treatment with IL-1β. Asterisk denotes difference from saline ($p<0.05$). **B**. Western blots show that mature NGF was detected in the Cerebrospinal fluid (CSF) collected from IL-1β infused rats compared to saline-infused controls. Blot is representative of three independent experiments. **C**. In situ zymogram showed

increased tPA activity with IL-1β infusion. Fresh frozen sections were covered with an in situ zymogram assay buffer containing 20 μg/mL plasminogen. Areas of tPA activity was detected in block spots on dark field of views. Images are representative of two independent experiments. Scale bars = 250μm.

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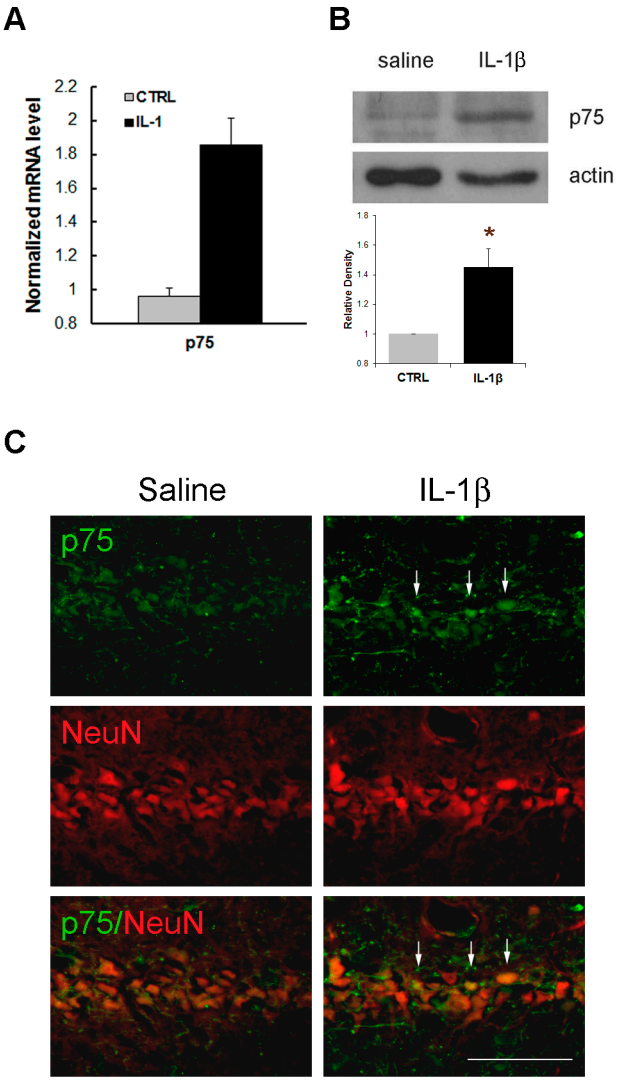
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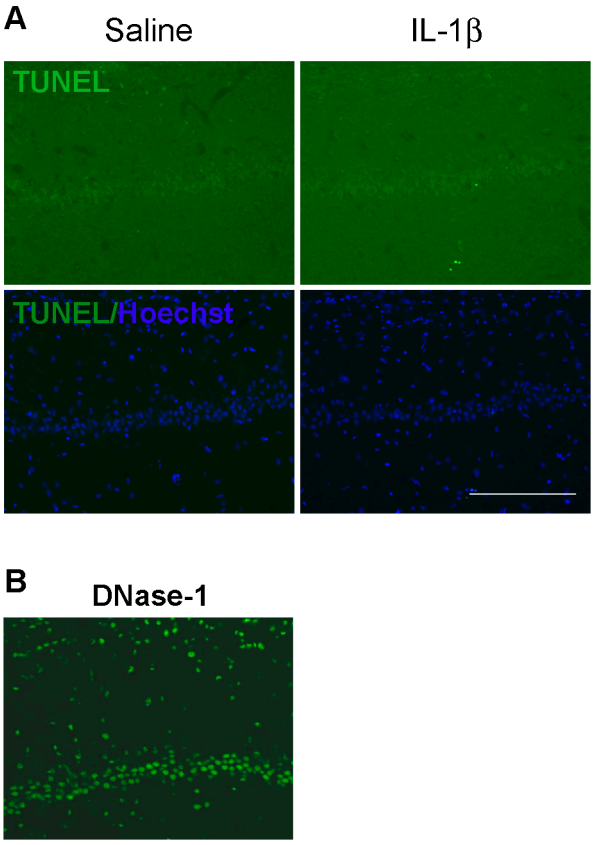
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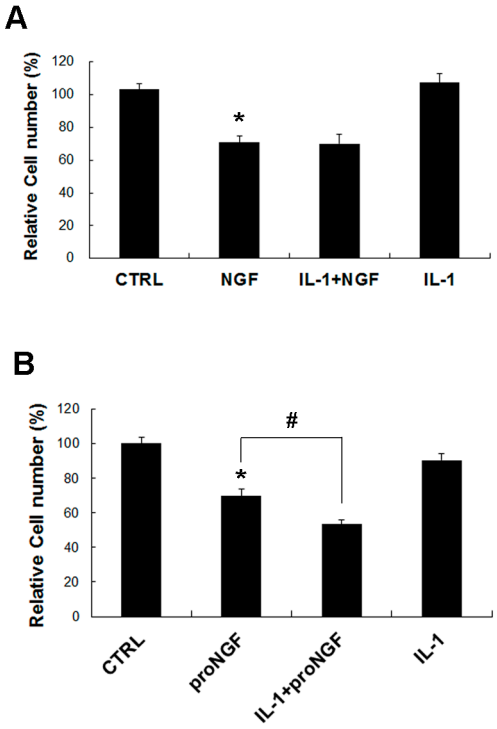
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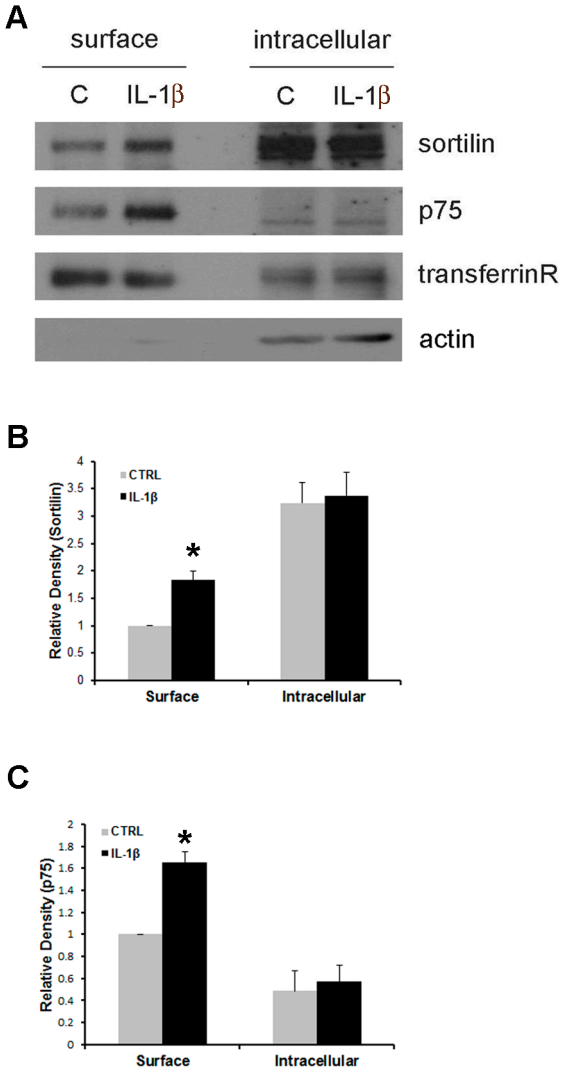
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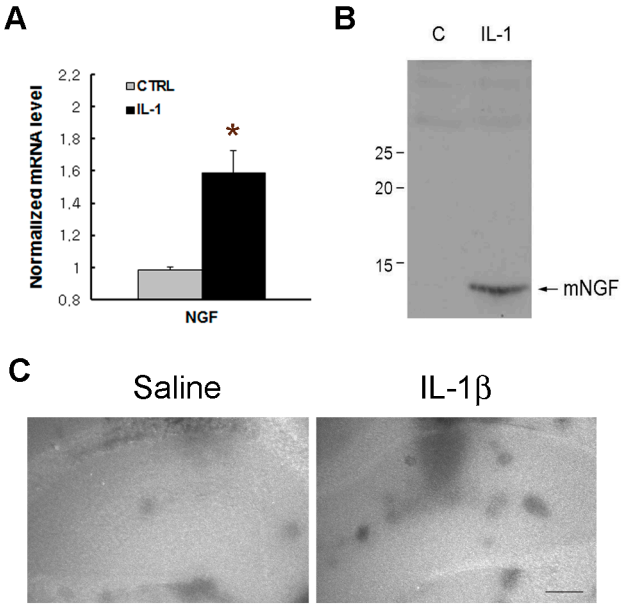
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