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Interleukin-1 β enhances neuronal vulnerability to proNGF-mediated apoptosis by increasing surface expression of p75^{NTR} and sortilin

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Abstract

Many types of injury such as seizure, ischemia, and oxidative stress cause upregulation of the p75 neurotrophin receptor (p75^{NTR}) 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-1 β (IL-1 β) are highly produced under these injury conditions and, in particular, are expressed rapidly in the rat hippocampus after seizure. IL-1 β 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-1 β infusion into the brain induces p75^{NTR} in neurons of the CA1 area of the hippocampus. While IL-1 β induction of p75^{NTR} is not sufficient to induce cell death, we demonstrate that IL-1 β 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-1 β exacerbates neuronal death following injury.

Keywords

interleukin-1; neurotrophin; brain injury; apoptosis; NGF; p75

IL-1 β 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-1 β 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 β exacerbates traumatic, ischemic or excitotoxic stimulated neurotoxicity (Yamasaki et al., 1995, Patel et al., 2003), and blocking IL-1 β with the receptor antagonist (IL-1ra) attenuates neuronal loss (Allan et al., 2005), suggesting that IL-1 β indirectly contributes to neuronal injury.

Our lab has recently reported that proinflammatory cytokines such as IL-1 β and TNF α regulate expression of the p75 neurotrophin receptor (p75^{NTR}) both in neurons and

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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-1 β 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-1 β may be involved in neurodegeneration by regulating the p75^{NTR} as well as ligands that promote neuronal death.

In this study, IL-1 β 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-1 β treatment and show that IL-1 β specifically exacerbated proNGF induced hippocampal neuronal death by recruiting the p75^{NTR} and sortilin receptors to the cell surface. This study, therefore, identifies a mechanism by which IL-1 β may enhance neuronal vulnerability following brain injury.

Experimental procedures

Materials

Human recombinant IL-1 β was a gift from Dr. Ron Hart, (Rutgers University, Piscataway, NJ) and human 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 = $\pm$ 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, 10ng (in 0.5 μ l) of IL-1 β was infused unilaterally into the hippocampus via the guide cannula at a rate of 0.5 μ l/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-1 β 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 μ m) 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 μ g/ml; Sigma) was added into PBS during the last wash to label nuclei. Cell death was examined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining following manufacturer's manual (Roche, Mannheim, Germany). Sections were mounted with anti-fading medium (ProLong Gold; Invitrogen) and were examined by fluorescence microscopy (Nikon). Images were obtained using MetaMorph and figures were assembled in Adobe Photoshop.

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).

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^{TM} 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'-TCACCATATCCGCGCACTGTA-3'), NGF (rat, forward: 5'-CAAGGACGCAGCTTTCTATCCTG-3', reverse: 5'-CTTCAGGGACAGAGTCTCCCTCT-3'), or actin (forward: 5'-TCATGAAGTGTGACGTTGACATCCGT-3', reverse: 5'-CTTAGAAGCATTGCGGTG 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% CO₂ 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 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 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 (biotinylated surface protein) and supernatants (intracellular pool) were analyzed by Western blot for p75^{NTR} (Upstate), sortilin (BD), and transferrin receptor (Invitrogen).

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 activity were detected as black spots on dark field of views, where the endogenous enzyme degraded the substrate. Quantification is expressed as the surface area of black spots, indicating enzymatic activity, relative to the total surface area of the hippocampus.

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), and cell counts indicate that the number of cells expressing p75^{NTR} was increased by IL-1β infusion (figure 1D). 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 saline

nor IL-1 β infusion induced TUNEL positive cells (Fig. 2), which is consistent with many studies demonstrating that IL-1 β alone does not induce neuronal cell death (Lawrence et al., 1998). The positive control of DNase I treatment showed that the assay would detect apoptotic neurons (figure 2B). Since IL-1 β infusion induced neuronal expression of p75^{NTR} but no apoptosis, these findings suggest that p75^{NTR} induction alone is not sufficient to mediate cell death.

IL-1 β releases NGF into the CSF

IL-1 β is also known to regulate NGF expression in the brain (Spranger et al., 1990, Yasuda et al., 2007), however those studies did not investigate which form of the NGF protein was secreted. We confirmed the *in vivo* regulation of NGF mRNA by IL-1 β in tissue samples taken from the dorsal hippocampus after IL-1 β or saline infusion analyzed by quantitative RT-PCR. Consistent with previous studies, IL-1 β increased NGF mRNA levels compared to saline (Fig 3A). Since proNGF has been shown to be involved in cell death as a ligand for p75^{NTR} following injury (Beattie et al., 2002, Volosin et al., 2008), we examined which forms of NGF were secreted following infusion of IL-1 β compared to untreated animals and to rats following pilocarpine-induced seizures. The cerebrospinal fluid (CSF) was collected and analyzed by Western blot. The CSF sample taken from IL-1 β infused rats showed no change in proNGF and increased levels of mature NGF compared to the CSF samples from control rats (Fig. 3B), while the seizure rats showed increased proNGF without cleavage to mature NGF in the CSF. Basal levels of proNGF were detected in control and IL-1 β -infused rats, suggesting that IL-1 β , in the absence of injury, increased NGF expression and the subsequent increased secretion of mature NGF, but not proNGF which is induced by seizures (Volosin et al., 2008) and other types of injury (Beattie et al., 2002).

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-1 β may alter proNGF processing enzyme activity to result in increased mature NGF rather than proNGF, *in situ* zymography for MMP7 and tPA was performed. In contrast to the seizure-induced injury, IL-1 β infusion showed no change in MMP7 activity (not shown), and increased tPA activity compared to the saline infused side (Figure 3C), suggesting that IL-1 β promotes tPA-mediated cleavage of proNGF to NGF. Taken together, these data suggest that IL-1 β induces p75^{NTR} and NGF expression, but does not prevent proNGF cleavage *in vivo* and therefore by itself is not sufficient to induce cell death.

IL-1 β exacerbates proNGF mediated hippocampal neuron death

IL-1 β increased the activity of tPA, a proNGF-cleaving enzyme, *in vivo*, preventing the ability to co-infuse IL-1 β and proNGF into the brain. Therefore, we used cultured neurons to investigate the functional consequences of IL-1 β -mediated p75^{NTR} upregulation. Hippocampal neurons express the p75^{NTR} and sortilin receptors (figure 4A), both of which are necessary for proNGF-induced apoptosis {Volosin, 2006 #1137}. Neurons were pretreated with IL-1 β 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. 4), consistent with previous studies (Friedman, 2000, Volosin et al., 2008). Interestingly, whereas IL-1 β did not affect NGF mediated cell death (Fig. 4B), IL-1 β pretreatment exacerbated proNGF induced cell death (Fig. 4C), suggesting that this cytokine may make neurons selectively more vulnerable to proNGF.

IL-1 β 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-1 β treatment. Biotinylation assays showed increased sortilin and p75^{NTR} on the cell surface in IL-1 β primed neurons (Fig. 5), while transferrin receptor levels were unchanged by IL-1 β treatment. These results suggest that IL-1 β elicits selective recruitment of sortilin and p75^{NTR} to the plasma membrane, which increases vulnerability to proNGF treatment.

Discussion

Many changes occur in the brain following injury or in neurodegenerative disease that contribute to neuronal loss, including the induction of proinflammatory cytokines. In this study we have specifically investigated the effects of IL-1 β in the absence of the numerous other changes associated with brain injury. Recently, our lab has demonstrated that IL-1 β increased the expression of p75^{NTR} in hippocampal neurons in culture, and our previous studies demonstrated that induction of p75^{NTR} in hippocampal neurons contributes to neuronal loss following seizure-induced injury (Troy et al., 2002). Moreover, seizures caused an alteration in the activity of the enzymes that regulate proNGF cleavage, leading to the stabilization of proNGF in the extracellular environment, which was crucial for neuronal death to occur (Le and Friedman, 2012). Additional studies have demonstrated that p75^{NTR} induced after many types of injury or in pathological disease 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. Since the induction of proinflammatory cytokines, specifically IL-1 β , 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-1 β 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-1 β . We found that IL-1 β recruited both p75^{NTR} and sortilin to the plasma membrane, making the neurons more vulnerable to cell death upon proNGF stimulation.

IL-1 β 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-1 β 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-1 β 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-1 β has 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 increased mature NGF, but not proNGF, was secreted following IL-1 β treatment. Moreover, IL-1 β alone was not sufficient to elicit the changes in the activity of proNGF cleaving enzymes seen following seizure that are necessary to stabilize proNGF in the extracellular environment (Le and Friedman, 2012). We had previously demonstrated that seizures caused a decrease in MMP7 activity, leading to decreased proNGF processing. In contrast, IL-1 β alone evoked no change in MMP7 activity, and actually increased tPA activity, which can also cleave proNGF (Lee et al., 2007). Since IL-1 β alone, in the absence of injury, did not inhibit the activity of the extracellular proNGF cleaving enzymes, proNGF could not be administered to the brain *in vivo* to assess the consequences of IL-1 β -mediated p75^{NTR} induction, therefore this analysis was performed on cultured hippocampal neurons.

IL-1 β exacerbates proNGF-mediated hippocampal neuron death

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

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-1 β recruits both sortilin and p75^{NTR} to the cell surface. Sortilin is mostly present in the cytosol (Sarret et al., 2003), and IL-1 β induction of sortilin trafficking to the plasma membrane may be a crucial factor for proNGF to cause the p75^{NTR}-mediated cell death observed following injury.

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

We confirmed that direct infusion of IL-1 β 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-1 β elicits induction of NGF mRNA, those studies did not clarify the forms of NGF protein that were produced. Here, we have shown that IL-1 β caused and increase in 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 (Le and Friedman, 2012). In contrast, IL-1 β 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-1 β 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-1 β 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-1 β is involved in p75^{NTR} regulation that can occur following injury or in disease, the elevated p75^{NTR} by IL-1 β 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 in injury or disease conditions. 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.

Many studies have demonstrated that IL-1 β alone does not initiate damage in healthy cells, although IL-1 β 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-1 β contributes to neuronal degeneration have been unknown. Here, we demonstrate that IL-1 β 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-1 β and proNGF have been reported (Griffin et al., 1989, Fahnstock et al., 2001). Thus, IL-1 β 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.

Conclusions

IL-1 β is a critical inflammatory cytokine induced in the brain that exacerbates neuronal loss following injury and in disease. Our study suggests that a mechanism by which IL-1 β can enhance neuronal vulnerability is by increasing the surface levels of p75^{NTR} and sortilin, thereby creating a cellular environment more vulnerable to proNGF-induced neuronal death following injury or in neurodegenerative disease.

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Highlights

- IL-1 β induces p75^{NTR} expression in hippocampal neurons *in vivo*
- IL-1 β increases tPA activity *in vivo*
- IL-1 β promotes secretion of NGF, not proNGF, *in vivo*
- IL-1 β enhances surface localization of p75^{NTR} and sortilin in hippocampal neurons
- IL-1 β increases vulnerability without directly inducing neuronal death

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

Rats were cannulated 7 days before infusion with IL-1 β (10ng). **A.** p75^{NTR} mRNA was induced by IL-1 β . Tissue was taken after 4 hr or 1d treatment with IL-1 β (mean $\pm$ 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-1 β 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-1 β infusion, brains were perfused, sectioned through the hippocampus, immunostained with anti-p75^{NTR} (green) and anti-NeuN (red). IL-1 β infusion increased p75^{NTR} expression (arrows) in the CA1 region of the hippocampus (right column) compared to saline infusion (left column). Scale bars = 50 μ m. n=6. **D.** Counts of p75^{NTR}-positive cells indicated a 3-fold increase following IL-1 β infusion.

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

A. TUNEL staining showed negative labeling with both saline (left column) and IL-1 β (right column) infusion, indicating that IL-1 β 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 = 50 μ m.

Figure 3. IL-1 β induces increased secretion of mature NGF

A. Bars indicate NGF mRNA level (mean $\pm$ SEM, $n = 3$) by qPCR after 4 h treatment with IL-1 β . Asterisk denotes difference from saline ($p < 0.05$). **B.** Western blots show cerebrospinal fluid (CSF) collected from IL-1 β infused rats compared to saline-infused controls or rats after pilocarpine-induced seizures. A short exposure shows the seizure-induced increase in proNGF without being cleaved, while the longer exposure shows the IL-1 β -induced increase in mature NGF in the CSF. Blot is representative of three independent experiments. **C.** In situ zymogram shows increased tPA activity with IL-1 β infusion. Fresh frozen sections were covered with an in situ zymogram assay buffer containing 20 μ g/mL plasminogen. Areas of tPA activity are indicated by black spots on a bright background. Images are representative of two independent experiments. Scale bars = 250 μ m. **D.** Quantification of the area showing tPA activity relative to the entire hippocampus in the control and IL-1 β infused hippocampus. Asterisk indicates significantly different from control by Student's t -test, $p < 0.01$.

Figure 4. IL-1 β primed neurons are more vulnerable to proNGF than NGF

A. Immunostaining of hippocampal neurons for sortilin and p75^{NTR} demonstrating colocalization of the receptors. **B.** Bars show relative cell number (mean $\pm$ SEM, $n = 4$ independent experiments) in hippocampal neuronal cultures. Cells were treated for 4–6 h with IL-1 β (10ng/mL), followed by overnight treatment with NGF (100ng/mL). IL-1 β did not exacerbate NGF-mediated neuronal death. Asterisks denote difference from untreated control ($p < 0.05$). **C.** Bars indicate relative cell number (mean $\pm$ SEM, $n = 4$ independent experiments) in hippocampal neuronal cultures treated with IL-1 β (10 ng/mL) for 4–6 hrs, followed by proNGF (1–10ng/mL). ProNGF elicited more cell death in IL-1 β 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 5. IL-1 β recruits sortilin and p75^{NTR} receptors to the plasma membrane

A. Cultured hippocampal neurons were treated with IL-1 β for 8h, incubated with biotin for 1h, and lysates were precipitated with streptavidin. Total biotinylated cell surface protein and 20 μ g of non-biotinylated intracellular proteins were analyzed by Western blot 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).