INTERLEUKIN-1 MEDIATED CELL-TYPE SPECIFIC SIGNALING IN

HIPPOCAMPAL NEURONS AND ASTROCYTES

BY

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ABSTRACT OF THE THESIS

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Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that is implicated in immune and inflammatory responses. In the central nervous system (CNS), IL-1β is synthesized and released during injury, infection, and many neurodegenerative diseases, but also under physiological conditions. Several IL-1-mediated signaling pathways and effects have been identified in hippocampal neurons and astrocytes, but their mechanisms have not been fully defined. IL-1 signaling requires the type one IL-1 receptor (IL-1RI) as well as IL-1 receptor accessory protein (IL-1RAcP) as a receptor partner. A novel isoform of the IL-1 receptor accessory protein, AcPb, has also been found in the CNS, but its role remains unclear. This thesis examined AcPb function in regulating IL-1β signaling. The results showed that IL-1β activated p38 MAPK but not NFκB in neurons. In astrocytes, IL-1β induced both p38 and NFκB pathways in regulating inflammatory responses. AcPb was not involved in mediating either p38 or NFκB in either cell type. In contrast, a physiological level of IL-1β treatment (0.01ng/ml) activated p-Src in neurons via AcPb *in vitro*. In addition, overexpression of AcPb in astrocytes was sufficient to induce p-Src mediated by IL-1β. Taken together, these results suggest that the restricted expression of AcPb in CNS neurons may mediate neuronal specific IL-1 pathways and outcomes, and that physiological and pathophysiological levels of IL-1β mediate particular neuronal functions via separate pathways.

Table of contents

Introduction

Inflammatory responses in the central nervous system (CNS) are associated with injury, infection and many acute and chronic neurodegenerative diseases such as stroke, Parkinson's, multiple sclerosis and Alzheimer's (Mrak and Griffin, 2001). During inflammation, pro-inflammatory mediators such as cytokines and chemokines are synthesized and released by local and invading immune cells. Interleukin-1 (IL-1) is one of the key pro-inflammatory cytokines released mainly by microglia (Giulian et al., 1986; Pearson et al., 1999) and astrocytes (Davies et al., 1999; Pearson et al., 1999) in response to CNS inflammation. IL-1 mediates inflammatory activity by stimulating release of pro-inflammatory cytokines and growth factors from astrocytes and microglia (Merrill and Benveniste, 1996). In the hippocampus, IL-1 acts upon neurons to inhibit synaptic strength and long-term potentiation (Katsuki et al., 1990; Bellinger et al., 1993; Murray and Lynch, 1998). The distinct functions of IL-1 are mediated through the same type I IL-1 receptor (IL-1RI), which is expressed and regulated by IL-1 in both hippocampal neurons and astrocytes (Friedman, 2001). However, the signaling pathways of IL-1 in these two cell types differ (Srinivasan et al., 2004). The mechanism behind the cell-type-specific IL-1 signaling is unknown and remains to be investigated.

IL-1R accessory protein (IL-1RAcP) is a required receptor partner in IL-1 signaling (Cullinan et al., 1998). AcPb, a novel isoform of IL-1RAcP, was recently identified to be mainly expressed in CNS (Smith et al., 2009). In this project, we will investigate the IL-1β mediated cell-type specific signaling pathways in hippocampal neurons and astrocytes and the role of AcPb in each pathway.

1. IL-1 cytokine family

Interleukin-1 is a family of cytokines that regulates immune and inflammatory reactions in a variety of cell types. The IL-1 gene family consists of three proteins: IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1ra). IL-1α and IL-1β are both receptor agonists that are synthesized as 33 kDa precursors and processed into mature forms of 17 kDa proteins by specific proteases (Giri et al., 1985). Although IL-1α and IL-1β are encoded by two distinct genes, they share sequence homology and structural similarity and bind to the same receptor, hence eliciting similar biological effects. In addition to IL-1α and IL-1β, IL-1ra, a natural IL-1 receptor antagonist, also maps to the IL-1 gene cluster on the long arm of human chromosome 2q, binding to IL-1 receptor, but not eliciting any actions. IL-1 is well known to be synthesized by multiple types of immune cells including monocytes, macrophages and neutrophils in the periphery, and it is involved in many types of inflammatory diseases such as rheumatoid arthritis (RA), periodontitis, diabetes mellitus, systemic sclerosis and cardiovascular diseases (Dinarello, 1994, 2005; Arend et al., 2008).

1.1 IL-1α

ProIL-1α is the biologically active precursor of IL-1α, which can bind to IL-1RI to activate cell signaling (Mosley et al., 1987). ProIL-1α lacks a signaling peptide (Gubler et al., 1986) and mostly remains in the cytosol after being synthesized. It is released and converted into mature IL-1α by proteases when cells die or are dying (Kobayashi et al., 1988). ProIL-1α is also processed by membrane bound proteases called calpains and released from cells in a calcium-dependent manner (Kobayashi et al., 1990; Carruth et al., 1991). The extracellular protein concentration of IL-1α is very low

except in severe diseases when it is released from dying cells or cleaved by calpains (Dinarello, 1996). Intracellular IL-1α expression is therefore thought to correlate with disease severity. A recent study has shown that IL-1α is a key mediator that is released from necrotic cells to provoke inflammation (Chen et al., 2007a). In addition to being secreted and exerting its actions extracellularly, proIL-1α also contains a functional nuclear localization sequence (NLS) and can be actively imported to the nucleus from the cytosol (Wessendorf et al., 1993). Intranuclear IL-1α has been reported to regulate gene expression and RNA splicing, leading to regulation of cell proliferation, migration and apoptosis in different cell types (Luheshi et al., 2009).

1.2 IL-1β

IL-1β is synthesized as proIL-1β and intracellularly cleaved to its mature form before being released from the cell (Gunther et al., 1989). Lacking a signaling peptide like IL-1α , IL-1β uses a unique translocation pathway that bypasses the traditional endoplasmic reticulum (ER) and Golgi route (Rubartelli et al., 1990). Unlike proIL-1α, proIL-1β is biologically inactive (Mosley et al., 1987; Gunther et al., 1989). IL-1β – converting enzyme (ICE), also known as caspase-1, is the most common protease that intracellularly processes IL-1β (Kostura et al., 1989; Dinarello, 1998). Recent studies also show that IL-1β precursor can be cleaved by caspase-8 under certain inflammatory conditions caused by lipopolysaccharide (LPS) or Poly (I:C) via toll-like receptor 3 (TLR 3) or TLR 4 activation (Maelfait et al., 2008). IL-1β is easily secreted compared to IL-1α, which is mainly cell-bound. The cleavage process and release of IL-1β requires a secondary extracellular stimulus, typically ATP, to activate the cell via surface receptor P2X7 (Solle et al., 2001). P2X7 receptors are ATP-gated ion channels that are widely

expressed on microglial, but not neuronal, membranes in the CNS (Sim et al., 2004). The expression of P2X7 in microglia is upregulated after cerebral occlusion (Melani et al., 2006) or in chronic CNS diseases such as Alzheimer's (Parvathenani et al., 2003; McLarnon et al., 2006). Microglia are known to be the main source of IL-1 in the CNS, and the unique expression of P2X7 in microglial cells may be involved in the regulation of IL-1β release. Under normal conditions, expression levels of IL-1β are very low and do not have a neurotoxic effect on brain tissue (Vitkovic et al., 2000). However, it can be rapidly induced by pro-inflammatory cytokines, such as Tumor Necrosis Factor alpha (TNFα), Interleukin-6 (IL-6) and IL-1β itself, or by lipopolysaccharide (LPS) in glial cells, both in vitro (Hetier et al., 1988; Lieberman et al., 1989) and in vivo by inflammatory processes or injury (Davies et al., 1999; Depino et al., 2005). The released mature IL-1β is able to bind to IL-1 receptors and remains biologically active. In fact, most known effects of IL-1β are mediated by IL-1β extracellularly binding to IL-1RI. ProIL-1β is also found in cell nuclei (Stevenson et al., 1992), but no intranuclear actions have been reported.

1.3 IL-1ra

IL-1ra was first identified as a natural IL-1 inhibitor from febrile patients' urine (Liao et al., 1984). IL-1ra is also synthesized from its precursor form, pro-IL-1ra. Unlike IL-1α or IL-1ß, pro-IL-1ra has a signaling peptide that can direct its secretion (Eisenberg et al., 1990). IL-1ra shares high amino acid homology with IL-1β and also has a high binding affinity to IL-1RI (Seckinger et al., 1987). However, IL-1ra is a natural antagonist that does not activate cells (Hannum et al., 1990; Dripps et al., 1991), probably due to the lack of a secondary binding site to the extracellular immunoglobin domains of

IL-1R (Evans et al., 1995). As a negative regulator in IL-1 signaling, IL-1ra is beneficial for a number of diseases caused by imbalances between IL-1β and IL-1ra, such as arthritis, diabetes mellitus and cardiovascular diseases (Arend et al., 2008). In the CNS, IL-1ra treatment has been shown to improve clinical outcome in patients with acute stroke (Emsley et al., 2005).

2. IL-1 receptors and receptor accessory proteins

To date, two primary types of IL-1 receptors have been identified: IL-1 receptor type I (IL-1RI) and IL-1 receptor type II (IL-1RII). Although the two IL-1 receptors are coded from two distinct genes, they share a significant homology (Sims et al., 1995). The extracellular sequence of both IL-1Rs contain three IgG-like domains for IL-1 binding, hence they are classified as members of the Ig superfamily.

The intracellular domain of IL-1RI was originally found to share sequence homology with the cytosolic domain of the *drosophila* protein Toll (Gay and Keith, 1991). Extracellularly, Toll has leucine-rich repeats (LRRs) instead of three Ig domains. Interestingly, they activate similar downstream proteins to regulate various functions: IL-1R activates IRAK and IκB/NFκB to regulate pro-inflammatory genes, while Toll activates a protein kinase Pelle (IRAK homologue) and Cactus (IκB homologue)/Dorsal (NFκB homologue) to regulate dorsoventral polarity (Letsou et al., 1991; Shelton and Wasserman, 1993). N protein, found in tobacco, was also shown to share a signaling domain with IL-1RI and Toll (Whitham et al., 1994). This domain was later named Toll-IL-1 receptor domain or TIR domain (O'Neill and Greene, 1998). With the discovery of Toll-like receptors in human as well (Medzhitov et al., 1997; Rock et al., 1998), the IL-

1R family thus expanded to become the IL-1R/TLR superfamily, all of which share conserved sequence and TIR domains.

2.1 IL-1RI

IL-1RI is an 80kDa protein (Sims et al., 1988; Sims et al., 1989) and is highly glycosylated on the cell membrane. Blocking of glycosylation sites reduces the ability of the receptor to bind IL-1 (Mancilla et al., 1992; Einstein et al., 1996). The intracellular site of IL-1RI contains a TIR domain. TIR domains mostly exist on cell-surface receptors, such as IL-1RI and Toll-like receptors. Two adapter proteins, myeloid differentiation factor 88 (MyD88) and MyD88-adapter like protein, also contain TIR domains. One important functional role of TIR domains is to interact with other TIR domain-containing proteins to form signaling complexes. MyD88 is a common adapter protein that is recruited to the IL-1/IL1RI complex to relay intracellular signals (Burns et al., 1998). MyD88 is also involved in other toll-like receptor (TLR) pathways. Almost all known functions of IL-1 are mediated through IL-1RI. Furthermore, mice lacking IL-1RI do not respond to IL-1 (Glaccum et al., 1997).

2.2 IL-1RII

IL-1RII is a 68kDa protein that was first cloned from mouse and human B cells and shares 28% amino acid homology in the extracellular domains with IL-1RI (McMahan et al., 1991). IL-1RII binds to IL-1β with a much higher affinity than IL-1α and IL-1ra (Scapigliati et al., 1989; McMahan et al., 1991). However, IL-1RII has a very short intracellular sequence that does not contain a TIR domain (McMahan et al., 1991) and is not able to recruit any downstream adapter proteins or relay cell signals. In

addition, IL-1RII and IL-1RI do not form heterodimeric complexes and they each bind to IL-1 independent of each other (Slack et al., 1993). Therefore, IL-1RII is also called a "decoy receptor" and serves as a negative regulator in the complex IL-1 signaling pathway (Stylianou et al., 1992; Colotta et al., 1993; Sims et al., 1993). The extracellular domain of IL-1RII can also be cleaved by proteases and exists as a soluble receptor form (sIL-1RII) (Arend et al., 1994). The shedded soluble IL-1RII is able to bind to interleukin-1 ligands, especially IL-1β (Arend et al., 1994), to sequester ligand and negatively regulate IL-1 signaling.

2.3 IL-1RAcP

Another functionally critical receptor partner that has been identified on the cell membrane is the IL-1 receptor accessory protein (IL-1RAcP) (Greenfeder et al., 1995; Liu et al., 1996). IL-1RAcP, IL-1RI and IL-1RII belong to the same Ig superfamily and are each composed of three extracellular IgG-like domains. The predicted molecular weight of the first identified murine IL-1RAcP is 66kDa with seven potential extracellular glycosylation sites (Greenfeder et al., 1995). The cytoplasmic sequence of IL-1RAcP also contains a TIR domain. IL-1RAcP does not bind to IL-1 itself, but it has been shown to increase the binding affinity of IL-1RI to IL-1β (Greenfeder et al., 1995), stabilize the IL-1 receptor forming complex with IL-1 (Wesche et al., 1998) , and further recruits downstream adapter proteins such as IL-1R associated kinase (IRAK) (Huang et al., 1997; Volpe et al., 1997). AcP knockout studies have established that AcP is an essential component not only in IL-1 signaling (Cullinan et al., 1998), but also in IL-33 signaling (Palmer et al., 2008). One splice-variant form of IL-1RAcP, recognized as soluble IL-1RAcP, and which codes only the extracellular domain of IL1-RAcP, was

shown to exist in different tissues, mainly in the liver (Greenfeder et al., 1995). The soluble IL-1RAcP is able to increase the binding affinity of sIL-1RII to IL-1β and decrease available IL-1β concentration to IL-1RI (Smith et al., 2003); therefore it serves as another negative regulator in the complex IL-1 signal. Soluble AcP also negatively regulates IL-33 signaling (Palmer et al., 2008).

3. Expression of IL-1 and receptors in the brain

Numerous studies have shown that IL-1 and IL-1R family members are expressed in healthy brains. The expression was not only region specific, but also cell type specific. Through radioligand binding studies, RT-PCR and southern blotting, both IL-1RI and IL-1RII have been detected in mouse and rat brains, particularly in the hippocampus and hypothalamus (Farrar et al., 1987; Parnet et al., 1994). In the mouse hippocampus, IL-1 receptor mRNA was strongly detected in granule cells in the dentate gyrus and moderately detected in the pyramidal cell layer of the hilus and CA3 region by in situ hybridization (Cunningham et al., 1991). IL-1RI mRNA was also detected in rat hippocampal pyramidal neurons by immunocytochemical analysis, but not in CNS glial cells until acutely induced by stab injury (Friedman, 2001). In rat, different brain regions were also reported to express high mRNA levels of IL-1RI, such as the basolateral amygdaloid nucleus, the medial and posterior thalamic nuclei, the ventromedial hypothalamic nucleus, and the trigeminal nucleus (Yabuuchi et al., 1994a; Ericsson et al., 1995). Both mRNA and protein levels of IL-1 receptor were strongly induced in the brain following cerebral ischemic injury (Wang et al., 1997) and mechanical injury (Yan et al., 1992; Toulmond and Rothwell, 1995). *In vitro*, both hippocampal astrocytes and

neurons express low levels of IL-1RI mRNA and protein, but expression is strongly induced in the presence of pro-inflammatory cytokines TNFα and IL-1 (Friedman, 2001). In contrast, IL-1RAcP is constitutively expressed throughout the brain in excess and is not subject to regulation under inflammatory conditions (Gabellec et al., 1996).

IL-1α and β were also extensively expressed in normal brains, at both mRNA and protein levels, in different brain regions and cell types (Breder et al., 1988; Hunter et al., 1992; Pitossi et al., 1997). However, the expression is particularly high during injury or neurodegenerative diseases (Loddick et al., 1998). Both in situ hybridization (Buttini et al., 1994) and northern blot analyses (Liu et al., 1993; Wang et al., 1994) showed that IL-1β mRNA was induced within 30 minutes in rat brain cortex following cerebral ischemia. Neurons and glia in areas such as the olfactory bulb, hippocampus, striatum and hypothalamus also manifest increased IL-1β mRNA following cerebral ischemia (Yabuuchi et al., 1994b) and kainic acid treatment (Yabuuchi et al., 1993). IL-1β protein was also upregulated in the brain tissue after cerebral ischemia (Hara et al., 1997; Hillhouse et al., 1998), mechanical injury (Woodroofe et al., 1991) and during CNS infection (Waage et al., 1989). Elevated IL-1 was also found within brain lesions in animal models of Alzheimer's diseases (Lim et al., 2000), multiple sclerosis (Kennedy et al., 1992) and Creutzfeldt-Jakob disease (Kordek et al., 1997). Direct injection of Interferon gamma (IFN-γ)/LPS to the rat brain also induces IL-1β mRNA (Higgins and Olschowka, 1991). Microglia are the main source of IL-1 production in the CNS upon stress, tissue injury, infection, inflammation or many disease processes (Giulian et al., 1986; Pearson et al., 1999; Spulber and Schultzberg, 2009). Astrocytes and infiltrating macrophages in the CNS also produce IL-1β, but at a much later disease stage (Pearson et

al., 1999; Mabuchi et al., 2000). IL-1 is also produced by neurons within the brain (Dinarello, 1994).

In addition to the two IL-1 agonists, IL-1ra can also be detected in the normal hippocampus (Yasuhara et al., 1997) and hypothalamus (Diana et al., 1999). Very low levels of IL-1ra were found in healthy conditions, but were greatly increased after focal ischemia (Wang et al., 1997). The presence of IL-1ra in the brain suggests a self-regulatory IL-1 signal. Because IL-1ra is a natural antagonist, the upregulation of IL-1ra following IL-1 increase after injury can minimize long-term detrimental effects of IL-1-induced inflammation.

4. IL-1 mediated functions in the CNS

The widespread expression of IL-1 and IL-1R family members in the healthy brain suggests a necessary and important role for IL-1 in normal CNS functions. IL-1 was also implicated in acute and chronic neurodegenerative diseases as well as normal brain development and aging.

Upon infection, injury or during acute degenerative diseases, IL-1 is rapidly produced and secreted in the brain to initiate immune responses that reduce the severity of insults. These inflammatory effects are mainly mediated through glial cells. CNS glial cells respond to elevated IL-1 by producing more inflammatory cytokines, growth factors, cell surface adhesion molecules, cyclooxygenases, matrix metalloproteases and chemokines that have autocrine effects or paracrine effects on neuronal functions. IL-1 can induce astrocyte and microglia proliferation *in vitro* (Selmaj et al., 1990; Thery and Mallat, 1993; Giulian et al., 1994; John et al., 2004), implying a role for IL-1 in gliosis.

Released growth and trophic factors from astrocytes may enhance neuronal survival

(Gadient et al., 1990; Silberstein et al., 1996), but neurotoxic agents and proinflammatory

cytokines released by microglia at the injury sites may cause neuronal death (Chao et al.,

1995a). Although inflammatory responses are healthy and necessary for normal brain

functions, prolonged exposure to IL-1, particularly at pathologically high levels, were

shown to be neurotoxic in pure hippocampal neuronal cultures (Araujo and Cotman,

1995). *In vivo*, IL-1 was shown to exacerbate neuronal damage that was induced by

cerebral injury (Stroemer and Rothwell, 1998), while blocking IL-1 signaling via IL-1ra

(Boutin et al., 2001), deleting IL-1 genes (Allan and Pinteaux, 2003) or knocking out IL-

1RI (Basu et al., 2005; Lazovic et al., 2005) is neuroprotective. On the other hand,

deletion of the IL-1ra gene greatly increased ischemia-induced brain damage (Pinteaux et

al., 2006).

In addition to its general roles in immunoregulation and inflammation, IL-1 also

plays a major role as a neuromodulator in the CNS, from neuronal plasticity, to

neuroendocrine regulation and sickness behavior (Dantzer et al., 1998). All these

functions are known to be mediated by direct actions of IL-1 on neurons. IL-1 was

originally identified as an endogenous pyrogen that acts on the hypothalamus

thermoregulatory center to elevate core body temperature. IL-1 was also found to

regulate normal physiological functions such as sleep behavior (Takahashi et al., 1997;

Krueger et al., 2001). IL-1 can also activate the hypothalamo-pituitary-adrenal (HPA)

axis to regulate the downstream neuroendocrine system (Dunn, 2000). IL-1 directly acts

on CNS neurons to induce sickness behavior in the form of increased weight loss,

depression, reduced food intake and decreased social activities (Neveu et al., 1998;

Konsman et al., 2002). At the cellular level, IL-1 can change neuronal activity through modulating the release of excitatory/inhibitory neurotransmitters, modifying the sensitivity of neurotransmitter receptors, or regulating the expression of neurotransmitter receptors and ion channels. For example, Low concentration IL-1β (1pM or 0.017ng/ml) was shown to enhance neuronal excitability by increasing cell depolarization (Desson and Ferguson, 2003). In contrast, high concentration IL-1 (3.5-50ng/ml) was shown to inhibit neuronal activity by increasing GABAergic inhibitory post-synaptic potential (Brambilla et al., 2007), decreasing excitatory glutamate release (Murray et al., 1997) and reducing the expression of AMPA receptors (Lai et al., 2006) and Ca^{2+} channels (Zhou et al., 2006). IL-1β (143pM or 2.38ng/ml) also induced neuronal hyperpolarization and enhanced synaptic inhibition (Zeise et al., 1992). The different neuromodulatory effects of IL-1 seem to be dose-dependent: at lower concentrations, IL-1 induces cell depolarization and but at higher concentrations, IL-1 induces hyperpolarization and synaptic inhibition (Desson and Ferguson, 2003).

Both IL-1 and IL-1RI are strongly present in the hippocampus and have an important role in mediating hippocampal neuronal actions. At pathophysiological levels, IL-1 produces profound detrimental effects on the hippocampus, such as impaired learning and memory processes (Oitzl et al., 1993; Aubert et al., 1995; Gibertini et al., 1995; Rachal Pugh et al., 2001). IL-1 also inhibits LTP, an underlying neural mechanism for memory (Bliss and Collingridge, 1993), in different hippocampal regions (Katsuki et al., 1990; Bellinger et al., 1993; Cunningham et al., 1996; Murray and Lynch, 1998; O'Connor and Coogan, 1999; Vereker et al., 2000). Although most previous work indicated inhibitory/detrimental effects of IL-1 on hippocampal plasticity at

pathophysiological concentrations, recent evidence also suggested that IL-1 may be required for the normal development and physiological functions of the hippocampus, particularly in regulating learning and memory formation. First, both *in vitro* and *in vivo* experiments showed that inducing LTP significantly up-regulated IL-1β gene expression in the hippocampus (Schneider et al., 1998; Coogan et al., 1999), and the normal maintenance of LTP or memory consolidation was impaired when IL-1 signaling was blocked, either by IL-1ra (Coogan et al., 1999; Spulber et al., 2009) or in an IL-1R knockout mouse model (Avital et al., 2003). Transgenic animal studies demonstrated that constitutive over-expression of IL-1ra in the mouse CNS impaired learning and long term memory compared to wild type mice (Oprica et al., 2005; Spulber et al., 2009). Furthermore, when IL-1β was administered at a lower concentrations (10ng/rat), it facilitated hippocampus-dependent memory in a passive avoidance test; in contrast, blocking of IL-1 signaling by IL-1ra infusion impaired memory in both the water maze and passive avoidance tests (Yirmiya et al., 2002). All of these studies suggest that IL-1 was required in maintaining normal hippocampal functions. A dual role for IL-1 in LTP and memory in the hippocampus has been proposed: IL-1 is required for normal hippocampal development and LTP under physiological conditions, and a slight increase in brain IL-1 levels can even improve memory; however, any deviation from physiological conditions, either with high pathophysiological doses or through total blockage of IL-1 signaling, IL-1 inhibits LTP (Ross et al., 2003) and impairs memory (Goshen et al., 2007).

5. Other members of the IL-1 receptor superfamily and their ligands

Studying the role of IL-1 can be challenging because both the IL-1 ligand and receptor families are expanding and some are not yet functionally defined. Some receptors share the same receptor accessory proteins for signaling, further complicating the IL-1 regulatory system.

IL-1 and IL-18 are the most classical and well-studied pro-inflammatory cytokines in the IL-1 cytokine family. In the last decade, different research groups have identified six new members of IL-1 cytokine family based on gene location, sequence homology, structural similarity, and receptor binding (Mulero et al., 1999; Barton et al., 2000; Kumar et al., 2000; Smith et al., 2000; Lin et al., 2001; Pan et al., 2001). The genes of these six novel members are located downstream of IL-1α and β on human chromosome 2, leading to the assumption that these genes may have evolved from one common ancestral gene. Different names have been assigned to these cytokines by several groups, but a new nomenclature for the IL-1 cytokine family has been proposed (Sims et al., 2001): IL-1F5-10 refer to the six newly discovered IL-1 members, while IL-1F1-4 correspond to IL-1α, IL-1β, IL-1ra, and IL-18, respectively. IL-33 (or IL-1F11) is the most recently identified member of the IL-1 cytokine family (Schmitz et al., 2005). All eleven cytokines mediate inflammatory or anti-inflammatory functions by binding to their corresponding receptors and receptor partners.

Other putative receptors have been reported to share sequence homology with IL-1RI. These receptors all contain extracellular Ig domains and intracellular TIR domains except for IL-1RII, which does not have a TIR domain (Figure 1). All receptors belong to the IL-1R family, which currently includes ten members (Sims, 2002). As mentioned

earlier, IL-1α and β bind to IL-1RI, and IL-1RAcP is required for IL-1 signaling. IL-1ra also binds to IL-1RI but does not elicit any effects, and therefore serves as a negative regulator for IL-1 signaling. IL-18R and IL-18RAcP (also called AcPL) form a receptor complex for IL-18 signaling. ST2 (Yanagisawa et al., 1993) is the receptor for newly identified cytokine IL-33, and IL-1RAcP is required for its signaling (Palmer et al., 2008). Recent studies have shown that the novel members of the IL-1 family, IL-1F6, IL-1F8 and IL-1F9, are ligands for IL-1 receptor related protein 2 (IL-1Rrp2), which also requires IL-1RAcP as a receptor partner (Towne et al., 2004; Blumberg et al., 2007). IL-1F5 appears to be genetically related to IL-1ra (Blumberg et al., 2007), and antagonizes the inflammatory effects induced by IL-1β, most likely via the orphan receptor SIGIRR (Costelloe et al., 2008). Unlike the other receptors, which have three extracellular Ig domains, SIGIRR has only one (Polentarutti et al., 2003). IL-1RAPL, TIGIRR and SIGIRR are orphan receptors and no ligands have yet been identified.   IL-1F7 binds to IL-18 binding protein in fluid and enhances its inhibitory effect on IL-18 (Bufler et al., 2002). IL-1F10 has been shown to bind to soluble IL-1RI (Lin et al., 2001).
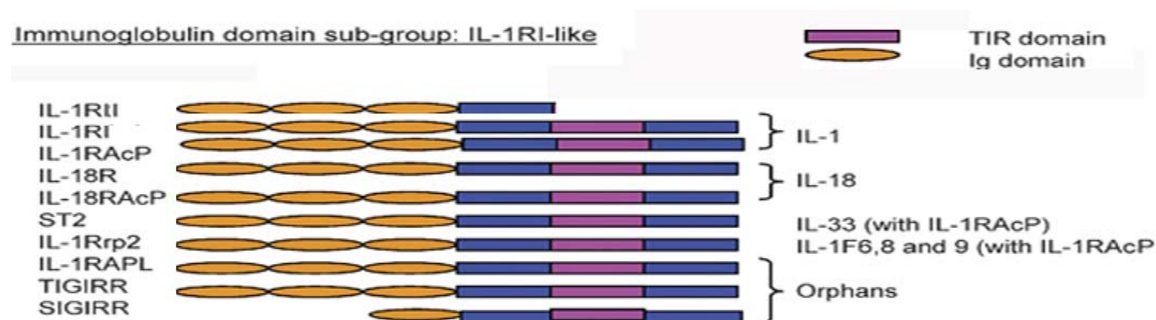

Figure 1. The IL-1R family. The IL-1R subfamily has IG domains extracellularly responsible for ligand binding. The ligands that bind are shown, and the second receptor chain for each ligand indicated. All other members except IL-1RII have intracellular TIR domain responsible for signaling.
modified from O'Neill LAJ (2008) Immunological Reviews 226: 10-18.

IL-1RAcP is a particularly interesting protein because it is a receptor partner for three receptors: IL-1RI, ST2 and IL-1Rrp2. As mentioned earlier, IL-1RI is highly expressed in the brain, particularly in the hippocampus and hypothalamus, and in both neurons and astrocytes. ST2 is preferentially expressed in T-helper 2 (Th2) cells and stimulates Th2 cell activation/maturation (Lohning et al., 1998). ST2 is also expressed in the brain cells, such as cortical astrocytes and microglia, but is not detected in neurons (Andre et al., 2005). IL-1Rrp2 and its ligands (IL-1F6, IL-1F8, and IL-1F9) are expressed mainly in epithelial cells and regulate pro-inflammatory signals in the skin and GI tract (Towne et al., 2004). IL-1Rrp2 cDNA has been cloned in both rat and human and was found to share a 42% amino acid identity with IL-1RI. Further, its mRNA was found to be highly expressed in the lung and epididymis by an RNase protection assay (Lovenberg et al., 1996). In situ hybridization analysis revealed non-neuronal localization of IL-1Rrp2 in the rat brain, mainly in the meninges, choroid plexus and perivascular cells (Lovenberg et al., 1996), suggesting an alternative inflammatory pathway mediated by IL-1Rrp2 in the brain. IL-1Rrp2 messenger RNA was later confirmed not to be in primary neuronal culture, but was constitutively expressed in mixed glial cells, particularly in astrocytes and microglia (Berglof et al., 2003). However, IL-1Rrp2 does not bind IL-1 (Lovenberg et al., 1996), and therefore is not alternatively activated by IL-1 in the brain.

6. Intracellular signaling following IL-1/IL-1R interaction

IL-1 signaling begins with IL-1 agonists (IL-1α and IL-1β) binding to IL-1RI. Crystallographic studies showed that IL-1RI interacts with IL-1 via all three Ig domains,

and the binding of IL-1 to the "receptor trigger site" of IL-1RI causes a conformational change of the receptor in extracellular domain 3 (Vigers et al., 1997), allowing IL-1RAcP to bind to IL-1RI. Computer modeling later revealed IL-1RI wraps around IL-1ß, and the outer edge of IL-1RI forms contact with IL-1RAcP (Casadio et al., 2001). As a consequence, the stability between IL-1 and IL-1RI is increased with the association of IL-1RAcP (Wesche et al., 1998). Binding of IL-1ra prevents the association between IL-1RI and IL-1RAcP, as well as IL-1 signaling (Schreuder et al., 1997). The IL-1/ IL-1RI/ IL-1RAcP complex can bind with the adapter protein MyD88 to further recruit IL-1 receptor associated kinases (IRAK) (Wesche et al., 1997). The autophosphorylation of IRAK4 and phosphorylation of IRAK1 subsequently recruit TNF receptor associated factor 6 (TRAF-6), which leads to the activation of transforming growth factor β-activated kinase-1 (TAK-1, a MAPKK kinase) and two classical downstream signaling pathways (Ninomiya-Tsuji et al., 1999). One of the pathways involves phosphorylation and degradation of the IκB subunit from NFκB, leading to the release and nuclear translocation of active transcription factor NFκB and subsequent immediate gene transcription (Ninomiya-Tsuji et al., 1999). Another pathway involves activation of mitogen-activated protein kinase kinases (MAPKKs), and further activation of three downstream MAPKs (ERK, JNK and P38) and additional transcription factors such as ATF and AP1, leading to nuclear gene transcription (O'Neill et al., 1990; Zhang et al., 1996; Ninomiya-Tsuji et al., 1999).

In addition to the two classical pathways, alternative IL-1 signaling has been reported in neurons (Viviani et al., 2003; Davis et al., 2006a), where they activate Src family kinases to regulate fast non-transcription-dependent neuronal activities. IL-1

signaling was further reported to be MyD88 independent in anterior hypothalamic neurons that activated PI3K and Akt to promote neuronal survival (Davis et al., 2006b). In hippocampal neurons, low concentrations of IL-1β (0.05ng/ml) enhances NMDA receptor functions, such as intracellular calcium increase and neuronal death, through Src-mediated phosphorylation of the NMDA receptor subunit NR2B, but blocking this pathway has no effect on IL-1 mediated gene induction of IL-6 (Viviani et al., 2003). In anterior hypothalamic neurons, IL-1β hyperpolarizes and inhibits neuronal firing within minutes. This fast electrophysiological effect is also IL-1RI and MyD88 dependent, but mediated through activation of ceramide and Src (Davis et al., 2006a). This ceramide and Src-mediated fast IL-1 signal pathway is also implicated in febrile response to IL-1β (Sanchez-Alavez et al., 2006).

Src is one of a family of kinases that have been shown to rapidly phosphorylate ion channels (Salter and Kalia, 2004) and thus have direct and fast neuronal responses that bypass gene regulation. Since Src regulates NMDA receptor phosphorylation and calcium influx, it can regulate neuronal LTP (Lu et al., 1998). Cellular Src (c-Src) is a normal protein kinase that is expressed by all cells. C-Src consists of four domains: the N-terminal unique domain, SH3 and SH2 domains, and the C-terminal tyrosine kinase domain (Koch et al., 1991; Salter, 1998). Neurons also express high levels of Src, in a specific isoform called n-Src (Brugge et al., 1985; Martinez et al., 1987). Unlike c-Src, n-Src contains a spliced cassette of six amino acids in the SH3 domain (Martinez et al., 1987), which may cause a shift in the mobility of Src protein on the SDS-PAGE gel (Brugge et al., 1985). There are two tyrosine phosphorylation sites in the C-terminal domain. Normally, Src is phosphorylated at Tyr527 site and binds intramolecularly to

the SH2 domain of c-Src, which locks c-Src in an inactive state and inhibits protein kinase activity. When Tyr527 is dephosphorylated or Tyr416 is phosphorylated, Src enzyme activity increases (Roussel et al., 1991; Cooper and Howell, 1993).

Atypical IL-1 signaling pathways have also been observed in astrocytes. IL-1β activates IFN response factor 3 (IRF3) and signal transducer and activator of transcription-1 (STAT-1) in human fetal astrocytes in culture, suggesting a role for IL-1 in anti-microbial and anti-viral innate immune responses. IL-1β also deactivates RhoA-ROCK pathway in mediating reactive astroglial phenotype and cytoskeleton proteins (John et al., 2004). IL-1β can also induce COX-2 and PGE2 by activating the protein kinase C (PKC) and MAPK pathways (Molina-Holgado et al., 2000).

7. Differences in IL-1ß signaling pathways in hippocampal neurons and astrocytes

In the inflammatory process, IL-1ß in astrocytes normally causes activation of NFκB and nuclear translocation to regulate inflammatory gene expression. However, IL-1β uses a different pathway in hippocampal neurons—p38 MAPK and CREB phosphorylation—regulating gene expression independent of NFκB (Srinivasan et al., 2004). What causes the selection of different pathways in neurons and astrocytes is not clear and remains to be addressed.

8. AcP and AcPb

AcP is an accessory protein that is required for downstream relay of IL-1 signaling. AcP is widely expressed in the brain, both in neurons and astrocytes. The

sequence analysis showed that AcP cDNA is 3355bp in length, with an open reading

frame (ORF) of 1710 bp (Greenfeder et al., 1995). A 570-amino acid-protein is coded

from the ORF, including a 20aa signaling peptide, 240aa extracellular domain, 29aa

transmembrane domain, and 181aa cytoplasmic domain (Greenfeder et al., 1995). A new

isoform of AcP, named AcPb, was recently identified and sequenced (Smith et al., 2009).

As shown in figure 2, the classical AcP cDNA contains 12 exons. AcPb consists of the

same first 11 exons as AcP as well as a unique, but longer, 12b exon. Exon 12b shares

about 35% amino acid homology with exon 12, and the TIR domain motifs are conserved

(Smith et al., 2009). In addition, there are about 140 extra amino acids in the C-terminal

with undefined functions. Preliminary data suggests that AcPb is expressed exclusively

in the brain and spinal cord (John Sims, Dirk Smith, personal communication). However,

the role of AcPb is not yet clear. Among the three receptors that share AcP as a signaling

partner, only IL-1RI is highly expressed in hippocampal neurons. Therefore, my

dissertation research has focused on comparing IL-1 signaling pathways in hippocampal

neurons and astrocytes, as well as their downstream gene regulation.
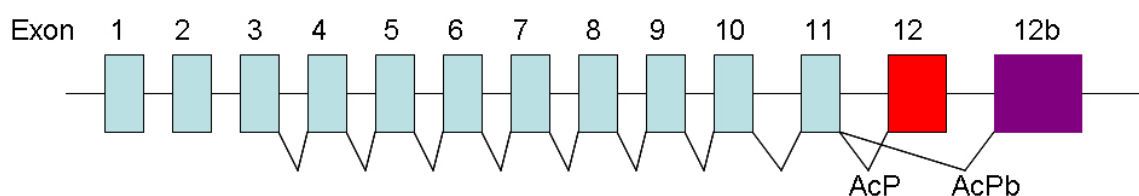

Figure 2. Schematic representation of AcP and AcPb genes. Shown here is a map of the human AcP gene: boxes represent exons, and straight lines in between boxes are introns. The bent lines at the bottom of the boxes represent translated mature AcP protein and the alternative splicing isoform, AcPb.

Research Aims

To characterize the roles of AcP and AcPb in cell-type specific IL-1 signaling pathways in hippocampal neurons and astrocytes. The research aims of this project were to:

a) Determine relative mRNA and protein expression levels of AcP and AcPb in cultured neurons and astrocytes.

b) Compare different cell signaling pathways in WT, AcP-/- and AcPb-/- neurons and astrocytes, focusing on the classical p38 MAPK and NFκB pathways. Since previous studies have shown that hippocampal neurons utilize the p38 pathway while astrocytes signal through NFκB and p38 MAPK, we will determine if the lack of AcP or AcPb has an effect on each pathway.

c) If AcPb does not have a role in the classical pathways, we will investigate the atypical pathways that were reported in neurons, such as p-Src, and determine if the presence of AcPb in neurons stimulates this pathway.

d) If our hypothesis that AcPb is not expressed in astrocytes is confirmed, we will examine whether overexpression of AcPb in astrocytes affects any of the signaling pathways.

e) Determine the biological effects of AcPb present uniquely in CNS neurons.

Materials and Methods

1. Animals used and description of knockout mice: Total AcP knockout and AcPb alone knockout mice were generated by Taconic Laboratory in collaboration with Amgen on a C57BL/6 background as previously described (Smith et al., 2009). Briefly, the original AcP knockout mice were generated by deletion of exons 4 and 5 and insertion of a neomycin sequence; therefore, both AcP and AcPb were knocked out. AcPb knockout mice were generated by replacing the entire AcPb specific exon 12b with a neomycin resistance gene, and the upstream 12 exons were left intact to maintain expression of normal full-length AcP. All mice were genotyped using primers covering either the inserted neomycin or the knocked out region before being mated for E16 or E19 cell culture. All animal studies were conducted using the NIH guidelines for the ethical treatment of animals with approval of the Rutgers Animal Care and Facilities Committee (Protocol No. 01-039).

2. Astrocyte culture: Pregnant rats or mice were sacrificed by exposure to CO_2 and soaked in 80% ethanol for 10 minutes. Rat embryonic day 21(E21) or mice embryonic day 19 (E19) fetuses were removed under sterile conditions and kept in PBS on ice. Hippocampi were dissected, dissociated by trituration, and plated on poly-D-lysine (0.1mg/ml)-precoated flasks in NM15 medium (Eagle's MEM with earle's salts and 2mM L-glutamine, 15% heat-inactivated fetal bovine serum, 6mg/ml glucose, 0.5 μg/ml penicillin and 0.5 U/ml streptomycin). Astrocytes were grown to confluence. Flasks were shaken at 450 rpm for 10 minutes to remove neurons and microglia, fresh medium was added, and the flasks were returned to the incubator for at least 2 hr. The flasks were then shaken overnight at 225 rpm to remove type 2

astrocyes and oligodendrocytes. The confluent astrocytes were exposed to cytosine arabinoside (0.1mM) for 3 days to eliminate any remaining non-astrocyte cell populations. Finally, the astrocytes were trypsinized and replated at subconfluent density. The purity of astrocyte cultures was above 99%, confirmed by immunostaining for other cell types.

3. Neuronal culture: Rat E18 or mouse E16 hippocampi were dissected, dissociated and plated in poly-D-lysine-(0.01ng/ml)- precoated plates. The medium consists of 1:1 mixture of Eagle's MEM (Invitrogen, CA) and Hams 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). Neurons were cultured for 5 days in serum free medium and subjected to cytokine treatments. The purity of neuronal cultures was above 95%, confirmed by immunostaining for other cell types.

4. Total RNA isolation and polymerase chain reaction (PCR)

   Total RNA of different samples was isolated with trizol reagent (Invitrogen, CA) as recommended by the manufacturer. Briefly, $1X10^6$ cells were lysed in 1ml trizol reagent, followed by chloroform extraction and isopropanol precipitation. The RNA was resuspended in nuclease-free water and quantified spectrophotometrically at 260/280 nm. 1 microgram of RNA was reverse transcribed at 42°C for 2 hours in a 20 µl reaction mixture using SuperScript^{TM} II reverse transcriptase (Invitrogen Carlsbad, CA). cDNA was amplified and quantified by regular PCR and real-time PCR. The following primer sequences are provided by Amgen: rat IL1R (forward:5'-CTACTTGGGTTCATTTGTCTCATTGTGCC-3', reverse: 5'-

TTCCACTTCCAGTAGACAAGGTCGGTGAAC-3'); mouse AcP specific primers (mAcP.cyto.foward 5'-TGTTTCCTATGCAAGAAATGTGGAAGAAGAGG-3', mAcP.cyto.rev: 5'-TGCTTGTCATTGCTAGACCACCTGG-3') and mouse AcPb specific primers and also used for cDNA genotyping (mAcP.cyto.foward: 5'-TGTTTCCTATGCAAGAAATGTGGAAGAAGAGG-3', mAcPb.1.rev: 5'-ATGGGGTTGCTCAAGCGACGGTACTCCAC-3'); mouse AcP knockout cDNA genotyping primers (mAcP.exon3.fwd.30: 5'-GACACGGGCAATTACACCTGCATGTTGAGG -3', mAcP.cyto.rev: 5'-TGCTTGTCATTGCTAGACCACCTGG-3'). The following primer sequences were designed using primer 3 software: mouse AcP cDNA genotyping primers from exon 4 (forward: 5'-ACCATGCGACAAATCCAAGT-3', reverse: 5'-CGGAACCAGAGCACATCTTT -3'); mouse IL-6 (forward 5'-GGAGAGGAGACTTCACAGAGG-3', reverse: 5'-GGAAATTGGGGTAGGAAGGA-3'); actin (forward: 5'-TCATGAAGTGTGACGTTGACATCCGT-3', reverse: 5'-CTTAGAAGCATTTGCGGTGCACGATG-3'); GAPDH (forward:5'-TTCTTGTGCAGTGCCAGCC-3', reverse:5'-CACCGACCTTCACCATCTTGT-3'). PCR reaction were performed in a 20 μl volume with goTaq Green Master Mix (Promega, Madison, WI) and 1μM primers according to manufacturer's recommendation. PCR reaction condition for IL1R, AcP and AcPb is: 1. 95ºC 5 minutes, 2. 95ºC 30 seconds, 50 ºC 30 seconds, 72 ºC 30senconds, 3. repeat cycle 2 for 34 times, 4.72 ºC 7 minutes. PCR products were resolved on 1% agarose gel and

stained with ethidium bromide. For realtime PCR, Roche 480 sybr green master mix was used according to manufacturer's recommendation.

5. Western blots: Cells were harvested using lysis buffer containing 120mM Tris pH 6.8, 2% SDS, and 10% glycerol. Proteins were quantified using the Bradford assay (Bio-Rad, Hercules, CA). Equal amount of protein samples were run on SDS polyacrylamide gels and transferred to nitrocellulose membrane. The blots were blocked in 5% non-fat milk in TBST for 1 hour, and incubated with primary antibody (1: 1000 in 5% milk in TBST) overnight at 4ºC. The primary antibodies are anti-phspho-IκBalpha (Cell Signaling Technologies, #9246) anti-IκBalpha (Cell Signaling Technologies, #9242), anti-phospho-p38 (Thr 180/Tyr 182) (Cell Signaling Technologies, #9215), anti-p38 (Cell Signaling Technologies, # 9212), anti-phospho-CREB (Cell Signaling Technologies, #9191), anti-CREB (Cell Signaling Technologies, #9197), anti-phospho-Src (Try 416) (Cell Signaling Technologies, 2101), anti-Src (Cell Signaling Technologies, #2110), anti-betaActin (Sigma # A5441). The blots were then washed 3 times with TBST, and incubated with secondary antibody (goat anti-mouse, or goat anti-rabbit labeled with horseradish peroxidase, Thermo Fisher Scientific, 1:5000) for 1 hour at room temperature. The membranes were developed using either the Pierce ECL Western blot substrate kit (Thermo Fisher Scientific, Rockford, IL) or Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Quantifications of immunoblots were performed on scanned images of the films using Adobe Photoshop software (San Jose, CA).

6. IP/co-IP Western Blot analysis

Cultured neurons or astrocytes were lysed in TNE buffer (50mM Tris, pH7.3, 137mM

NaCl, 2mM EDTA) containing 0.2% triton, 60mM octylglucoside and protease inhibitor cocktail. Proteins were quantified using the Bradford assay (Bio-Rad, Hercules, CA). 500μg proteins were incubated with primary antibody anti-AcP/b M215 (10ng/ml, provided by Amgen, Seattle, WA) overnight on a rocking platform at 4°C. Protein G-sepharose was added to the lysates and kept for an additional 3 hours at 4 °C. The lysates were then centrifuged at 14,000 rpm for 15 minutes and washed with TNE buffer three times and distilled water once. The pellets were resuspended in 1x loading buffer with beta-mecaptoethanol. The samples were then run on SDS-PAGE, transferred to nitrocellulose membrane, and probed using anti-AcP P2 anti-rabbit antibody (1:1000, provided by Amgen, Seattle, WA) which detects both AcP and AcPb. 50μg proteins from astrocytes transfected with AcP or AcPb plasmids were used as positive controls.

7. Cell transfection: Primary rat/mouse astrocytes were nucleofected using the Rat Astrocyte Nucleofector^{TM} kit/Mouse Astrocyte Nucleofector^{TM} kit (Amaxa Biosystems, Cologne, Germany), program T-20 by Amaxa according to manufacturer's instructions. In brief, 2 to 4 million astrocytes were suspended in 100 μl of supplemented nucleofector solution in each transfection. The cell suspension was then mixed with 3-5μg AcP, AcPb, empty vector DNA plasmids (Provided by Amgen, Seattle, WA), 3-5μg kB-driven-luciferase plasmid and/or 1μg GFP plasmid (Amaxa Biosystems, Cologne, Germany). Within 15 minutes of cell suspension, DNA supplemented cells were transfered to an amaxa cuvette and nucleotransfected with program T-20. Cells were then resuspended with warm NM15 medium to minimize cell loss and replated on poly-D-lysine pre-coated dishes. Two hours after

transfection, fresh medium NM-15 was applied to cell culture. 1-3 days after transfection, cells were exposed to different IL-1β treatment for different experimental purposes.

8. Luciferase activity assay: 24 hours after nucleotransfection, astrocytes were treated with IL-1β (10ng/ml, generously provided by Dr. Ronald Hart, Dept. of Cell Biology and Neuroscience, Rutgers University, NJ, USA) for 4 hours. Protein lysates were then collected in reporter lysis buffer (Promega, E 4030, Madison, WI). Protein lysates were centrifuged at 14,000 rpm x 2 minutes at 4°C and supernatants were stored at -80 °C. Lysates were warmed at room temperature for 30 minutes and equal amount of protein was mixed with luciferase substrate and subjected to luminescence reading immediately.

9. Immunocytochemistry

Primary neurons and astrocytes were cultured in poly-D-lysine-precoated-Lab-tek chamber slide (Nunc). Cells were subjected to IL-1β (10ng/ml) treatment for 2 hours, followed by fixation with 4% paraformaldehyde for 30 minutes. Fixed cells were blocked in PBS/10% goat serum and permeabilized with PBS/0.3%Triton X-100, then exposed to anti-p65 (1:1000, Santa Cruz, CA) or anti-AcP/b P2 antiserum antibody (1:1000, provided by Amgen, Seattle, WA) overnight at 4°C in PBS. Slide wells were then washed 3x in PBS, exposed for 1 hr at room temp to goat anti-rabbit secondary antibodies coupled to Alexa 555. After secondary antibody, cells were washed 3x in PBS, followed by DAPI (10μg/ml, Invitrogen, Oregon, USA) application. Cells were then coverslipped with anti-fading medium (ProLong Gold,

Invitrogen, Oregon, USA) and imaged on a Nikon TE200 Inverted Epifluorescent microscope. Images were analyzed with Adobe Photoshop software (San Jose, CA).

10. Statistical analysis

All *in vitro* data were expressed as mean ± SEM of three or more independent experiments conducted on different cell cultures. Student's t-test was used to analyze two comparisons. One-way analysis of variance (anova) followed by Tukey-Kramer post-hoc tests were used for analysis of groups of three or more. For all statistical analyses, a value of $p < 0.05$ was considered significant.

Results

1. AcP is expressed in both hippocampal astrocytes and neurons while AcPb is only expressed in hippocampal neurons but not astrocytes.

Previous reports from our laboratory indicated that IL-1RI was present in both rat hippocampal neurons and astrocytes in culture (Friedman, 2001), and that IL-1β stimulated different signaling pathways in these two cell types (Srinivasan et al., 2004). IL-1RAcP is a known, required receptor partner in IL-1 signaling (Cullinan et al., 1998). A new isoform of AcP, AcPb, was recently identified exclusively in CNS neurons and was found to negatively regulate gene expression (Smith et al., 2009). Thus, we first determined whether AcPb is responsible for the distinct IL-1β signaling seen in neurons and astrocytes.

To study IL-1β signaling, we first examined the expression of IL-1RI and AcP/b in neurons and astrocytes. IL-1RI mRNA was detected by RT-PCR in mouse

hippocampal neurons and astrocytes (Figure 3A), in accordance with previous results from rat cell culture experiments (Friedman, 2001). To identify the role of AcPb in IL-1β signaling in hippocampal neurons and astrocytes, we also examined the expression of AcP and AcPb. The mRNA level of AcP and AcPb was determined by PCR analysis using primers designed specifically against the last two exons. AcP was detected in both neurons and astrocytes, but AcPb was found only in neurons (Figure 3A). Realtime PCR quantification (Figure 3B) shows that hippocampal neurons expressed both AcP and AcPb, with slightly more AcP than AcPb. In contrast, AcPb was not detected by 35 cycles in astrocytes, and AcP levels were 7 times above that in neurons. Since AcP is also a co-receptor for ST2 in IL-33 signaling and for IL-1Rrp2 in IL-1F6, IL-1F8, and IL-1F9 signaling, we wanted to determine whether ST2 and IL-1Rrp2 are expressed in CNS neurons. Previous studies showed that IL-1Rrp2 is not detected in CNS neurons (Berglof et al., 2003). We examined mRNA expression of ST2, and found that ST2 was strongly expressed in astrocytes but barely detected in neurons (Figure 3A). The low expression of ST2 (Figure 3A) and IL-1Rrp2 (Berglof et al., 2003) in CNS neurons suggested that the presence of AcPb in neurons may only regulate IL-1 signaling in the brain.

We next examined protein expression of the receptors in cultured neurons and astrocytes. Immunocytochemical analysis with mIL-1R P2 antiserum showed that both hippocampal neurons and astrocytes expressed type one IL-1 receptor (Figure 4A). To examine expression of AcP isoforms, IP-Western analysis was used with anti-pan-AcP antibodies that recognize both AcP and AcPb proteins at different sizes. The result

showed that both AcP and AcPb proteins were present in neurons, but only AcP was detected in astrocytes (Figure 4B).

2. Genotype confirmation in cultured neurons and astrocytes.

Previous knockout studies have demonstrated that IL-1RAcP is an essential component in IL-1 signaling (Cullinan et al., 1998). To characterize the unique role of AcPb in cell signaling, especially in hippocampal neurons expressing AcPb, we utilized total AcP knockout and AcPb alone knockout mice generated by Taconic Laboratory in collaboration with Amgen (Smith et al., 2009).

To confirm the success of knockouts, cDNA from cultured WT, total AcP-/- and AcPb-/- neurons and astrocytes were amplified via PCR using primers specific to the knockout regions shown in Fig 5A. The results showed that AcP-/- neurons and astrocytes lack AcP amplicon using primers covering exon 4 and 12, while both WT and AcPb-/- cells have intact AcP amplicon, confirming that AcPb knockout does not affect the expression of classic AcP. WT neurons express AcPb, and AcP-/- neurons may express truncated AcPb that can still be detected by using primers from exon 11 and 12b; in contrast, AcPb-/- neurons and astrocytes do not manifest an AcPb band (Figure 5B). IP/western results from brain lysates also confirmed that AcPb-/- only deleted AcPb but not classical AcP, while AcP-/- was a total knockout lacking both AcP and AcPb (Figure 5C). Proteins in AcP and AcPb-expressing EL4 cells migrate differently from brain lysates, likely due to differential glycosylation.

3. AcPb does not inhibit NFκB activation by IL-1 in hippocampal neurons and astrocytes

The NFκB signaling pathway is known to be activated by IL-1β in hippocampal astrocytes but not in neurons (Srinivasan et al., 2004). Preliminary data from Amgen suggested that the expression of AcPb in addition to AcP may have inhibitory effects on IL-1 regulated gene induction compared to expression of AcP alone (John Sims, Dirk Smith, personal communication). We hypothesized that the presence of AcPb in neurons repressed the NFκB pathway, leading to inhibition of some gene expression. We therefore examined if IL-1β can induce NFκB pathway when neurons lack AcPb using cells cultured from AcP-/- and AcPb-/- mice.

IκB is the inhibitory subunit that normally binds the NFκB heterodimer and prevents its nuclear translocation and gene transcription activity (Beg and Baldwin, 1993). Upon upstream signal activation, IκB is rapidly phosphorylated and released from NFκB, followed by ubiquitination and degradation of total IκB protein (Beg et al., 1993). The phosphorylation of IκB is, therefore, a measure of activated NFκB pathway. Protein lysates from all three genotypes of cultured astrocytes or neurons were subjected to Western blot analysis and probed for p-IκB and total IκB. Figure 6A showed that in WT astrocytes, IκB was phosphorylated quickly in 5 to 10 minutes, accompanied by reduction of total IκB. AcPb-/- astrocytes had a similar phosphorylation pattern compared to WT. However, total AcP knockout in astrocytes abolished IκB phosphorylation and degradation induced by IL-1β, suggesting that only classic AcP but not AcPb was required for NFκB activation in astrocytes. In contrast, no IκB phosphorylation was

detected in neurons of any genotype (Figure 6B), suggesting that AcPb did not inhibit NFκB activity in neurons and removing AcPb had no effect on this pathway.

NFκB subunits p65 and p50, have previously been shown to translocate to the nucleus following IL-1β stimulation in astrocytes (Friedman et al., 1996). To confirm that NFκB was translocated to nuclei after IκB phosphorylation, cultured cells were stained for NFκB p65 subunit and imaged using fluorescence microscopy. Nuclear translocation of the p65 subunit was observed in WT and AcPb-/- astrocytes, but not in AcP-/- astrocytes or any of the three genotypes of neurons (Figure 6C and 6D), consistent with our data on IκB phosphorylation.

To further confirm NFκB activity in astrocytes, WT, AcP-/- and AcPb-/- astrocytes were transfected with kB-driven-luciferase plasmids. 24 hours after plasmid transfection, astrocytes were treated with IL-1β (10ng/ml) for 4 hours, and equal amounts of protein were used for luciferase assay. Luciferase activity was induced by IL-1β in WT astrocytes; this induction was abolished in AcP-/- astrocytes but remained unchanged in AcPb-/- astrocytes (Figure 6E).

IL-1β induces expression of a number of genes via activation of NFκB, including IL-6 (Libermann and Baltimore, 1990) and NGF (Friedman et al., 1996). To determine the roles of AcP and AcPb in IL-1β mediated signaling in hippocampal astrocytes, real-time PCR was used to quantify IL-6 mRNA expression from WT, AcP-/- or AcPb-/- astrocytes treated with or without IL-1β (10ng/ml) for 6 hours. The experiment (Figure 6F) demonstrated that IL-1β induced IL-6 by 17 folds in WT astrocytes, and the absence of AcP totally abolished this induction. Consistent with our earlier findings that AcPb is

not expressed in astrocytes, IL-6 induction by IL-1β in AcPb-/- astrocytes was similar to what was seen in WT astrocytes.

Our results in neurons showed that deletion of AcPb did not induce IL-1β -mediated p-IκB, suggesting AcPb did not inhibit NFκB signaling pathway in neurons. In astrocytes, no AcPb expression is present, but strong NFκB signaling was induced by IL-1β. We next determined if overexpression of AcPb in astrocytes would inhibit IκB phosphorylation. We transfected excess AcPb into WT astrocytes and found no effect on IL-1β induced p-IκB signal compared with empty vector transfection (Figure 7).

In summary, the NFκB pathway is activated by IL-1β in astrocytes, but not neurons. AcP is required for IL-1β mediated NFκB pathway in astrocytes. The presence or absence of AcPb does not affect this pathway in either astrocytes or neurons.

4. AcPb does not mediate IL-1β- induced – p38 signaling in hippocampal neurons and astrocytes

Besides NFκB signaling, IL-1 can also trigger activation of the c-Jun N-terminal kinase (JNK)/p38 mitogen activated protein kinase (MAPK) family, which translocates into the nucleus and activates several transcription factors of the basic leucine zipper family, such as c-Jun and c-Fos (O'Neill, 2002; Dunne and O'Neill, 2003). IL-1β has been shown to activate p38 but not NFκB signaling pathway in hippocampal neurons (Srinivasan et al., 2004). However, it is not clear whether the p38 pathway is also activated by IL-1β in hippocampal astrocytes. We next examined the phosphorylation of

p38 in both neurons and astrocytes and the roles of AcP and AcPb in this pathway in both cell types.

Consistent with previous studies from our lab in rat neurons (Srinivasan et al., 2004), p38 phosphorylation was induced by IL-1β at 5 minutes and peaked at 30 minutes in WT mouse neurons. Eliminating total AcP abolished this induction, while eliminating AcPb did not change this regulation (Figure 8A). A dose-dependent induction of p-p38 by IL-1β at 5 minutes was also observed in WT and AcPb-/- neurons, but not AcP-/- neurons (Figure 8B). These results showed that AcPb did not play a critical role in p38 activation by IL-1β.

In WT astrocytes, p38 phosphorylation was also strongly induced at 10 minutes (Figure 9A). No phosphorylation of p38 was induced by IL-1β in AcP-/- astrocytes, and a similar induction pattern to wildtype astrocytes was observed in AcPb-/- astrocytes, again confirming the essential role of AcP but not AcPb in IL-1β signaling in astrocytes.

Previous data from our lab showed that the transcription factor cAMP-response element-binding protein (CREB) was activated by IL-1β downstream of p38 MAPK in rat hippocampal neurons (Srinivasan et al., 2004). To assess whether CREB was also activated in astrocytes, as well as the roles of AcP and AcPb in this regulation, we performed a Western blot analysis of IL-1β treated WT, AcP-/- and AcPb-/- astrocytes. In WT astrocytes, CREB was phosphorylated by IL-1β at 10 and 20 minutes (Figure 9A). Knocking out total AcP but not AcPb alone abolished this induction (Figure 9A). In rat neurons, CREB is phosphorylated downstream of p38 MAPK, inhibition of p38 activity by SB203580 also inhibited CREB phosphorylation (Srinivasan et al., 2004). To test if CREB is also downstream of p38 in hippocampal astrocytes, cells were treated with p38

inhibitor SB203580 for 30 minutes prior to IL-1β treatment. The result showed that both p38 and CREB phosphorylation were inhibited by the SB inhibitor in astrocytes (Figure 9B), suggesting that CREB phosphorylation is also p38 dependent in hippocampal astrocytes. In contrast, SN-50, an inhibitor of NFκB nuclear translocation, did not inhibit p-38, p-CREB or p-IκB (Figure 9B).

In summary, p38 was activated by IL-1β, followed by CREB phosphorylation in both neurons and astrocytes. Only classical AcP was responsible for this pathway in both cell types. AcPb did not function in regulating this signaling pathway.

5. Src phosphorylation by IL-1 in neurons requires AcPb

Thus far, AcPb has not been found to play any significant role in IL-1β-mediated induction of the p38 or NFκB pathways, either at different dosages or time points. We next examined an atypical signaling pathway (p-Src) that has been reported in CNS neurons. Previous studies reported that Src activity was induced by low doses of IL-1β (0.05ng/ml) by 6 minutes in rat hippocampal neurons (Viviani et al., 2003). To confirm this signaling pathway in our culture system, cultured E18 rat hippocampal neurons were exposed to IL-1β at different doses for 5 minutes and examined for p-Src (Tyr416). IL-1 induced Src phosphorylation at a low dose (0.01ng/ml), but not at higher doses (Figure 10A and 10B). To test if AcPb plays any role in IL-1 induced Src activation, WT, AcP-/- and AcPb-/-neurons were treated with IL-1 (0.01ng/ml) for different time periods. The results (Figure 10C) showed that 0.01ng/ml IL-1 induced Src phosphorylation in WT neurons at 5 minutes, peaked at 20 minutes and stayed stable for at least 40 minutes. In

comparison, there was no IL-1β induced p-Src in either AcP-/- or AcPb-/- neurons,

suggesting that AcPb is necessary for IL-1β to activate p-Src.

6. The effects of overexpressing AcPb in WT astrocytes on p-Src and p-p38 signaling pathways

Our neuronal data showed that AcPb is required for p-Src induction by IL-1β.

We next investigated if this pathway is activated by IL-1β in astrocytes that do not

express AcPb. IL-1β did not induce Src phosphorylation, either at 5 minutes at different

doses (Figure 11A), or at 0.01ng/ml at different time points (Figure 11B). However,

when WT astrocytes were transfected with AcPb, IL-1β was able to activate p-Src,

compared with no p-Src induction in WT astrocytes (Figure 11A and Figure 11B).

Unlike neurons, which show p-Src induction by IL-1β (0.01ng/ml) at all timepoints, WT

astrocytes overexpressing AcPb showed p-Src induction at later timepoints (20, 30 and

40 minutes). At five minutes, this induction occurred in neurons with low-dose IL-1β

(0.01ng/ml), but astrocytes required higher doses. More interesting, the same experiment

also showed that 0.01ng/ml IL-1β was sufficient to induce p-p38 at 20, 30, and 40

minutes, which was inhibited when AcPb was overexpressed in WT astrocytes. This

result demonstrates that expression of AcPb in WT astrocytes is sufficient to induce p-

Src, but also reduces regulation of p-p38 induced by IL-1β at lower doses (Figure 11B)

but not higher doses (Figure 11A).

7. The effects of overexpressing AcPb in total AcP-/- astrocytes on p-Src, p-p38 and p-IκB signaling pathways

Thus far, my results have shown that AcPb is necessary for p-Src induction by IL-1β in neurons, and overexpressing AcPb in WT astrocytes is sufficient to induce the p-Src pathway by IL-1β. However, the question remained whether AcPb alone in astrocytes would be adequate for IL-1β to induce p-Src. We next examined the same signaling pathways in total AcP-/- astrocytes transfected with AcP alone or AcPb alone. The results (Figure 12A and 12B) showed that restoration of AcPb but not AcP in total AcP-/- astrocytes was sufficient for IL-1β (0.01ng/ml) to induce p-Src. In addition, the same experiment showed that low dose IL-1β (0.01ng/ml) induced p-p38 in astrocytes expressing AcP alone but not AcPb alone (Figure 12A and 12C). We next examined if IL-1β was able to induce p-p38 in astrocytes expressing AcPb alone at a higher dose (10ng/ml). When AcP-/- astrocytes were reconstituted with AcPb alone, IL-1β (10ng/ml) induced p-p38, although to a lesser degree when compared to AcP alone expression (Figure 12D). In contrast, AcP-/- astrocytes that were reconstituted with AcPb alone were not able to activate p-IκB compared with AcP-/- astrocytes reconstituted with AcP alone (Figure 12D).

In **summary**, we have found that AcP but not AcPb was expressed in astrocytes. Both NFκB and p38 signaling pathways were activated by IL-1β in wildtype astrocytes. Lack of total AcP but not AcPb alone abolished both pathways. Expression of AcPb in total AcP-/- astrocytes partially recovered regulation of the p38 pathway but not the NFκB pathway, as opposed to the full regulation of both pathways by AcP reconstitution.

In contrast, overexpression of AcPb in wildtype astrocytes reduced p38 signaling but had

no effect on NFκB signaling, suggesting a possible competitive role between AcPb and

AcP in which the presence of AcPb reduces p38 signaling. In contrast, we found that

both AcP and AcPb were expressed in neurons. Knocking out total AcP abolished p38

signaling, but the absence of AcPb only weakened p-p38 induction in neurons. No NFκB

signaling was observed in WT, AcP-/- or AcPb-/- neurons, suggesting that AcPb did not

inhibit NFκB signaling and deleting AcPb in neurons was not sufficient to recover NFκB

signaling.

More important, low dose IL-1β (0.01ng/ml) induced an alternative pathway –

phospho-Src in neurons but not astrocytes, suggesting that physiological and

pathophysiological levels of IL-1β mediate separate pathways in neurons. AcPb was

required for IL-1β-induced-Src phosphorylation in neurons, which may explain the

restricted expression pattern of AcPb in CNS neurons. In addition, overexpression of

AcPb was sufficient for IL-1β to induce p-Src signaling in astrocytes. Overall, these data

suggest a new possible role of AcPb in mediating IL-1β signaling.

A summary of the results on IL-1β mediated different cell signaling in neurons

and astrocytes are shown in Table 1.

Discussion

AcPb plays a specific role in neuronal IL-1β signaling and functions

IL-1 is a pro-inflammatory cytokine that has essential roles in many mammalian cell functions, such as immune defense, inflammatory reactions, neuronal plasticity and epilepsy. Almost all known IL-1 functions signal through the IL-1RI/IL-1RAcP receptor complex. However, distinct signaling cascades have been described in different cell types with no mechanistic explanation. For example, our lab has previously shown that hippocampal neurons and astrocytes use different signaling pathways induced by IL-1β. Alternative IL-1 signaling pathways were also reported in hippocampal neurons from other labs, such as phosphorylation of Src induced by IL-1β. With the discovery of AcPb, a new isoform of AcP, we were able to describe a possible mechanism governing the different IL-1β signaling pathways in hippocampal neurons and astrocytes. In accordance with previous reports in rat hippocampal neurons, we found that phospho-Src was also induced by IL-1β in mouse hippocampal neurons. More interesting, our results indicate for the first time that AcPb is necessary for the induction of phospho-Src by IL-1β in hippocampal neurons, one possible reason for the unique presence of AcPb in CNS neurons.

Src activity has been long implicated in neuronal long term potentiation (LTP) (Lu et al., 1998; Salter, 1998; Huang et al., 2001). LTP is a persistent enhancement in signal transduction between two neurons when electrically stimulated simultaneously, and it is one underlying neural mechanism for learning and memory (Bliss and Collingridge, 1993). Hence, the presence of AcPb in hippocampal neurons may have a specific role in mediating IL-1-regulated LTP via the Src pathway. In addition,

overexpressing AcPb in WT or total AcP-/- astrocytes was sufficient to induce p-Src in response to IL-1β treatment. Again, these data confirmed that AcPb is not only necessary but also sufficient for IL-1β to induce Src phosphorylation, even in cells like astrocytes that normally do not have IL-1β-induced Src activity or LTP activity.

Our data showed that AcPb is only expressed in CNS neurons, which suggests that AcPb plays a role in mediating neuron-specific IL-1 signaling and functions in the CNS. We found that Src was phosphorylated in hippocampal neurons but not astrocytes when cells were exposed to a relatively low dose of IL-1β (0.01ng/ml). Studies have shown that small amounts of IL-1 are required for LTP, while higher dosages inhibit LTP (Ross et al., 2003). In addition, Src family kinases were strongly implicated in mediating LTP induction and Src activity was also increased by stimulation that produces LTP (Lu et al., 1998; Salter, 1998; Huang et al., 2001). The unique presence of AcPb in hippocampal neurons suggests that AcPb may be specifically involved in mediating Src activity and IL-1-induced LTP.   The N-terminal unique domain of Src has been shown to mediate binding of this kinase to ion channel receptors (Fuhrer and Hall, 1996).   Src is a family of kinases that can rapidly phosphorylate ion channels (Salter and Kalia, 2004) and thus directly mediate fast neuronal responses that bypass gene regulation. Therefore, IL-1 may enhance LTP through activating Src activity at lower doses, followed by direct and fast phosphorylation of the NMDA receptor (Viviani et al., 2003). This postulate also agrees with a previously proposed model of a reverse-U-shape effect of IL-1 in the hippocampus (Goshen et al., 2007): IL-1 is required for LTP, learning and memory formation, and at physiologically low levels it may even increase LTP and memory formation. However, at pathophysiological levels, IL-1 inhibits LTP and impairs novel

memory formation. The inhibitory effects on LTP and memory could be due to Src being dephosphorylated by high concentrations of IL-1. If Src is directly involved in regulating LTP via AcPb at physiological IL-1 concentrations, we would expect to see a relatively high level of p-Src in the healthy hippocampus, where IL-1 is maintained at a physiological level. In comparison, mice lacking IL-1RI, AcPb or overexpressing IL-1ra would likely have less p-Src in the hippocampus and have trouble with hippocampus-dependent learning and memory tasks, such as maze tests.

One example of an inhibitory effect of high concentration IL-1 on LTP is that during fever, when IL-1 levels are known to be strongly increased in the hypothalamus, IL-1 may also increase in the hippocampus as reported in aged rats by heat exposure (Katafuchi et al., 2003). At this high concentration, IL-1 inhibits LTP, possibly leading to effects such as attenuated learning and thought processes.

At a pathologically high dose (10ng/ml), IL-1β induced p38 MAPK phosphorylation in neurons, which depends on both AcP and AcPb. Deletion of AcPb weakened, but did not suppress induction of p-p38 in neurons, suggesting that AcPb is partially contributing to the induction of p-p38. In addition, overexpression of either AcP alone or AcPb alone in total AcP-/- astrocytes was adequate for the restoration of p38 phosphorylation induced by IL-1β, although to a lesser degree in AcPb versus AcP expressing astrocytes. These data suggest that AcP/AcPb may be redundant in activating p38, and it is not the main purpose of AcPb being present in neurons.

Possible dose-dependent actions of IL-1 signaling in neurons

The mechanism for the distinct neuronal responses to IL-1β at physiological and pathophysiological doses, as well as the role of IL-1β in these situations remains unclear. One possible explanation is that, at low concentrations, IL-1β may constantly stimulate neurons through the Src pathway, promoting neuronal excitability by directly phosphorylating ion channel receptors. Here, we observed high levels of total Src expression in neurons, and thus one role of Src in neurons may be to maintain a certain level of excitability/responsiveness. A physiological role for IL-1β in enhancing neuronal excitability was also suggested by other groups. One study showed that pre-exposure to lower levels of IL-1β (0.05ng/ml) potentiated NMDA-receptor mediated Ca^{2+} influx (Viviani et al., 2003), suggesting IL-1 enhanced neuronal excitability. Another study showed that physiological levels of IL-1 (1pM or 0.017ng/ml) enhanced neuronal depolarization (Desson and Ferguson, 2003). This speculation could also explain why pre-injection of lower dosages of IL-1β prolonged bicuculline methiodide-induced seizure time in mice (Vezzani et al., 2000). The constitutive neuronal activity/excitability may also be involved in memory formation and retention, because total blockage of IL-1 signaling was shown to impair normal maintenance of LTP and memory consolidation (Avital et al., 2003; Spulber et al., 2009). In contrast, administering low concentrations of IL-1 facilitated hippocampus dependent memory formation (Yirmiya et al., 2002).

Interestingly, we found that IL-1β also induced phosphorylation of p38 at physiological levels, but the role of this pathway remains ambiguous. It does not necessarily need AcPb, and it is not neuron-specific. p38 MAPK has been implicated in

neuronal death induced by glutamate, but not from trophic withdrawal, and inhibiting p38 can attenuate neuronal death (Cao et al., 2004). Low-dose IL-1β was shown to increase neuronal cell death induced by NMDA via a Src kinase-dependent pathway (Viviani et al., 2003). It is likely that at lower concentrations, IL-1β induces p-Src to maintain a certain level of neuronal excitability, but it also increases neuronal vulnerability to insults. Meanwhile, IL-1β also induces p-p38, which might potentiate NMDA-induced excitotoxicity.

At pathophysiological concentrations, IL-1β may hyperpolarize neurons (Desson and Ferguson, 2003), increase neuronal vulnerability to damage and lead to excitotoxicity. As previously shown, high doses of IL-1 itself did not induce neuronal death, but did exacerbate injury-induced neuronal damage (Stroemer and Rothwell, 1998). In neuronal cultures, high-dose IL-1 is not toxic up to 24 hours (Araujo and Cotman, 1995; Chao et al., 1995b). However, *in vivo*, higher concentrations do result in cell death, possibly through the release of neurotoxic factors from astrocytes via the MAPK/NFκB pathways (Pinteaux et al., 2009) such as nitric oxide (NO), which mediates cell death through NMDA receptors (Chao et al., 1995b). Another astrocyte-released factor is NGF (Friedman et al., 1990), which may mediate neuronal death via p75^{NTR}. Although short exposure to IL-1 in neuronal culture is nontoxic, long term exposure (72 hours) to high doses of IL-1 (500ng/ml) can become neurotoxic (Araujo and Cotman, 1995). In our system, high concentrations of IL-1 (10 to 100ng/ml) induced strong phosphorylation of p38 but not JNK or ERK, followed by activation of the transcription factor CREB. CREB regulates expression of various genes, which may include calcium channels, AMPA receptors, and neurotransmitters. At lower doses, IL-1β also induced p-

p38 but did not cause neuronal death. It is possible that p-38 induced at lower dosages is not as robust as at higher dosages (Figure 8B). It is also possible that CREB is not activated downstream of p38 when exposed to lower concentrations of IL-1β, which leads to de-regulation of various genes.

Proposed models of IL-1β mediated signaling in neurons and astrocytes

We found that IL-1β signaling in neurons and astrocytes differed depending upon the dose concentration and the expression of different receptor accessory proteins *in vitro*. Proposed models of IL-1β signaling pathways in neurons and astrocytes are presented in Figures 13 and 14.

In neurons, we observed that only low dose IL-1β activates p-Src and only in the presence of AcPb. It is likely that when IL-1β was applied at low concentrations, IL-1RI forms a complex with both AcPb and AcP, which lead to the induction of p-Src. At the same time, one IL-1RI may also form a smaller complex with one AcP or one AcPb, enough to induce p-p38 signaling, which may explain why the lack of AcPb weakened p-p38 induction by IL-1β in neurons and why the expression of AcP alone or AcPb alone in astrocytes was sufficient for IL-1β to induce p-p38. At pathophysiological concentrations, saturated IL-1β may cause the IL1R/AcP/AcPb complex to separate into two smaller complexes, IL-1R/AcP and IL-1R/AcPb. Under such conditions, only the p38 signaling pathway, but not the Src pathway, is induced by IL-1β in neurons.

Furthermore, the role of AcPb in the receptor complex remains unclear. One possibility is that the presence of AcPb in the IL-1RI/AcP complex changes IL-1 receptor

conformation and increases binding affinity for IL-1β at low concentrations. If this is true, overexpression of only the extracellular or transmembrane domain should be enough to increase binding affinity, and it would not necessarily require the AcPb-specific intracellular domain. Since the extracellular and transmembrane domains are the same for both AcP and AcPb, we would assume that overexpressing AcP alone in astrocytes could also increase the receptor binding affinity for IL-1β and should also induce p-Src with low-dose IL-1β treatment. However, this is not what we found. Another explanation could be that recruiting AcPb to the IL-1R/AcP complex results in different intracellular adaptor proteins interacting with the complex. Although AcPb alone does not recruit MyD88 or IRAK (Smith et al., 2009), it may recruit other adaptor proteins that can activate Src directly. Alternatively, the presence of AcPb may partially disguise the AcP intracellular domain, leading to weakened p38 MAPK pathway activation but not NFκB signaling. Another possibility is that AcPb does both: extracellularly, it increases the binding affinity of IL-1β to its receptor and intracellularly, it recruits different adaptor proteins and kinases that activate p-Src.

A second possible model is that at low concentration, IL-1β induced Src phosphorylation at the membrane. Src has been shown to directly phosphorylate ion channel receptors on the cell membrane (Salter and Kalia, 2004), therefore, it may remain proximal to the cell membrane. In addition, there is a myristoylation site at the N-terminal of Src (Salter, 1998), which localizes Src to the cell membrane. High concentration IL-1β may cause the IL-1 receptor complex to be internalized quickly, followed only by robust p38 phosphorylation, but not Src phosphorylation through the signals transduced by receptor-mediated endocytosis. p38 is also phosphorylated by IL-

1β at low concentrations, which may happen at the membrane site or there may be some receptor internalization at low concentrations.

A third possible model is that high levels of IL-1β induce expression of Src-specific phosphatases, such as PP1 and PP2 (Bain et al., 2007), which leads to subsequent de-phosphorylation of Src but not p38. In contrast, low concentrations of IL-1β only activate Src but not its phosphatases. Therefore, we see both Src and p38 phosphorylation at physiological IL-1β concentrations, but only p38 phosphorylation at pathophysiological concentrations.

In the astrocytes, AcP but not AcPb is normally expressed, leading to activation of both p38 and NFκB. When AcPb was overexpressed in astrocytes, it not only induced Src phosphorylation by IL-1β, but also attenuated p38 phosphorylation by IL-1β (0.01ng/ml), suggesting a signaling switch caused by the presence of AcPb. A proposed model in astrocytes is shown in Figure 14. The linkage protein between IL-1RAcP and Src has not been identified, but our results indicate that it is AcPb specific. Since the C-terminal of AcPb protein is longer than that of AcP protein, it could result in recruiting different or additional adapter protein kinases that activate Src instead of the classical MAPK or NFκB pathways. Indeed, biochemical studies by the group who first identified AcPb have shown that AcPb does associate with IL-1RI in a ligand-dependent manner. However, IL-1 fails to recruit MyD88 and IRAK4 via AcPb, resulting in a failure to activate the classical MAPK pathways –JNK and ERK (Smith et al., 2009).   It may also explain why the p38 MAPK pathway is weakened in astrocytes overexpressing AcPb.

In accordance with the first neuronal model I proposed, the signal switch may occur when AcPb was expressed, with some AcP being recruited to the IL-

1RI/AcP/AcPb complex and thus inducing p-Src. In addition, we found that the p-p38

signal was attenuated, possibly due to less AcP available to form a complex with IL-1RI

alone. The weakened p-p38 signal in astrocytes overexpressing AcPb may explain the

inhibitory role of AcPb on some gene regulation found in cortical neurons (Smith et al.,

2009). This result combined with our neuronal data showed that normal levels of AcPb

in neurons did not inhibit, but rather contributed to p38 activation as seen in WT neurons,

but overexpression of AcPb could have inhibitory effects on genes regulated through p38.

In addition, we found that overexpression of AcPb alone in AcP-/- astrocytes was

sufficient for IL-1β to induce p-Src, probably by forming a complex of one IL-1RI and

multiple AcPb. Overexpression of AcPb may change the stoichiometry between IL-1RI

and AcPb, which may also explain why high-dose IL-1β also activates p-Src in astrocytes

overexpressing AcPb, in contrast with low-dose IL-1β activating p-Src in neurons. This

could be due to IL-1RI forming a complex with multiple AcPb when AcPb is

overexpressed, despite the different doses of IL-1β.

Expression of AcPb alone in total AcP-/- astrocytes was sufficient for IL-1 to

induce p-p38 at 10ng/ml but not 0.01ng/ml, probably due to the dissociation constant

(Kd) of IL-1β to IL-1RI. The dissociation constant is commonly used to describe binding

affinity between a ligand and its receptor; the lower the Kd, the stronger the binding

affinity. The Kd of IL-1β to IL-1RI in rat astrocytes is about 60pM or 1ng/ml (Juric and

Carman-Krzan, 2001). At IL-1β concentrations below the Kd, IL-1RI is unoccupied

more of the time and unable to activate p38 but may be sufficient to activate Src. At high

doses, the receptor is occupied by IL-1β most of the time, leading to more p38 activation.

In addition, the induction of p38 by IL-1β is weaker in astrocytes expressing AcPb alone

compared with those in AcP alone astrocytes.   Overexpressing AcPb in WT astrocytes

attenuated p38 activation by IL-1β, due to either competitive binding between AcP and

AcPb that lead to relatively weaker p38 induction, or competition between the receptors

for different signaling: with the presence of AcPb, IL-1β activates p-Src but attenuates p-

p38.

What is downstream of Src activity?

Src is well studied as a proto-oncogene protein because the SH2 domain binds to

phosphorylated tyrosine in receptor tyrosine kinases, especially in growth factor receptors

such as EGF receptor and PDGF receptor, and regulates cell proliferation. The SH3 and

SH2 domains of Src can also recruit intracellular signaling proteins, which may activate

other signaling cascades downstream of Src that are important for gene regulation, cell

division, metabolism and cytoskeletal architecture. However, it is not clear which

downstream signaling of Src is activated in neurons. Src is known to enhance NMDA

receptor functions (Ali and Salter, 2001; Salter and Kalia, 2004), suggesting a role in

regulating LTP. One of the signaling cascades downstream of Src could be Ras-GAP-

MAPK because Ras and GAP also share SH2 and SH3 domains, which were suggested

as possible intracellular interacting partners with Src (Koch et al., 1991), or perhaps

because Ras-GAP-MAPK has been implicated in regulating LTP (Brambilla et al., 1997).

In addition to Src effects at the membrane, genes downstream of Src may also be

activated following Src phosphorylation, leading to LTP regulation through NMDA and

AMPA receptors and calcium channels. NMDA receptor NR2B was known to be

directly phosphorylated by Src, but its gene regulation is not clear. AMPA receptor

GluR1 subunit is a possible candidate because the AMPA receptor is involved in LTP, and phosphorylation and surface expression of the GluR1 subunit is regulated by IL-1 (Lai et al., 2006). Gene expression of calcium channels is another possibility since this protein is involved in LTP and its protein expression was shown to be regulated by IL-1(Zhou et al., 2006).

Because Src activity potentiates NMDA-stimulated Ca^{2+} influx, Src may regulate Ca^{2+} -mediated immediate early genes, such as BDNF, c-fos and cpg15 (Takasu et al., 2002). BDNF enhances NMDA receptor function (Lin et al., 1998; Levine and Kolb, 2000), and was implicated in inducing LTP in hippocampal slices (Ji et al, 2010). Src may regulate BDNF gene transcription in a positive feedback loop to potentiate LTP. CREB has also been shown to regulate BDNF (Tao et al., 1998), and is also implicated in LTP regulation (Chen et al., 2007b). Since IL-1 also plays an important role in regulating LTP, IL-1 may modulate LTP formation by activating Src, followed by CREB and its downstream gene regulation of BDNF.

Why is NFκB not activated by IL-1 in neurons?

Our original hypothesis that the presence of AcPb inhibits the IL-1-mediated-NFκB pathway in neurons was proven to be null. Previous results from our lab showed that TRAF6, a key adaptor protein in IL-1-mediated-NFκB activation (Cao et al., 1996), was abundant in hippocampal astrocytes but not detected in neurons (Srinivasan et al., 2004). TRAF6 has been shown to interact with IRAK (Cao et al., 1996), and activates IKKs through TAK1 (a MAPKK kinase), NIK (NFκB inducing kinase), and MEKK1(MAPK kinase/ERK kinase kinase) in IL-1 signaling (Lee et al., 1997;

Ninomiya-Tsuji et al., 1999), suggesting an essential role for TRAF6 in the NFκB

pathway. It is also interesting that a dominant negative TRAF6 blocked NFκB activation

induced by IL-1β but not by TNFα (Cao et al., 1996), in accordance with studies from our

lab that found that NFκB is activated in neurons by TNFα but not IL-1β (Choi and

Friedman, 2009). The absence of TRAF6 in hippocampal neurons is one explanation for

why IL-1 did not induce NFκB. Another theory states that CNS neurons are "immune

privileged" during viral infection by restricting NFκB activation (Massa et al., 2006),

because NFκB induces the expression of class I major histocompatibility complex (MHC

I) (Kaltschmidt et al., 1995). MHC I presents part of the infectious virus to cytotoxic T

cells that target and kill the infected cells to diminish viral replication.   This strategy

comes at a high cost for post-mitotic neurons because CNS neurons have finite numbers.

Therefore, it seems likely that neurons do not activate NFκB in response to IL-1 for an

overall more beneficial effect, and the cellular context, such as lack of TRAF6,

determines the way they respond. However, the role of NFκB in CNS neurons is

controversial. Some groups found that NFκB was constitutively active in hippocampal

neurons both *in vitro* and *in vivo*, possibly induced by glutamate *in vivo* (Kaltschmidt et

al., 1994). However, this study did not show any endogenous gene regulation induced by

NFκB or inhibited when lacking NFκB and the purity of their neuronal cultures was not

clear. Using pure neuronal cultures and a transgenic mouse carrying a β-galactosidase

transgene driven by κB, Massa et al. (2006) showed that inflammatory cytokines and

glutamate do not induce NFκB in neurons. Like Massa et al., we have never observed

constitutive NFκB activity in hippocampal neurons (2006).

Future directions

Biochemical studies showed that IL-1β does not recruit MyD88 and IRAK4 via AcPb (Smith et al., 2009). However, the original paper did not characterize which adapter proteins were recruited to the receptor complex when both AcP and AcPb were expressed. Future experiments could determine if the activation of Src by IL-1β in hippocampal neurons is MyD88 dependent, and if IRAK1, IRAK2, IRAK4 or other protein kinases were recruited to the IL-1RI/AcP/AcPb receptor complex in our culture system. The data presented in this thesis demonstrated that AcPb is required in neurons for IL-1-induced Src activity. A future experiment would be to test if AcPb is sufficient for Src pathway in neurons. Since our AcP deficient-mice were total AcP knockout, we plan to overexpress AcPb only in total AcP-/- neurons, and examine if IL-1β induces p-Src. However, when AcPb is overexpressed, the result may differ from neurons that express normal level of AcPb only. The ideal experiment is to develop AcP alone knockout mice in the future. Data from astrocytes suggested that AcPb is not only necessary but also sufficient to induce Src activation by IL-1β. A next step for this project could be to determine if IL-1RI forms complex with AcP and AcPb separately or together in one big complex when treated with low versus high doses of IL-1β. Developing new antibodies against AcP alone or AcPb alone would be helpful in investigating this question. We can also pull down AcPb alone, and test if Src is associated with AcPb in one complex.

There may be other signaling pathways activated by IL-1β at different doses and time points other than what we tested, and they could also be AcPb dependent in neurons. For example, *in vivo* data showed that AcPb-deficient mice were more vulnerable to local

inflammatory challenges in the CNS, suggesting a neuroprotective role for AcPb (Smith et al., 2009). The PI3K/p-Akt pathway was also reported to be activated by IL-1 in neurons (Davis et al., 2006b). Therefore, it may worth investigating the specific effects of AcPb on the p-Akt pathway.

To directly identify the role of AcPb in Src activity and IL-1β -induced LTP, our future experiments include examining the phosphorylation of NMDA receptor NR2B subunits by IL-1β with or without Src inhibitors, as well as downstream functions such as IL-1β -potentiated Ca^{2+} influx and IL-1-induced LTP in WT versus in AcPb-deficient neurons.

AcP is a common receptor partner for IL-1RI, ST2 and IL-1Rrp2. Although we and others have shown ST2 and IL-1Rrp2 not to be constitutively expressed in brain neurons, it has yet to be determined if cytokine treatment in vitro or under inflammatory conditions *in vivo* can cause an upregulation of these two receptors in the brain, especially in the neurons that express AcPb. It would be crucial to understand if AcPb in CNS neurons also regulates inflammatory signaling cascades induced by other ligands through these two receptors.

Differential effects of IL-1α and IL-1β have been reported in primary neurons (Tsakiri et al., 2008). This study focused on the role of AcPb in IL-1β-regulated signaling. However, it is possible that an alternative neuronal signaling pathway, which does not involve AcPb, is activated by IL-1α. And it is worth investigating if the differential effects of IL-1α and IL-1β result from recruiting different IL-1R partners (AcP or AcPb or both).

IL-1 is upregulated in numerous inflammatory diseases in the periphery and neurodegerative diseases in the CNS. In the periphery, most therapeutic strategies focus on negatively regulating IL-1 signaling in inflammatory responses, such as using soluble IL-1ra, soluble IL-1RII, or TIR domain mimetics (Barksby et al., 2007). Central injection of IL-1ra also significantly inhibits neurodegeneration induced by cerebral ischaemia (Rothwell and Hopkins, 1995). However, since IL-1 has an essential role in regulating other CNS effects, such as sleep, body temperature, learning, and LTP, it is not ideal to broadly inhibit IL-1 actions. In addition, short term inflammatory responses are beneficial to tissue functions. Since most *in vivo* neurodegeneration is induced by astrocyte-released neurotoxic factors such as NO, chemokines and cytokines, another possible IL-1 therapeutic strategy could be to attenuate inflammatory responses induced by IL-1 in astrocytes, but without affecting neuronal functions. Here, we found AcPb expression reduced IL-1β -regulated p38, but not NFκB, signaling in astrocytes, describing a novel component of IL-1 biology specific to glial function that may have profound implications for future IL-1 drug development therapies.

Conclusions

IL-1β mediates different intracellular signals and functions in CNS neurons versus other cell types. IL-1β signals through IL-1 receptor and IL-1 receptor accessory protein (AcP). We have found that AcPb, a novel isoform of AcP, is only expressed in hippocampal neurons but not astrocytes. The restricted expression of AcPb in neurons mediates a neuronal specific IL-1 signaling pathway – p-Src –which may have an important role in regulating LTP and memory. The downstream signal cascades of Src and its direct implication in LTP remain to be investigated. In addition, overexpression of AcPb in astrocytes induces p-Src, but attenuates p-p38 by IL-1β, suggesting a possible therapeutic aspect in IL-1 biology.

Figure 3. mRNA expression of AcP and AcPb in primary mouse hippocampal neurons and astrocytes. (A) RT-PCR amplification of AcP, AcPb, IL-1RI and ST2 mRNAs from hippocampal neurons and astrocytes. (B) Relative expression of AcP and AcPb mRNA levels in hippocampal neurons and astrocytes quantified by Realtime PCR. The neuronal expression level of AcP was normalized to 1, all other expressions were relative to neuronal AcP, using GAPDH as internal control (data from three different experiments with triplicate loading each).

A.
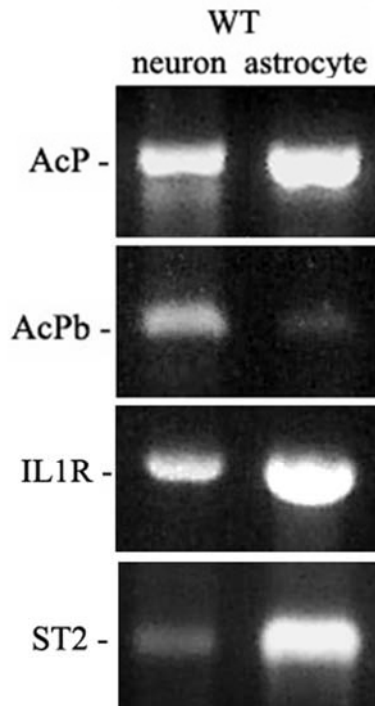
B.
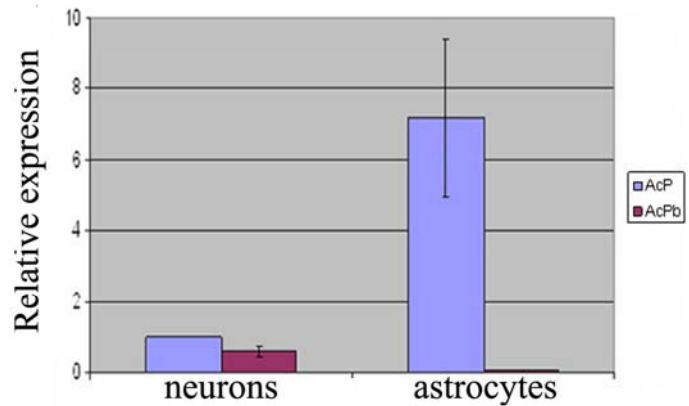
Figure 4. Protein expression of AcP and AcPb in cultured hippocampal neurons and astrocytes. (A) Immunostaining of IL-1RI in cultured hippocampal neurons and astrocytes. (B) IP-Western blots for AcP and AcPb in hippocampal neurons and astrocytes. 50μg protein lysates from astrocytes transfected with AcP or AcPb were used as positive control on the left two lanes. 500μg protein lysates from neurons or astrocytes were immunoprecipitated with a pan-AcP monoclonal antibody. Precipitations were analyzed by immunoblot for a pan-AcP anti-serum antibody.

A.
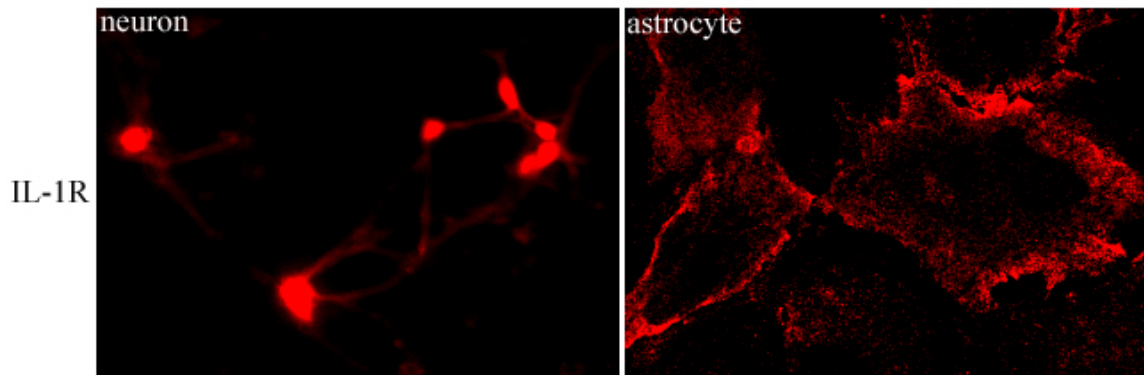
B.
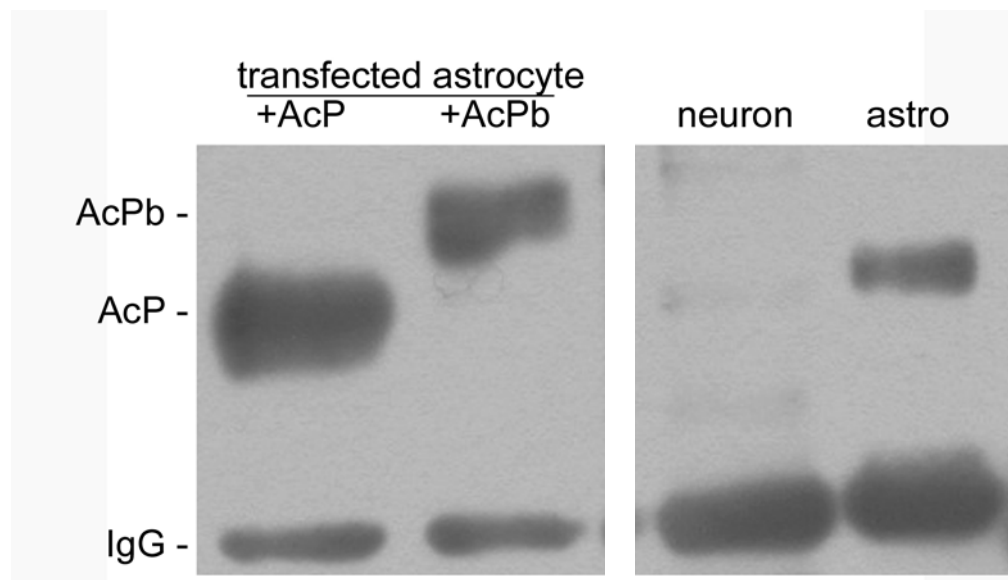
Figure 5. Confirmation of AcP and AcPb knockout. (A) A schematic diagram of AcP and AcPb knockout. The bars covering exon 4 and 5 and exon 12b are the specific knockout regions. Primers specific for AcP or AcPb were shown as arrows with the amplicon sizes on the right. (B)RT-PCR primers specific for AcP or AcPb as shown in (A) were used to amplify cDNA generated from WT, AcP-/- or AcPb-/- neurons and astrocytes. (C) Immunoprecipitations of the whole brain lysates from WT, AcPb-/- or AcP-/- were performed with anti-pan-AcP antibody and immunoblotted with a polyclonal pan-AcP antibody. EL4 cells transfected with AcP or AcPb were used as positive control.

A.

B.

C.
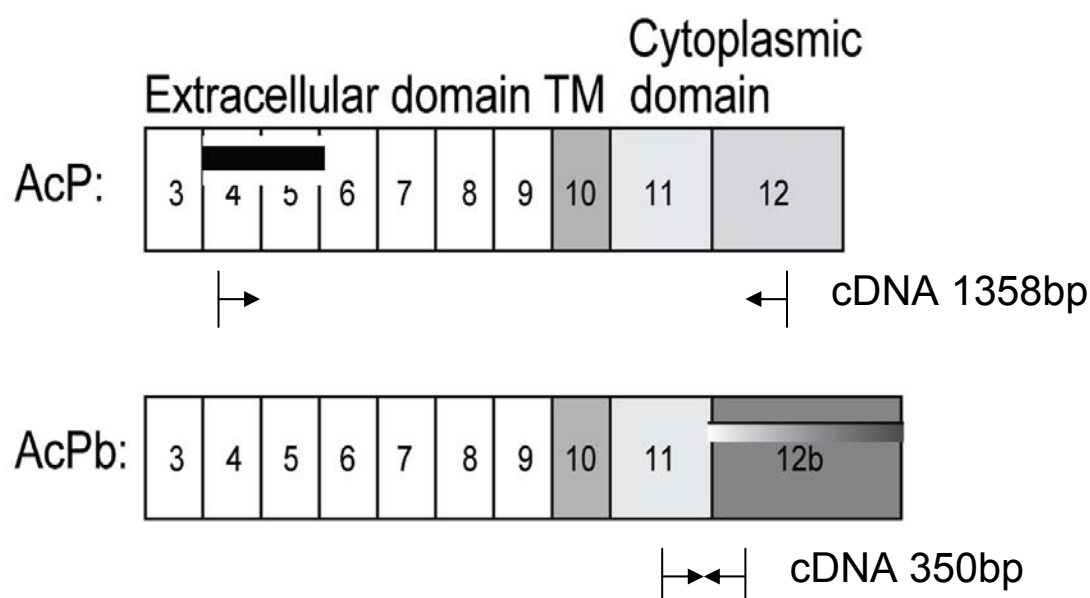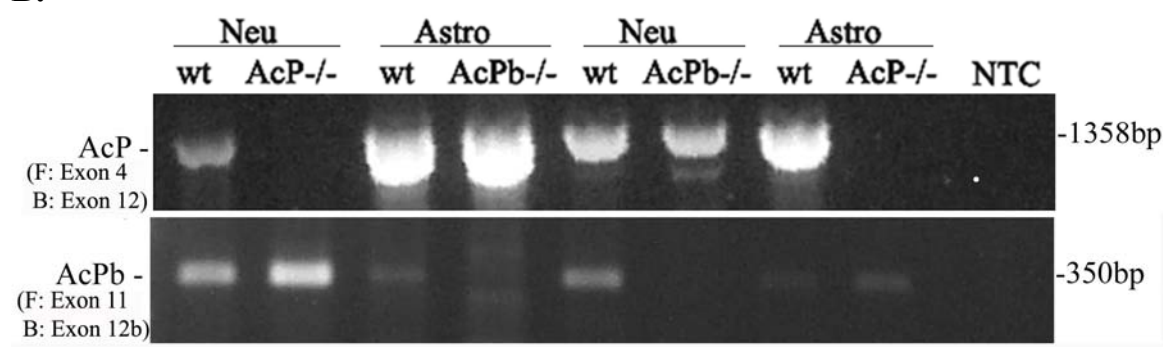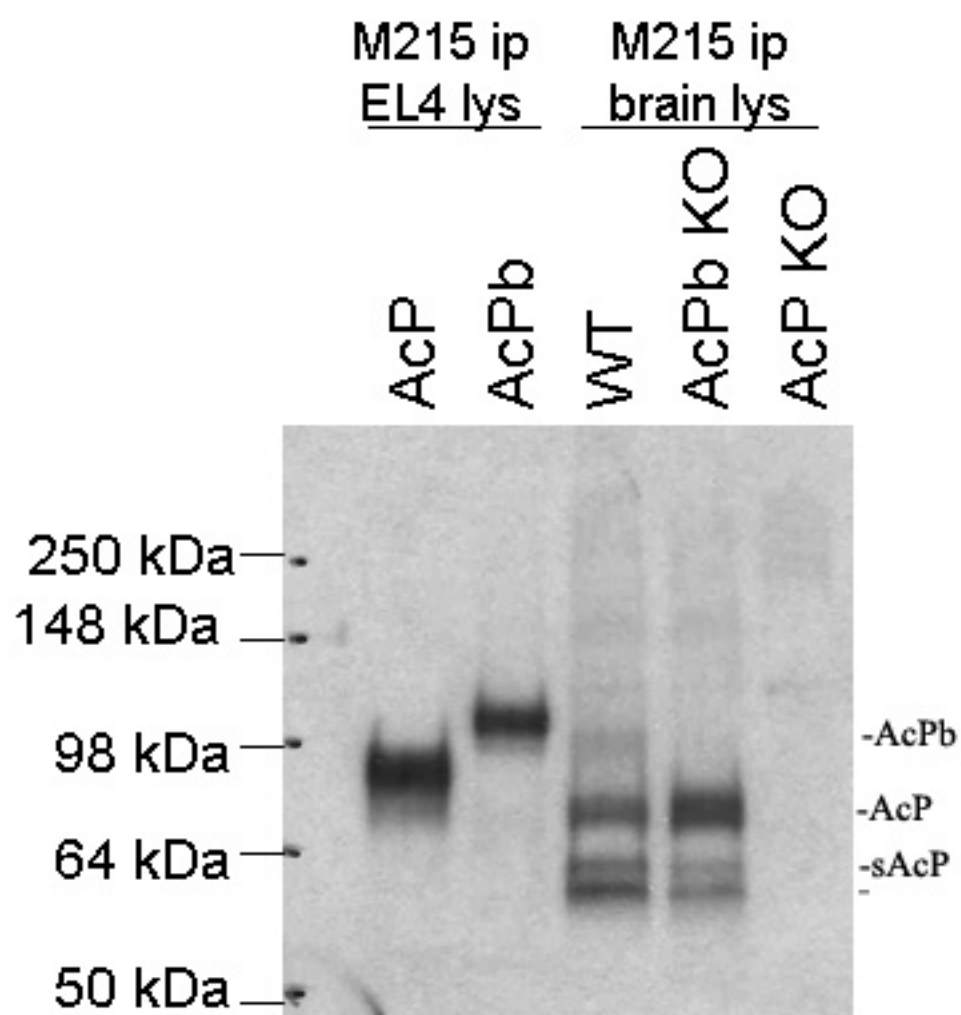
Figure 6. NFκB signaling pathway is activated in hippocampal astrocytes but not neurons. Cultured E19 astrocyte or E16 neuron protein lysates were analyzed by Western blot for phospho-IκB, with total IκB or beta actin as load control. (A) Phosphorylation of IκB in WT and AcPb-/- astrocytes but not AcP-/- astrocytes. B) No phosphorylation of IκB was detected in WT, AcP-/- or AcPb-/- neurons. p-IκB in astrocytes was used as positive control.   (C) and (D) p65 NFκB subunit is translocated to nucleus in WT and AcPb-/- astrocytes but not AcP-/- astrocytes or neurons of any genotype. Cultured hippocampal astrocytes (C) and neurons (D) were treated with IL-1β (10ng/ml) for 2 hours, fixed with 4%PFA, and immunostained for p65 (red). Cell nuclei were labeled with DAPI (blue). (E) kB-luciferase activity is induced by IL1β in WT and AcPb-/- astrocytes, but not AcP-/- astrocytes. Astrocytes were transfected with κB-driven luciferase reporter plasmids. 24 hours after transfection, cells were treated with IL-1β (10ng/ml) for 4 hours. Protein lysates were analyzed for luciferase activity. Data are from four different experiments with triplicate loading each. Student's t-test was used to evaluate data from cells treated with or without IL-1β. *P< 0.01. (F) IL-6 mRNA was upregulated by IL1β (10ng/ml) in WT and AcPb-/- astrocytes but not AcP-/- astrocytes by realtime-PCR analysis. Graph depicts 6 hour IL-1β treatment.
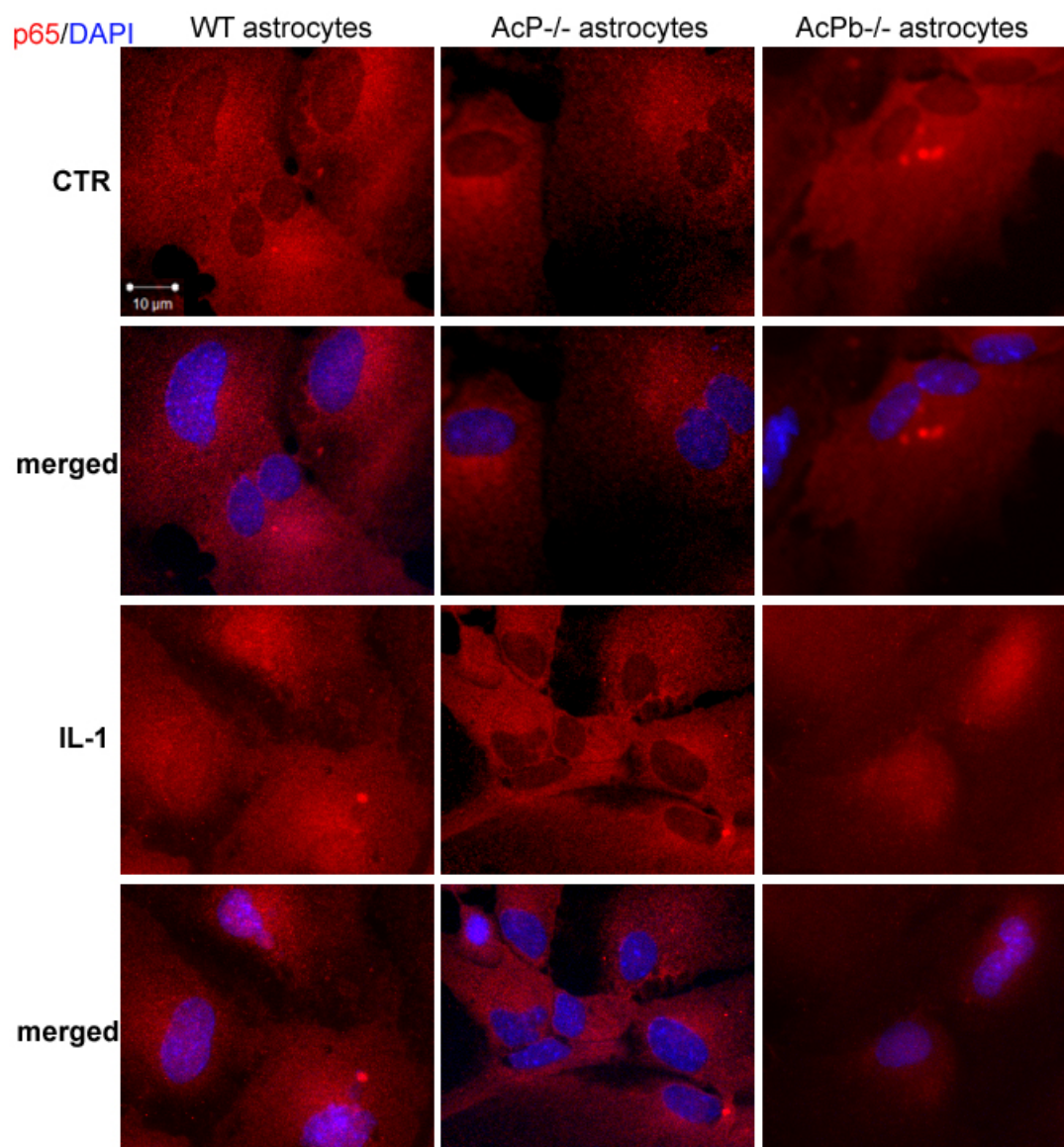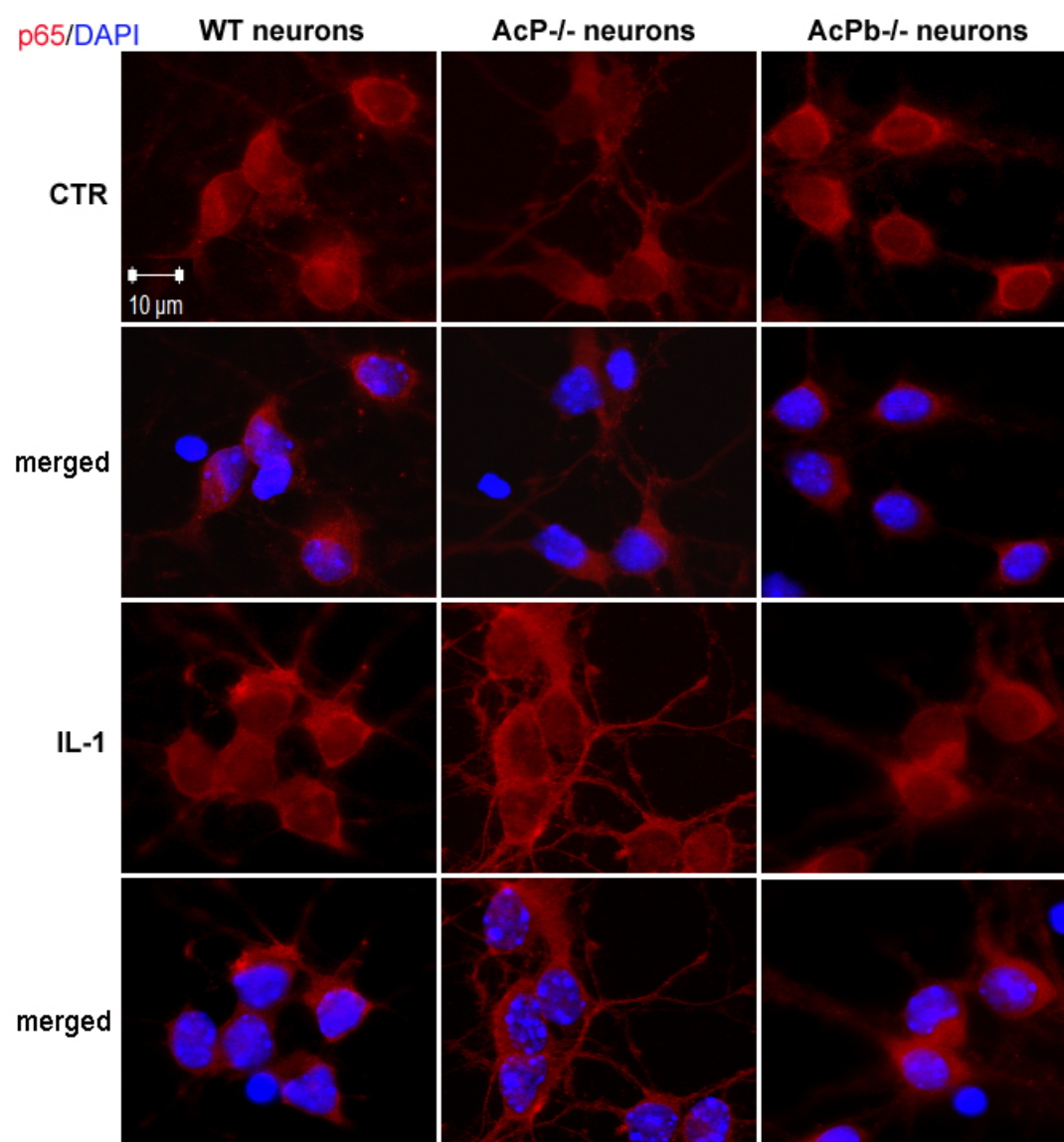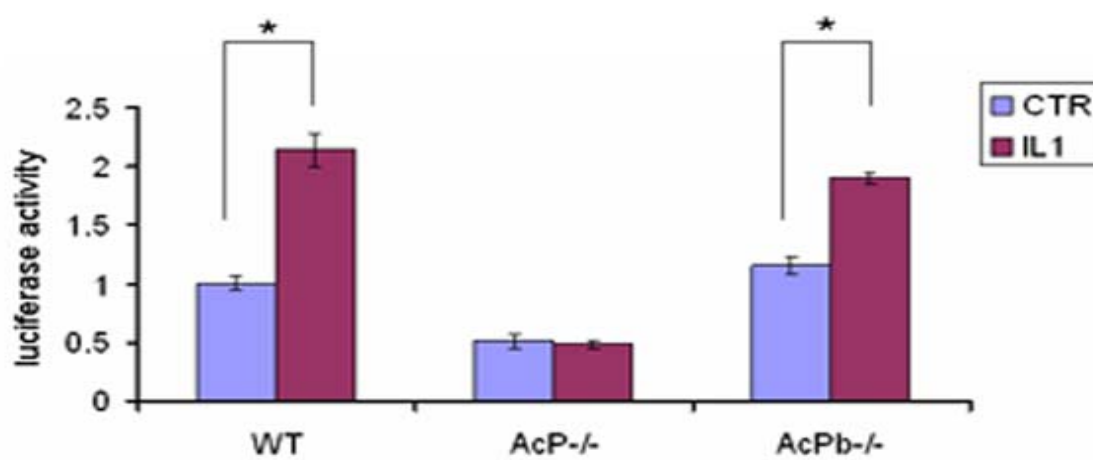
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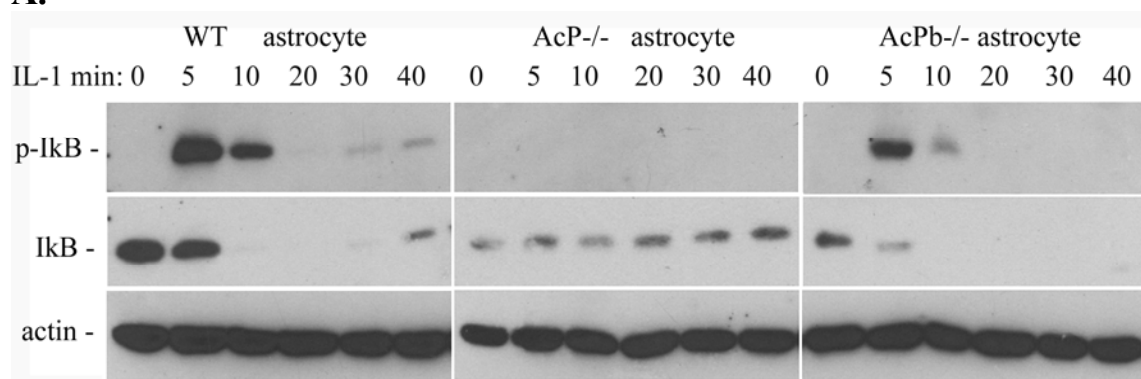
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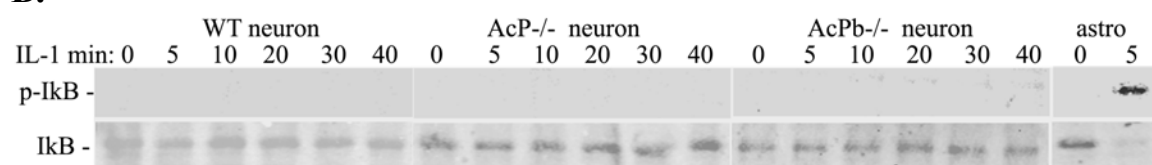
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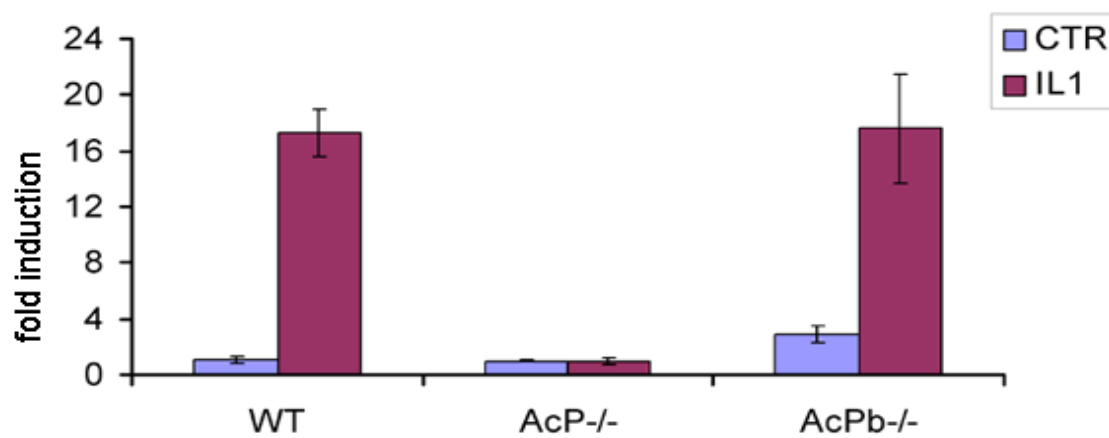
Figure 7. Overexpressing AcPb in WT astrocytes does not inhibit IL-1β -induced IκB phosphorylation. WT astrocytes were transfected with AcPb or an empty vector. 48 hours after transfection, cells were treated with IL-1β (10ng/ml) for 5 minutes. Protein lysates were immunoblotted for p-IκB and AcPb, then stripped and reprobed for IκB and actin as control.
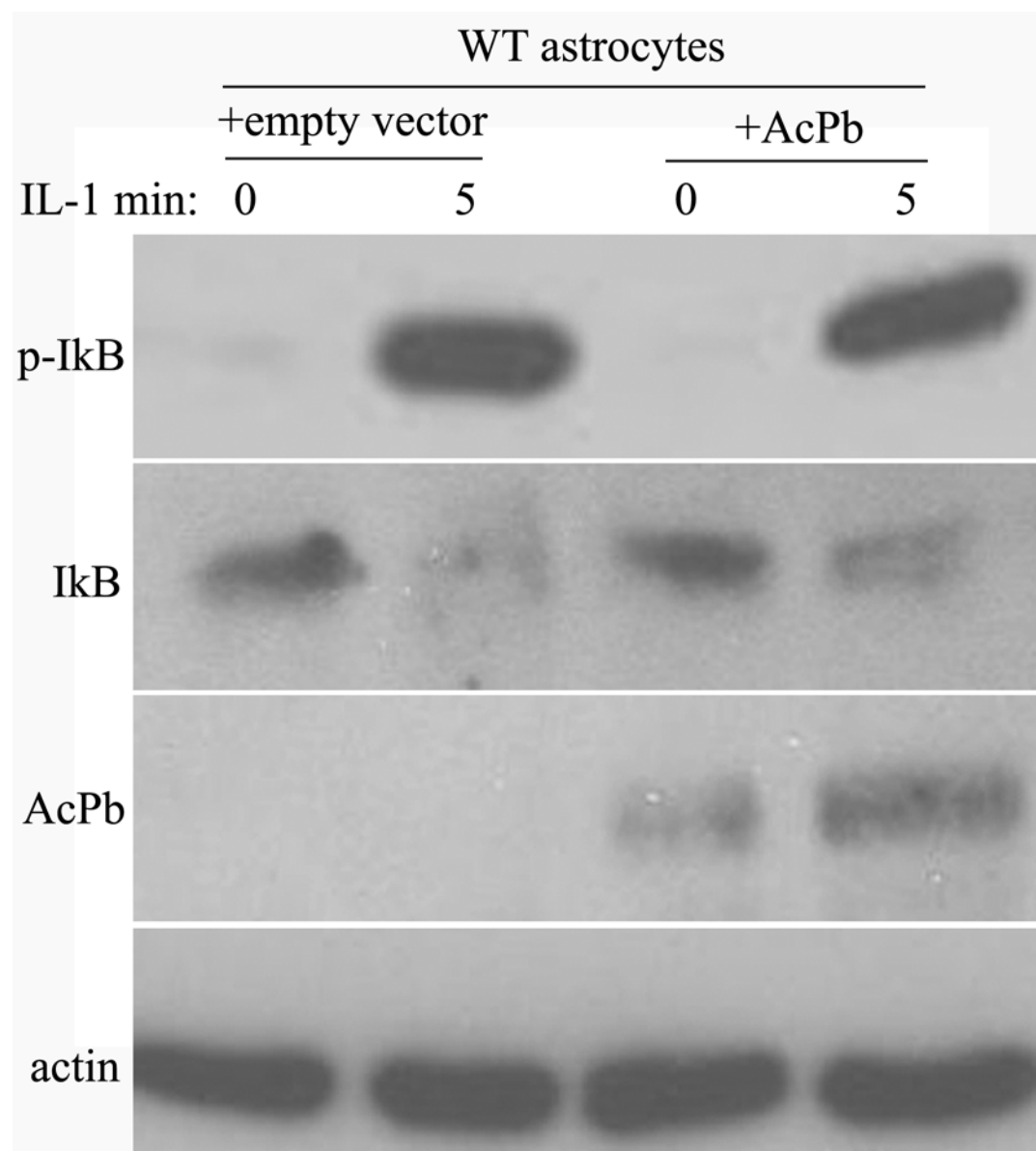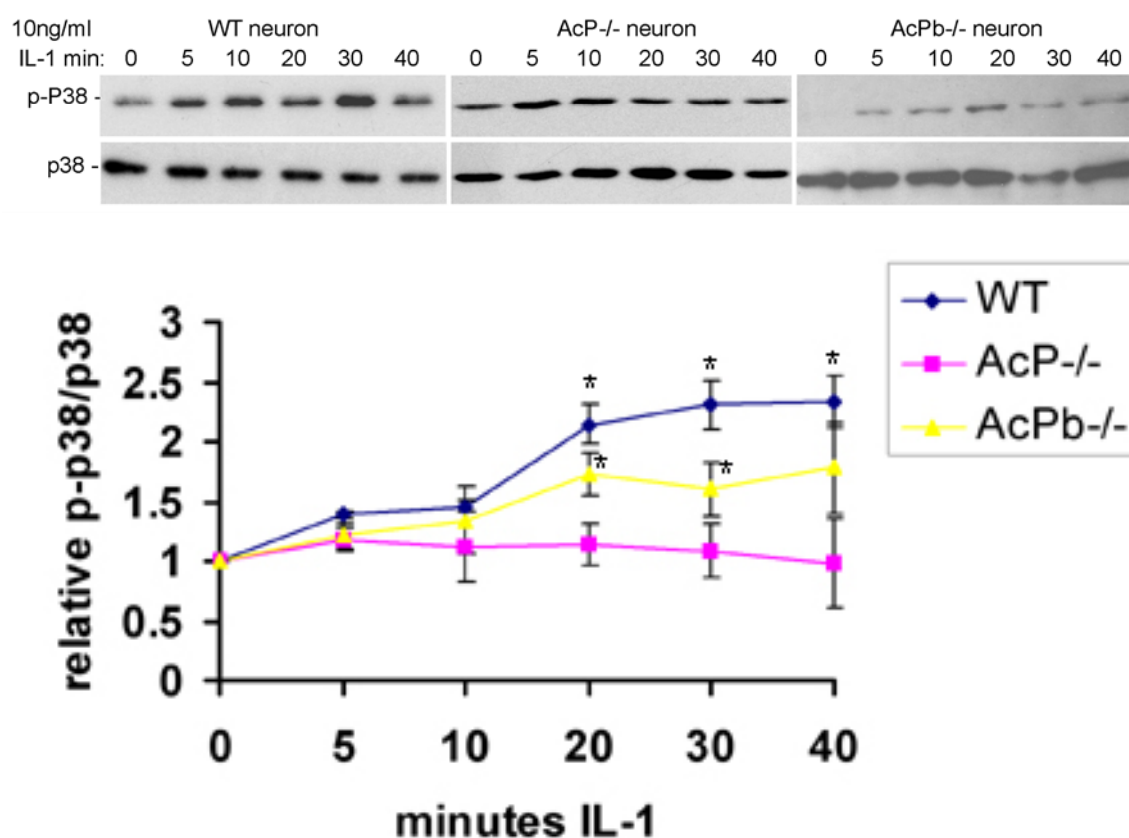
Figure 8. IL-1β induced p38 phosphorylation in hippocampal neurons independent of AcPb. (A) Time course treatment of IL-1β (10ng/ml) induced p38 phosphorylation in WT and AcPb-/- neurons, but not AcP-/- neurons. Quantification of blots from three different experiments as in (A). Densitometric values were expressed as p-p38/total p38, and then normalized to untreated cells (0 minutes). The significance was determined by one way ANOVA with Tukey's post-hoc analysis. * indicates values significantly different from control at $p<0.05$. (B) IL-1β induced p38 phosphorylation at 5 minutes in a dose dependent manner in WT and AcPb-/- neurons, but not AcP-/- neurons.
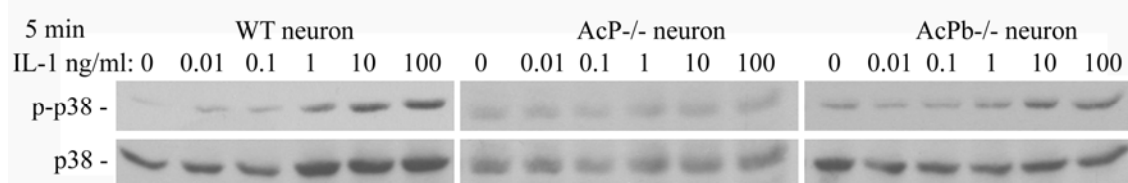
A.

B.

Figure 9. IL-1β induced p38 phosphorylation in hippocampal astrocytes, followed by CREB phosphorylation, in an AcP-dependent manner. (A) IL-1β (10ng/ml) treatment induced p38 and CREB phosphorylation in WT and AcPb-/- astrocytes, but not AcP-/- astrocytes. (B) Phosphorylation of both p38 and CREB was inhibited by p38 inhibitor SB203580 (20μM), but not by SN-50 (10μM), an inhibitor of NFκB nuclear translocation in both rat astrocytes (upper panels) and mouse astrocytes (lower panels).

A.
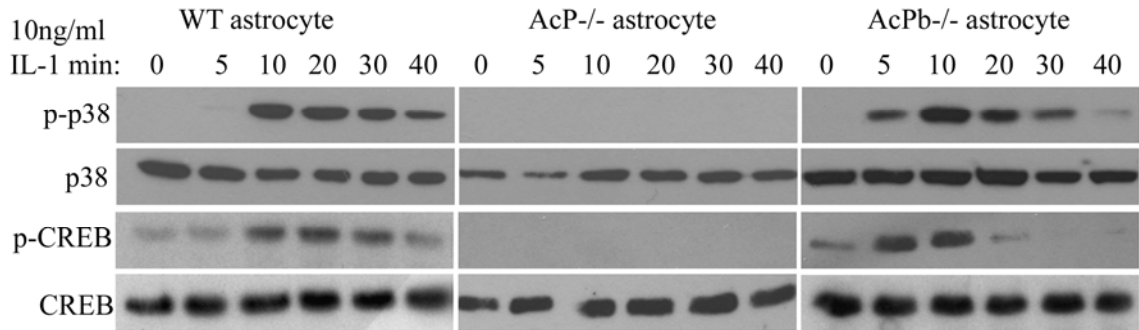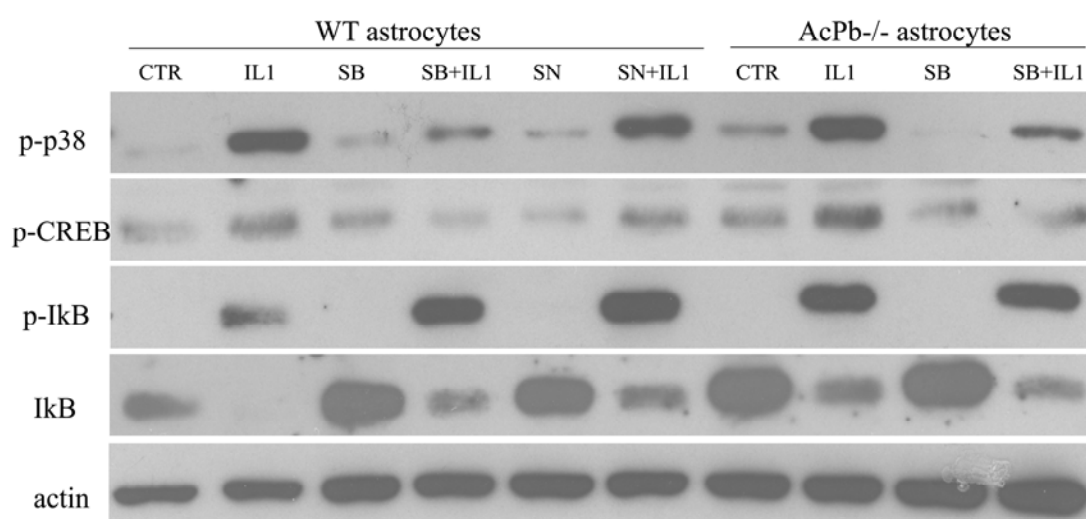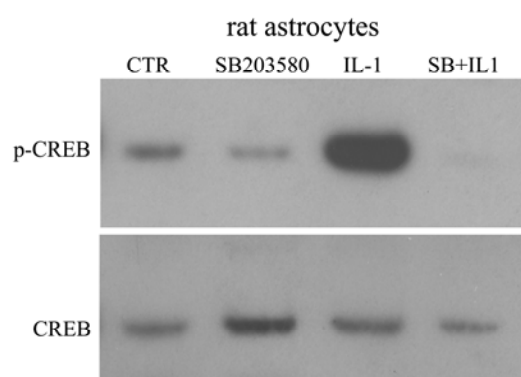
B.

Figure 10. Src phosphorylation by IL-1β (0.01ng/ml) in hippocampal neurons is dependent on AcPb. (A) Dose response treatment of IL-1β applied to cultured rat hippocampal neurons for 5 minutes. Protein lysates were immunoblotted for p-Src and p-p38, and reprobed for total Src and p38. (B) Quantification of blots from three different experiments as in (A). Densitometric values were expressed as p-Src/total Src or p-p38/total p38, and then normalized to untreated cells (0 ng/ml). The significance was determined by one way ANOVA with Tukey's post-hoc analysis. * indicates values significantly different from control at $p < 0.05$. (C) Time course of Src phosphorylation induced by IL-1β in mouse hippocampal neurons. At low concentration (0.01ng/ml), Src is phosphorylated by IL-1β in WT neurons, but not AcP-/- or AcPb-/- neurons.

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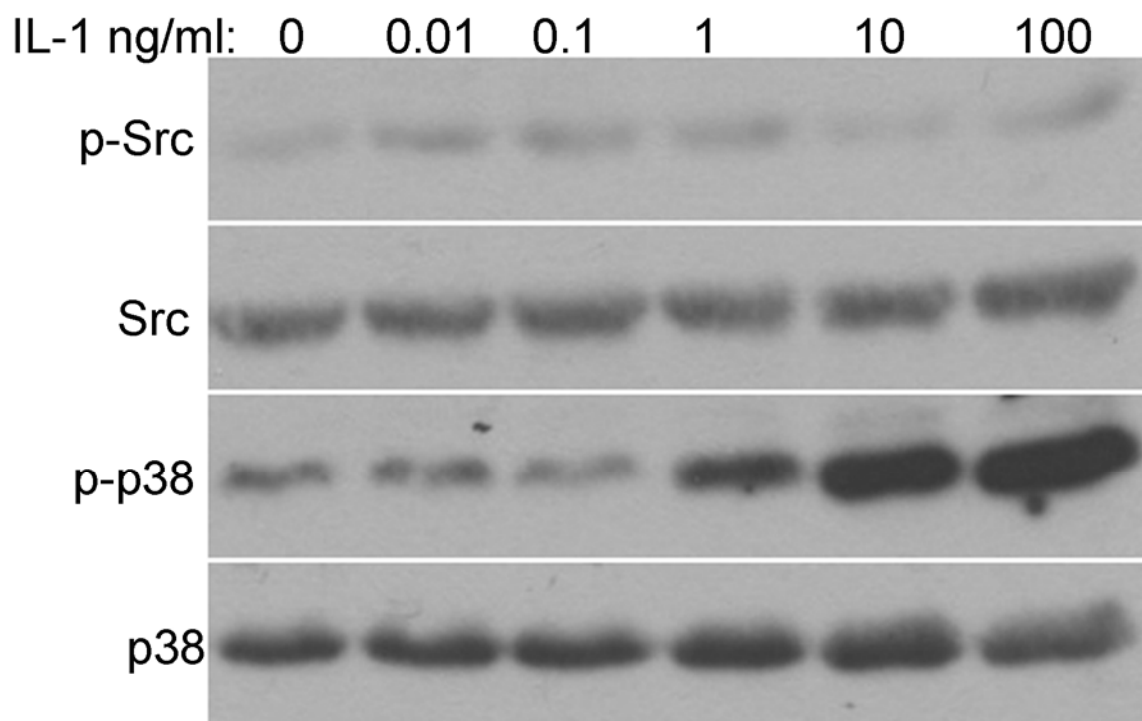
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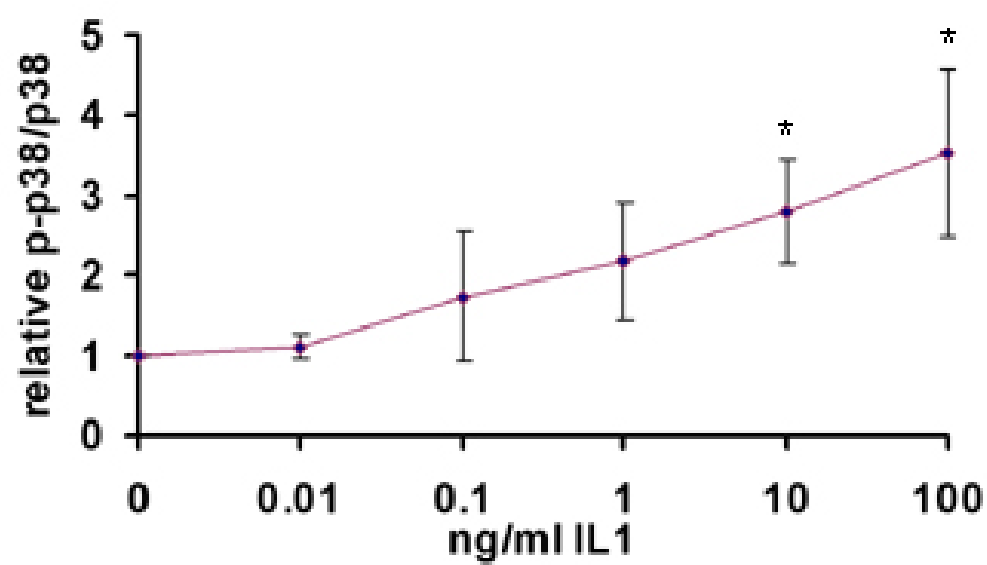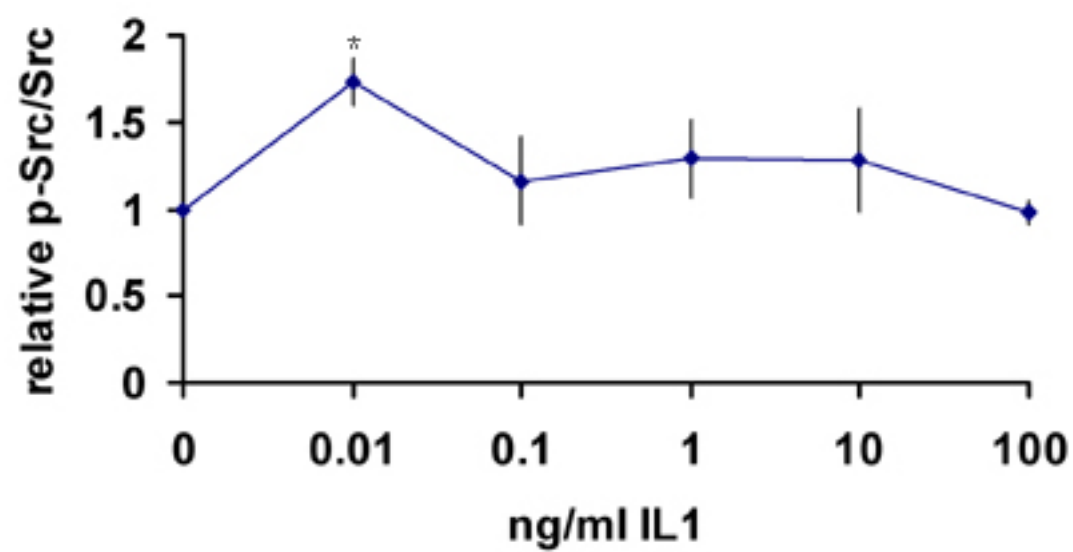
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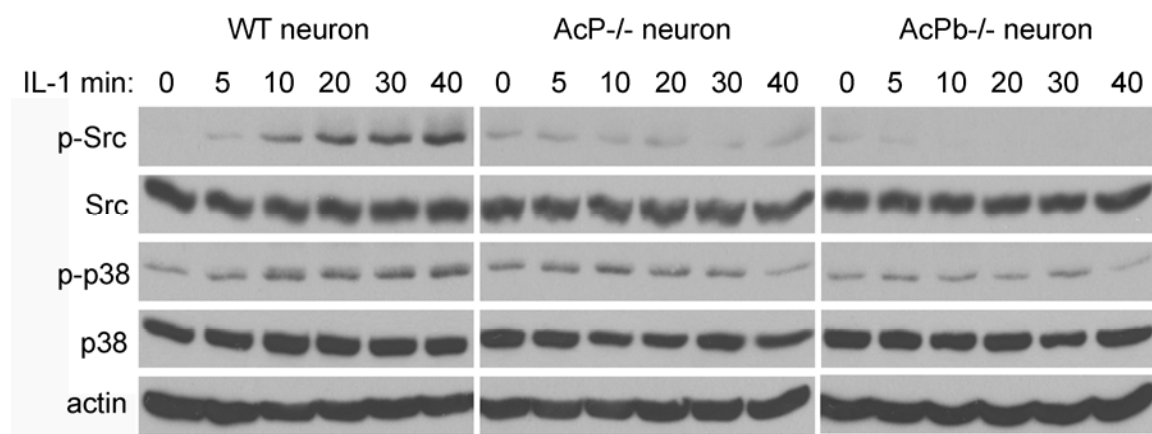
Figure 11. The effects of over-expressing AcPb in WT astrocytes on IL-1β -induced p-Src and p-p38 signaling pathway. A). Dose response treatment of IL-1 at 5 minutes in WT astrocytes with and without AcPb transfection. IL-1 induced Src phosphorylation in astrocytes transfected with AcPb compared to no transfection, but had no effect on p38. B). Time course treatment of IL-1β (0.01ng/ml) in WT astrocytes with and without AcPb transfection. Expression of AcPb in WT astrocytes enabled the IL-1β induced Src phosphorylation, but inhibited IL-1β induced p38 phosphorylation.

A.
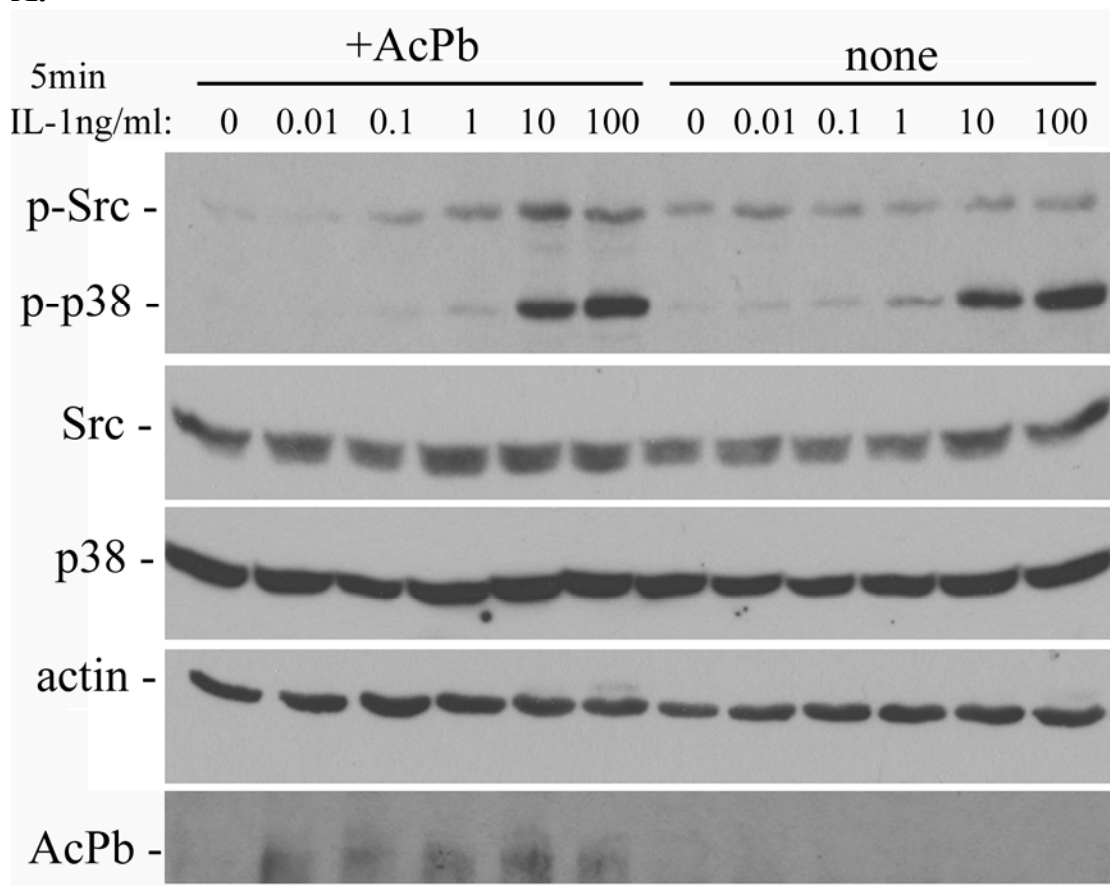
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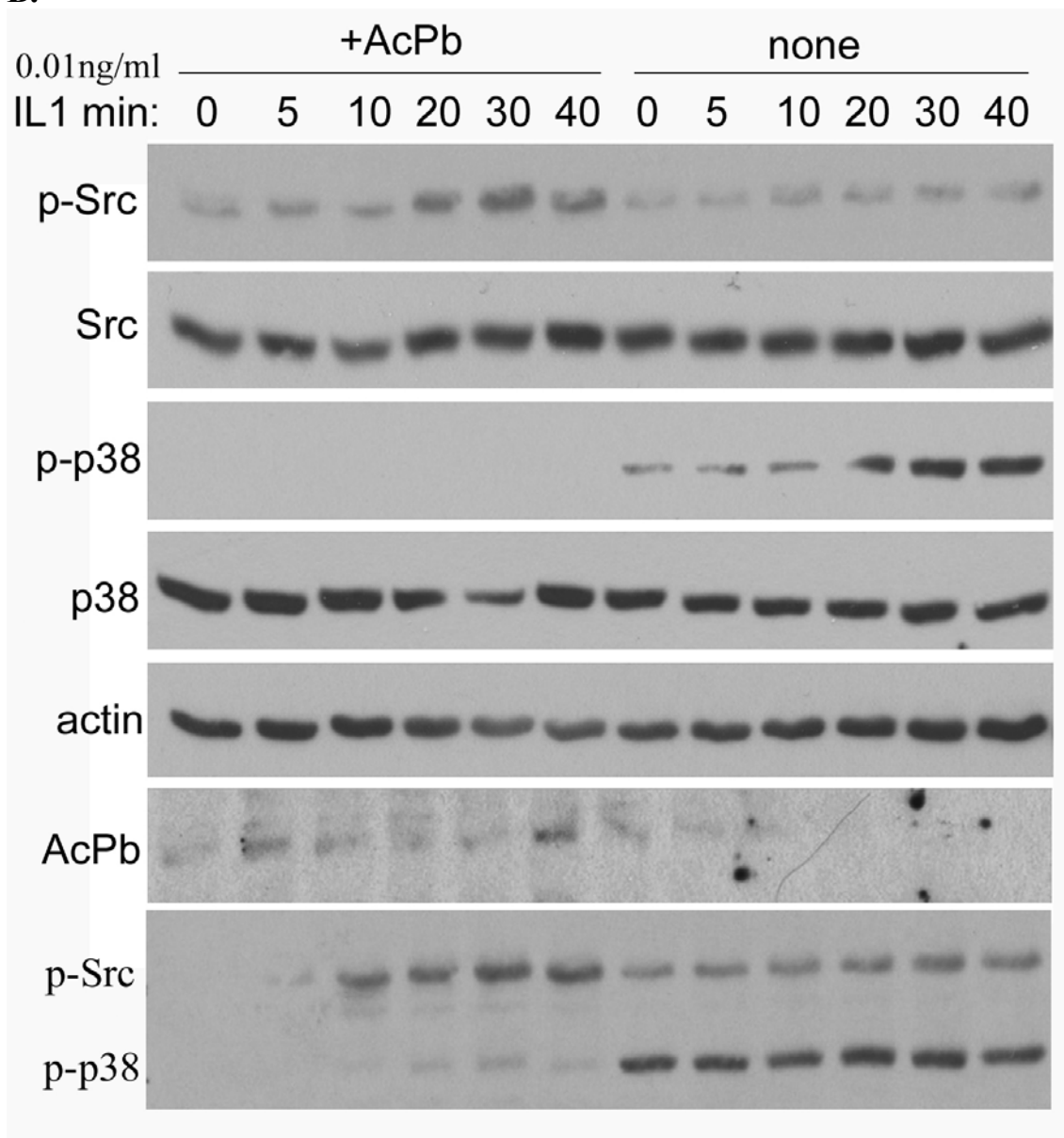
Figure12. The effects of over-expressing AcPb in total AcP-/- astrocytes on IL-1β-induced p-Src, p-p38 and p-IκB signaling pathway. (A) IL-1 (0.01ng/ml) induced p-Src in AcP-/- astrocytes restored with AcPb but not AcP. (B) and (C) are quantification of blots from three different experiements as in (A). Densitometric values were expressed as p-Src/total Src or p-p38/total p38, and then normalized to untreated cells (0 minutes) (D) IL-1 (10ng/ml) induced p-p38 in AcP-/- astrocytes restored with either AcP or AcPb, but only induced p-IκB in AcP-/- astrocytes restored with AcP.

A.
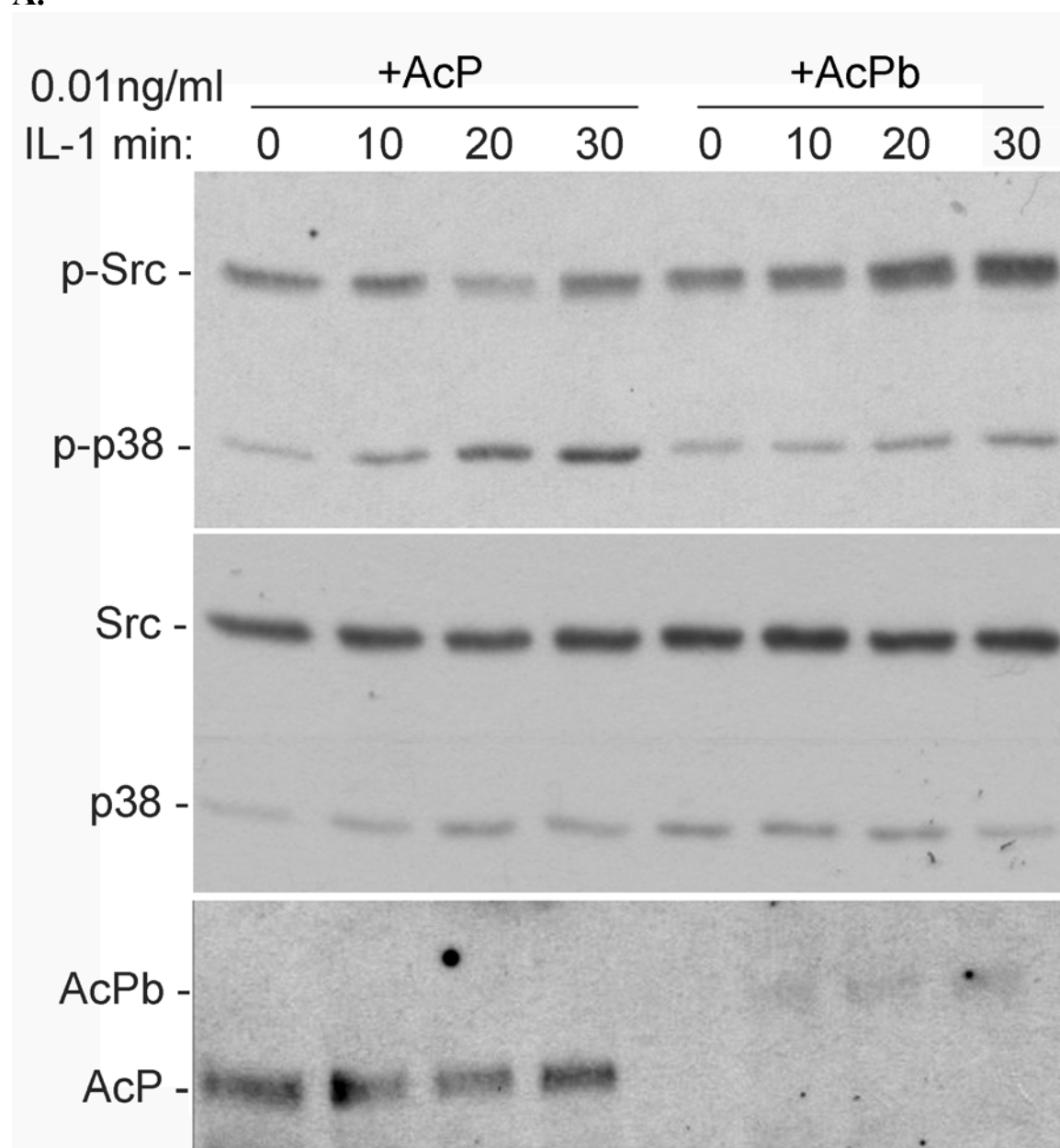
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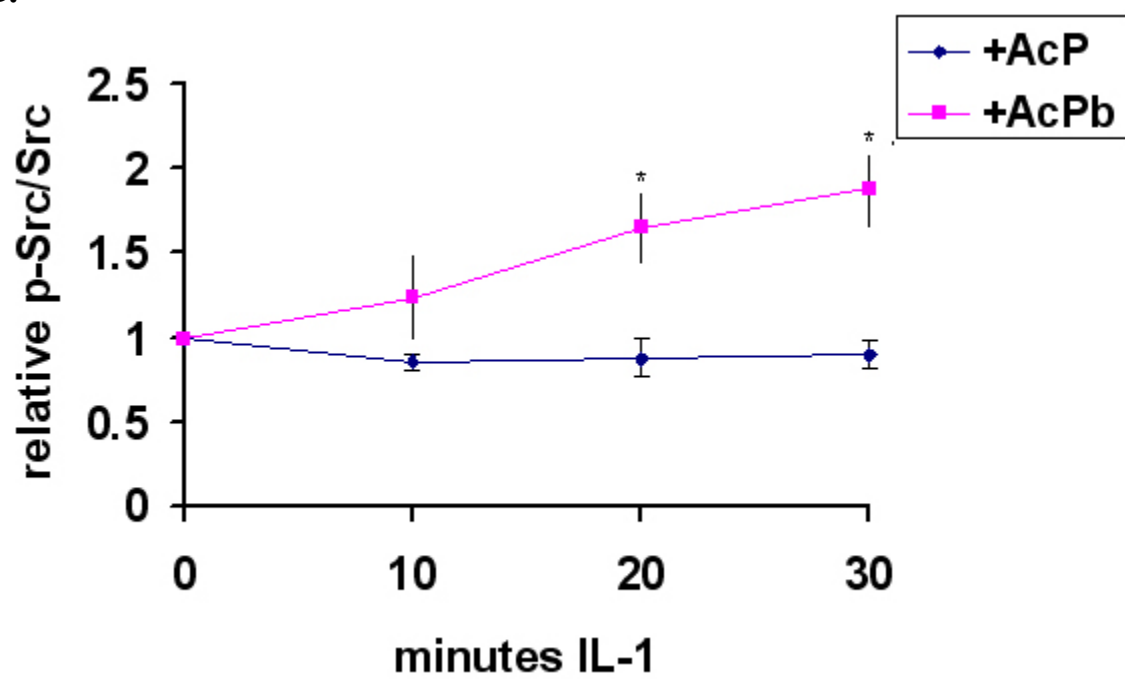
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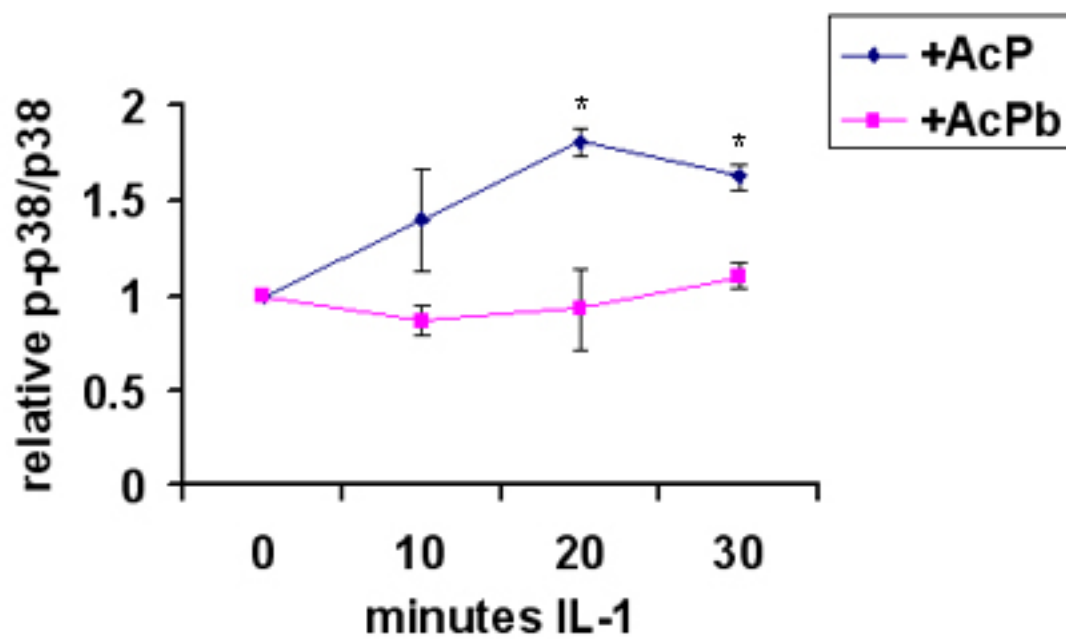
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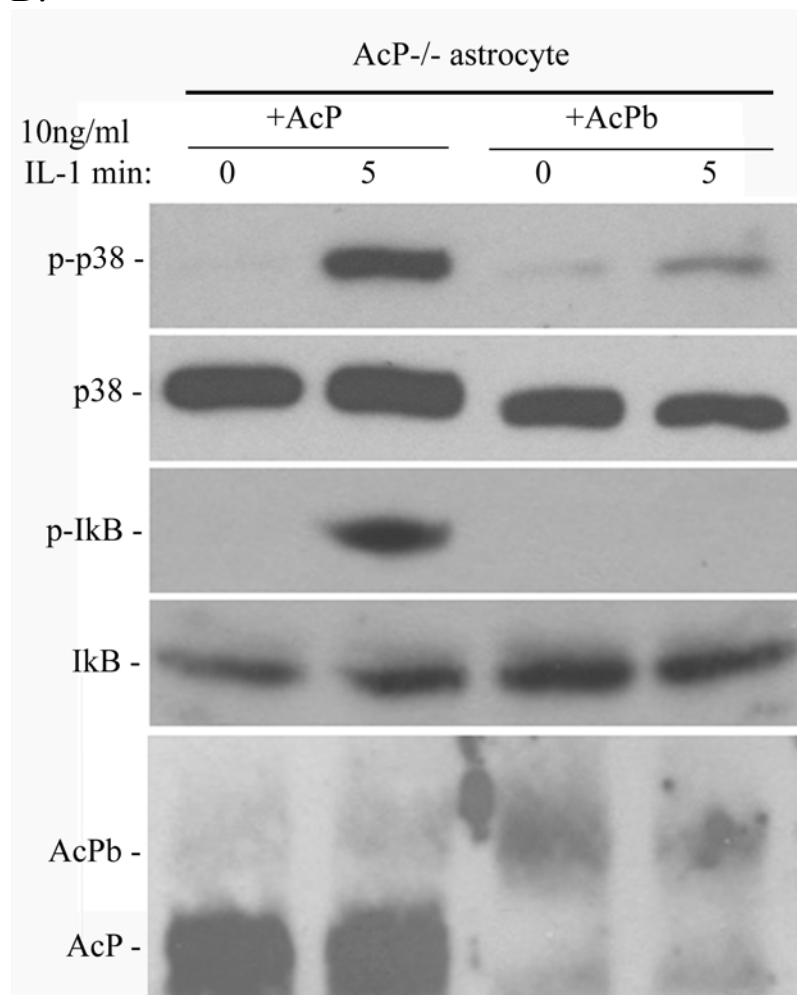
Figure13. Proposed model of neuron-specific IL-1β signaling pathways. Physiological concentrations of IL-1β (0.01ng/ml) bind to IL-1RI, which forms a complex with both AcP and AcPb to increase the binding affinity. The receptor complex recruits unidentified adaptor proteins and kinases, leading to the activation of Src kinase. Src kinase directly phosphorylates the NMDA receptor, causing potentiated calcium influx mediated through the NMDA receptor, and possibly increased LTP. At the same time, IL-1RI forms a small complex with AcP alone, which recruits known adaptor proteins and kinases such as MyD88 and IRAK4, leading to activation of p38MAPK followed by gene transcription. AcPb may also form a small complex with IL-1RI upon IL-1β binding, leading to the activation of the p38MAPK pathway. At pathophysiological concentration (10ng/ml), saturated IL-1β causes the receptor complex to separate, leading to more robust p38MAPK signaling and absence of Src signaling.
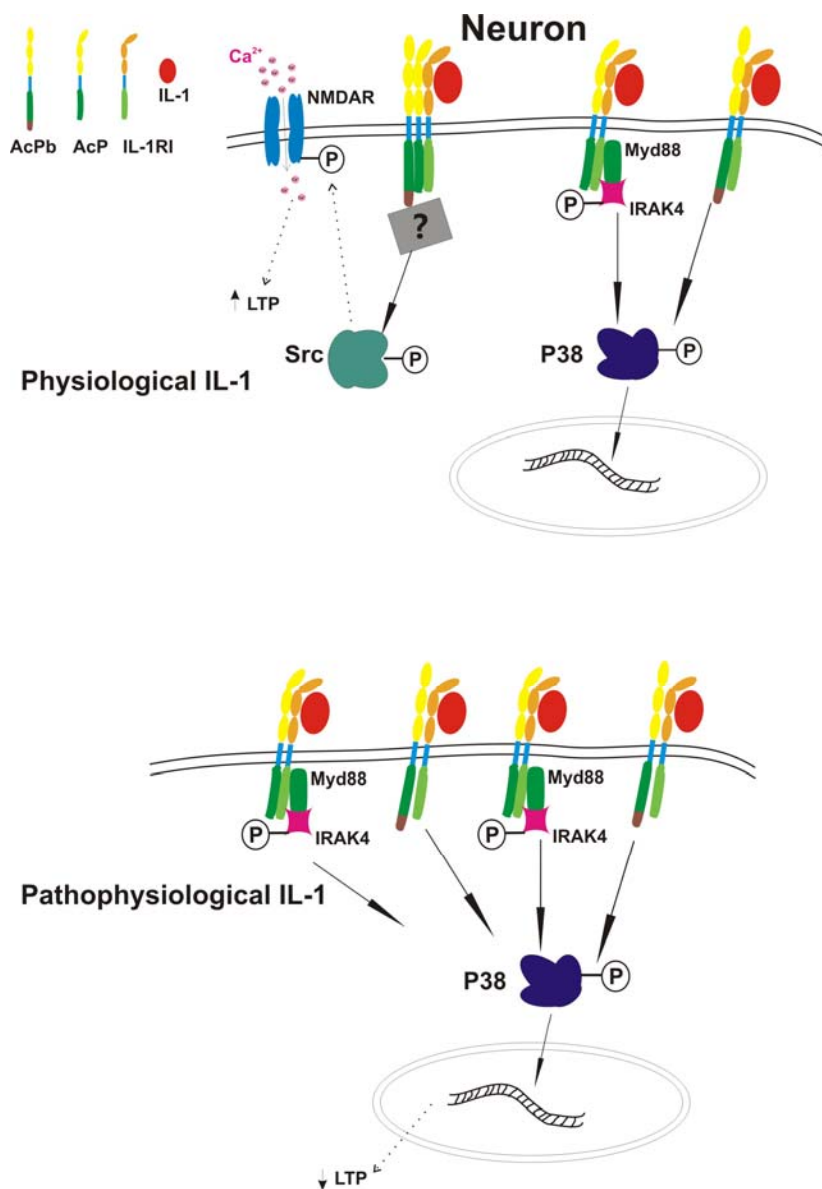
Figure 14. Proposed model of IL-1β signaling pathways in astrocytes. IL-1β activates both p38 and NFκB signals through the IL-1RI/AcP complex to mediate inflammatory gene regulation. When AcPb is overepxressed in astrocytes by transfection, some AcP is recruited to IL-1RI/AcPb to form a complex that induces p-Src but weakens p38 signaling. Excess AcPb may also form a complex with IL-1RI, which is sufficient for activation of Src.
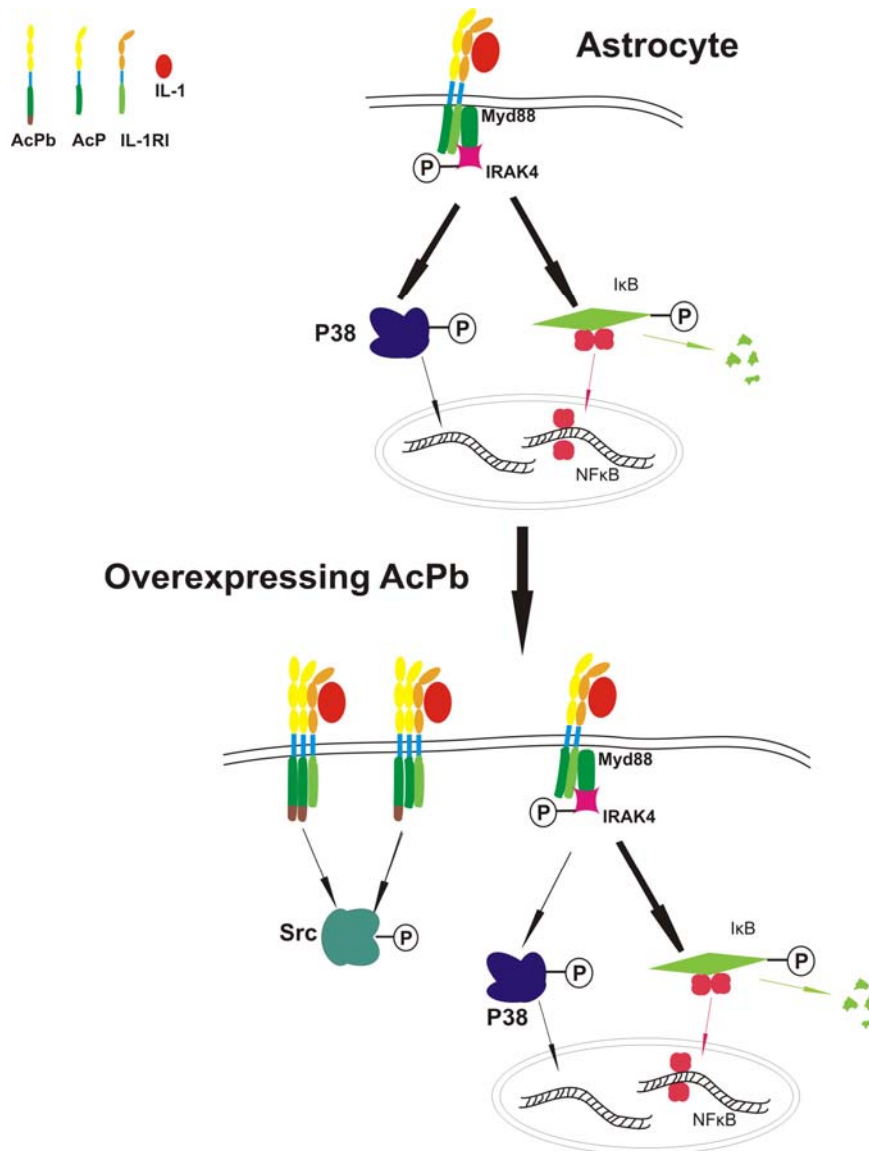
Table 1. Summary of IL-1β mediated cell signaling in mice hippocampal neurons and astrocytes. Black arrows represent strong inductions by IL-1β, grey arrows represent weak inductions by IL-1β.

Cell type	Neuron			WT astrocyte		AcP-/- astrocyte	
	WT	AcP-/-	AcPb-/-		+AcPb	+AcP	+AcPb
expressing / signaling	AcP AcPb		AcP	AcP	AcP AcPb	AcP	AcPb
P-Src	↑	–	–	–	↑	–	↑
P-38	↑	–	↑	↑	↑ (grey)	↑	↑ (grey)
P-IκB	–	–	–	↑	↑	↑	–

Green = endogenous protein
Red = exogenous protein

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