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INTERLEUKIN-1beta AND NMDA RECEPTOR: A BRIDGE BETWEEN INFLAMMATION AND GLUTAMATERGIC SYSTEM

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PREFACE

Interleukin (IL)-1β, originally described as an immune cell mediator in the periphery, has been involved in the modulation of several neurological functions and dysfunctions (Rothwell and Hopkins, 1995; Viviani et al., 2007). IL-1β can be expressed and produced by brain cells, albeit at low level in the healthy central nervous system (CNS). As well, IL-1β receptor (IL-1RI) and all components of IL-1 signalling are expressed in both neurons and glia.

IL-1β is involved in processes like regulation of sleep-wake cycle, control of synaptic activity, LTP maintenance/inhibition, and is implicated in several pathological conditions like ischemia, excitotoxic injury, Alzheimer's disease, HIV-dementia complex, epilepsy, neuropathic pain. Recently IL-1β has been indicated as important mediator in neuroendocrine and neurobehavioral stress response (Goshen and Yirmya, 2009) and to play a role in psychiatric disorders like schizophrenia (Meyer, 2011). While the initial trigger for acute injury or chronic disease may differ between neurological disorders, the resulting pathology may involve overlapping, if not identical, mechanisms. As such, a better understanding of the molecular mechanisms that underlie the action of this cytokine within the CNS might facilitate the development of promising therapeutics in the field of CNS disorders.

The biochemical pathways by which this cytokine contribute to brain dysfunction and injury remains largely unidentified. Substantial evidence suggests the existence of a reciprocal functional interaction between IL-1β and NMDA receptors (NMDARs) (Fogal and Hewett, 2008; Hagan et al., 1996; Loddick and Rothwell; 1996 Vezzani et al., 1999).

NMDARs are glutamate-gated ion channel widely expressed in CNS and play key roles in excitatory synaptic transmission. They are essential mediators of many forms of synaptic plasticity and molecular mechanisms of cognition (Aamodt and Costantine-Paton, 1999; Bliss and Collingridge, 1993). NMDARs are also key mediators of glutamate exicitotoxicity associated in acute neurological traumas as stroke, or in chronic neurodegeneration disease, including Huntington's disease, Alzheimer's diseases (Triller and Coquet, 2005). Based on these observations, in 2003 we hypothesized the existence of a functional relationship between IL-1β and the NMDAR that could in a way provide a molecular mechanism to several features common to both neurodegenerative and psychiatric disorders. We actually

demonstrated, that recombinant IL-1β induces the activation of Src family kinases and the subsequent phosphorylation at Tyr-1472 of GluN2B subunit of NMDAR (Viviani et al., 2003) in primary hippocampal neurons. The activation of this pathway potentiates NMDA-induced intracellular Ca^{2+} increase and also exacerbates NMDA-induced neuronal death *in vitro* (Viviani et al., 2003).

Thus, these results confirmed our hypothesis suggesting that hippocampal neurons exposed to IL-1β are more susceptible to glutamatergic excitation through the NMDA receptor component. Furthermore, these findings suggest that the recruitment of IL-1β/NMDAR cross talk could provide the missing link to understand the events implicated in the convergence of these to systems.

Due to: (i) the relevance of these two systems in the regulation of neuronal functions and in inducing susceptibility of neuronal impairment and decline, and (ii) the potential therapeutic implications, we thought to better define the molecular mechanisms that regulate the IL-1β/NMDAR cross talk by using both *in vitro* and *in vivo* approaches.

1. INTRODUCTION

1.1. INTERLEUKIN-1β (IL-1β) AND THE RELATED SIGNALLING COMPLEX: RELEVANCE IN THE MODULATION OF NEURONAL FUNCTIONS AND IN CENTRAL NERVOUS SYSTEM DISEASE

1.1.1. The IL-1 family

The canonical interleukin-1 family is composed of three closely related proteins that are products of different genes: two agonists with high sequence homology, interleukin-1α (IL-1α) and interleukin-1β (IL-1β), and a naturally occurring antagonist IL-1 receptor antagonist (IL-1ra) (Dinarello, 1994; 1996; 1998; 2002). IL-1α and IL-1β are synthesized as large precursor proteins. Most of IL-1α (90%) remains in the cytosol of cells in its precursor form or is transported to the cell surface where it remains membrane associated (Endres et al., 1989; Lonnemann et al., 1989). This membrane bound form may become activated and released following cleavage by an extracellular protease, perhaps now acting as a paracrine messenger to adjacent cells (Endres et al., 1989; Lonnemann et al., 1989; Dinarello and Wolff, 1993). However, considering the intra-nuclear localization of pro-IL-1α (Grenfell et al., 1989; Curtis et al., 1990), it has been suggested that intracellular pro-IL-1α may directly function as a gene regulator (Maier et al., 1990; Kawaguchi et al., 2006). In contrast to IL-1α, proteolytic cleavage performed by the IL-1β-converting enzyme (ICE) also known as caspasi-1 is required for the biological activity of IL-1β (Cerretti et al., 1992; Thornberry et al., 1992). IL-1β is then released by the cell into the extracellular space. Hence, it is unknown exactly how IL-1β is secreted, but it has been suggested that this can occur via exocytosis, active transport by a multi-drug resistance transporter, and/or following cell death (Hogquist et al., 1991; Griffiths et al., 1995; Singer et al., 1995; Ferrari et al., 1997; MacKenzie et al., 2001; Le Feuvre et al., 2002a; 2002b; Andrei et al., 2004; Bianco et al., 2005; Brough and Rothwell, 2007).

There are multiple levels of regulation of IL-1 production and activity, including

transcription, translation, cleavage and cellular release (Watkins et al., 1999). A diverse range of stimuli can affect these processes and lead to changes in IL-1 expression at the mRNA and/or protein level (Allan et al., 2005).

Both mature IL-1α and IL-1β can exhibit an essentially identical repertoire of functions by binding a specific 80 kDa plasma membrane receptor, IL-1 receptor type I (IL-1RI) which then associates with IL-1 accessory protein (IL-1RAcP) to form a complex that allows intracellular signalling (Sims et al., 1988; Korherr et al., 1997). There is also a type II IL-1 receptor (IL-1RII); however, it lacks the intracellular-signalling domain, so no downstream signal is initiated when IL-1 binds. IL-1RII functions biologically as a sink for IL-1β, it has a 10-100 fold lower affinity for IL-1α, and has been termed a decoy receptor (Colotta et al., 1993). All three receptor molecules, IL-1RI, IL-1RII and IL-1RAcP, can be shed from the cell membrane and therefore exist in soluble forms, sIL-1RI, sIL-1RII and sIL-1RAcP, respectively. Although most studies indicates that sIL-1RI functions as a decoy receptor, it has been proposed that signal transduction could be initiated if membrane-bound IL-1 were to bind sIL-1RI and subsequent association with IL-1RAcP occurred. sIL-1RII and sIL-1RAcP both functions as inhibitors of IL-1-mediated signal transduction, by sequestering pro-IL-1β and IL-1RI respectively. Furthermore, sIL-1RII can associate with IL-1AcP, thereby preventing the formation of an IL-1/IL-1RI/IL-1RAcP tri-molecular signalling complex (Subramaniam et al., 2004). The existence of these complex regulatory mechanisms for IL-1 indicates the potential biological importance of this molecule (Allan et al., 2005).

The third element of IL-1 family, IL-1ra, is produced by the same cells that express IL-1, and there are three intracellular isoform (icIL-1ra1, icIL-1ra2, icIL-1ra3) and one secreted isoform (sIL-1ra). The secreted isoform functions as a competitive antagonist that binds IL-1RI but does not trigger the signal transduction, whereas the intracellular isoforms have poorly defined roles at present (Malyak et al., 1998).

All members of the IL-1 family (i.e., IL-1, IL-1ra, IL-1RI, IL-1RII, and IL-1RAcP) are expressed in the healthy central nervous system (CNS). Low levels of IL-1β immunoreactivity have been detected throughout the brain of rodents, with highest expression occurring in the hippocampus, hypothalamus, and basal forebrain (Breder et al. 1988; Lechan et al. 1990; Molenaar et al., 1993). IL-1RI has also been shown

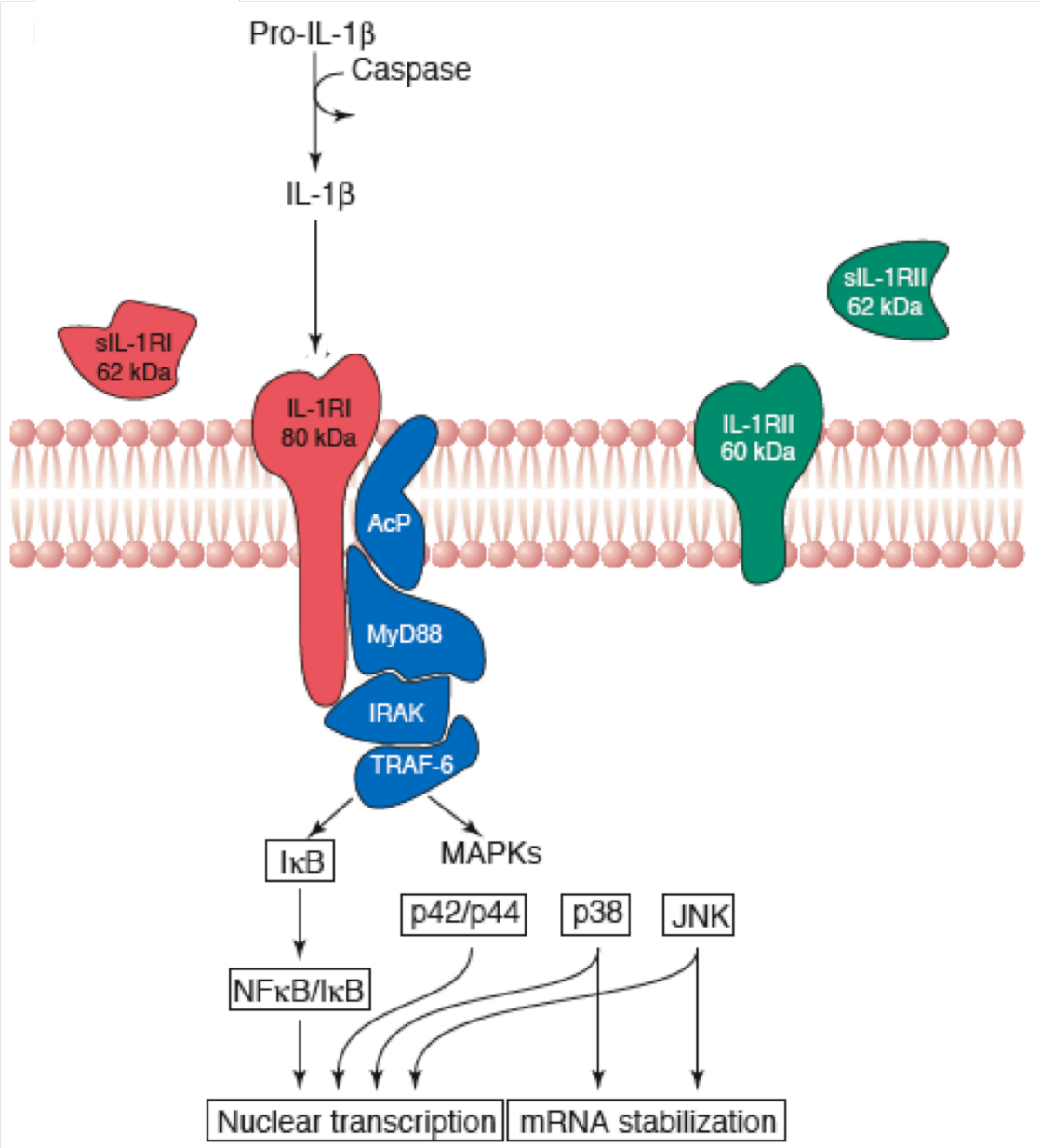
to be expressed throughout the brain, with highest levels found in cerebral cortex and hippocampus (Farrar et al., 1987; Takao et al., 1990; French et al., 1999). The cell types capable of synthesizing IL-1 - microglia (Giulian et al., 1986; Hetier et al., 1988; Yao et al., 1992), astrocytes (Lieberman et al., 1989; Knerlich et al., 1999; Zhang et al., 2000), oligodendrocytes (Blasi et al., 1999), and neurons (Lechan et al., 1990; Takao et al., 1990; Watt and Hobbs, 2000) - also express the signalling receptor (Ban et al., 1993; Cunningham and De Souza, 1993; Wong and Licinio, 1994; Tomozawa et al., 1995; Blasi et al., 1999; French et al., 1999; Hammond et al., 1999; Friedman, 2001; Pinteaux et al., 2002; Wang et al., 2006). Finally, IL-1RAcP - the protein necessary for signal transduction via IL-1RI to occur - is also expressed in the rat brain under normal physiological conditions with particularly high levels in the hypothalamus, cortex, hippocampus, and cerebellum (Liu et al., 1996; Ilyin et al., 1998).

Under normal conditions, the levels of IL-1 are low, both in the circulation and in the CNS, whereas upon infection, injury or other types of insults/stimuli, an abrupt but transient increase in the IL-1 levels occurs.

1.1.2. IL-1 signalling

IL-1 binds to its receptors with high affinity and hence only low concentrations are required for a biological response (Allan et al., 2005). Indeed, IL-1 can elicit responses on cells with a low receptor number (<100 per cell) because it activates a complex cascade resulting in signal amplification (O'Neill and Dinarello, 2000; O'Neill, 1995). Activation of IL-1RI by IL-1 ligands results in association with IL-1RAcP and recruitment of the adaptor protein myeloid differentiation factor 88 (MyD88) by the intracellular domain of the IL-1RI. This, in turn, leads to recruitment of IL-1R-associated kinase (IRAK I and II), which complexes with the IL-1RAcP (Huang et al., 1997), and activation of other proteins, including tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6), and the nuclear factor kappa B (NF-κB) inducing kinase (O'Neill, 1995). Phosphorylation and degradation of the NF-κB

inhibitor, IKB, by IKB kinases (IKKα and IKKβ) results in release of NFκB and its subsequent translocation into the nucleus (Di Donato et al., 1997). Once within the nucleus, NFκB binds to its consensus sequence on a target gene promoting transcription and upregulation of expression (Figure 1). IL-1 also activates the protein kinase pathway. To date, three major protein kinases have been identified that are responsive to IL-1 (O'Neill and Greene, 1998). These include p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK and c-Jun N-terminal kinase 1 (JNK1) (Figure 1). IL-1β seems to promote MAPK signalling pathway and CREB activation in hippocampal neurons. On contrary, IL-1β activates NFκB in hippocampal astrocytes (Srinivasan et al., 2004), indicating a distinct and specific effect on both these cell population. The majority of the work conducted on unravelling the signalling pathways activated by IL-1 has been carried out on peripheral cells. It is assumed that similar mechanisms are responsible for most IL-1 actions in the CNS. However, important exceptions are emerging, some of which involve the cells of the nervous system, this suggests the importance of defining the signalling of IL-1β in a cell-type specific manner. The current consensus is that IL-1β signalling has an absolute requirement for MyD88 as MyD88-deficient cells are apparently unresponsive to IL-1β (Adachi et al., 1998). However, it has been shown that the IL-1R/IL-1RAcP complex is capable of signalling in the absence of MyD88 in anterior hypothalamic neurons (Kenny and O'Neill, 2008). It was revealed that p85 subunit of PI3-kinase binds directly to IL-1RI when the receptor is phosphorylated leading the activation of Akt in the absence of MyD88. The activation of this pathway seems to have a neuroprotective effect in response to IL-1β.



Adapted from: Rothwell, N.J. and Luheshi, G.N. 2000. *Interleukin 1 in the brain: biology, pathology and therapeutic target*. TINS 23: 618-625.

Figure 1. Signalling of IL-1β

The binding of IL-1β to IL-1RI in the immune system leads to its association with the IL-1R accessory protein (IL-1RAcP) and the myeloid differentiation primary response protein 88 (MyD88) to form the core of the IL-1β/IL-1RI signalling complex. IL-1 activates the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways.

1.1.3. IL-1β as neuromodulator of the central nervous system disease

IL-1β has been proposed as a novel neuromodulator involved in the communication between glia and neurons (Allan and Rothwell, 2001; Allan et al., 2000; Chatterton, 2002; Coogan and O'Connor, 1997), opening up new perspective in the current view of brain behavior. This cytokine, locally produced by glial cells as a consequence of central nervous system (CNS) diseases and/or in response to neuronal activities (Coogan and O'Connor, 1997), exerts a profound impact on neuronal functionality.

IL-1β has been implicated in the exacerbation of neuronal damage caused by excitotoxic, ischemic, traumatic brain injury (Allan and Rothwell, 2001; Yamasaki et al., 1995), and viral infection (AIDS dementia complex: HAD) (Bagetta et al., 1999; Gallo et al., 1999; Viviani et al., 2001), in seizures (Vezzani et al., 2000). These physiological and pathological conditions are associated with an increased expression of IL-1β in the brain (Taishi et al., 1997; Schneider et al., 1998; Vezzani et al., 1999, 2000; Allan and Rothwell, 2001). Furthermore, administration of the naturally occurring antagonist of IL-1β, namely IL-1 receptor antagonist (IL-1ra), inhibits motor and electroencephalographic seizures (Vezzani et al., 2000), and reduces neuronal damage caused by ischemic, excitotoxic, and traumatic brain injury (Yamasaki et al., 1995; Loddick and Rothwell, 1996; Allan et al., 2000). In addition, there is experimental evidence demonstrating the action of IL-1β in neurodegenerative diseases. In patients with Alzheimer's, there is an increase in the levels of IL-1 in post-mortem brain tissue (Griffin et al., 1989) and in the CNS tissues of patients with this disease (Cacabelos et al., 1991). In amyotrophic lateral sclerosis has been observed an increased expression of IL-1β and related cell death, the latter inhibited by IL-1ra (Troy et al., 1996).

High level of pro-inflammatory IL-1β has been also associated with cognitive decline, impairment of memory, and recently has been linked to the development of psychiatric disorders (McAfoose and Baun, 2009; Allan and Rothwell, 2001). For example, the increase of IL-1β in the CNS (via icv administration, or caused by pathogens) adversely affects the processes of memory consolidation in hippocampal and causes the onset of symptoms similar to depression (Thomas et al., 2005), attenuated by the antagonist of the event type I receptor of IL-1 (IL-1ra) (Pugh,

2000; Hayley et al., 2005) or by manipulating of genes family of IL-1 (Avital et al., 2003).

Increased CSF levels of pro-inflammatory cytokines such as IL-1β have also been noted in schizophrenic patients (Söderlund et al., 2009). Some reports further suggest that schizophrenia is associated with reduced potency to mount anti-inflammatory responses in the CNS, as supported by findings of reduced gene and/or protein expression of sIL-1RA (Toyooka et al., 2003). Furthermore, schizophrenic patients have repeatedly been shown to display allelic variants in IL-1 and IL-1RA (Katila et al., 1999; Xu and He, 2010; Zanardini et. al, 2003). Since many of the cytokine gene/promoter polymorphisms are known to directly influence protein synthesis, it is likely that such genetic variants exert a functional impact on cytokine protein networks in affected subjects.

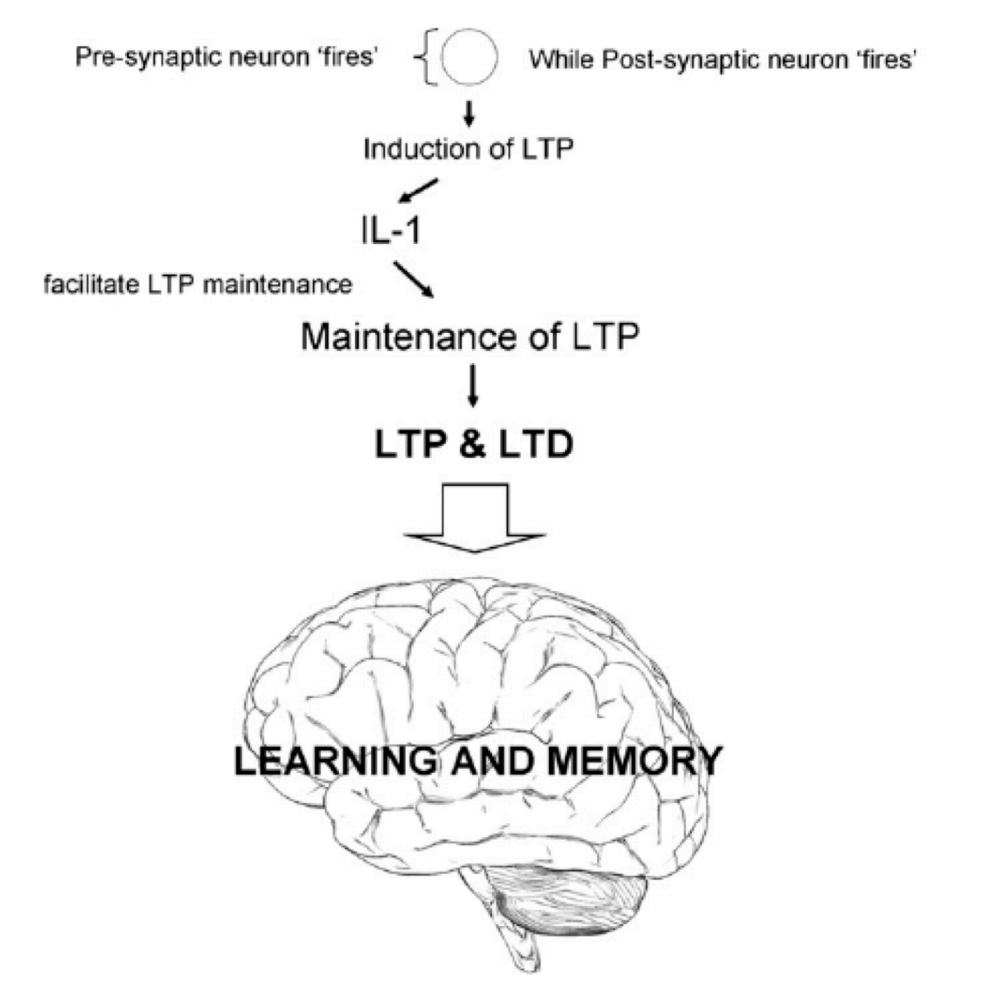
1.1.4 Cytokine model of cognition: IL-1β influences cognitive function by affecting synaptic plasticity

Molecular investigations in experimental rodent model have also confirmed that IL-1β can exert appreciable influence on various form of synaptic plasticity, which is recognized to provide an important neuronal substrate for multiple aspects of learning and memory (Bauer et al., 2007). It is therefore feasible that altered IL-1β activity may exert its cognitive effects by modulating synaptic architecture and functions.

Previous research has suggested that IL-1β can modulate synaptic transmission in the hippocampus and appears to inhibit LTP induction (Bellinger et al., 1993; Cunningham et al., 1996; Katsuki et al., 1990; Murray and Lynch, 1998). Failing to confirm such findings, Schneider et al. (1998) demonstrated that increased local production of IL-1β in the hippocampus plays a role in LTP maintenance. As noted by Schneider et al. (1998), this alternative finding might have occurred because of the high concentration levels used in previous studies, which were more comparable to levels seen during pathological and inflammatory states then normal physiological

conditions. Subsequent work (Coogan et al., 1999; Ross et al., 2003) has confirmed that at least under physiological conditions IL-1β is required for LTP maintenance, whereas, higher concentration of IL-1β, under pathophysiological conditions, inhibits LTP. Together these findings suggest that IL-1 plays an intimate role in synaptic plasticity and that through these mechanisms possibly an important role in memory consolidation (Figure 2).

Cognitive-behavioural studies in animals have repeatedly shown that IL-1β influences various types of hippocampal-dependent memory (Brennan et al., 2003; Yirmiya et al., 2002). Moreover, research by Depino et al. (2004), has recently demonstrated that endogenous IL-1a also participates in hippocampal memory processing. As reviewed by Pugh et al. (2001), there is considerable evidence to suggest that IL-1 might under physiological conditions play a role in memory consolidation processes. However, during stress, aging and disease, IL-1 appears to elicit memory impairment (Pugh et al., 2001). In support of these findings, IL-1β has recently been demonstrated to play a dual role in hippocampal-dependent memory processes (Avital et al., 2003; Goshen et al., 2007b). More specifically, it was demonstrated by these authors that the involvement of IL-1β in hippocampal-dependent memory follows an inverted U-shape pattern, in that basal levels of IL-1β are required for normal memory function, and any deviation from this physiological range (either deletion or elevation) results in impaired memory (Avital et al., 2003; Goshen et al., 2007b). Research by Avital et al. (2003), further demonstrated that impaired memory in IL-1 receptor type 1 knockout mice, coincided with deficits in synaptic plasticity. Moreover, as recently shown by Young et al. (2007), increased levels of IL-1 disrupts an LTP-associated spinal learning paradigm (Grau et al., 2006), suggesting that IL-1 over-expression might impair LTP-associated learning processes possibly throughout the neuroaxis (Deak, 2007). Although not fully understood, these findings provide strong evidence to support a direct link between synaptic plasticity, IL-1 and cognitive functioning.



Adapted from: McAfoose, J. and Baune, B.T. 2008. *Evidence for a cytokines model of cognitive function*. Neurosci Behav Reviews 33: 355-366.

Figure 2. Schematic illustration of the involvement of IL-1 in Hebbian synaptic plasticity, such as LTP and LTD

Essentially this process can be divided into three stages. In stage 1, the simultaneous neuronal activity (firing) of both pre-synaptic and post-synaptic neurons results in the induction of LTP. This induction of LTP leads to the production of IL-1 (stage 2). Finally in stage 3, LTP maintenance is fine-tuned by the overall level of expression of IL-1 (facilitates) thus influencing the consolidation of learning and memory.

1.2 NMDA RECEPTOR (NMDAR)

1.2.1 Structure, subunit composition and functional properties of NMDAR

N-methyl-D-aspartate receptors (NMDARs) are glutamate-gated ion channels widely expressed in the central nervous system (CNS) that play key roles in excitatory synaptic transmission. NMDARs mediate aspects of CNS development and are essential mediator of many forms of synaptic plasticity and neurotoxicity (Paoletti and Neyton 2007).

NMDARs are heteromeric complexes incorporating different subunits within a repertoire of three subtypes: GluN1, GluN2 and GluN3. There are eight different GluN1 subunits generated by alternative splicing from a single gene, four different GluN2 subunits (A, B, C and D) and two GluN3 subunits (A and B); the GluN2 and GluN3 subunits are encoded by six separate genes (Dingledine et al., 1995). Expression of functional recombinant NMDARs in mammalian cells requires the co-expression of at least one GluN1 and one GluN2 subtype. The stoichiometry of NMDARs has not yet been established definitely, but the consensus is that NMDARs are tetramers that most often incorporate two GluN1 and two GluN2 subunits of the same or different subtypes (Dingledine et al., 1995). The GluN3 subunit assembles with GluN1 and GluN2, resulting in a receptor with diminished activity (Das et al., 1998), and GluN3 subunit assembles with GluN1 alone to create a functional glycine receptor (Chatterton et al., 2002).

The GluN2A and GluN2B subunits are the major and most widespread GluN2 subunits. The GluN2B subunit predominates early in development and then gradually decreases, whereas expression of GluN2A is low shortly after birth but continues to increase. Therefore, GluN2B is the major subunit during the early period of a neuron's life, whereas GluN2A is predominant in the later stages, suggesting that the GluN2B to GluN2A switch is responsible for the transition of a synapse from a more plastic to a less plastic state (Wenthold et al., 2003). GluN2C is restricted primarily to the cerebellum and is expressed later in development (Paoletti et al., 1997). In

contrast, GluN2D is predominantly expressed early in development and is localized mainly in the thalamic and hypothalamic nuclei and in the brainstem (Paoletti et al., 1997).

NMDAR subunits all share a common membrane topology characterized by a large extracellular N-terminus, a membrane region comprising three trans-membrane segments (TM1, 3 and 4) plus a re-entrant pore loop (M2), an extracellular loop between TM3 and TM4, and a cytoplasmic C-terminus, which varies in size depending upon the subunit and provides multiple sites of interaction with numerous intracellular proteins (Dingledine et al., 1995; Mayer, 2006) (Figure 3).

The extracellular region of NMDAR subunits is organized as a tandem of two domains. The N-terminal domain (NTD; first 350 amino acids) plays an important role in subunit assembly (Meddows et al., 2001); in GluN2A and GluN2B, the NTD also contains binding sites for allosteric inhibitors such as Zn^{2+} and ifenprodil. The second domain comprises the pre-TM1 region and the TM3–TM4 loop (~150 amino acids each) contains the agonist binding site (Figure 3).

The activation of NMDARs requires the simultaneous binding of two co-agonists: glutamate and glycine (or D-serine). The agonist binding domain (ABD) binds glycine in GluN1 and GluN3, whereas GluN2 ABDs bind glutamate (Furukawa et al., 2005; Yao and Mayer, 2006) (Figure 3).

The sequences of the regions lining the pore are highly conserved in GluN2 subunits and, accordingly, permeation properties (i.e. single-channel conductance, ionic selectivity), as well as affinity for the pore blocker Mg^{2+}, vary little among the different GluN1/GluN2 receptor subtypes. By contrast, incorporating the GluN3 subunit markedly decreases single-channel conductance, Ca^{2+} permeability and Mg^{2+} block (Sasaki et al., 2002).

The opening of the ligand-gated cation channel exhibits a profound voltage dependence because the channel is blocked by physiological concentrations of Mg^{2+} at resting membrane potentials. A partial depolarization of the plasma membrane is required to relieve the Mg^{2+} block, which allows NMDARs to sense simultaneous inputs of several presynaptic cells and behave as coincidence detectors (Paoletti et al., 1997).

A distinctive property of NMDAR is that the activated channel is highly permeable to

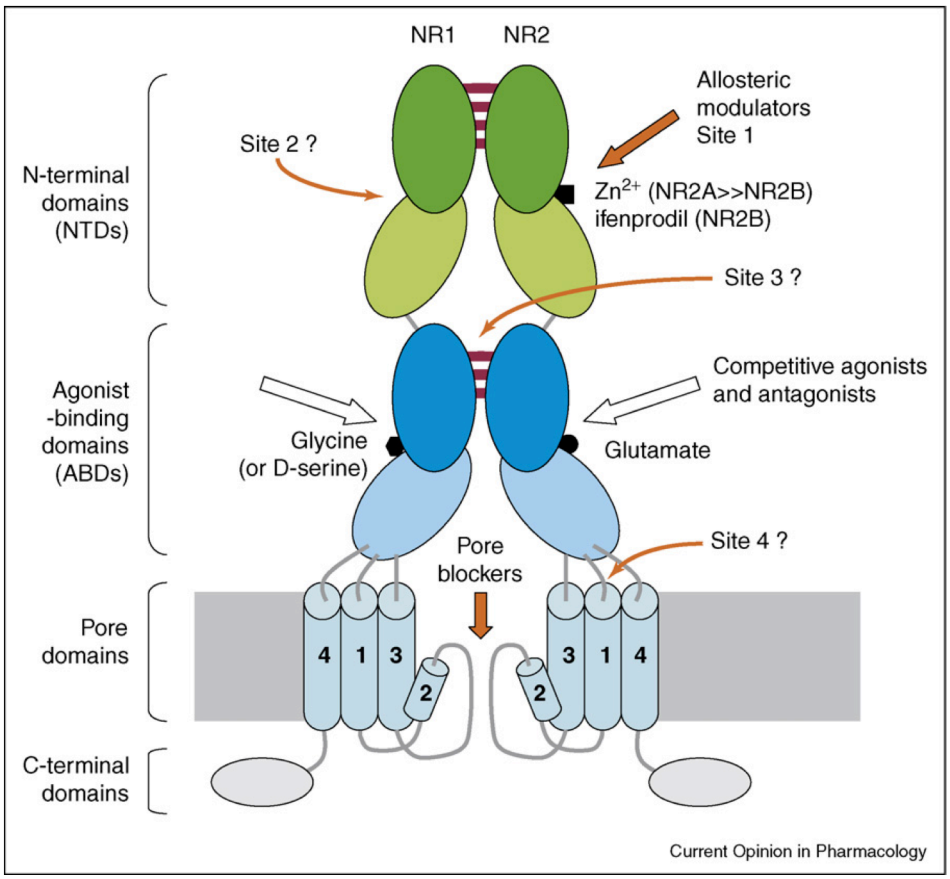
Ca^{2+} ions. This ability to flux Ca^{2+} couples the NMDAR to intracellular signal transduction pathways and is key to the expression of NMDAR functions.

The functional properties of the receptor complex depend on the specific subunit composition and the stoichiometry in which subunits combine to form the channel (Cull-Candy et al., 2001).

Incorporation of different GluN1 splice variants into NMDAR complexes influences such properties as modulation by Zn^{2+}, polyamines and protein kinase C (PKC) (Groc et al., 2009). The GluN2A subunit confers a lower affinity for glutamate, distinctly faster kinetics, greater channel open probability and more prominent Ca^{2+}-dependent desensitization than does the GluN2B subunit, which confers slower channel kinetics and reduced open probability. The GluN2C and GluN2D subunits are characterized by low conductance openings and reduced sensitivity to Mg^{2+} block (Cull-Candy et al., 2004). The GluN3 subunit confers reduced Ca^{2+} permeability and reduced surface expression (Cull-Candy et al., 2004).

NMDARs form an extended complex that connects with scaffolding proteins, adaptor proteins and signalling enzymes (Sheng et al., 2001; Husi et al., 2000), these molecules interact with the receptors and modulate their function, by providing a structural/organizational role, or by linking the receptors to downstream signalling events.

Extensive evidence demonstrates that NMDA-receptor signalling depends on the receptor phosphorylation state regulated by Src family Kinase (Salter and Kalia, 2004), alternative splicing and assembly into heteromeric channels (Swope et al., 1999; Cull-Candy et al., 2001). Exciting new research provides evidence that synaptic NMDA-receptor function is additionally regulated by redistribution of receptors into, and away from the synapse.



Adapted from: Paoletti, P. and Neyton, J. 2007. *NMDA receptor subunits: function and pharmacology*. Curr Opin Pharmacol 7: 39-47.

Figure 3. Potential sites for ligand binding at NMDARs

Most NMDAR are believed to assemble as tetramers, associating two GluN1 and two GluN2 subunits in a 'dimer of dimers' quaternary architecture. For clarity, only one of the two GluN1/GluN2 heterodimers is shown. The extracellular region of each subunit is organized as a tandem of two domains, the NTD and the ABD. In the extracellular region, the subunits dimerize at the level of the ABDs and probably also at the level of the NTDs. The GluN2 ABD binds glutamate, whereas the GluN1 ABD binds the co-agonist glycine (or D-serine). White arrows indicate binding sites for competitive agonists and antagonists. Thick orange arrows indicate sites known to bind allosteric modulators such as endogenous zinc (GluN2A and GluN2B NTDs) or ifenprodil-like compounds (GluN2B NTDs), both acting as non-competitive antagonists. The ion-channel domain also forms binding sites for pore blockers such as endogenous Mg^{2+}, MK-801, memantine or ketamine, acting as uncompetitive antagonists. Thin orange arrows indicate putative modulatory sites, which can bind either positive or negative allosteric modulators. The only known NMDAR antagonists that display strong subunit selectivity are the GluN2 NTD ligands Zn^{2+}, which selectively inhibits GluN2A-containing receptors at nanomolar concentrations, and ifenprodil-like compounds, which selectively inhibit GluN2B-containing receptors.

1.2.2 Tyrosine phosphorylation: the Src family kinases

Members of the Src family kinases (SFKs) were originally believed to regulate cell proliferation and differentiation (Stehelin et al., 1976). In the central nervous system (CNS) these kinases are expressed in differentiated, post-mitotic neurons, and are involved in many cellular functions, such as neuronal differentiation, neuritis out-growth, ion channel activity and synaptic transmission (Wang and Salter, 1994; Wanget al., 2004).

NMDA receptor functions are regulated by SFKs, in particular the two members Src and Fyn are important modulators of NMDAR.

The involvement of Src in the potentiation of the NMDA receptor is demonstrated by these observations (Yu et al., 1997):

- increasing the concentration of Src or favoring their activation through specific peptide, induce, at the cellular level, an increase in excitatory post-synaptic current evoked by the NMDAR;
- the increased activity of the NMDAR can be prevented by specific inhibitors of Src;
- the Src phosphorylates the receptor subunits GluN2A and GluN2B of NMDAR (Suzuki et al., 1999).

The NMDAR subunits GluN2A and GluN2B are tyrosine phosphorylated, and GluN2B is the main tyrosine-phosphorylated protein in the postsynaptic density. The GluN1 subunit seems not to be phosphorylated on tyrosine. The carboxy-(C)-terminal tails of GluN2A and GluN2B contain about 630 and 650 amino acids, respectively, with each C-terminal tail containing 25 tyrosine residues. SFK-mediated phosphorylation sites in the C-terminal tails that have been verified in biochemical studies are: Y1292, Y1325 and Y1387 in GluN2A, and Y1252, Y1336 and Y1472 in GluN2B. Y1472 has been shown by a phosphospecific antibody to be phosphorylated in the brain (Salter and Kalia, 2004).

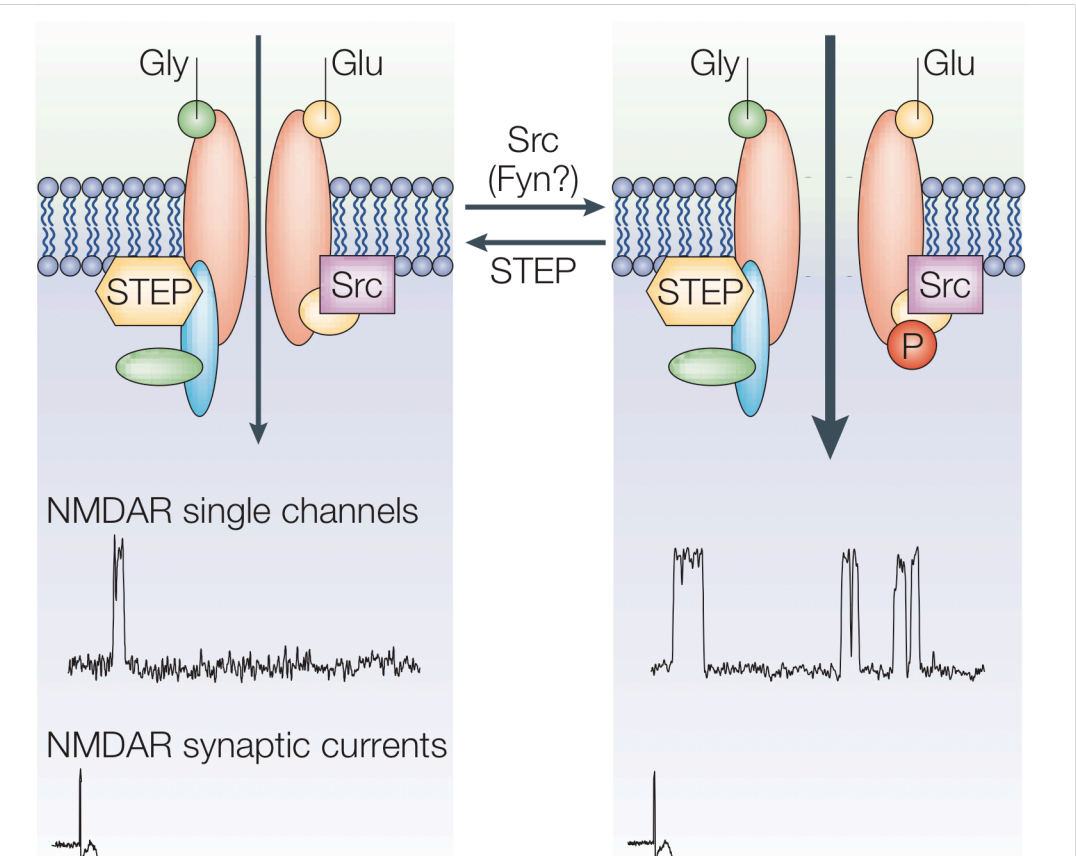
The increased phosphorylation of one or more tyrosine residues of NMDAR facilitates the opening of the channel by enhancing the probability of glutamate and glycine to bind the NMDA receptor and prolongs the opening times (Yu et al., 1997).

Electrophysiological studies showed that NMDAR currents in neurons are potentiated

by increasing Src activity or inhibiting PKC activity, this confirms the role of Src on NMDA receptor functions (Salter and Kalia, 2004) (Figure 4). In fact, recent evidence indicates that PKC action may involve the activation of protein tyrosine kinase 2 signalling cascade upstream of Src kinase (Lu et al., 1999; Grosshans and Browning, 2001).

Signalling pathways upstream of the regulation of NMDARs by Src are beginning to be identified and characterized in the CNS. It is becoming evident that Src act as a point of convergence for multiple, diverse signalling pathways that alter NMDAR function, such as Gq-coupled G-protein-coupled receptor (GPCR) cascades, or, how demonstrated in our laboratory, such as cytokine receptor pathway (Figure 5). In particular, recombinant IL-1β, by binding IL-1RI, potentiates the rise in intracellular Ca^{2+} ($[Ca^{2}]_i$) that is induced by NMDA stimulation in cultured hippocampal neurons (Viviani et al., 2003). Pre-treating the neurons with an IL-1 receptor antagonist prevents the IL-1β-induced potentiation of NMDAR responses, showing that the effect of IL-1β on NMDAR responses is mediated by IL-1RI. A role for SFKs in signalling downstream of IL-1RI was revealed by application of the SFK inhibitor, PP2, which prevents IL-1β-induced enhancement of the NMDA-mediated rise in $[Ca^{2+}]_i$ (Viviani et al., 2003). In addition, treatment of the neurons with IL-1β increased the amount of active Src associated with the NMDAR complex and the tyrosine phosphorylation of Y1472 in the GluN2B C-terminal tail (Viviani et al., 2003). These effects were prevented by SFK inhibition, whereas inhibition of SFKs did not affect IL-1RI function *per se* (Viviani et al., 2003). These findings indicate that SFKs probably act downstream of IL-1RI activation to upregulate NMDAR function.

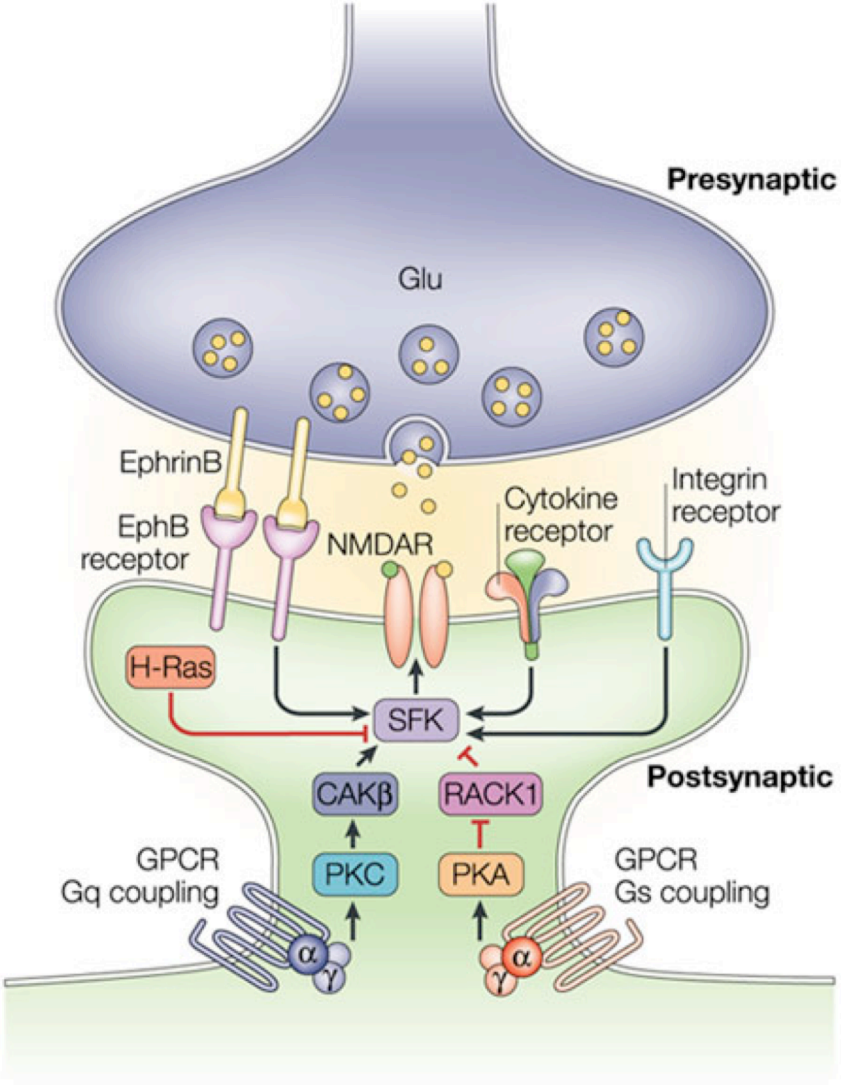
Another potential function of SFK-mediated tyrosine phosphorylation of GluN2 subunits is in the trafficking of NMDARs to and from the cell surface (Salter and Kalia, 2004).



Adapted from: Salter, M.K., Kalia, L. 2004. *Src kinases: a hub for NMDA receptor regulation*. Nat Rev Neurosci 5: 317-328.

Figure 4. Regulation of NMDAR gating by the balance of tyrosine phosphorylation and dephosphorylation

Illustration of the opposing actions of the tyrosine kinase Src and phosphotyrosine phosphatase STEP (striatal enriched tyrosine phosphatase) on the activity of NMDARs. Src enhances NMDAR single-channel gating, resulting in increased NMDAR-mediated synaptic currents in neurons. Src might phosphorylate NMDAR subunits and/or other proteins in the NMDAR complex. Whether endogenous Fyn, or other Src family kinases (SFKs), regulates NMDARs remains to be tested directly with kinase-specific reagents. STEP61 activity leads to dephosphorylation of Src substrates, thereby reversing Src-mediated upregulation of NMDAR channel gating and resulting in decreased NMDAR-mediated currents.



Adapted from: Salter, M.K., Kalia, L. 2004. *Src kinases: a hub for NMDA receptor regulation*. Nat Rev Neurosci 5: 317-328.

Figure 5. Convergence of signalling pathways on Src family kinases (SFKs)
SFKs act as a crucial intermediary in multiple signalling pathways that modulate NMDARs. These pathways include: Gq-coupled G-protein-coupled receptor (GPCR) cascades, which signal through protein kinase C (PKC) and cell adhesion kinase-β (CAKβ) to activate Src and upregulate NMDAR (*N*-methyl-D-aspartate receptor) function; a Gs-coupled cascade, which might signal through PKA to relieve the inhibition of Fyn by RACK1 (receptor for activated C kinase 1), resulting in upregulation of NMDARs; a receptor protein tyrosine kinase pathway involving the EphB receptor, which leads to activation of SFKs and upregulation of NMDARs; pathways mediated by H-Ras, which lead to inhibition of SFK activity and thereby to depression of NMDAR activity; a cytokine receptor pathway that increases NMDAR activity; and an integrin receptor pathway that leads to enhancement of NMDAR functions.

1.2.3 Trafficking of NMDAR

Until recently, NMDA receptors were considered to be tightly associated with the synaptic cytostructure and locked tightly into the postsynaptic complex. This notion derived, in part, from the observation that NMDA receptors exhibit high resistance to detergent extraction from PSDs (Allison et al., 1998; Kennedy, 2000). Interest in the possibility that synaptic NMDA receptors are dynamically organized in the postsynaptic complex and move laterally into and out of synaptic sites emerged, in part, from knowledge that other ionotropic receptors diffuse rapidly within the membrane in response to activity-dependent changes. It was known for nearly two decades that block of neuromuscular junctions with α-bungarotoxin induces rapid diffusion of functional ACh receptors from extra-junctional sites to the blocked junction (Young and Poo 1983; Weiss et al., 1986). Moreover, inactivated receptors diffuse rapidly from junctions to peri-junctional sites, where they reside for hours before internalization (Akaaboune et al., 1999).

A recent study indicates that NMDA receptors also undergo rapid lateral translocation between synaptic and extrasynaptic sites (Tovar and Westbrook, 2002). In this study, a physiological approach was taken: the quasi-irreversible, use-dependent channel blocker MK-801 was used to inactivate selectively synaptic NMDA receptors in response to release of glutamate at hippocampal synapses. Surprisingly, although initially completely blocked, NMDA-receptor-mediated synaptic responses recovered by 40% within 20 min of MK-801 washout. By contrast, when synaptic and extra-synaptic receptors were blocked by co-application of NMDA and MK801, synaptically evoked NMDA-receptor responses did not recover. This finding suggests that recovery is not due to unbinding of blocker or insertion of new receptors but, rather, that functional NMDA receptors can diffuse laterally from extra-synaptic to synaptic sites. The result supports a model in which NMDA receptors are in rapid equilibrium between an immobilized, PSD-95-associated state and a mobile state.

Furthermore, NMDAR do traffic from the endoplasmic reticulum (ER) to the synaptic compartment through dendritic vesicle transport (Newpher and Ehlers, 2008; Stephenson et al., 2008; Wenthold et al., 2003). GluN1 and GluN2 subunits of NMDAR assembled in the endoplasmic reticulum (ER) to form functional channels

(Moyer et al., 1994; McIlhinney et al., 1998; Ozawa et al., 1998). Assembled NMDA receptors are targeted selectively to the postsynaptic side of glutamatergic synapses and appear, together with AMPA receptors, at nascent synapses within one or two hours of initial axo-dendritic contact (Friedman et al., 2000). At mature synapses, NMDA receptors are delivered within hours of experience-dependent synaptic activation (Quinlan et al.,1999) and are reciprocally regulated by changes in synaptic activity (Rao and Craig, 1997; Liao et al., 1999; Watt et al., 2000).

GluN1 receptor might play an active role in controlling the delivery of NMDA receptors to synapses.

Finally, NMDARs may be also removed from the synaptic and extra-synaptic plasma membrane of neurons through endocytosis processes (Barria and Malinowa, 2002; Lan et al., 2001, Roche et al., 2001, Snyder et al., 2001, Li et al. , 2002; Nong et al., 2003, Scott et al. 2004; Lavezzari et al., 2004; Washbourne et al., 2004).

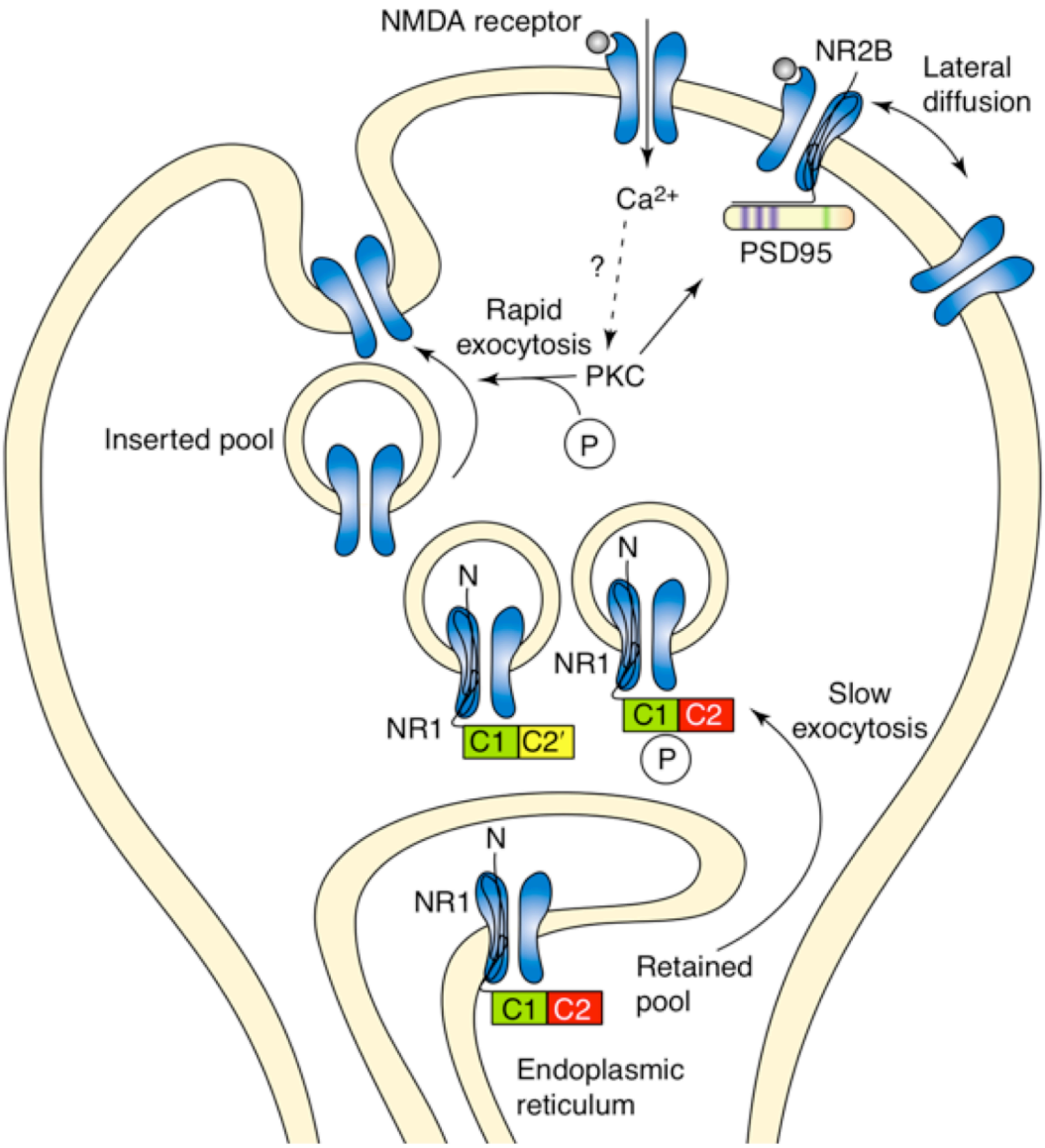
In brief, NMDAR surface content and distribution are regulated by (i) their insertion/internalization to/from the plasma membrane by exocytosis/endocytosis, respectively, and (ii) their lateral diffusion within the plasma membrane.

Trafficking of NMDARs to and from the cell surface are mediated by SFKs.

Tetanic stimulation activates Src kinase activity (Lu et al., 1998) and causes LONG-TERM POTENTIATION of NMDAR currents in area CA1 of the hippocampus69. Tetanic stimulation also reduces the intracellular pool of NMDARs in neurons in hippocampal CA1 slices70, which is presumed to be accompanied by an increase in the number of NMDARs on the neuronal surface. Inhibition of endogenous SFKs in hippocampal slices prevents both the potentiation of NMDAR responses and the decrease in intracellular NMDARs after tetanus, indicating that both require SFK activity (Grosshans et al., 2002). So, SFK-mediated tyrosine phosphorylation of NMDAR subunits might stabilize NMDARs on the cell surface and thereby increase NMDAR responses.

Studies on recombinant NMDARs have indicated that the association of NMDARs with the CLATHRIN-MEDIATED ENDOCYTOSIS machinery, a complex of proteins involved in the removal of receptors from the cell surface, is regulated by Src-mediated tyrosine phosphorylation of NMDAR subunits (Vissel et al., 2001). The C-tails of both GluN2A (Vissel et al., 2001) and GluN2B (Roche et al., 2001) contain a putative

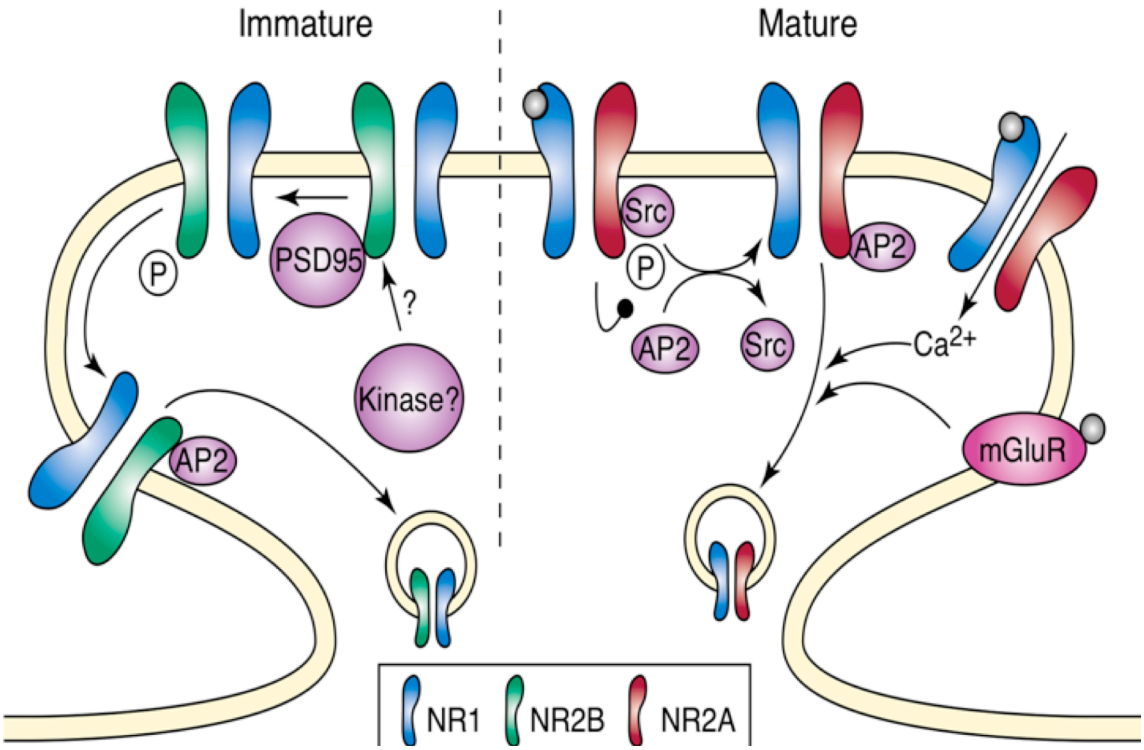
tyrosine-based internalization signal motif - a consensus binding motif for the µ2 subunit of the adaptor protein complex AP2, which associates with endocytic clathrin-coated vesicles. According to the crystal structure of the µ2 subunit, phosphorylation of the tyrosine in the µ2 consensus binding motif might prevent binding of the motif to µ2 (Marsh and McMahon, 1999). So, phosphorylation of the tyrosine residue in the motif could prevent the assembly of the clathrin-mediated endocytosis machinery at the NMDAR and thereby prevent the internalization of NMDARs.



Adapted from Carroll, R.C., Zukin, R.S. 2002. *NMDA-receptor trafficking and targeting: implications for synaptic transmission and plasticity*. Trends Neurosci 25: 571-577.

Figure 6. Trafficking of NMDA receptors

NMDA-receptor moves in the cell by diverse mechanisms: 1) exocytosis of assembled NMDA receptors from the endoplasmic reticulum, 2) exotcytosis of NMDA receptors, likely to be docked near the plasma membrane, to synaptic sites, and 3) dispersion of NMDA receptors from synaptic sites (lateral diffusion). PSD (postsynaptic density protein) 95 and other scaffolding proteins promote NMDA-receptor surface expression. Gray spheres represent glutamate.



Adapted from: Carroll, R.C., Zukin, R.S. 2002. *NMDA-receptor trafficking and targeting: implications for synaptic transmission and plasticity*. Trends Neurosci 25: 571-577.

Figure 7. Mechanisms of NMDA-receptor internalization
Whereas GluN1–GluN2B receptors predominate at immature synapses, GluN1–GluN2A receptors predominate at mature synapses. Both the GluNRA and GluN2B subunits contain motifs that bind the endocytic adaptor protein AP2 involved in the clathrin-dependent endocytosis machinery. Whereas the internalization of GluN2B is regulated by its association with PSD95, the ability of NR2A to associate with AP2 is modulated by dephosphorylation of tyrosine residues in its C-terminal tail. Activation of metabotropic glutamate receptors (mGluRs) also leads to the internalization of NMDA receptors; however, the mechanism of this is unknown.

1.2.4 NMDAR, synaptic plasticity and toxicity

Synapses that use glutamate as their transmitter mediate most excitatory neurotransmission in the central nervous system (CNS). Both ligand-gated ion channels (ionotropic receptors) and G protein-coupled receptors (metabotropic receptors) sense the glutamate released from presynaptic terminals and transduce it into electrical or biochemical responses. A key property of the glutamatergic synapse is its plasticity, which enables the developing and mature brain to modify the properties of neural circuits in a long-term fashion and respond adequately to changing needs in the environment. This plasticity allows neuronal connectivity to be regulated at the level of individual synapses as well as in entire synaptic networks over time courses that range from seconds to months (Abbott et al., 2003; Turrigiano et al., 2000; Abraham et al., 1996; Zito et al., 2002; Bliss and Collingridge, 1993; Feldman et al., 1998). During neural development, glutamatergic synapses initially form, and then either stabilize and mature or are eliminated in order to shape neural networks. Once established, most glutamatergic synapses retain the potential for considerable plasticity during later stages of development and into adulthood (Feldman et al., 1998; Katz and Shatz, 1996; Cohen-Cory, 2002). In principle, the strength of a synapse can be modified pre-synaptically by altering transmitter release or post-synaptically by modifying the number, efficacy or stability of postsynaptic receptors. A number of recent developments have uncovered the importance of postsynaptic mechanisms for plasticity at many CNS synapses, and demonstrated that dynamic changes in the receptor complement at the postsynaptic membrane constitute a fundamental means to generate and remodel a plastic neural network, both by 'making' functional synapses and by strengthening or weakening those already formed (Barry and Ziff, 2002; Luscher et al., 2000; Malinow and Malenka, 2002).

While glutamate receptors of the AMPA subtype (AMPARs) mediate most of the rapid excitatory transmission in the mature brain, NMDA-type glutamate receptors (NMDARs) initiate many forms of synaptic plasticity and participate in long-term homeostatic and adaptive brain processes. For instance, NMDAR activity is required for the establishment and refinement of neural circuits during development by

contributing to the formation and maturation of dendritic processes, dendritic spines and synaptic connections themselves (Nikonenko et al., 2002; Cline, 2001; Constantine-Paton et al., 1990). In mature networks, the activation of NMDARs mediates the forms of plasticity such as long-term potentiation (LTP) and long-term depression (LTD) that are considered the cellular basis for memory formation and storage, and are involved also in pathological processes (Hebb, 1949; Dudek and Bear; 1992; Martin et al., 2000). Activation of the highly calcium-permeable NMDAR causes the insertion or removal of AMPARs, resulting in changes in synaptic strength, most notably at CA1 hippocampal synapses (Malinow and Malenca, 2002). Therefore, additional mechanisms are needed to stabilize activity and keep it within an optimal working range (commonly referred to as synaptic homeostasis) and to modify the thresholds at which synaptic stimulation induces LTP and LTD (metaplasticity). Regulation of the synaptic abundance of NMDARs provides a cell biological mechanism that may account for these additional forms of synaptic plasticity (Abbott and Nelson, 2000, Abrahm and Bear; 1996).

A common denominator of plasticity at glutamatergic synapses is the need for prior synaptic or cellular activity. Diverse forms of plasticity are generated by different patterns of synaptic activation, many of them involving NMDARs (Malenka and Nicoll; 1993; Kirkwood et al., 1996; Craig, 1998; Shouval et al., 2002), which requires a tight control over the quality and magnitude of NMDAR-dependent signals – most notably Ca^{2+} influx. This task can be accomplished both by regulating the numbers and subtypes of NMDARs present at a synapse, and by modifying NMDAR composition – and thereby properties – over time periods ranging from minutes to months (Cull-Candy et al., 2001; Carroll and Zukin; 2002; Wenthol, 2003).

Control over the magnitude of NMDAR Ca^{2+} influx is a key event in the control of neurotoxicity. An excessive Ca^{2+} entry is a crucial mediator of glutamate excitotoxicity. Excitotoxicity can be defined as cell death ensuing from the toxic actions of glutamate (Lucas and Newhouse, 1986; Rothman and Olney, 1957). The resulting calcium overload is particularly neurotoxic, leading to activation of enzymes that degrade proteins, membranes and nucleic acid (reviewed in Berliocchi et al., 2005). Excitotoxicity that is mediated by NMDARs has been implicated in neuronal death in many pathological conditions, including CNS ischaemia, trauma and

neurodegeneration.

1.3. CONVERGENCE OF IL-1β AND NMDA RECEPTOR

Recent studies showed that IL-1β affects various classic neurotransmitter systems (Rothwell and Hopkins, 1995; Grazia de Simoni et al., 1995). In particular, substantial evidence suggests the existence functional interaction between IL-1β and NMDA receptors (NMDARs). IL-1β plays a key role in the effects mediated by NMDA. It is observed that the expression of this cytokine in astrocytes and microglia increased during NMDA-dependent stimulation (Pearson et al., 1999), while the use of NMDA receptor antagonists suppress the expression of the cytokine after ischemic injury (Jander et al., 2000).

We and others have demonstrated that the production of IL-1β could regulate NMDA receptors function, whose activation results in an increase in intracellular Ca^{2+} (Viviani et al., 2003, Kawasaki et al., 2008). In particular, in primary culture of hippocampal neurons recombinant IL-1β, by binding IL-1 receptor type I (IL-1RI), increases NMDAR functions through the activation of tyrosine kinases and subsequent phosphorylation at Tyr-1472 of the GluN2B subunit of the NMDAR. These events induce a sustained elevation of intracellular Ca^{2+} in neurons and, in our in vitro experimental conditions: prolonged NMDAR stimulation and relatively high concentrations of IL-1β, neuronal death (Viviani et al., 2003). The sustained intracellular Ca^{2+} and the alteration of the levels of glutamate are key events in excitotoxicy. Nevertheless, the maintenance of the intracellular Ca^{2+} homeostasis together with the maintenance of extracellular glutamate concentrations, are fundamental for cellular functions and survival. It is thus conceivable that a different balance between IL-1β and glutamate may alter NMDAR functions (i.e. LTP, synaptic plasticity and then cognition) without necessarily inducing neuronal death.

1.3.1 Gp120 in neurons/glia co-cultures: a suitable *in vitro* model to study the convergence of il-1β and NMDAR

Gp120 is one of the envelope glycoproteins of human immunodeficiency virus-1 (HIV), which displays the interesting characteristic of activating glial cells to release IL-1β and triggering neuronal death through the overactivation of NMDAR. This protein is implicated in the neurodegenerative events associated with HIV infection; in fact transgenic mice that express the viral protein gp120 develop neuropathological features similar to those found in patients with HIV-associated dementia (HAD) (Toggas et al., 1994). HAD is a common neurological disorder observed after infection with HIV. Clinically, HAD is characterized by disabling cognitive impairment, including poor concentration and memory impairment; motor dysfunction, such as loss of fine motor control, poor balance, tremors, speech problems; and behavioral changes including apathy and lethargy (Rothenhausler 2006; Ances and Ellis 2007). The histopathological alterations observed are: widespread reactive astrocytosis, myelin pallor, neuronal degeneration, structural discontinuity of blood-brain barrier (BBB), infiltration of blood-derived macrophages, resident activated microglia and multinucleated giant cells (Kaul et al., 2001). It is now believed that gp120 shed by the virus may activate microglia (and astrocytes) and stimulate the release of neurotoxins, the nature of which is still unknown. The mechanism by which gp120 acts through activated glia may include increased production of potentially detrimental factors such as cytokines, excitotoxic amino acids, free oxygen radicals and bioactive lipid mediators as well as inhibition of the production or action of neurotrophic/protective factors (Dreyer et al., 1990; Heyes et al., 1991; Genis et al., 1992; Toggas et al., 1994; Lipton and Rosenberg, 1994; Lannuzel et al., 1995; Lipton, 1998). Previous studies have shown that exposure to gp120 induces IL-1β production in microglia and/or astrocytes *in vitro* (Merril et al., 1992), and administration of gp120 in the brain of adult rat increases the expression of IL-1β mRNA (Ilyin & Plata-Salaman, 1997) and gene product (Bagetta et al., 1999). Importantly, IL-1β mRNA levels are also increased in postmortem brain tissue from HIV-infected patients diagnosed with HAD as compared to non-demented HIV-infected patients (Zhao et al. 2001), substantiating a role for IL-1β in the

pathogenesis of HAD.

Exposure of glia cells to gp120 induces both ROS and IL-1β formation (Viviani et al., 2001; Corasantini et al., 2001). Furthermore, in primary hippocampal neurons in co-culture with glia cells, gp120 increases intracellular calcium ($[Ca^{2+}]_i$) that precedes neuronal death (Viviani et al., 2001; Corasantini et al., 2001). While an anti-IL-1β neutralizing antibody protects from gp120-induced neuronal injury, the antioxidant trolox prevents both increased IL-1β release and neurodegeneration, suggesting ROS to be the initiator of this cascade (Viviani et al., 2001). The neurotoxicity of gp120, when injected intracerebroventricularly, is prevented by concomitant injection of IL-1 receptor antagonist (IL-1ra) (Bagetta et al., 1999). IL-1ra prevents also the impairment of memory consolidation induced by gp120 (Pugh et al., 2000).

This information suggests that the glia-derived IL-1β is a key substance in gp-120-induced neuronal impairment and death.

The mechanism(s) by which IL-1β signalling might contribute to the progression of gp120-induced neuronal injury are not completely understood.

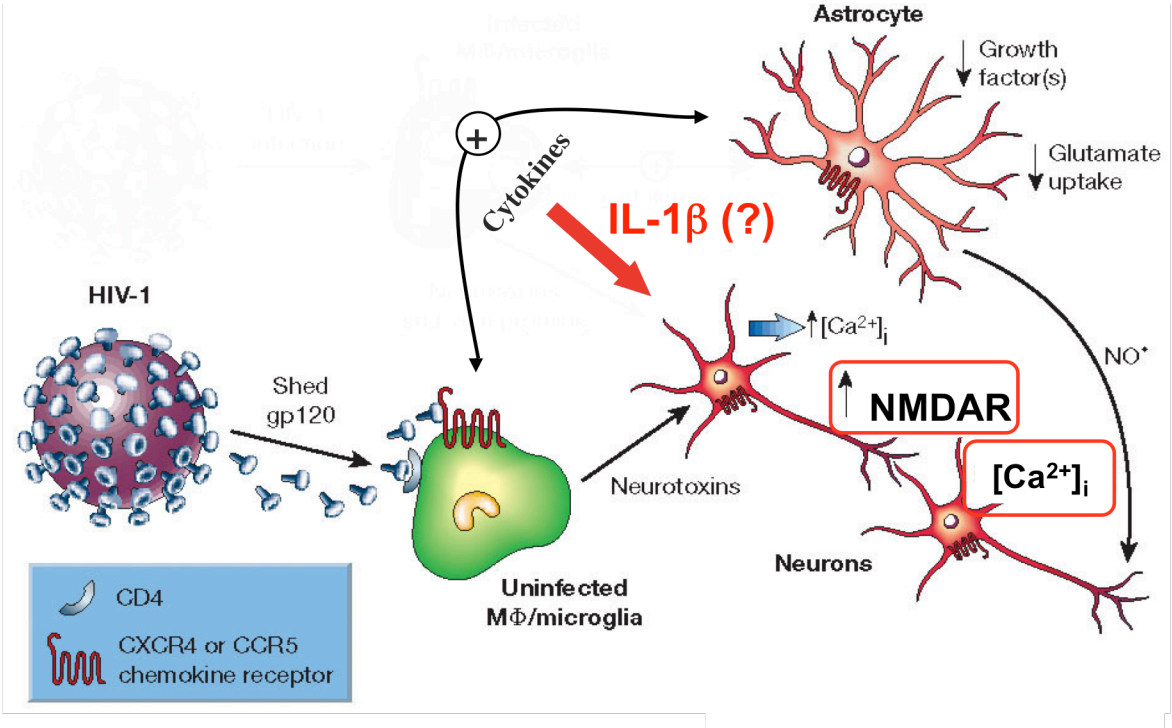
It is well established that over-stimulation of NMDAR represents a key event in HAD. The mechanism underlying cells death involves excessive Ca^{2+} entry in neurons *via* NMDAR associated cation channel since NMDA antagonist prevent both the rise of intracellular level of Ca^{2+} and the cytotoxicity (Dreyer et al., 1990; Lipton et al., 1991) suggesting an excitotoxic, glutamate-mediated, type of death (Corasantini et al., 2001).

Interestingly, gp120 neurotoxicity *in vivo* (Toggas *et al.* 1996) and *in vitro* (Lipton 1992) is decreased by NMDAR antagonism, providing a potential link between IL-1β and excitotoxicity.

Being IL-1β and the NMDAR, key points in gp120 mediated neurotoxicity, it is therefore possible that the relationship between IL-1β/NMDAR play an important role in the development and progression of HAD, and in the induction of neurodegenerative features like decreased synaptic density, spine loss, dendritic simplification, and neuronal loss.

On the basis of these considerations, in this study we used gp120 to induce the endogenous production of IL-1β in response to a pathological trigger and to better define the molecular and functional interaction between this cytokine and the

NMDAR in an *in vitro* model of neurotoxicity.



Adapted from: Kaul, M., Lipton, S.A. 1999. *Chemokines and activated macrophages in HIV-gp120-induced neuronal apoptosis.* Proc Natl Acad Sci USA 96: 8212-8216.

Figure 8. Current model of HIV-related neuronal damage involving gp120 and cell–cell signalling

gp120 stimulation of glia cells enhances their production cytokines, among which IL-1β. These cytokines stimulate gliosis. Reactive glia may then release of free-radical nitric oxide (NO•), which in turn may react with superoxide to form the neurotoxic molecule peroxynitrite. Arachidonate released from macrophages impairs astrocyte clearing of the neurotransmitter glutamate and thus contributes to excitotoxicity. Neuronal injury is mediated predominantly by overactivation of NMDAR-coupled ion channels that allow excessive influx of Ca^{2+}. This in turn triggers a variety of potentially harmful enzymes, free-radical formation and release of glutamate. Glutamate subsequently overstimulates NMDARs on neighbouring neurons, initiating further injury. This final common pathway to neurotoxicity can be blocked by NMDAR antagonists. For certain neurons, depending on their exact repertoire of ionic channels, this form of damage can also be ameliorated by voltage-activated calcium channel antagonists or non-NMDA glutamate antagonists. **In red is our working hypothesis: is IL-1β involved in the exacerbation of neuronal damage due to the overactivation of NMDAR?**

1.3.2. Early-life experiences shape immune response, synaptic plasticity and behaviour: a suitable *in vivo* model to study the convergence of IL-1β and NMDAR

A plethora of epidemiological studies in humans and experimental work in animals emphasizes the critical impact of the early-life environment in shaping postnatal physiological, emotional and behavioral functions (Meyer, 2011).

The concept of "early life developmental programming of adult diseases" refers to the phenomenon whereby specific environmental factors acting during sensitive prenatal or early postnatal developmental periods can modulate or "program" the normal course of development, with the result that adult outcomes are significantly and often permanently altered (Bennet and Gunn, 2006).

Accumulating evidence suggests that such early-life programming also exists for the development and functions of the immune system (Merlot et al., 2008; Bilbo and Schwarz, 2009). Perinatal exposure to infection and/or immune activation through other stimuli like stress, is one of the prominent environmental factors with known impact on postnatal immune functions (Bilbo and Schwarz, 2009). For example, prenatal maternal exposure to the bacterial endotoxin lipopolysaccharide (LPS) or the pro-inflammatory cytokine IL-6 in rats leads to enhanced microglial densities and elevation of peripheral and central pro-inflammatory cytokine levels in the offspring (Borrell et al., 2002; Samuelsson et al., 2006; Romero et al., 2010). Such inflammatory changes can persist even until adulthood (Borrell et al., 2002; Samuelsson et al., 2006; Romero et al., 2010), suggesting that immune challenge early in life can permanently alter postnatal immune functions. Further to the precipitation of overt immune dysfunctions, which may be evident even under basal conditions, early-life exposure to infection and/or immune activation may also induce sensitizing or preconditioning effects (Bilbo and Schwarz, 2009; Meyer, 2011).

Notably, early-life infection and/or immune activation which could also induced by stress, not only shapes the function as of the immune system, but it also influences reactivity to stress, disease susceptibility, and, increased vulnerability to cognitive and/or neuropsychiatric disorders, including Alzheimer's, Parkinson's, schizophrenia,

and autism (Hornig et al., 1999; Nelson and Willoughby, 2000; Rantakallio et al., 1997; Shi et al., 2003). In this scenario, an increased cytokine exposure during key periods of brain development may also act as a "vulnerability" factor for later-life pathology, by sensitizing the underlying neural substrates and altering the way that the brain responds to a subsequent immunological and stressful challenge in adulthood. In turn, this altered immune response has significant and enduring consequences for behavior, including social, cognitive, and affective abilities (Bilbo et al., 2009).

Pro-inflammatory activity has been postulated also in early attachment-figure separation and has been suggested to contribute to the passive behavior of separated pups (Hennessy et al. 2010). Indeed, Coe at al. (1988) found that a 24 hours separation procedure produced a dramatic and prolonged increase in natural immune response. Furthermore, it has been shown that, in rats, a single prolonged episode of maternal deprivation (MD) during the neonatal period [24 hours, postnatal day (PND) 9–10] induced diverse behavioural alterations later in life that resemble certain psychotic and depressive-like symptoms (Ellenbroek, 2003; Ellenbroek et al 2004; Ellenbroek and Riva, 2003; Marco et al., 2009).

In this same model, it have recently described sex-dependent alterations in developing hippocampal neurons and glial cells in MD neonatal rats, with males being more markedly affected (Llorente et al., 2009; Llorente et al., 2008).

Finally, variations in maternal care not only influence the development of behavior in the offspring, but determine alterations in molecular players of cellular plasticity like NMDA and AMPA receptors (Pickering et al., 2006; Roceri et al., 2002). These effects are specific from the anatomical and temporal point of view and might represent one of the mechanisms through which early adverse life events determine long-term impairment in brain function and plasticity. For all these characteristics, to assess the possibility that the functional cross talk between IL-1b/NMDAR could be recruit *in vivo* we adopted a maternal deprivation paradigm of stress early in life (Collaborative work with Maria Paz Viveros).

2. AIM

Aim of this thesis has been to characterize, from a molecular and structural point of view, the bidirectional interplay between IL-1β and the NMDA receptor.

In the first part of the study we used an *in vitro* model of neurotoxicity characterized by endogenous production of IL-1β and over-stimulation of NMDAR. We evaluated the role of IL-1β in several important events that are involved in the regulation of NMDAR functions: phosphorylation and trafficking.

The IL-1β signalling has been well characterized in the peripheral cells of the immune system and this information has been hypothetically extended to the CNS, without considering the peculiarities of this system. Neurons are strategically complex cells. Their responsiveness lies in the information of highly specialized compartments composed of unique repertoires of selectively distributed protein complexes. Thus, we analysed the molecular composition and the subcellular distribution of the components of IL-1β signalling, the possibility that IL-1β/IL-1RI signalling could be regulated by re-distributing the IL-1RI complex between different neuronal compartments in dependence to IL-1β and NMDA simulation.

In the last part of this study we have evaluated whether the crosstalk between IL-1β/IL-1RI/NMDAR could has been recruited also *in vivo.* For this purpose we used an animal model of early life stress. Stressful challenge experienced in early-life could shape the development and functions of the immune system. These events could change the immunoreactivity in the brain for the remainder of the lifespan and play a fundamental role in promoting susceptibility to central nervous system dysfunctions from poor cognitive disabilities to neuropsychiatric disorders (Hornig et al., 1999; Nelson and Willoughby, 2000; Rantakallio et al., 1997; Shi et al., 2003). The alteration of cognitive and behavior functions are due to an alteration of glutamatergic system.

This implicates that in this model the IL-β/NMDAR cross talk could be recruited.

3. MATERIALS AND METHODS

3.1. CELLS CULTURES

3.1.1. Primary cultures of glial cells

Primary cultures of glial cells were prepared from 1- to 2-day-old newborn rats (Sprague-Dawley) (Charles River, Calco, Italy). All animal care procedures were in accordance with the local Animal Care Committee, and no weight loss or death was observed after receipt of rats in our animal facility. Pregnant rats were housed over wood chip bedding, acclimatized to a 12 hours light-dark cycle and allowed food and water ad libitum. All efforts were made to minimize the suffering of animals. Cerebral hemispheres were freed from the meninges and mechanically disrupted. Cells were dispersed in a solution of trypsin 2.5% and DNase 1%, filtered through a 100-μm nylon mesh and plated (140000 cells per 35-mm dish) in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 0.6% glucose, streptomycin (0.1 mg/ml), and penicillin (100 international units/ml). Glial cultures were fed twice a week and grown at 37°C in a humidified incubator with 5% CO_2.

3.1.2. Primary cultures of hippocampal neurons

Neuronal cultures were established from the hippocampus of 18-day rat fetuses. Briefly, brains were removed and freed from meninges, and the hippocampus was isolated. Cells were then dispersed by incubation for 5 min at 37°C in a 2.5% trypsin solution followed by trituration. The cell suspension was diluted in Neurobasal media supplemented with 1% B-27 (Invitrogen, Carlsbad, CA) and plated onto polyornithine-coated coverslips at a density of 80000 cells per coverslip.

3.1.3. Sandwich co-cultures of hippocampal neurons and glial cells

Three days before treatment with gp120, coverslips were transferred to dishes containing a glial monolayer in neuron maintenance medium. Coverslips were inverted so that the hippocampal neurons faced the glia monolayer; both cell preparations were immersed in the same culture medium. Paraffin dots adhering to the coverslips supported them above the glia, creating a narrow gap that prevented the two cell types from contacting each other but allowed the diffusion of soluble substances (Figure 1). These culture conditions allowed us to grow differentiated neuronal cultures with 96% homogeneity, as assessed by immunocytochemistry of microtubule-associated protein 2 and glial fibrillary acidic protein (Molecular Probes, Eugene, OR). Hippocampal neurons were used after a culturing period of 14 days.

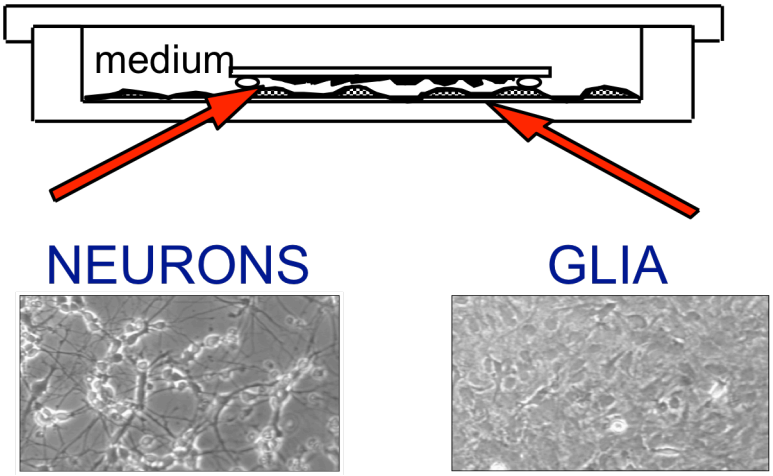


Figure 1. Model of sandwich co-culture of primary hippocampal neurons and glia cells
Primary hippocampal neurons were plated on glass coverlips and then turned over glia monolayer seeded in 24-well plate. The two different cell populations are separated by small paraffin dots at the edges of the coverslip, in this way the two cell populations face each other without touching.

3.2. TREATMENTS

3.2.1. HIV-gp120 / IL-1ra / Ifenprodil / Ca-pYEEIE

Recombinant HIV-gp120 protein type IIIB (Bartels, Carlsbad, CA) was used in all the experiments. Neurons were exposed to drugs in their culture medium (Neurobasal media supplemented with 1% B-27). Treatment with the glycoprotein was mainly performed in the presence of glia. To this purpose, primary hippocampal neurons were transferred on a glial monolayer for 3 days before exposure to gp120. Glass coverslips with primary hippocampal neurons were detached from the glial monolayer at the end of the exposure and assessed for NMDAR Tyr phosphorylation and trafficking, Ca^{2+} homeostasis, cell fractionation, confocal microscopy, and cell death.

1 μg/ml human recombinant IL-1ra (R&D Systems, Minneapolis, MN), 10 μM ifenprodil hemitartrate (Tocris, Bristol, UK) were incubated with gp120 and kept in the incubation medium until the end of the treatment. In contrast, 10 μg/ml caffeic acid-pYEEIE (Ca-pYEEIE; Tocris) was delivered only in primary hippocampal neurons, prior to gp120 treatment, by means of the lipid base transfection reagent Chariot (Active Motif, Rixensart, Belgium) according to supplier instructions. Briefly, caffeic acid-pYEEIE was incubated with Chariot dilution at room temperature for 30 min to allow the formation of the Chariot-molecule complex. Neuronal cells without glia were then overlaid with the Chariot-macromolecule complex solution diluted in Neurobasal and incubated at 37°C for 1 hour. At the end, complete growth medium was added, and incubation continued for another hour. Ca-pYEEIE loaded neurons were then washed to remove extracellular Ca-pYEEIE and transferred to the glial monolayer for gp120 exposure. Control neuronal cultures were run in parallel under each experimental condition and incubated for the appropriate times in the presence or absence of glia.

3.2.2. IL-1β / NMDA

Rat recombinant IL-1β and NMDA (Sigma Chemical Co. St. Louis, MO, USA) were used in all the experiments. For localization studies, neurons were exposed to drugs in ACSF buffer (137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 4.2 mM $NaHCO_3$, 5 mM glucose, 10 mM Hepes): NMDA was applied to neurons for 10 min, cells were then washed, and incubated for further 20 min in ACSF; IL-1β was applied for 30 min. Control neuronal cultures were run in parallel in each experimental condition and incubated for the appropriate times in ACSF and conditioned medium.

3.3. METHODS

3.3.1. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from glial cells by guanidinium thio-cyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). RT-PCR reactions were performed as previously described (Corsini et al., 1996). In preliminary experiments, RNA concentrations and PCR cycles were titrated to establish standard curves to document linearity and to permit semi-quantitative analysis of signal strength (40 ng and 200 ng for β-actin and all cytokines, respectively). The PCR products were made visible by UV illumination after electrophoresis through 1.5% agarose at 80 V and staining in Tris borate EDTA buffer 1X, containing 0.5 μg/ml ethidium bromide. Gels were photo- graphed with type 667 film (Polaroid, Cambridge, MA, USA). The image of the PCR products was acquired with a Nikon CCD video camera module (Nikon, Melville, NY, USA). The optical density of the bands was calculated, and the peak area of a given band was measured by means of the Image 1.61 program for digital image processing (Wayne Rasband, Research Service Branch, NIH, Bethesda, MD,

USA).

3.3.2. IL-1β assay

IL-1β release was measured by means of an interleukin-1L rat ELISA system (Quantikine, R&D Systems, Abingdon, UK).

3.3.3. Western blotting

Gel electrophoresis and transfer

The separation of proteins by sodium dodecyl sulphate (SDS) ployacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (1970). Samples were prepared by adding SDS sample buffer and reducing agent, the sample mix was heated at 37°C for 10 min to gently denature the proteins. The samples were loaded onto a 6% SDS-PAGE gels and electrophoresed at 60V for approximately 2 hours. Following electrophoresis the gel was transfer into a cassette, on top of a sponge pad and two pieces of filter paper. The membrane was placed on the top of the gel and any air bubbles were removed to maximise protein transfer. Two pieces of filter paper were placed on the top of the membrane, and further sponge pad placed on the top of this. The membrane was pre-wet for 15 sec in 100% methanol, rinsed with distilled H_2O and then soaked with transfer buffer (0.192 M glicine, 0.025 M Tris, MeOH 20%, pH 8.3) together with the sponge pads and the pieces of filter paper.

Proteins were transferred from gel onto membrane at constant current (230 mA) for 2 hours at 4°C. The successful transfer of proteins from the gel to the membrane was verified using Ponceau S staining. After this, the membrane was rinsed in TBS buffer, (10 mM Tris-HCl, 150 mM NaCl, pH 8) to removed transfer buffer and stain. Following this, protein-free sits on the blot were blocked for 2 hours at room temperature (RT) in a solution of i-Block (Invitrogen, Carlsbad, CA) TBS-T (TBS buffer with 0.1% TWEEN-20).

Primary and secondary antibodies

The blot was treated with a primary antibody overnight at 4°C. Antibodies were diluted in i-Block TBS-T, the optimal dilution was performed for each antibody. Following 8 washes with TBS-T, the blot was treated with the secondary antibody (HRP-conjugate) with constant rotation for 1 hour. The blot was then washed 8 times with TBS-T.

Signal detection

The chemiluminescent western blotting system ECL (Enhanced-Chemiluminescence, Amersham-Pharmacia Biotech, UK) was used for specific detection of secondary antibody.

3.3.4. Determination of the cytosolic-free Ca^{2+} concentration $[Ca^{2+}]_i$

At the end of exposure to gp120, neurons on glass coverslips were detached from glial cells and loaded with 10 μM Fura 2-AM (Sigma) for 1 hour at 37°C in the treatment culture medium supplemented with 1% bovine serum albumin, 0.1% pluronic (Sigma). $[Ca^{2+}]_i$ measurement was performed in neurons only in Hepes buffer, pH 7.4, as previously described (Vivaini et al., 2001). The Fura 2 fluorescence ratio signal was measured in a PerkinElmer Life Sciences 50 B double wavelength fluorometer and calibrated in terms of $[Ca^{2+}]_i$ as described by Grynkiewicz et al., 1995.

3.3.5. Subcellular fractionation

The subcellular compartments from rat hippocampus was purified as previously described (Gardoni et al., 1998). To isolate the different fractions from rat hippocampus, a modification of the method of Carlin et al. (1980) was used. Animals were killed, and brain areas were dissected within 2 min. Hippocampi were rapidly

dissected and pooled. Homogenization was carried out by 10 strokes in a Teflon-glass homogenizer in 4 ml/g wet weight cold 0.32 M sucrose containing 1 mM HEPES pH 7.4, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in the presence of a complete set of protease inhibitors (Complete™, GE Healthcare, Mannheim, Germany). The homogenized tissue was centrifuged at 1000x*g* for 10 min.

The obtained supernatant corresponds to low speed supernatant fraction (S1) and the pellet corresponds to P1, nuclei-associated membrane (P1). The resulting supernatant was centrifuged at 3000x*g* for 15 min to obtain the supernatant (cytosol, S2) and the pellet (crude membrane fraction, P2). The pellet was solubilized in 2.4 ml/g of 0.32 M sucrose containing 1 mM HEPES pH 7.4, 1 mM $NaHCO_3$, and 0.1 mM PMSF, layered on a sucrose gradient (0.85-1.0-1.2 M), and centrifuged at 82500x*g* for 2 hours. The fraction (synaptosomes, Syn) between 1.0 and 1.2 M sucrose was removed, diluted with an equal volume of 1% Triton X-100 in 0.32 M sucrose containing 1 mM HEPES, 1 mM $NaHCO_3$, and 0.1 mM PMSF, and stirred at 4°C for 15 min followed by centrifugation at 82500x*g* for 30 min. The pellet was solubilized, layered on a sucrose gradient (1.0-1.5-2.1 M), and centrifuged at 100000x*g* at 4°C for 2 hours. The fraction between 1.5 and 2.1 M was removed and diluted with an equal volume of 1% Triton X-100 and 150 mM KCl. The pellet corresponds to TIF (Triton insoluble fraction) and the supernatant corresponds to PSD (post-synaptic density). PSDs were finally collected by centrifugation at 100000x*g* at 4°C for 30 min.

Triton insoluble postsynaptic fraction (TIF) was purified from primary hippocampal neurons and from hippocampi of control and maternal deprived rats as previously described with minor modifications (Gardoni et al., 2006). Briefly, primary hippocampal cultures and hippocampi were homogenized in ice-cold sucrose 0.32 M containing 1 mM Hepes, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM $NaHCO_3$, 0.1 mM PMSF, at pH 7.4. The homogenized tissue was centrifuged at 13000x*g* for 15 min to obtain a crude membrane fraction. The pellet was resuspended in buffer containing 75 mM KCl and 1% Triton-X 100 and centrifuged at 100000x*g* for 1 hour. The pellet was homogenized in a glass-glass potter in 20 mM Hepes. Then, an equal volume of

glycerol was added and this fraction, referred as Triton insoluble fraction (TIF), was stored at -80°C until processing. TIF fraction was used instead of the classical postsynaptic density (PSD) because the amount of the starting material was very limited. All purifications were carried out in presence of a complete set of protease inhibitors (Complete™, GE Healthcare, Mannheim, Germany) and of both Ser/Thr- and Tyr-phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA).

3.3.6. Immunofluorescence labelling and image acquisition

Hippocampal neurons were fixed in 100% methanol at -20°C for 15 min. Primary (1:100) and secondary (1:200) antibodies were applied in GDB buffer (30 mM phosphate buffer (pH7.4) containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl). Fluorescence images were acquired using Zeiss LSM510 confocal microscope. Pharmacologically treated neurons were chosen randomly for quantification from three to five independent experiments by taking from each experiment from three to five coverslips.

3.3.7. Immunoprecipitation

Neuronal lysates (200 μg proteins) were incubated overnight at 4°C in a RIA buffer containing: 200 mM NaCl, 10 mM EDTA, 10 mM Na_2HPO_4, 0.5% NP-40, 0.1% SDS, NaF 10 mM, with antibody against GluN2B (antibody dilution 1:200), GluR1 (1:200) or IL-1RI (1:200). Samples were at first solubilized in RIA buffer in presence of 1% SDS, and only subsequently diluted ten times in RIA buffer to obtain a final 0.1% SDS concentration. Protein A-agarose beads (Sigma-Aldrich), washed in the same buffer, were added, and incubation continued for 2 hours. The beads were collected by centrifugation and washed five times, sample buffer for SDS-PAGE was added,

and the mixture was boiled for 5 min. Beads were pelleted by centrifugation, and supernatants were applied to 6% SDS-PAGE.

3.3.8. Pull-down assay

The IL-1RI and PSD-95 fragments were subcloned downstream of glutathione *S*-transferase (GST) in the *Bam*HI and *Hind*sites of the expression plasmid pGE-KG by PCR using Pfu polymerase (Stratagene) on a IL-1RI cDna template or on PSD-95 cDNA template. The inserts were fully sequenced with ABI Prism 310 Genetic Analyser (ABI Prisma). IL-1R(CD) and PSD-95(PDZ1-2) GST-fusion proteins were expressed in BL21 Escherichia coli, purified on glutathione agarose beads (Sigma-Aldrich) as described previously (Gardoni et al., 2001).
Aliquots of neuronal homogenates (200 μg) were diluted with TBS (10 mM Tris and 150 mM NaCl) and 0.1% SDS to a final volume of 1 ml and incubated (1 hour, 37°C) with glutathione agarose beads saturated with fusion proteins or GST alone. After the incubation period, the beads were extensively washed with TBS and 0.1% Triton X-100. Bound proteins were resolved by SDS-PAGE and subjected to immunoblot analysis with polyclonal anti-IL-1RI antibody.

3.3.9. Surface expression assays

As previously described, cross-linking experiments by means of bis (sulfosuccinimidyl) suberate (BS^3) (Pierce, Rockford, IL) were performed (Mauceri et al., 2004). Briefly, following a wash incubation of 20 min at 37°C, cultures were incubated with 1 mg/mL BS^3 in saline solution for 10 min with agitation at 37°C. Plates were then washed three times with harvest buffer before harvesting neurons. Ethanolamine is present in the harvest buffer to quench any unreacted BS^3.

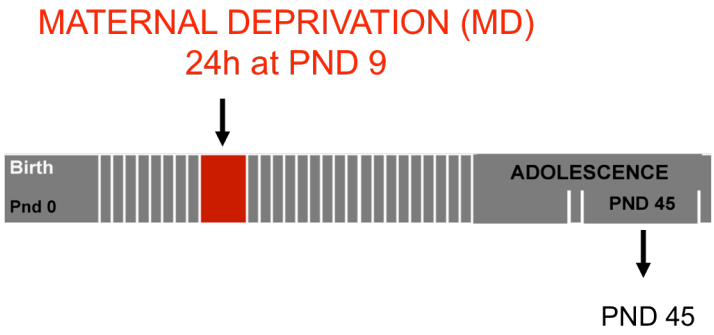
3.3.10. Viability assay

Neuronal cell death was monitored over 6, 24, 48, and 72 hours. In a set of experiments neurons were loaded with IL-1ra, and exposed to 600 pM gp120 for 72 hours. Cell viability was measured by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Denizot and Lang, 1986). MTT tetrazolium salt was dissolved in serum-free medium to a final concentration of 0.75 mg/ml and added to the cells before the end of the experiment for 3 hours at 37°C. The medium was then removed and the formazan was extracted with 1 N HCl:isopropanol (1:24). Absorbance at 560 nm was read on a Multiscan reader.

3.4 MATERNAL DEPRIVATION PROTOCOL

The maternal deprivation experiments were performed by the laboratory of Prof Maria Paz Viveros Departamento de Fisiologia, Facultad de Biologia, Universidad Complutense, Madrid, Spain). The maternal deprivation (MD) protocol took place on post natal day 9 (PND 9) as previously described (Llorente et al., 2008). In brief, on PND 9, half of the litters were submitted to 24 hours of MD (four litters), that is, dams removed from their home-cages at 09.00 h and pups left undisturbed in their corresponding home-cage (in the same room) for 24 hours, until PND 10, when dams returned to their corresponding home-cages. Control animals (six litters) were submitted to a similar manipulation: dams were briefly removed both on PND 9 and 10, but dams were immediately returned to their home cages. Rat pups were henceforth left undisturbed for some days to allow the consolidation of the short-term MD effects on our parameters of interest.

Animals were sacrificed by rapid decapitation during the dark phase of the cycle (09.00–14.00 h) at PND 45. The brains were rapidly extracted, the hippocampi were isolated and stored at −80°C until their use.



Biochemical analysis:

- phosphorylation at Tyr-1472 of GluN2B subunit in homogenate (western blotting)
- expression of IL-1RI anf GluN2B in triton insoluble fraction (western blotting)
- interaction between IL-1RI and GluN2B in triton insoluble fraction (immunoprecipitation

3.5. QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of confocal experiments was performed using Laserpix software (Biorad, CA, USA). Image acquisition, quantification of the fluorescence signal and co-localization analysis were performed by investigators who were 'blind' to the experimental conditions. Quantification of western blot analysis was performed by means of computer-assisted imaging (Quantity-One^{R} System; Biorad, CA, USA).
Statistical significance of differences was determined by one-way or two-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's test). A significance level of 95% ($p<0.05$) was accepted.

3.6. ANTIBODIES

Polyclonal IL-1R antibody was purchased from Santa Cruz Biotechnology; α-CaMKII monoclonal antibody and polyclonal GluA1 antibody were purchased from Chemicon International, Inc., (Temecula, CA); monoclonal PSD-95 antibody was purchased from Affinity BioReagents Inc. (Golden, CO); polyclonal pTyr-1472-GluN2B antibody were purchased from Calbiochem (Nottingham, UK), polyclonal anti-β-tubulin was purchased from Covance (Berkeley, CA); monoclonal anti-GluN2A was purchased from Zymed Laboratories Inc.; monoclonal anti-synaptophysin was purchased from Sigma-Aldrich; polyclonal MyD88 was purchased from Imgenex (San Diego, CA, USA) and from ProScience, (Poway, CA, USA); polyclonal IL-1RAcP was purchased from Genetex (CA, USA); polyclonal anti-GluN2A, polyclonal anti-GluN2B, and AlexaFluor 488, 555, and 568 secondary antibodies were purchased from Molecular Probes (Eugene, Oregon).

4. RESULTS

4.1. RELEVANCE OF GLIAL CELLS AND ROLE OF ENDOGENOUS IL-1β ON Tyr-1472 PHOSPHORYLATION OF GluN2B SUBUNITS OF NMDAR

It has been previously reported the existence of an interaction between interlukin-1β (IL-1β) and NMDA receptor (NMDAR) (Viviani et al., 2003). In primary cultures of hippocampal neurons recombinant IL-1β, by binding IL-1 receptor type I (IL-1RI), increases NMDAR functions through the activation of Src family tyrosine kinases and subsequent phosphorylation at Tyr-1472 of GluN2B subunit of NMDAR. These events induce a sustained elevation of intracellular Ca^{2+} in neurons and neuronal death (Viviani et al., 2003).

To better define the functional and molecular aspects of this crosstalk, we thus investigated the effect of native IL-1β, generated in an *in vitro* model of neurotoxicity on NMDAR and the impact on neuronal organization and survival. For this purpose, we used a sandwich co-culture of primary hippocampal neurons and glia treated with gp120, one of the envelope glycoproteins of HIV virus.

First of all, IL-1β mRNA expression was measured 2, 4 and 24 hours after treatment with gp120 600 pM in primary glial cells. As shown in Figure 1A, gp120 increased IL-1β mRNA expression after 2 and 4 hours of treatment. IL-1β mRNA returned to control levels within 24 hours from the treatment (Figure 1A). As consequence, an increase of the release of IL-1β was also evident, and still detectable after 48 hours of glia cells to gp120 600 pM (Figure 1B, IL-1β pg/ml, control 62.4±3.19, gp120 139±18 *n*=6, *p<0.05 gp120 *versus* control).

Hippocampal neurons co-cultured with glia were then treated with 600 pM gp120 for 6 and 24 hours, and GluN2B subunit Tyr phosphorylation was evaluated. At the end of the treatment, neurons were separated by glia and lysed; phosphorylation at GluN2B Tyr-1472 was assessed with a specific antibody. A significant increase of GluN2B Tyr-1472 phosphorylation was evident 24 hours after gp120 treatment (Figure 2A, **p<0.01 gp120 *versus* control), whereas 6 hours of treatment was ineffective (Figure 2A). This effect was specifically limited to phosphorylation processes because GluN2B protein level was not affected at the above time points

(Figure 2A).

The effect of gp120 on GluN2B Tyr-1472 phosphorylation in neurons required the presence of glia, because it was undetectable when primary hippocampal neurons were exposed alone to 600 pM gp120 for 24 hours (p1472 immunostaining % of control: controls 100±31.6, gp120: 88±33.0 *n*=3). The need of glia for gp120 to trigger Tyr-1472 phosphorylation of the GluN2B subunit together with the promotion of IL-1β release suggest the possible involvement of this cytokine in the observed effect. Hippocampal neurons co-cultured with glia were then incubated with 600 pM gp120 in the presence or absence of 1 µg/ml IL-1 receptor antagonist (IL-1ra) and Tyr-1472 phosphorylation in neurons was assessed. IL-1ra *per se* did not affect basal p1472 immunostaining but prevented gp120-induced increase of Tyr-1472 phosphorylation (Figure 2B; **p<0.01 *versus* control, §p<0.05 *versus* gp120), supporting our hypothesis.

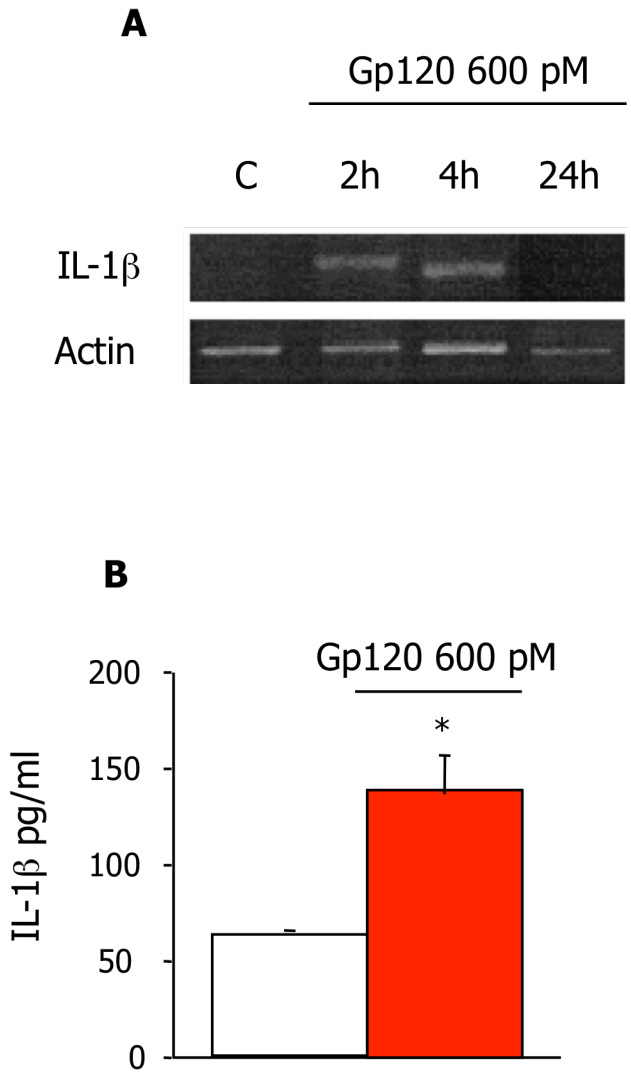


FIGURE 1. Gp120 effect on IL-1β expression and production in glia cells

A. IL-1β mRNA expression in glial cells exposed or not to gp120 600 pM for 2, 4 and 24 hours.

B. IL-1β protein level in cultured medium of glia cells in the presence or absence of gp120 600 pM, measured 48 hours after the treatment. Values are mean ± S.E. of 6 independent experiments (IL-1β pg/ml, control 62.4±3.19, gp120 139±18, *p<0.05 gp120 *versus* control; ANOVA followed by Tukey's test).

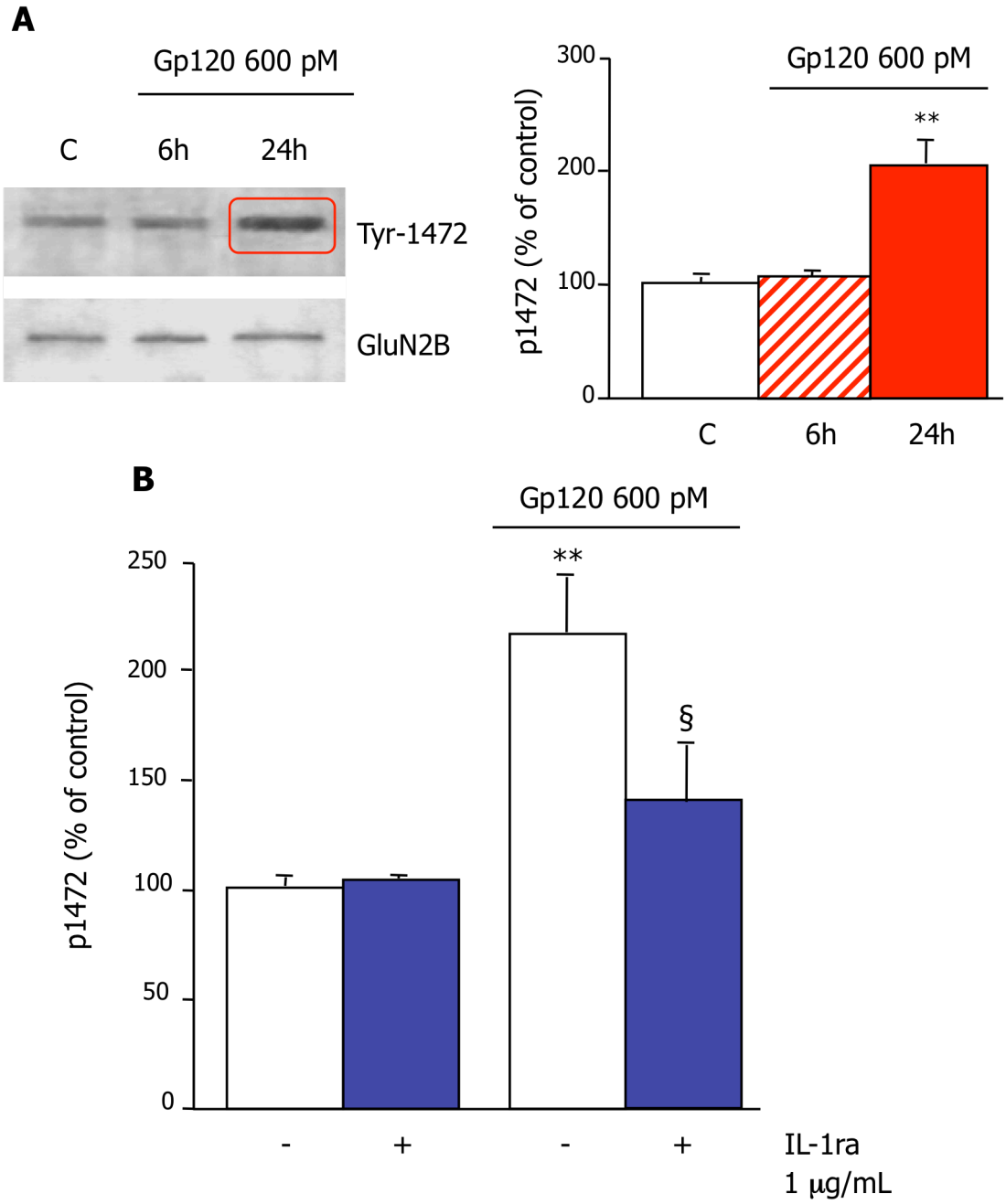


FIGURE 2. Gp120 effect on Tyr-1472 phosphorylation of GluN2B subunits

A. Representative western blotting and quantification of GluN2B-Tyr-1472 phosphorylation after exposure for 6 and 24 hours of hippocampal neurons to gp120 in the presence of glia. At the end of treatment, neurons were detached from glia and lysed for western blotting. Values are mean ± S.E. of 3 independent experiments (**p<0.01 gp120 *versus* control; ANOVA followed by Tukey's test).

B. Quantification of GluN2B-Tyr-1472 phosphorylation after exposure of hippocampal neurons in the presence of glia ± IL-1ra to gp120 for 24 hours. Values are mean ± S.E. of 3 independent experiments (**p<0.01 gp120 *versus* control, §p<0.05 gp120 + IL-1ra *versus* gp120; ANOVA followed by Tukey's test).

4.2. RELEVANCE OF GLIA CELLS AND ROLE OF ENDOGENOUS IL-1β ON INTRACELLULAR Ca^{2+} ($[Ca^{2+}]_i$) HOMEOSTASIS INDUCED BY Tyr-1472 PHOSPHORYLATION OF GluN2B SUBUNITS OF NMDAR

Tyr phosphorylation is a well recognized pathway adopted from neurons to up-regulate NMDA receptor function (Salter and Kalia, 2004) and has been involved in pathological conditions such as ischemic neuronal death (Takagi et al., 1997; Cheung et al., 2000). Neuronal injury consequent to gp120 is also predominantly mediated by over-activation of NMDAR-coupled ion channels that allows excessive Ca^{2+} influx and a general imbalance of its homeostasis (Kaul et al., 2001, Haughey et al., 2002). We thus evaluated whether gp120-induced GluN2B Tyr phosphorylation, through the release of IL-1β, could be implicated in the altered $[Ca^{2+}]_i$ homeostasis in neurons. Primary hippocampal neurons were treated with 600 pM gp120 in the presence or absence of glia, separated at the end of treatment and monitored for $[Ca^{2+}]_i$. The exposure to 600 pM gp120 in the presence of glia significantly increased neuronal $[Ca^{2+}]_i$ (Figure 3A) after 24 hours. This effect was caused predominantly by Ca^{2+} influx through the GluN2B-NMDA receptor channels because the addition of 10 μM ifenprodil, a channel blocker that binds only to the GluN2B subunit of NMDA receptors, inhibited the rise of gp120-induced neuronal $[Ca^{2+}]_i$ (Figure 3A).

To investigate whether GluN2B Tyr phosphorylation was implicated in gp120 modulation of the NMDAR-dependent Ca^{2+} response, we examined the rise of gp120-induced neuronal $[Ca^{2+}]_i$ in the presence of Ca-pYEEIE, a peptide inhibitor for Src family SH2 domain (Park et al., 2002). Thus, primary hippocampal neurons were loaded in the absence of glia with 10 μg/ml Ca-pYEEIE, washed to remove extracellular Ca-pYEEIE, and then exposed for 24 hours in the presence of glia to 600 pM gp120. This experimental approach allowed us to act on neurons without interfering with the Src family signal transduction in glia. The classical tyrosine kinases inhibitors PP1 and PP2 could not be used for this purpose because of the reversibility of their action upon removal (Wang et al., 2004; Osterhout et al., 1999). As shown in Figure 3B, in Ca-pYEEIE pretreated neurons, $[Ca^{2+}]_i$ levels were not

altered by gp120 treatment. As expected, treatment with Ca-pYEEIE also prevented Tyr-1472 phosphorylation (data not shown).

As previously observed for GluN2B-induced phosphorylation, gp120 ability to increase $[Ca^{2+}]_i$ requires the presence of glia because 24 hours of treatment with 600 pM gp120 did not affect basal $[Ca^{2+}]_i$ in neurons exposed alone (Figure 3C). Again, gp120-induced $[Ca^{2+}]_i$ increase in neurons was prevented by 1 μg/ml IL-1ra (Figure 3C), confirming the role of IL-1β and IL-1RI as possible mediators of gp120 action on the NMDA receptor.

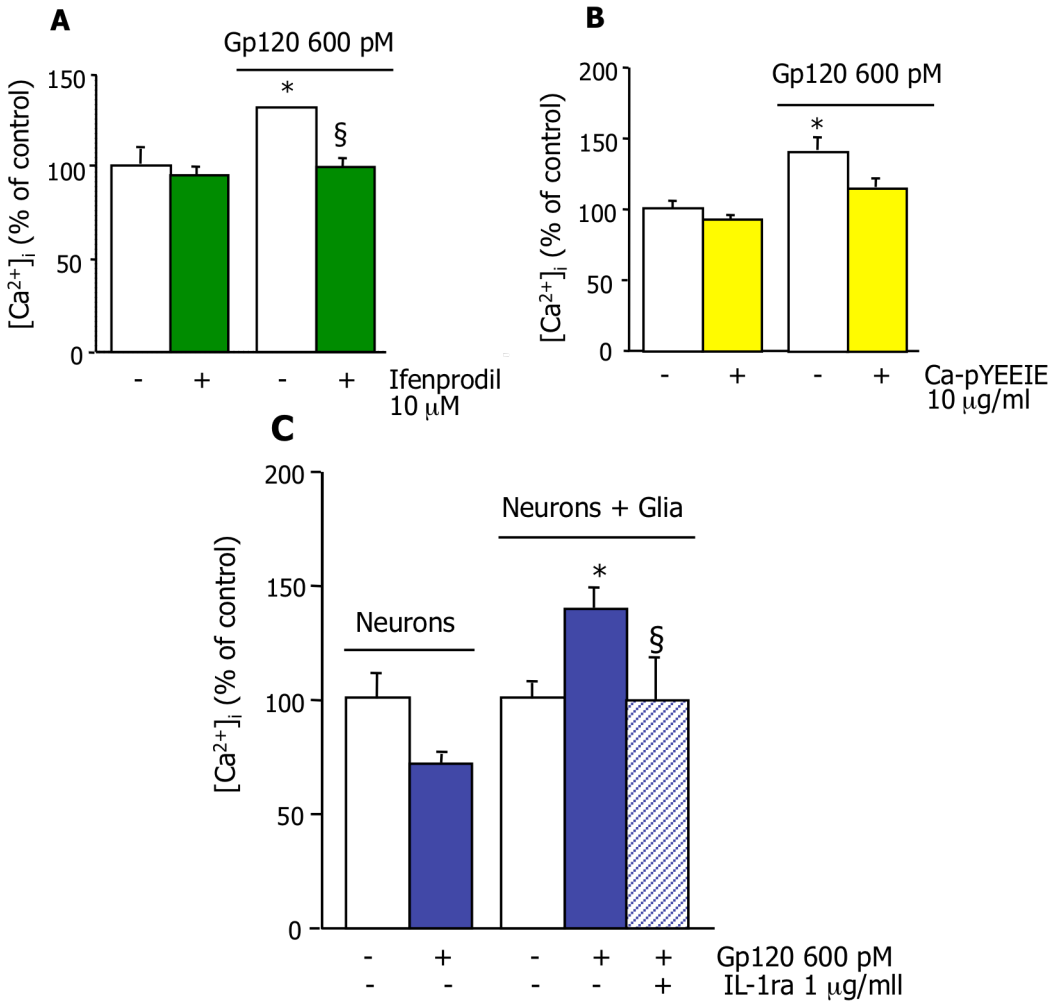


FIGURE 3.Involvement of glia, IL-1β and GluN2B subunit Tyr phosphorylation, and IL-1β in gp120-induced $[Ca^{2+}]_i$ increase in primary cultures of hippocampal neurons

A. Hippocampal neurons were exposed to gp120 for 24 hours in the presence of glia with or without 10 mM ifenprodil. At the end of treatment, hippocampal neurons were detached from the glial monolayer and assayed for $[Ca^{2+}]_i$. Values are means ± S.E. of 9 independent samples and represent the $[Ca^{2+}]_i$ levels in neurons at the end of treatment (*p<0.05 gp120 *versus* control, §p<0.05 gp120 + ifenprodil *versus* gp120; ANOVA followed by Tukey's test).

B. Hippocampal neurons were loaded with Ca-pYEEIE, washed to remove extracellular Ca-pYEEIE and then exposed to gp120 for 24 hours in the presence of glia. Values are means ± S.E. of 15 independent samples and represent the $[Ca^{2+}]_i$ levels in neurons at the end of treatment (*p<0.05 gp120 *versus* control; ANOVA followed by Tukey's test).

C. Hippocampal neurons were exposed to gp120 for 24 hours in the presence (right) or absence (left) of glia. IL-1ra was added in part of the co-cultures (striped column). At the end of treatment, co-cultured neurons were detached from glia and assessed for $[Ca^{2+}]_i$. IL-1ra did not affect $[Ca^{2+}]_i$ levels *per se* ($[Ca^{2+}]_i$ % of control: 112±9.64). Values are means ± S.E. of 9 independent samples and represent the $[[Ca^{2+}]_i$ levels in neurons at the end of treatment (*p<0.05 gp120 *versus* control, §p<0.05 gp120 + IL-1ra *versus* gp120; ANOVA followed by Tukey's test).

4.3. DISTRIBUTION OF GluN2B SUBUNITS OF NMDAR, IL-1RI AND IL-1RI CORE SIGNALLING PROTEINS IN HIPPOCAMPAL NEURONS

The ability of IL-1ra to prevent Tyr-1472 phosphorylation and NMDA receptor over-activation induced by both recombinant and endogenous IL-1β, suggests the recruitment of IL-1 receptor type I (IL-1RI).

In the immune system the binding of IL-1β to IL-1RI leads to its association with IL-1R accessory protein (IL-1RAcP) (Korherr et al., 1997) and the myeloid differentiation primary response protein 88 (MyD88) (Burns et al., 1988) to form the *core* of IL-1β/IL-1RI signalling complex. However, little information is currently available concerning the molecular composition of the members of the IL-1RI complex, or the subcellular distribution and functional crosstalk with NMDARs in neuronal cells.

The distribution of IL-1RI, IL-1RAcP, MyD88 and GluN2B subunit together with the pre- and post-synaptic markers synaptophysin and PSD-95, was thus investigated in different subcellular compartments purified from adult rat hippocampi by means of western blotting (Gardoni et al., 1998).

Subcellular fractionation showed that IL-1RI, MyD88 and IL-1RAcP were present in all the tested fractions but, although IL-1RI and MyD88 were particularly enriched in the post-synaptic density (PSD) fraction (Figure 4), together with PSD-95 (Figure 4), only traces of IL-1RAcP were present in the post-synaptic Triton-insoluble fraction (TIF) and PSD (Figure 4). As expected, the pre-synaptic protein synaptophysin was present in all subcellular compartments analyzed, but not in the PSD or in the TIF-purified fractions, whereas PSD-95 was enriched in the post-synaptic fractions (PSD and TIF) (Figure 4). In these same samples the partition pattern of GluN2B closely resembled IL-1RI being similarly distributed in the different subcellular fractions and enriched at the post-synapse (Figure 4). The enrichment of both GluN2B subunits of NMDAR and IL-1RI at TIF fraction has been confirmed in primary hippocampal neurons as well (data not shown).

The distribution patterns of IL-1R complex members were also examined by confocal

microscopy in cultured hippocampal neurons (Figure 5); PSD-95 was used as a marker of post-synaptic structures. Confocal imaging showed that IL-1RI is distributed along dendrites and enriched in the post-synaptic compartment, as shown by the high degree of co-localization with PSD-95 (Figure 5, left panels, 34.3±3.7%). MyD88 was uniformly distributed along the neurons and moderately co-localized with PSD-95 (Figure 5, right panels, 15.6±2.8%). IL-1RAcP labeling was intense and diffuse in the somatic cytoplasm of cultured neurons, low and diffuse along the dendrites, and hardly co-localized with PSD-95 (Figure 5, central panels, 4.1%±1.9%;). Overall, these data suggest that there is a different subcellular distribution of the members of the IL-1R complex protein in neurons, with IL-1RI (and, to a lesser extent, MyD88) being enriched at the post-synaptic sites together with the GluN2B subunit of NMDA receptor.

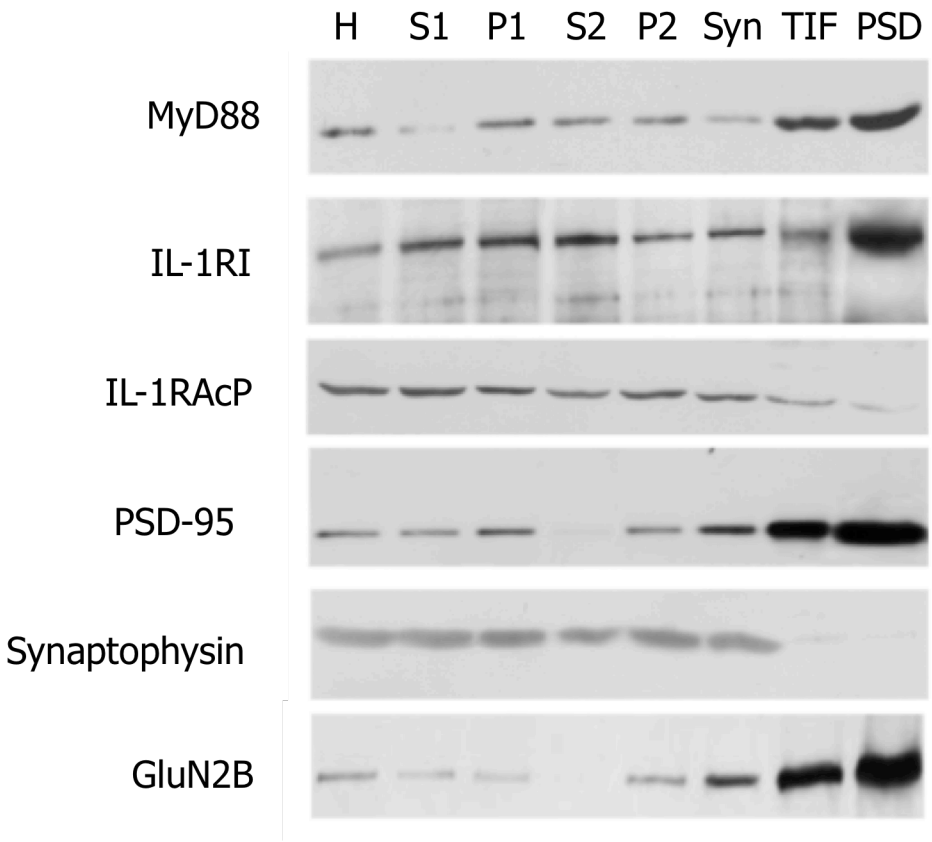


FIGURE 4. Characterization of IL-1RI, IL-1RAcP, MyD88 and GluN2B subcellular distributions in rat hippocampi

IL-1RI, IL-1RAcP and MyD88, and GluN2B together with markers of the pre-synaptic compartment (synaptophysin) and post-synaptic side (PSD-95) were analyzed in various rat hippocampus subcellular compartments by means of western blotting. H = homogenate; S1 = low-speed supernatant; P1 = nuclei-associated membranes; S2 = high-speed supernatant; P2 = crude membrane fraction; Syn = synaptosomes; TIF = Triton- insoluble postsynaptic fraction; PSD = post-synaptic density.

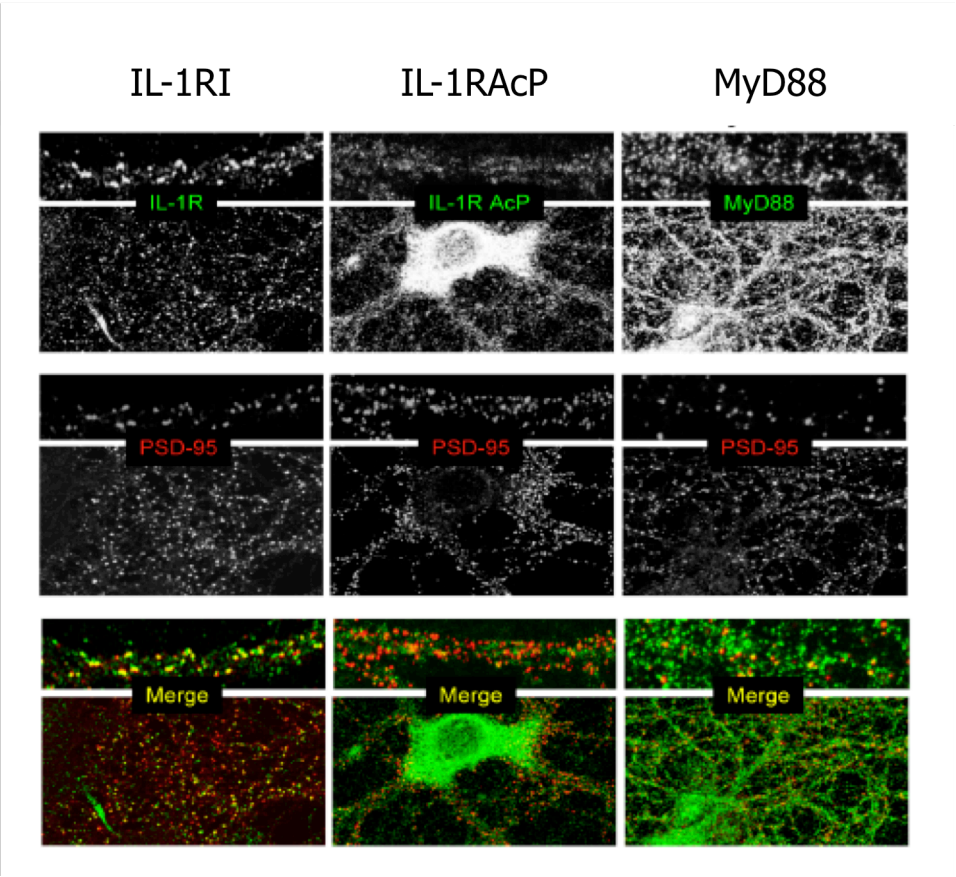


FIGURE 5. Distribution of IL-1RI, IL-1RAcP and MyD88 in primary cultures of hippocampal neurons

Hippocampal neurons at *days in vitro* 14 (DIV14) were immunolabelled for IL-1RI, IL-1RAcP and MyD88 (upper panels), and PSD-95 as a post-synaptic marker (middle panels). The bottom panels show the merged images. Scale bar: 10 μM. High-magnification images are shown at the top of each panel.

4.4. MOBILIZATION OF GluN2B SUBUNITS OF NMDAR AND IL-1RI IN PRIMARY HIPPOCAMPAL NEURONS

It is well known that the distribution and trafficking of receptors and ion channels along different neuronal subcellular compartments are involved in modulation of neuronal activity; in particular synaptic activity is strictly related to the ability of neuronal cells to dynamically adjust the content of receptors at the synaptic sites in response to various stimuli (Newpher et al., 2008; Groc et al., 2009).

We have examined whether the recruitment of IL-1 signalling and the activation of NMDA system following a toxic stimulus promote the synaptic localization of GluN2B subunit of NMDAR and IL-1RI.

TIF was obtained from the control and 24 hours gp120-treated neurons and protein levels of GluN2B and IL-1RI were measured in the homogenate and TIF. The same amount of proteins from homogenate and TIF was loaded on SDS-PAGE gels for western blotting analysis. As shown in Figure 6A, gp120 treatment significantly increased GluN2B immunostaining in TIF without affecting the total GluN2B protein level in the homogenate (**$p<0.01$; +47.2±8.1%, gp120 *versus* control expressed as GluN2B ratio TIF/homogenate). No alteration of both PSD-95 and αCaMKII immunostaining in both homogenate and TIF was observed following gp120 treatment.

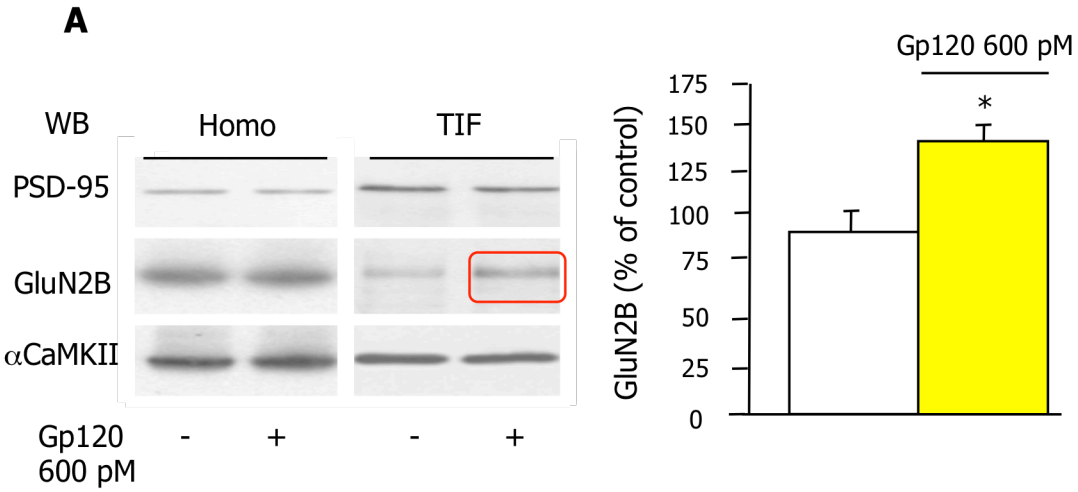
To further confirm these data by confocal labeling, primary hippocampal neurons were treated for 24 hours with gp120, fixed and double-labeled for GluN2B and PSD-95 (Figure 6B). In control neurons (Figure 6B, upper panels), GluN2B signal was of a greater intensity in the soma, but signals were clearly seen also in PSD-95 positive "spine-like" puncta as well as, in the dendritic shaft. In addition, GluN2B immunofluorescence in "spine-like" structures overlapped NR1 immunosignal, further confirming he presence of the NMDA receptor complex in the post-synaptic site (data not shown). 24 hours gp120 treatment produced a more pronounced GluN2B punctuated pattern associated with a higher co-localization degree with PSD-95 (Figure 6B). Quantification of GluN2B relative fluorescence intensity in spine *versus* dendritic shaft (Figure 6C) revealed that gp120-treated neurons exhibited an increased fluorescent signal in these spine-like structures (Figure 6C, **$p<0.01$,

gp120 *versus* control). In addition, quantification of GluN2B punctuated staining revealed a significant increase of GluN2B immunoreactivity in PSD-95-positive dendritic spines compared with control values (Figure 6C; **p<0.01, gp120 *versus* control). Both sets of data suggest a promotion of NMDA GluN2B subunit redistribution to synaptic sites by gp120.

To assess whether the effects on GluN2B localization were mediated by IL-1β, gp120 treatments were performed in the presence of IL-1ra. Figure 6C shows that IL-1ra prevented gp120-induced trafficking of the NMDA receptor GluN2B subunit (§§p<0.01 and §p<0.05, gp120 + IL-1ra *versus* gp120); both GluN2B relative fluorescence in spines *versus* dendrites and GluN2B immunoreactivity in PSD-95 positive dendritic spines were significantly reduced by IL-1ra. IL-1ra *per se* did not affect GluN2B subcellular distribution (Figure 6C) or cell survival (% of cell survival, control: 100±7.2; 24 hours IL-1ra: 97.4±4.45).

We also analyzed whether the GluN2A subunit of NMDA receptor, lacking the Tyr-1472 phosphosite was mobilized in a model of toxicity characterized by over-production of IL-1β like that of gp120 (Figure 7A). No alteration of GluN2A immunostaining in TIF was observed following gp120 treatment (Figure 7A) suggesting a specific effect on GluN2B subunit.

We also evaluated the IL-1RI mobilization to the synapse. The protein levels were measured in the homogenate and TIF by western blotting analysis (Figure 7B). The treatment with gp120 of hippocampal neurons in presence of glia, enriched the post-synaptic site with IL-1RI, without affecting the total amount of IL-1RI in homogenate. No alteration of PSD-95 levels in both homogenate and TIF was observed following gp120 treatment (Figure 7B).


A
WB
Homo
TIF
PSD-95
GluN2B
αCaMKII
Gp120 600 pM
- + - +
Gp120 600 pM
GluN2B (% of control)
175
150
125
100
75
50
25
0
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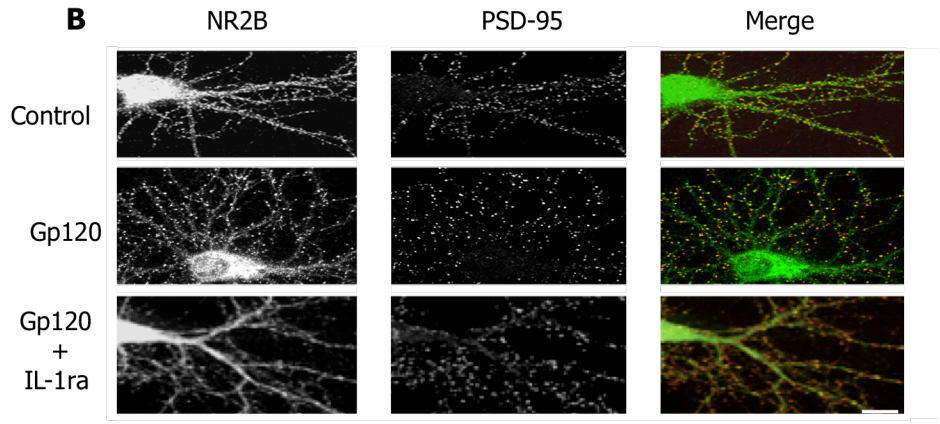

B
NR2B
PSD-95
Merge
Control
Gp120
Gp120 + IL-1ra

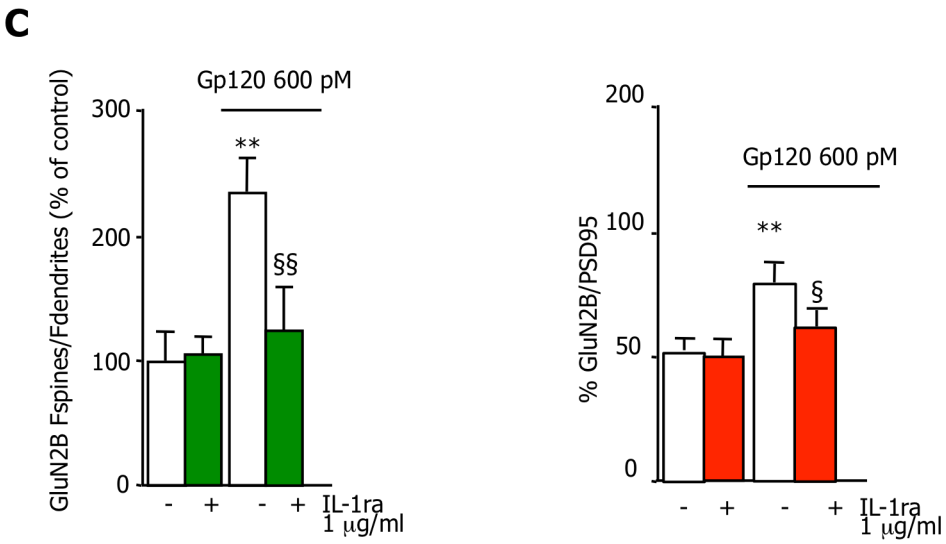

C
Gp120 600 pM
GluN2B Fspines/Fdendrites (% of control)
300
200
100
0
**
§§
- + - + IL-1ra
1 μg/ml
Gp120 600 pM
% GluN2B/PSD95
200
100
50
0
**
§
- + - + IL-1ra
1 μg/ml

FIGURE 6. Effect of gp120 on GluN2B and IL-1RI subcellular localization

A. Western blotting analysis performed in the homogenate (Homo) and in the TIF fraction obtained from control and gp120-treated hippocampal cultures. The same amount of protein was loaded in each lane. gp120 treatment leads to a higher GluN2B localization in the TIF fraction (*p<0.01; *versus* control; ANOVA followed by Tukey's test) leaving unaffected αCaMKII and PSD-95 immunostaining in the TIF fraction. Values are means ± S.E. of 8 independent samples and represent the GluN2B TIF/Homo ratio staining expressed as percentage of control neurons.

B. Hippocampal neurons were either left untreated (control) or treated for 24 hours with gp120 in the presence of glia + IL-1ra, fixed, and immunolabeled for GluN2B (left panels) or PSD-95 (middle panels). Scale bar, 10 μm. Merge data are shown on the right. Areas of overlap in merge panels appear yellow. 12 neurons for each experimental condition were analyzed.

C. Quantification of the confocal experiments. gp120 administration results in a redistribution of GluN2B into spine-like clusters; IL-1ra treatment interferes with gp120-mediated GluN2B trafficking toward spines. The ratio of spines to dendrites fluorescence was computed and averaged (**p<0.01, gp120 *versus* control; §§p <0.01, gp120 + IL-1ra *versus* gp120). Gp120 treatment also causes a significant increase in the percentage of GluN2B clusters of the total number of PSD-95-positive spines compared with control values (**p<0.01, gp120 *versus* control; §p<0.01, gp120 + IL1ra *versus* gp120) (ANOVA followed by Tukey's test).

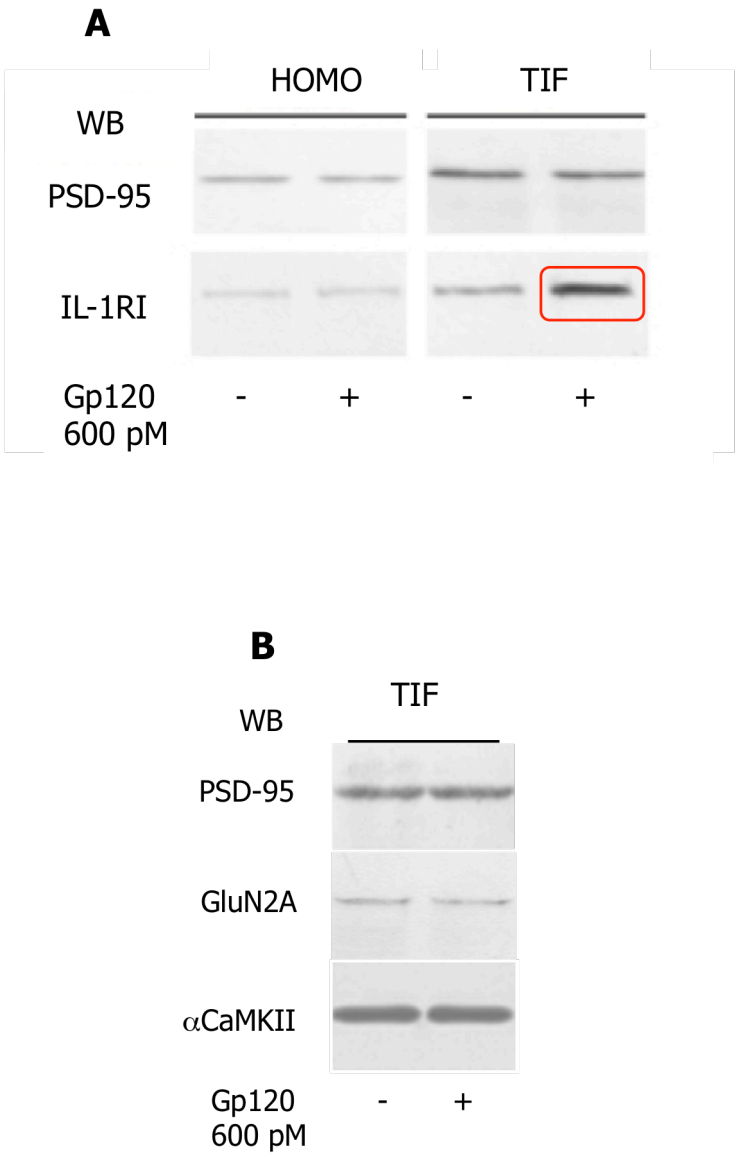


FIGURE 7. Effect of gp120 on IL-1RI and GluN2A subcellular localization

A. Western blot analysis performed in the Homo and in the TIF fraction obtained from control and gp120-treated hippocampal cultures. The same amount of protein was loaded in each lane. gp120 treatment leads to a higher IL-1RI localization in the TIF fraction leaving unaffected PSD-95 immunostaining in the TIF fraction.

B. Western blotting analysis performed in the TIF fraction obtained from control and gp120-treated hippocampal cultures. The same amount of protein was loaded in each lane. gp120 treatment does not affect GluN2A, PSD-95, and αCaMKII localization in the TIF fraction.

4.5. TREATMENTS WITH IL-1β AND NMDA INDUCE IL-1RI, BUT NOT IL-1RACP AND MYD88, SYNAPTIC LOCALIZATION

The model adopted using gp120 to study the relationship existing between IL-1RI and NMDAR underlies a central role of both IL-1β and NMDA signalling in the observed effects. We thus investigated whether IL-1β and NMDA stimulation contribute to the re-distribution of IL-1RI complex members in different neuronal compartments.

Primary hippocampal neurons were treated with IL-1β 0.05 ng/ml for 30 min, a concentration also known to enhance NMDAR activity (Viviani et al, 2003). A modified fractionation method was performed to obtain a TIF postsynaptic compartment from hippocampal primary neurons instead of tissues and the presence of IL-1RI was evaluated together with IL-1RAcP and MyD88 in this subcellular fraction by western blotting analysis. As shown in Figure 8A, treatment of primary hippocampal neurons with IL-1β significantly increased the amount of IL-1RI in the TIF fraction (Figure 8A, *$p<0.05$, IL-1β *versus* control) without affecting the synaptic distribution of both IL-1RAcP and MyD88 (Figure 8A). IL-1RI enrichment at synapses following IL-1β treatment was also confirmed by confocal microscopy (Figure 8B), which shows an increased co-localization of IL-1RI with PSD-95 (**$p<0.01$, IL-1β *versus* control).

Subcellular localization experiments have been performed also in primary hippocampal neurons triggered with NMDA 50 μM for 10 min in ACSF buffer after which the cells were washed and incubated for further 20 min in ACSF buffer. NMDA significantly enriched hippocampal TIF with IL-1RI (Figure 9A, *$p<0.05$, NMDA *versus* control), without changing the synaptic abundance of both IL-1RAcP and MyD88 (Figure 11A). Again confocal microscopy (Figure 9B) shows an increased co-localization of IL-1RI with PSD-95 following NMDA treatment (*$p<0.05$, NMDA *versus* control), thus confirming IL1RI enrichment at the post-synapses.

The increase in IL-1RI receptors at the post-synaptic site may be due to new synthesis and delivery and insertion of receptors from the endoplasmic reticulum to post-synaptic membrane, or to lateral diffusion from adjacent compartments

(Newpher et al., 2008; Groc et al., 2009), and this was addressed by carrying out surface expression assays using the non-cleavable, membrane-impermeable cross-linking agent BS^3 (Mauceri et al., 2004).

Primary hippocampal neurons were treated with IL-1β 0.05 ng/ml or NMDA 50 μM and then exposed to BS^3, to link all the proteins of plasma membrane; then they are lysed and blotted to check only the intracellular amount of IL-1RI.

The intracellular amount of IL-1RI was reduced by NMDA but not by IL-1β (Figure 10, *$p<0.05$, NMDA *versus* control).

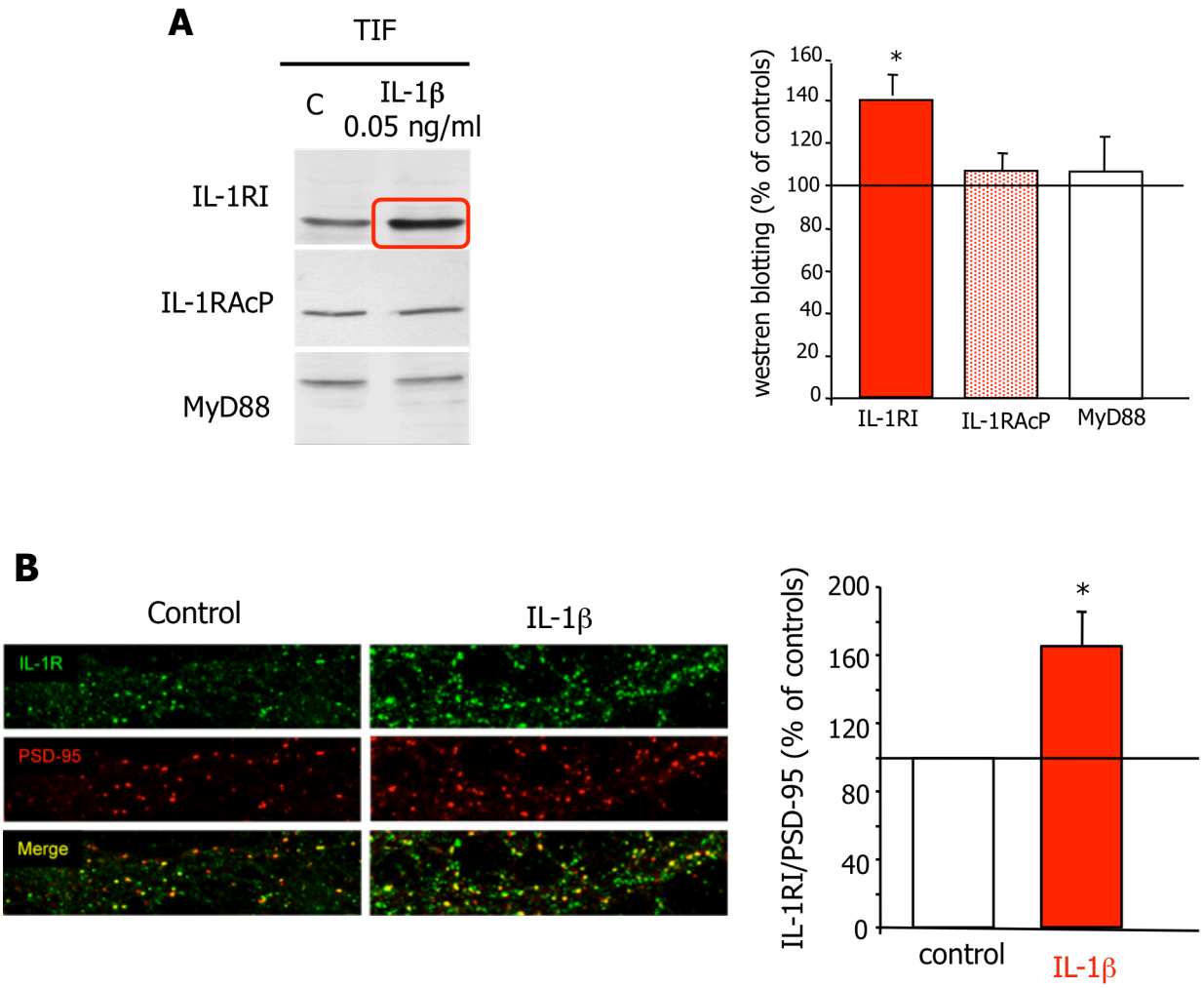


FIGURE 8. Effect of IL-1β on IL-1RI subcellular localization

A. Western blotting analysis of the TIF fraction obtained from control, IL-1β-treated (0.05 ng/ml). The same amount of proteins was loaded in each lane. IL-1β increases IL-1RI localization in the Triton-insoluble fraction (TIF) (*p<0.05, IL-1β *versus* control; ANOVA followed by Tukey's test) leaving unaffected IL-1RAcP and MyD88 levels. Values are means ± S.E of 4 independent experiments.

B. Hippocampal neurons were either left untreated (control) or treated with IL-1β (0.05 ng/ml, 30 minutes) fixed, and immunolabeled for IL-1RI (green) and PSD-95 (red) as a postsynaptic marker. Data are expressed as percentage of IL-1RI co-localization with PSD-95 (AIM4.2 software, Zeiss) (**p<0.01, IL-1β *versus* control; ANOVA followed by Tukey's test). White arrows indicate PSD-95 positive clusters in the merge panel. Scale bar: 5 μM.

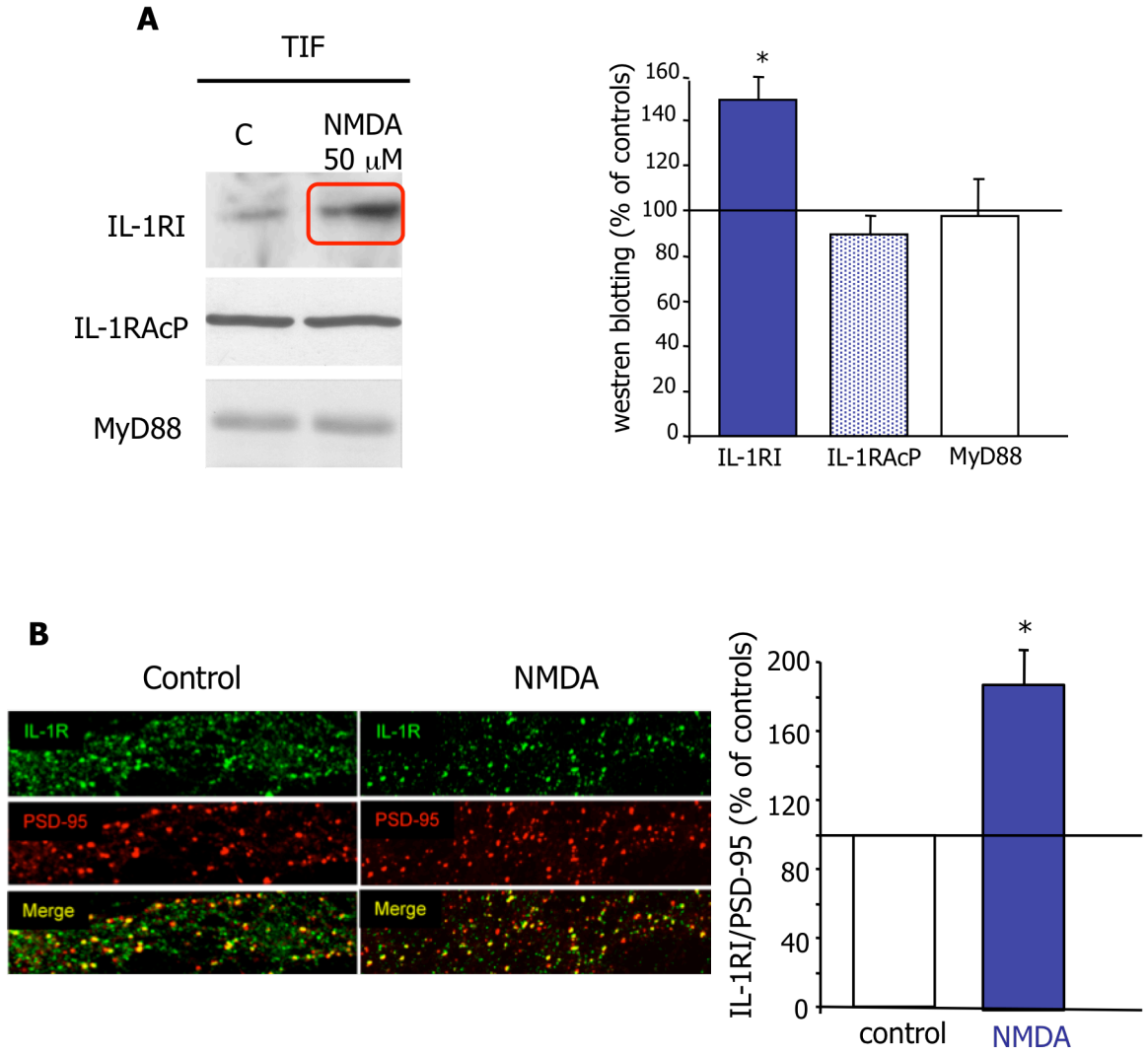


FIGURE 9. Effect of NMDA on IL-1RI subcellular localization

A. Western blotting analysis of the TIF fraction obtained from control, NMDA-treated (50 μM). The same amount of proteins was loaded in each lane. NMDA increases IL-1RI localization in the Triton-insoluble fraction (TIF) (*p<0.05, NMDA *versus* control; ANOVA followed by Tukey's test) leaving unaffected IL-1RAcP and MyD88 levels. Values are means ± S.E of 4 independent experiments.

B. Hippocampal neurons were either left untreated (control) or treated with NMDA (50 mM) fixed, and immunolabeled for IL-1RI (green) and PSD-95 (red) as a postsynaptic marker. Data are expressed as percentage of IL-1RI co-localization with PSD-95 (AIM4.2 software, Zeiss). (**p<0.01, NMDA *versus* control; ANOVA followed by Tukey's test). White arrows indicate PSD-95 positive clusters in the merge panel. Scale bar: 5 μM.

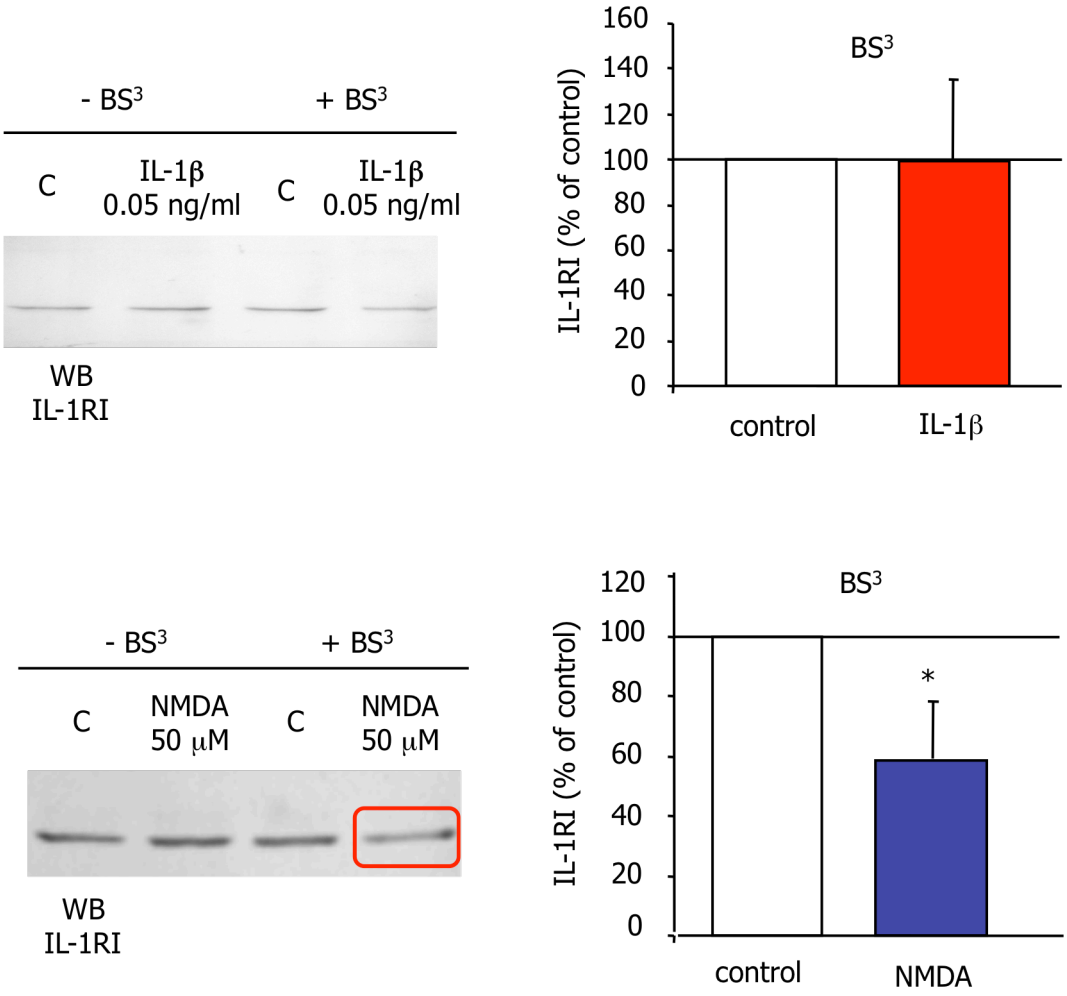


FIGURE 10. Effect of IL-1β and NMDA on IL-1RI subcellular localization
Western blotting of IL-1RI from control, IL-1β-treated (0.05 ng/ml) and NMDA-treated (50 μM) hippocampal cultures exposed (+BS^3 lanes) or not (-BS^3 lanes) to the cross-linking agent BS^3 (*$p<0.05$, NMDA *versus* control; ANOVA followed by Tukey's test). IL-1RI high-molecular-weight complexes that didn't enter the gel are not shown.

4.6. INTERACTION BETWEEN IL-1RI AND GluN2B SUBUNIT OF NMDA RECEPTOR

The enrichment of both IL-1RI and GluN2B at the post-synaptic sites induce us to verify if these two proteins could directly interact. To prove this hypothesis, we performed co-immunoprecipitation experiments between components of the IL-1RI complex and NMDA receptor, as well as AMPA receptor to assess the specificity of interaction.

Protein homogenates (200 μg) from rat hippocampi were immunoprecipitated (Gardoni et al., 2001) with antibodies specific for IL-1RI, for the GluA1 subunit of AMPA receptors, or for the GluN2B subunit of the NMDA receptor. Each sample was then evaluated for the presence of: i) IL-1RI, IL-1RAcP and MyD88; ii) the GluN2B subunit and iii) PSD-95. Figure 11A shows that, in hippocampal lysates, IL-1RI not only co-precipitated with IL-1RAcP and MyD88, but also with GluNRB. In line with this, GluN2B co-precipitated with IL-1RI, thus confirming the association between these components (Figure 11A, right lane). Finally, none of the members of the IL-1R complex was detectable in the immunocomplex of the GluA1 subunit of AMPA receptors (Figure 11A).

The association between IL-1RI and GluN2B was confirmed by a pull-down assay based on a fusion protein of the cytoplasmic domain of IL-1RI with GST (GST-IL-1Rcd) (Figure 11B), which contained the C-terminal 369-569 aa domain of IL-1RI. As a positive control, we used a GST-PSD-95 (PDZ1-2) fusion protein that has been previously shown to bind the GluN2B subunit of NMDA receptors (Gardoni et al., 2001). Lysates from rat hippocampal neurons were applied to affinity beads and extensively washed, after which the bound material was resolved by SDS-PAGE and underwent immunoblotting analysis using an antibody raised against GluN2B. Figure 11B shows that both IL-1Rcd and PSD-95 (PDZ1-2) associated with the GluN2B subunit, thus confirming a specific association between IL-1RI and GluN2B.

We also tested whether IL-1β and/or NMDA modulated the interaction between IL-1RI and the GluN2B subunit of the NMDA receptor (Figure 12). IL-1RI was immunoprecipitated from total lysates of primary hippocampal neurons treated or not with NMDA, 50 μM, or IL-1β, 0.05 ng/ml, and assayed for GluN2B by means of

western blotting (Figure 12). The results show that only NMDA significantly increased the interaction between IL-1RI and GluN2B (Figure 12, *p< 0.05, NMDA *versus* control).

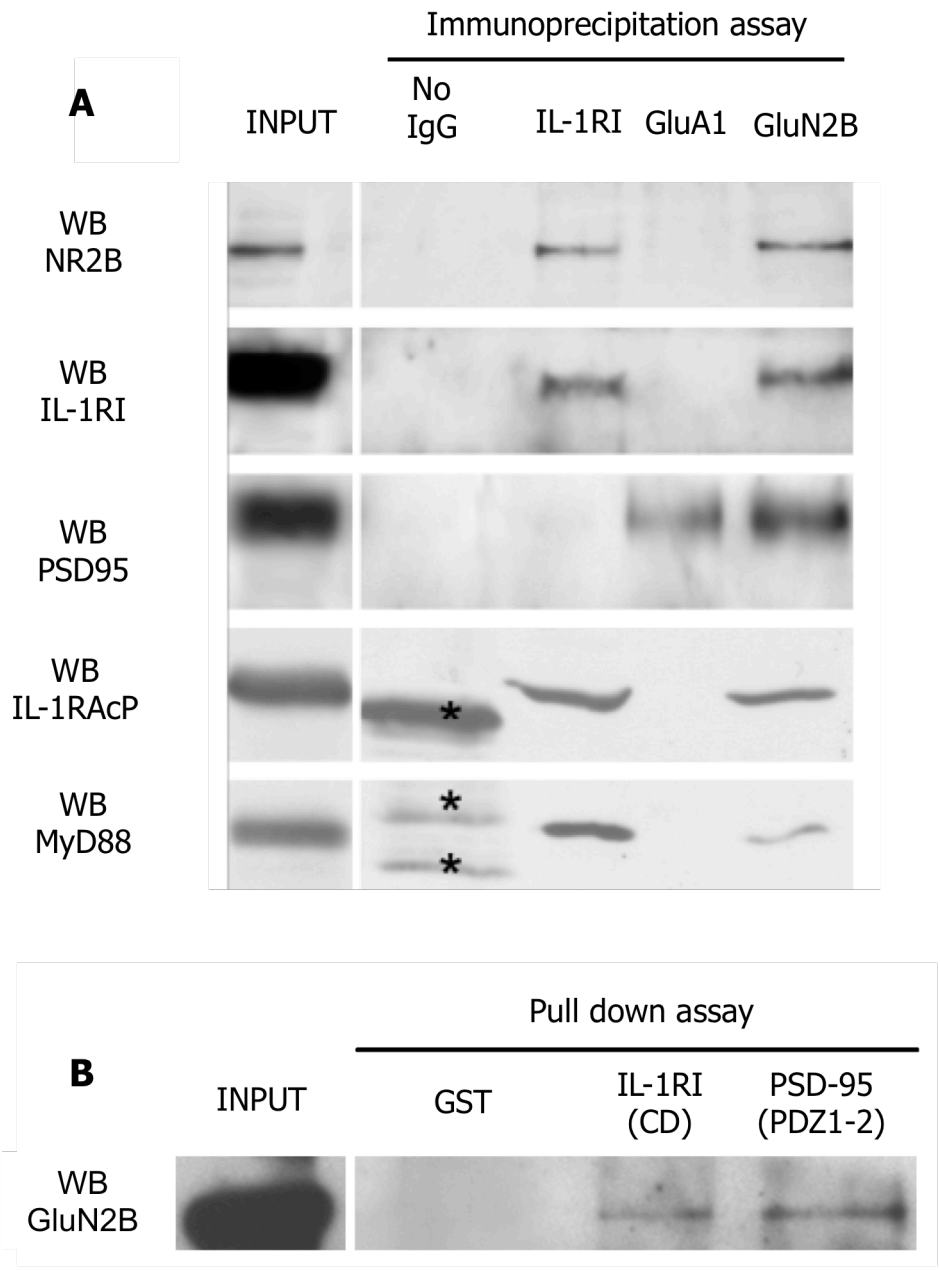


FIGURE 11. Interaction between IL-1RI and the GluN2B subunit of NMDA receptors

A. Total homogenate was immunoprecipitated (i.p.) with antibodies against IL-1RI, GluA1 or GluN2B, and the presence of GluN2B, IL-1RI, PSD-95, IL-1RAcP and MyD88 in the immunocomplex was evaluated by means of western blotting. IL-1RI, IL-1RAcP and MyD88 co-precipitated with GluN2B but not with GluA1. (*) Nonspecific bands were detected in the No IgG lane.

B. GST-IL-1R(CD) and GST-PSD-95(PDZ1-2) fusion proteins, and GST alone were incubated in a pull-down assay with total homogenate from rat hippocampus. The western blotting analysis was performed using the GluN2B antibody.

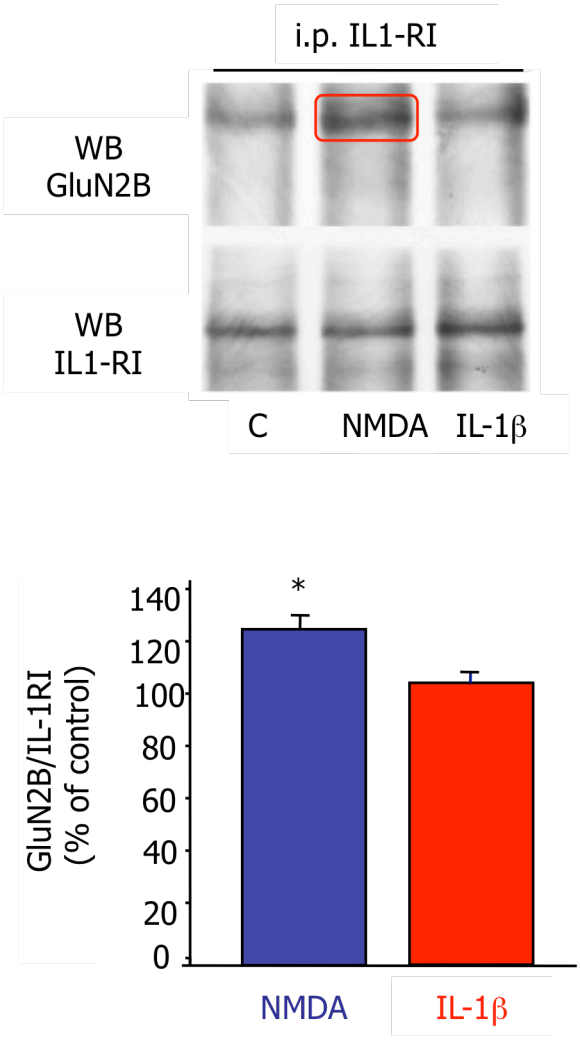
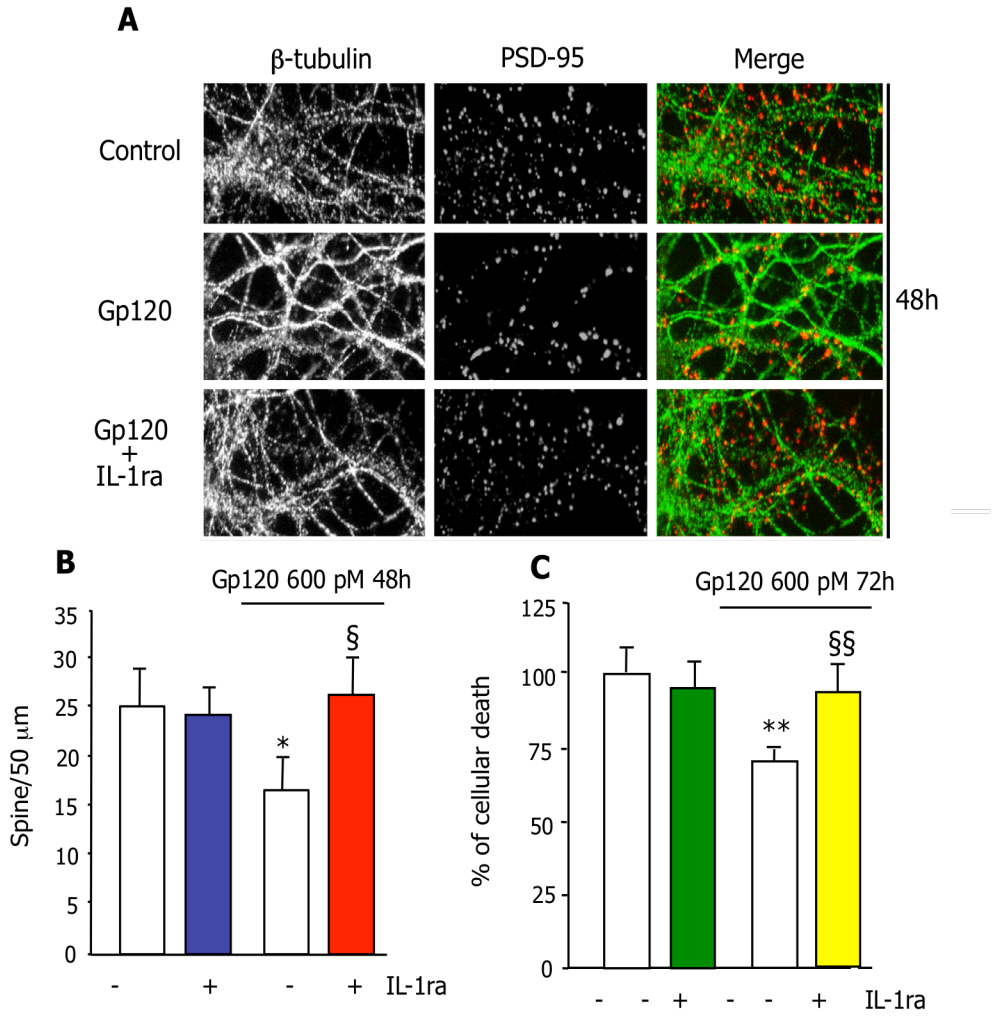


FIGURE 12. Effect of NMDA on interaction between IL-1RI and the GluN2B
Hippocampal cultures were exposed in the absence or the presence of IL-1β (30 minutes, 0.05 ng/ml) or NMDA (10 minutes, 50 μM). Neuronal lysates were immunoprecipitated with anti-IL-1RI, and the presence of GluN2B and IL-1RI in the immunocomplex was evaluated by means of western blotting. Treatment with NMDA but not with IL-1β led to a significant increase in the IL-1β/GluN2B complex (*p<0.05, NMDA *versus* control; ANOVA followed by Tukey's test).

4.7. EFFECT THE IL-1β/IL-1RI/NMDAR CROSS TALK ON PSD-95 POSITIVE CLUSTERS AND NEURONAL DEATH

This set of results shows that the recruitment of the cross talk between IL-1β/IL-1RI/NMDAR contributes to alter the post-synaptic organization We then evaluated the biological role of this event monitoring the spine density and finally neuronal death.

Under our experimental conditions, no effect on PSD-95 positive clusters was observed after 24 hours of gp120 treatment (PSD-95 clusters/50 μm, controls: 27±3.2; gp120 600 pM: 25±5,n=12). On the other hand, 48 hours of gp120 treatment produced a significant reduction of total PSD-95 clusters (Figure 13, A and B; from 24.9±4.56 to 16.5±3.28 protrusions per 50 <μm dendrite length; *p<0.05 *versus* control) suggesting the occurrence of spine loss at this time point. Differently, dendrites appear generally undamaged, as evidenced by co-staining with α-tubulin used in all experiments to check for normal dendritic branching following gp120 treatment (Figure 13A). Loss of PSD-95 clusters was counteracted in neurons loaded with IL-1ra (Figure 13, A/B, §<p 0.05 *versus* gp120). To further strengthen this data and quantify the effective neuronal damage, we monitored gp120-induced cell death in hippocampal neurons. No significant neuronal death was found after 6 and 24 hours of gp120 treatment (respectively, 99.6±2.49 and 91.6±5.12% of cell survival). On the other hand, 48 and 72 hours gp120 treatment resulted, respectively, in about 20% (p<0.01 *versus* control) and 30% (**p<0.01 *versus* control) of neuronal death (Figure 13C). IL-1ra 1 μg/ml prevented gp120-induced neuronal death (72 hours, Figure 13C; §§, p<0.01 *versus* gp120).



FGURE 13. Loss of PSD-95 positive clusters and neuronal death after gp120 treatment: modulation by Ca-pYEEIE and IL-1ra

A. Hippocampal neurons were either left untreated (control) or treated for 48 hours with gp120 in the presence of glia, fixed, and immunolabeled for β-tubulin (left panels) or PSD-95 (middle panels). Merge data are shown on the right. Areas of overlap in merge panels appear yellow.

B. Number of PSD-95-positive clusters per 50 mm of dendrite length in neurons, loaded or not with IL-1ra, after 48 hours of treatment with gp120 in the presence of glia (*p<0.05 *versus* control; § p<0.05 *versus* gp120).

C. Hippocampal neurons were loaded with IL-1ra and then exposed to gp120 in the presence of glia for 72 hours. MTT test was performed to assay cell viability. gp120-induced cell death was prevented by and IL-1ra. Values are means ± S.E. of 2 independent experiments in triplicate, **p<0.01 *versus* control; §§p <0.01 *versus* gp120).

4.8. IS IL-1β AND NMDAR FUNCTIONAL INTERACTION RELEVANT *IN VIVO?* THE MODEL OF EARLY LIFE STRESS

Recently it has been demonstrated that neuroinflammation experienced during early life can change immunoreactivity in the brain for the remainder of the lifespan and seems to play a fundamental role in promoting susceptibility to central nervous system dysfunctions from poor cognition to frank disabilities like depression and schizophrenia (Bilbo et al., 2009; Meyer et al., 2011). These observations give rise to the so called "immune origins of neurodevelopmental disorders hypothesis" (Meyer et al., 2011), whose molecular mechanisms remain almost as much a mystery. Fundamental in this process is the production of cytokines and among these IL-1β (Bilbo et al., 2009). Due to the relevance of both IL-1β and NMDA pathway in the cognition (Bilbo et al., 2009; McAfoose and Baune, 2008) we evaluated the relationship between IL-1RI and NMDAR in a model of maternal deprivation, thanks to our collaboration with Prof Maria Paz Viveros (Departamento de Fisiologia, Facultad de Biologia, Universidad Complutense, Madrid, Spain). The laboratory of Prof Maria Paz Viveros performed a single prolonged episode (24 hours) of maternal deprivation (MD) during the neonatal period at post-natal day 9 (PND9) of rats and then we evaluated the long-lasting modifications induced by maternal deprivation at PND45 examining the key events of IL-1β/NMDAR crosstalk, as studied in our *in vitro* models. In view of the abundant sex differences that have been found in this MD model (Viveros et al., 2009) the possible existence of sexual dimorphisms was also investigated throughout the study, the analyses have been performed both in male and in female rats.

The phosphorylation at Tyr-1472 of GluN2B subunit of NMDAR was assessed with a specific antibody in homogenate of hippocampus of control and MD rats; 24 hours of maternal deprivation at PND9 significantly increases the phosphorylation at Tyr-1472 of GluN2B evaluated at PND45 in male rats (Figure 15A, control: 100±12; MD: 137±16; *$p<0.05$, MD *versus* control). No changes in Tyr-1472 of GluN2B subunits has been found in homogenate of hippocampus of female rats following MD (control: 100±11.1; MD: 98±13.2).

To examine the role of MD in promoting the synaptic localization of IL-1RI, TIFs were obtained from hippocampi of control and MD rats, the protein levels were measured by western blotting analysis. 24 hours of MD significantly increased IL-1RI immunostaining in TIF without affecting the total IL-1R protein level in the homogenate of male rats (Figure 15B, control: 100±3.87; MD: 124±6.2; **p<0.01; MD *versus* control). There are no differences in the amount of IL-1RI at post-synaptic sites in control and MD female rats. (control: 100±4.39; 90.54±3.67).

We have previously demonstrated that IL-1RI enriched at the synapse of primary hippocampal neurons, can bind to the GluN2B subunit of the NMDAR. To assess a possible modulation of the interaction between IL-1RI and NMDAR at the synapse by MD, proteins of TIFs fraction from control and MD hippocampi were immunoprecipitated with an antibody against GluN2B and the presence of IL-1RI in the immunocomplex was evaluated by western blotting. As shown in Figure 15C, IL-1RI co-precipitates with GluN2B in all TIFs but maternal deprivation significantly increases the amount of IL-1RI associated to GluN2B in male rats (Figure 15C, control: 100±14; MD: 249±41, **p<0.01; MD *versus* control). The interaction didn't change in female rats after MD (control: 100±19.8; MD: 121±10.37).

These results provide a preliminary indication that early stress life modulates (i) GluN2B subunit phosphorylation, at a site relevant for NMDAR over activation induced by IL-1β, (ii) the trafficking of IL-1RI and (iii) its interaction with GluN2B subunits.

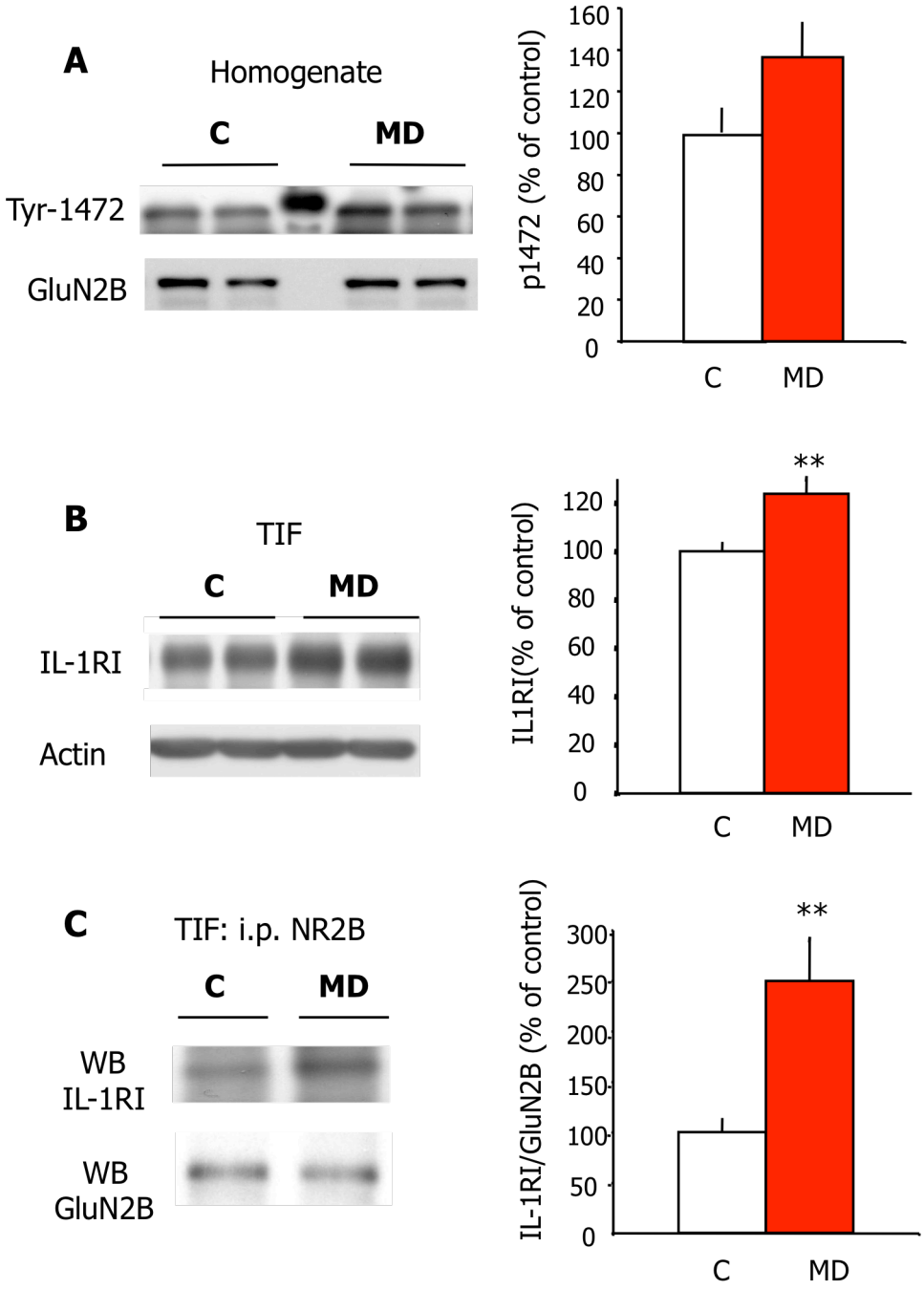


FIGURE 15. Effect of MD on Tyr-1472 phosphorylation of GluN2B, IL-1RI localization and its interaction with GluN2B.

A. Representative western blotting and quantification of GluN2B-Tyr-1472 phosphorylation performed in the hippocampus homogenate of control and MD male rats. Values are mean ± S.E. of 6 independent sample for each experimental group (*p<0.05 MD *versus* control; ANOVA followed by Tukey's test).

B. IL-1RI western blotting analysis of the TIF fraction obtained from control and MD male rats hippocampi. Values are mean ± S.E. of 6 independent sample for each experimental group (**p<0.01 MD *versus* control; ANOVA followed by Tukey's test).

C. TIFs obtained from control and MD male rats hippocampi were immunoprecipitated with anti-GluN2B, and the presence of GluN2B and IL-1RI in the immunocomplex was evaluated by means of western blotting. Values are mean ± S.E. of 6 independent sample for each experimental grou (**p<0.01 MD *versus* control; ANOVA followed by Tukey's te

5. DISCUSSION

The main outcome of this work is the characterization, from a molecular and structural point of view, of the bidirectional interplay between IL-1β/IL-1RI signalling and the NMDA receptor. We demonstrated that, in primary hippocampal neurons endogenous IL-1β released by activated glia potentiates the functions of NMDAR, and increases the synaptic localization of both NMDAR and IL-1RI; NMDA activation increases the amount of IL-1RI into the membrane where it specifically interacts with GluN2B subunits of NMDAR. Thus, the recruitment of this cross talk results in an altered organization of the synapse. This event occurs both *in vitro* and *in vivo*, in a model of maternal deprivation.

We have previously reported the existence of a functional interaction between IL-1β and NMDAR. In primary hippocampal neurons recombinant IL-1β induces the activation of Src family kinases and subsequent the phosphorylation at Tyr-1472 of GluN2B subunit of NMDAR (Viviani et al., 2003). The activation of this pathway potentiates NMDA-induced intracellular Ca^{2+} increase and also exacerbates NMDA-induced neuronal death (Viviani et al., 2003). These results suggest that hippocampal neurons exposed to IL-1β are more susceptible to glutamatergic excitation through the NMDA receptor component. The data also support previous *in vivo* evidences suggesting that this cytokine is a pathological mediator of pathological condition that depend primarily on NMDAR function (Yamasaki et al., 1995; Loddick and Rothwell, 1996, Meyer, 2011; Bilbo et al., 2009). As such, a better understanding of the molecular mechanisms that underlie the action of this cytokine within the CNS might facilitate the development of promising therapeutics in the field of CNS disorders.

We therefore continued our studies to evaluate whether IL-1β/NMDAR cross talk could have also been recruited by an endogenous modulation. To this purpose we used in an *in vitro* model of neurotoxicity characterized by endogenous production of IL-1β and over-activation of glutamatergic system and deregulation of intraneural calcium level,. A sandwich co-culture of primary hippocampal neurons and glia cells were exposed to gp120, an envelope glycoprotein of HIV virus. Gp120 stimulation induces glia activation, release of pro-inflammatory cytokines (among these IL-1β), and alteration of glutamate homeostasis (Kaul et al., 2001); all these events have a relevant impact on functions and survival of neuronal cells. The first set of

experiments shows that IL-1β, released by activated glia after gp120 exposure, increases tyrosine phosphorylation of NMDA receptor GluN2B subunit in rat hippocampal neurons. Previous results obtained in our laboratory have shown that recombinant IL-1β induced Tyr-1472 phosphorylation of the GluN2B subunit of the NMDAR occurs through the activation of Src kinases (Viviani et al., 2003). In accordance, we observed that Ca-pYEEIE, an inhibitor for Src family SH2 domains (Takasu et al., 2002), prevents the effects induced by endogenous IL-1β. In particular, Ca-pYEEIE was loaded only in neurons, prior to the exposure to gp120 in the presence of glia. This experimental approach allows us to act on neurons without interfering with Src family signalling transduction in glia, strengthening our results.

The activation of this pathway leads to a sustained elevation of intracellular calcium $[Ca^{2+}]_i$ in neurons and the stabilization of the NMDAR GluN2B subunit at the synaptic sites. The elevation of $[Ca^{2+}]_i$ in neurons occurs through the GluN2B subunit, in fact ifenprodil, a blocker that selectively binds to this subunit, inhibited this effect.

Both tyr-phosphorylation of NMDA receptor and neuronal $[Ca^{2+}]_i$ increase are prevented by the IL-1 receptor antagonist (IL-1ra) and requires the presence of glia, since the effects are not evident when neurons are exposed to gp120 alone. These results underline the relevance of endogenously produced IL-1β and the recruitment of IL-1RI.

Electrophysiological recordings from neurons show that NMDA currents are governed by a balance between tyrosine phosphorylation and dephosphorylation (Kalia et al., 2004). Whether phosphorylation causes the increase in NMDA receptor gating still remains unclear (Salter and Kalia, 2004). On the other hand, tyrosine phosphorylation of GluN2 subunits might also prevent the removal of signalling molecules from the NMDAR complex by protecting the subunits against degradation from the calcium-activated protease, calpain (Rong et al., 2001). Furthermore, studies on recombinant NMDA receptors indicate that their association with the clathrin-mediated endocytosis machinery, a complex of proteins involved in the removal of receptors from the cell surface, is regulated by Src-mediated tyrosine phosphorylation of NMDAR subunits (Vissel et al., 2001; Roche et al., 2001). The GluN2B Tyr-1472 consensus domain is part of the internalization signal motif, a

binding domain for the adaptor protein complex AP2, which associates with endocytic clathrin-coated vesicles. In addition, Src family tyrosine kinases have been shown to interact with NMDA receptors by binding to the scaffolding protein PSD-95 (Kalia and Salter, 2003). This interaction is strictly correlated to tyrosine phosphorylation of the NMDA receptors subunits (Rong et al., 2001; Collingridge et al., 2003). Finally, recent results show that stabilization of GluN2B-containing receptors at the synapse is dynamically regulated by binding to a PDZ protein such as PSD-95 and internalization through an interaction with AP-2 (Prybylowski, et al., 2005). With this view, GluN2B-PDZ protein interaction may keep Tyr-1472 phosphorylated, and consequently unable to interact with AP-2 (Prybylowski, et al., 2005). The final result of this event is an increased localization of GluN2B within the postsynaptic compartment, probably through prevention of degradation of GluN2B-containing synaptic receptors (Collingridge et al., 2004). Alternatively, tyrosine phosphorylation of GluN2 subunits could be involved in the trafficking of NMDA receptors to the cell surface (Dunah et al., 2004). Our results suggest that all these mechanisms may be recruited by endogenous IL-1β, since IL-1β increases the enrichment of GluN2B subunit in spine.

IL-1β, released by glia, enhances the presence of GluN2B subunit at the post-synaptic membrane but not of the GluN2A, this result indicates that the effect of this cytokine on NMDAR is subunit specific.

The ability of IL-1ra to prevent Tyr-1472 phosphorylation and NMDAR over-activation induced by both recombinant (Viviani et al., 2003) and endogenous IL-1β, as observed in this thesis, suggests the recruitment of IL-1 receptor type I (IL-1RI).

IL-1RI, together with its accessory protein IL-1RAcP and MyD88, is well characterized in peripheral cells of natural immunity (Xiaoxia and Jinzhong, 2005). However, little information is currently available concerning the molecular composition of the members of the IL-1R complex, or their subcellular distribution and functional cross talk with NMDARs in neuronal cells (Viviani et al., 2007; Brikos et al., 2007; Tsakiri et al., 2008). This is a major gap in our knowledge of the pathological mechanisms involving IL-1β/IL-1RI in neurons that may be relevant to therapeutic interventions in the central nervous system (CNS). We studied the molecular composition and the distribution among different subcellular

compartments of the members of IL-1 signalling in the central nervous system. We observed that IL-1RI and its *core* signalling complex are differently localized in hippocampal neurons. The receptor is particularly enriched in the post-synapses sites, MyD88 shows a more spread distribution in different neuronal compartments, even if nicely expressed at synaptic sites, while IL-1RAcP results to be only barely detectable at synapses compared to cell soma and dendrites.

Most important, the distributions pattern of GluN2B subunit and IL-1RI are closely similar, also GluN2B subunit is enriched at the post-synaptic sites.

This observation leads us to hypothesize that IL-1RI could directly interact with the GluN2B subunit of NMDAR. To prove this hypothesis, we performed co-immunoprecipitation experiments between components of the IL-1RI complex and NMDA receptor, as well as AMPA receptor to assess the specificity of interaction. IL-1RI, and its accessory proteins, interacts with GluN2B subunits of NMDAR but not with GluA1 subunits of AMPAR, indicating that there is a direct and specific interaction between the GluN2B subunit of the NMDA receptor and IL-1RI.

It is well known that the localization of synaptic NMDA receptor and its interaction with scaffolding and signalling proteins are not static, but change dynamically in response to different type of stimuli (Collin and Zukin, 2007). Starting from the observation that IL-1RI specifically interact with GluN2B subunit at the synapse and that IL-1β enriches GluN2B at this site, we hypothesize that also IL-1RI could be mobilized between different subcellular compartments. Thus, we focus our attention on the modulation of IL-1RI complex trafficking and its interaction with GluN2B subunit in response to different stimuli. Given the relationship between IL-1RI and NMDAR, we investigated whether IL-1β and NMDA stimulations contribute to the re-distribution of IL-1RI complex members in different neuronal compartments and modulate their interaction.

Both the treatments with IL-1β and NMDA induce an enrichment of IL-1RI at the post-synaptic sites, but none of above considered stimuli, alters the synaptic distribution of IL-1RAcP and MYD88. Although this effect, only NMDA significantly increased the interaction between IL-1RI and GluN2B.

The increase of IL-1RI receptors at the postsynaptic site, driven by IL-1β and NMDA, can be due to new synthesis and delivery of receptors from the endoplasmic

reticulum or to lateral diffusion from adjacent compartments (Perez-Otano and Ehlers, 2004; Newpher and Ehlers, 2008). We used the crosslinker BS^3 allows to discriminate which mechanism is involved. The reduction in intracellular IL-1RI after NMDA exposure, together with its increase in the synaptic fraction, suggests that NMDAR activation favours the membrane insertion of new IL-1RI. Alternatively, the increase in IL-1RI in the synaptic membrane may be attributable to stabilization of the complex with NMDAR (within the core of the PSD), which could prevent lateral movement and/or endocytosis. In either case, a new pool of receptors would be made available. On the contrary, IL-1β possibly enriches IL-1RI at post-synaptic sites, promoting its lateral translocation (i.e. membrane diffusion) from extra-synaptic sites; however, this probably does not occur within the core microdomain of the PSD, as suggested by the unchanged levels of IL-1RI associated with the NMDAR complex.

IL-1β modulates NMDAR functions and receptors trafficking, inducing an enrichment of the post-synaptic membrane with both NMDAR and IL-1RI. NMDA as well contributes to these events, furthermore NMDA enhances the interaction between IL-1RI and GluN2B subunit of NMDAR. This suggests a new molecular mechanism by means of which IL-1β system and NMDA system may contribute to an alteration of the synaptic organization.

The stimulation of NMDA and IL-1 receptors might thus concur to create a post-synaptic microdomain where the interaction between these two receptors are facilitated. The biological consequences of these events could be the loss of spine density and the exacerbation of neuronal death. In fact, endogenous IL-1β released by activated glia, in concomitance with a glutamatergic stimulation, reduces neuronal survival, the effect is prevented by IL-1ra. In particular, in our model of gp120 we observed a very slow progression towards degeneration. This allowed us to monitor its development. We observed that neuronal death was preceded by a reduction of the number of the spine without affecting dendrites morphology. This event seems to be the first signal of neuronal demise, since no signal of concomitant dendritic damage was evident. Again all these events are prevented by IL-1ra suggesting the pivotal role of IL-1β and the activation of IL-1RI. All these results open us the possibility that the alteration of the synaptic setting driven by IL-1β

system and NMDA system could represent the first event to induce reduction of the number of spines or alternatively could also explain the alteration of synaptic activity and plasticity, and neuronal decline. This hypothesis has to be furtherly evaluated in the future.

In the second part of this study we have evaluate whether the crosstalk between IL-1RI/NMDAR could has been recruited also *in vivo.* To this purpose we use an animal model of early life stress. It has been performed a long-time (24 hours) event of maternal deprivation (MD) during at post-natal day 9 (PND9) of rats and then the long-lasting modifications induced by maternal deprivation were evaluated at PND45 considering significant parameters involved in IL-1β/IL-1RI/NMDAR cross talk. In this model the alterations of cognitive and behavioural functions observed in adulthood is due to a stress-induced modification of immune response and alteration of glutamatergic system. This implicates that in this model the relation between IL-β signalling and NMDAR system could be involved in the alteration of behavioural and cognitive functions.

MD at PND9 significantly modulates three key events of IL-1β/IL-1RI/NMDAR connection; MD increases the phosphorylation at Tyr-1472 of the GluN2B, increase the levels of IL-1RI at the synapse and the amount of IL-1RI associated to GluN2B. This modulation still evident at PND45, specifically in the hippocampus, while no variation occurs at the prefrontal cortex. Thus, an early life stress induces a long-lasting modifications in synaptic setting by altering IL-1RI/NMDAR interaction. These final results reveal that the dynamic and functional interaction between IL-1RI and NMDAR is enduringly modulated early in life.

In this model, Viveros et al. (Llorente et al 2009; 2008) evidenced sex-dependent alterations in developing hippocampal neurons and glial cells in MD neonatal rats, with males being more markedly affected. Accordingly, IL-1RI/NMDAR functional and dynamical interactions occur only in males.

The enrichment of IL-1RI at the synapse of hippocampal neurons may contribute to prime the neuronal synapse to the action of IL-1β and could provide a molecular basis on the critical role for the immune system in early life programming of later in life brain functions and behaviour

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