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THE DEVELOPMENT OF GLUTAMATERGIC NEURONS IS SHAPED BY IL-1β

Settore scientifico disciplinare BIO/14

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Abstract

Abstract

The developing brain is exquisitely sensitive to immune system activation that, shaping the organism's response to infections, may impact the development of the nervous system increasing susceptibility to behavioural and neurological diseases later in life.

Experimental studies, in particular, link dysregulated production of pro-inflammatory cytokines (i.e. IL6 and IL-1β) to the onset of neurodevelopmental diseases later in life.

Neonatal immune activation, through cytokines production, can have direct long-term effects on neuronal function by interfering with neurotransmitter function, altering the expression of synaptic proteins, producing differential neuronal activation. We addressed this specific topic in vitro by means of primary hippocampal neurons shortly exposed to a pulse of IL-1β (0,05 ng/ml for 30 min) at different developmental stages, 3,7, and 14 days in vitro (DIV). Maturation of the glutamatergic system and any possible interference by IL-1β has been evaluated in terms of expression of NMDAR subunits (GluN2A and GluN2B) and AMPAR subunits (GluA1 and GluA2), distribution at the post-synaptic site and ability to control intracellular Ca^{2+} homeostasis at full maturation (DIV 21). The exposure of hippocampal neurons to IL-1β at DIV 7 leads to a reduced GluN2A expression at DIV 21 that result in an increased GluN2B/GluN2A ratio, favouring the GluN2B-containing NMDARs. This effect is coupled to a reduced mRNA GluN2A transcription and is prevented blocking the Src family of tyrosine kinases suggesting the involvement of a GluN2B over-activation induced by IL-1β.

These alterations, confirm at the post-synapses, are not evident when neurons are exposed to the cytokine at DIV 3 and 14, suggesting that IL-1β influences neuronal development only in a specific vulnerable period of growth. The effect is specifically operated by IL-1β, since no effects were assessed using another pro-inflammatory cytokine, TNF-α.

The different NMDAR composition and distribution observed after IL-1β treatment at DIV 7 is affected by the control of calcium homeostasis, with a reinforced GluN2B subunit contribution coupled with a weaker GluN2A subunit contribution.

Increased expression of GluN2A over a prevalence of NMDAR sharing a GluN2B subunit is believe to be a crucial factor to prompt synaptic spine maturation from stubby to mushrooms spines. Accordingly to the delayed GluN2B/GluN2A shift, IL-1β treatment at DIV 7 favours an higher amount of stubby and a lower amount of mushroom-shaped spines compared to controls.

Our findings suggest the hypothesis that a transient increase of IL-1β during the early postnatal neuronal development leads to "long-term" functional and structural alteration of the

glutamatergic system development providing a molecular link between neuroinflammation and “long-term” alteration of neuronal activity that could potentially predispose to neurodevelopmental disorders later in life.

Introduction

Developmental origins of Health and Disease (DoHaD)

The development of the central nervous system is a complex and critical process whose alteration may have long-lasting effects on brain structure and function, potentially resulting in neurodevelopmental disorders (Heyer et al., 2017).

Over the past 60 years, it has become clear that the prenatal and early postnatal environment interacts with an individual's genetic makeup to shape myriad aspects of long-term physiology. Endogenous and exogenous signals and experiences during perinatal period, including environmental factors, maternal stress, nutrition, trauma or infection may profoundly modulate or "program" developing neuronal circuits, with the result that adult outcomes are significantly affected (Bilbo and Schwarz, 2009; de Boo and Harding, 2006; Fatemi and Folsom, 2009; Owen et al., 2005).

These findings define the developmental origins of health and disease (DOHaD) paradigm.

An increasing number of studies suggest, in particular, an association between the long-term exposure to low doses of environmental factors and the onset neurodevelopmental diseases later in life, such as Autism spectrum disorder (ASD), Schizophrenia and Attention deficit/Hyperactivity disorder (ADHD) (Heyer et al., 2017).

Although these disorders exhibit an early onset, there may be a specific developmental period that if disrupted increase the risk for developmental diseases. Compelling epidemiological, pharmacological and toxicological evidence shows indeed that there are several vulnerable periods of growth and development during which the CNS is more sensitive and increase the risk for neurodevelopmental diseases later in life (Cameron and Demerath, 2002; Gluckman et al., 2007; Heindell, 2008; Bilbo and Schwarz, 2009; Swanson et al., 2009). These sensitive time-windows may vary depending on the outcome measured, the affected brain regions and the mechanisms of action of the environmental factor (Heyer et al 2017).

Although different factors may contribute to DoHaD common and shared pathways potentially link to neurodevelopmental diseases (NDDs) have been identified such as are oxidative stress, immune system dysregulation, thyroid hormone disruption and altered neurotransmitter system. Among these, immune system dysfunction is particular intriguing for its critical role in brain development, influence on behavioral outcomes such as learning and memory and possible correlation in developmental diseases such as autism and Schizophrenia. Neurotoxicants such as pesticides, various component of air pollution, phthalates and polychlorinates biphenyls are considered so far as potential risk factors for ASD with an impact on immune or inflammatory pathways (Lin et al., 2016; Bilbo et al., 2017).

Perinatal programming by immune activation

A link between perinatal infection and neuropsychiatric disorders have been first proposed in 1981 when Thomas Clousten suggested an infectious origin to what he described as ‘‘adolescent insanity’’. Since then, many researchers have noted the strong relationship between early-life infection and the later-life onset of neurodevelopmental disorders (Fruntes and Limosin, 2008; Cai et al., 2000; Meyer et al., 2006; Pang et al., 2003; Urakubo et al., 2001; Bilbo and Schwarz, 2012).

ADHD, one of the most common childhood neurobehavioral disorders, is characterized by dysfunction of dopamine and noradrenaline systems and, in addition, involves an immune system dysregulation (Heyer et al., 2017).

Schizophrenia results from aberrations during fetal development and a correlation to infections was proved. This pathology is indeed more prevalent in cities than rural areas, where infectious pathogens are less easily transmitted (Brown and Susser, 2002).

Evidences also support a link between clinical immune response dysregulation and neurological disturbances later in life.

Altered cytokine levels have consistently been detected in the blood and brain of ASD patients (Goines and Ashwood, 2013). Accordingly, elevated levels of fetal pro-inflammatory cytokines IL-6, IL-1β and TNFα can produce pathological changes similar to those observed in ASD and schizophrenia (Fatemi and Folsom, 2009; Heyer et al., 2017).

In order to determine the mechanisms underlying such changes a large number of animal models of early-life immune activation have been developed and characterized.

In one study for example has been characterized the impact of neonatal E. coli infection in rats on later-life brain and behavior. E. Coli is known as the primary cause of infection in premature infants in the US and it has been associated with significant delays and alteration in neurodevelopment (Adams-Chapman and Stoll, 2006). Infection of rat pups on postnatal day 4 with E. coli markedly increased circulating cytokines (IL-1β, TNFα, IL-6) in 48 h and has been related to physiological and behavioral changes, including an increased vulnerability to cognitive impairments in the adulthood (Bilbo et al., 2005, 2008, 2010, 2012).

Other examples that highlight the importance of perinatal inflammation in programming brain development and behavior are the studies of the long-term effects of maternal immune activation (MIA). Severe infection during pregnancy can predispose offspring to a variety of psychiatric and neurodevelopmental disorders, including autism spectrum disorders and schizophrenia (Atladottir et al., 2010; Brown, 2012; Spencer et al., 2017). Maternal treatment with polyriboinosinic-polyribocytidylic acid (Poly I:C), a common used viral mimetic, in both

early and late gestation leads to sensory-motor gating deficits and working memory impairments in rats (Meehan et al., 2017; Meyer et al., 2014, 2017). Lasting effects are more evident when MIA occurs in the late gestation, suggesting that the precise timing of MIA can influence the nature or the severity of behavioral abnormalities in the offspring (Meyer et al., 2006; Spencer et al., 2017).

Furthermore, studies in primates, demonstrated that MIA with Poly I:C induces behavioral changes in the offspring, including behaviors relevant to both ASD and SZ (Machado et al., 2015), alters prefrontal cortex dendritic morphology (Weir et al., 2015) and evidence of elevated cytokine levels at one year of age in the offspring, a profile that is also reflected in elevated cytokines at 4 years (Rose et al., 2017).

In particular, cytokines levels correlate to the impaired behavioral outcomes in a dose-response fashion. The most convincing data in support of the involvement of elevated levels of immune mediators like pro-inflammatory cytokines comes from animal models where blunting the production of maternal specific pro-inflammatory cytokines (i.e. IL6; IL-1β) by means of KO mice or neutralizing antibodies prevent later in life behavioural outcome in the offspring (Rose et al., 2017).

Environmental factors targeting the maternal, fetal or neonatal system can therefore induce lasting physiological, behavioral neuroimmune changes in the growing organism through mechanisms known as perinatal programming. Such programming can have long-term negative consequences on adult health and thus shape the organism's susceptibility to infections, neuroimmune and psychiatric diseases (Spencer et al., 2017).

Immune modulation of brain functioning

It is firmly establish that the immune system can modulate brain functioning and behavioural processes through its crucial role in remodelling and sculpting the brain (Yirmiya and Goshen, 2011). In the brain the immune processes are not identical to those occurring in the periphery. The brain has resident immune cells, namely microglia, which produce cytokines and other inflammatory molecules in response to disturbances in homeostasis, in a manner similar to peripheral immune cells. Other central nervous system (CNS) cells, including perivascular macrophages, astrocytes, endothelial cells, oligodendrocytes, and neurons also produce cytokines and chemokines and express their receptors, during normal brain function as well as in response to injury, infection, or illness (Figure 1). In addition to resident immunocompetent cells, there are multiple pathways by which peripherally derived immune factors can affect the brain, and in turn, by which the brain can affect peripheral immune responses. These include the autonomic nervous system (ANS), activation of the ''stress axis'' (the hypothalamic–pituitary–adrenal (HPA) axis), and cytokines, chemokines, leukocytes that travel or signal across the blood brain barrier (BBB).

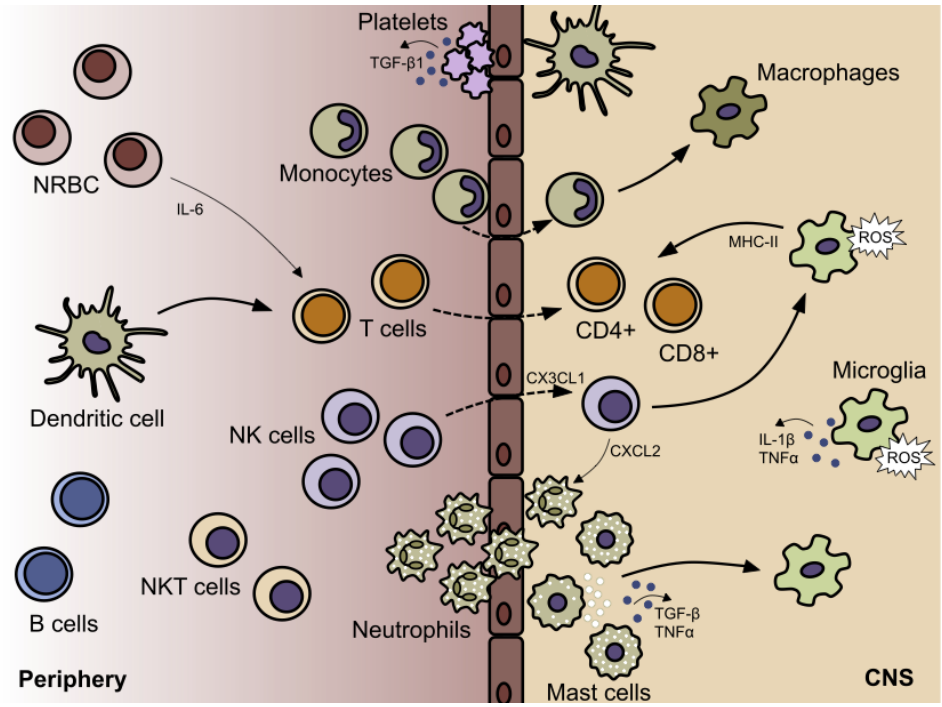


Figure 1. Activation of immune cells into the injured brain. (Lai et al., 2017).

Under normal, quiescent conditions immune system positively regulates neuroplasticity and neurogenesis, promoting learning, memory and hippocampal long-term potentiation (LTP) (Yirmiya and Goshen, 2011).

Instead in condition under which the immune system is strongly activated by infection, injury or by severe stressfull condition, glia and the other brain immune cells change their morphology and functioning and secrete high levels of pro-inflammatory cytokines and prostaglandins. The production of these inflammatory mediators disrupts the delicate balance needed for the neurophysiological actions of immune processes and produces direct detrimental effects on memory, neuronal plasticity and neurogenesis (Yirmiya and Goshen, 2011).

The immune system modulates therefore learning and memory processes through the release of cytokines.

Cytokines have known to be neuromodulators within the brain during infectious and inflammatory processes; however, they are also constitutively expressed in healthy brain tissue and regulate such homeostatic mechanisms and behaviours as sleep, memory, and metabolism (Farrar et al., 1987; Vitkovic et al., 2000; Yirmiya and Goshen, 2011). One observation that demonstrate the profound effects cytokines can have on brain function is in the expression of sickness behaviour (Dantzer et al., 1998; Dantzer and Kelley, 2007). Sick animals exhibit several well-characterized behavioural changes, including reductions in food and water intake, activity, exploration, increased sleep, and reduced social and sexual interactions (Hart, 1988). These sickness behaviours are not mediated by the infectious pathogens themselves, but rather they are a critical component of the immune response orchestrated by the immune system via the release of cytokines (Dantzer et al., 1998; Dantzer and Kelley, 2007). Cytokines induce physiological and behavioural changes via their actions within the brain (Dantzer et al., 1998).

Perinatal immune activation may influence neuronal function and related behavioural outcome through two different pathways:

- Reprogramming the adult immune response by priming microglia and macrophages to sustain an exaggerated response upon subsequent challenges, occurring also later in life (indirect pathway)
- By directly disrupting the development of neuronal pathways relevant for the control of cognition and behaviour (i.e. altered neurotransmitter functions, impaired long-term activation, altered spine structure and morphology) (Bilbo et al., 2012) (Figure 2).

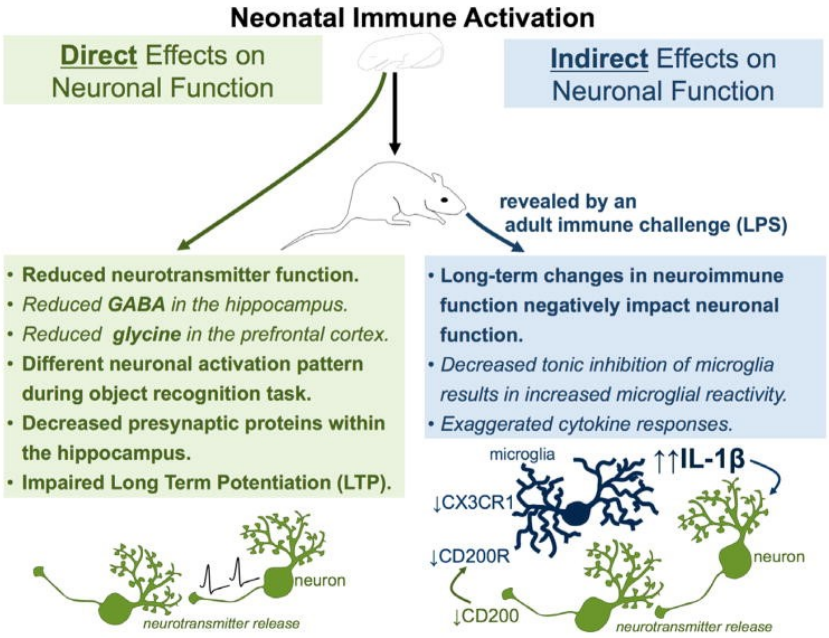


Figure 2. Neonatal immune activation can have direct long-term effects on neuronal function or indirect long-term effects on neuronal function via alterations in neuroimmune function (Bilbo at al., 2012).

Neonatal immune activation directly affects neuronal function by reducing neurotransmitter function (including GABA in the hippocampus and glycine in the prefrontal cortex), decreasing the expression of presynaptic proteins in the hippocampus, inhibiting long-term potentiation, and producing a differential neuronal activation pattern during a learning task such as the novel object recognition task. Neonatal immune activation indirectly alters neuronal function by producing long-term changes in neuroimmune function that in turn negatively affect neuronal function. Decreased tonic inhibition of microglia via altered expression of neuronal inhibitory signals, including fractalkine (via its receptor CX3CR1) and CD200, also results in exaggerated cytokine responses, which impact neuronal function.

The glutamatergic system

The Glutamatergic system is, among the neuronal pathways, the most critical to normal execution of cognitive processes of memory and learning.

Excitatory synapses are integral component of neurons and allow information to travel coordinately through the nervous system to adjust behaviour to environmental stimuli and to control body functions, memories and emotions. Synapses communication is required for proper brain physiology, and slight perturbation of synapse function can lead to brain disorders.

Glutamate-gated ion channels are essential mediators of brain plasticity and are capable to convert specific pattern of neuronal activity into long-term changes in synapses structure and function that are thought to underlie higher cognitive functions. This process, known as synaptic plasticity, refers to the ability of synapses to adapt to different contexts and enabling learning and memory processes.

In particular, for the Glutamatergic system, prototypic forms of synapses plasticity are the Long Term Potentiation (LTP), induced by repeated synaptic activity that promotes the activation of NMDARs, and the Long Term Depression (LTD), induced by low-frequency stimulation that is thought to contribute to refining memory processes in the brain (Lepeta et al., 2017).

In this way glutamatergic synaptic activity controls several processes such as neuronal development, synapses formation, their maturation and elimination.

Glutamate mediate its excitatory effects via several ionotropic and metabotropic receptors subclasses. Metabotropic glutamate receptors (mGluRs) indirectly modulate post-synaptic ion channels, consist of G-protein coupled receptors (mGluR1-8), which are further subdivided according to their activation by either (±)1-amino-cyclopentane-trans-1,3-dicarboxylic acid (trans-ACPD) or L(+)-2-amino-4-phosphonobutyric acid (L-AP4), and cause slower synaptic responses that can either increase or decrease post-synaptic potentials (Miladinovic et al., 2015).

Post-synaptic ionotropic glutamate receptors (iGluRs) are glutamate-gated ion channels and include *N-methyl-D-aspartate* receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4- isoxazole propionic acid receptors (AMPARs), and kainate receptors. Like other ligand-gated channel receptors, iGLuRs are formed from the association of several protein subunits that combine in various ways to produce a large number of receptor isoforms. The subunit composition of these receptors is cell type, brain region specific and developmentally regulated (Traynelis et al 2010, Lepeta et al., 2016). AMPA receptors are critical to fast excitatory neurotransmission, whereas NMDA receptors mediate much of the slow post-synaptic excitatory potentials. The

Kainate receptor, instead, have a less clearly understood role in neuronal signaling that include both pre- and post- synaptic modulation of excitatory neurotransmission (Lepeta et al., 2016).

NMDA Receptors

NMDA receptors are glutamate-gated ion channels that form tetrameric complexes and play a critical role in excitatory neurotransmission in the CNS, important for neuronal development and synaptic plasticity.

Calcium influx, produced by the opening of NMDARs, induces a cascade of events crucial for these processes, resulting in synapse potentiation. This phenomenon occurs by increasing the size of the dendritic spine head and of the underlying PSD that allows more glutamate receptors to localize at the site, providing a stronger response to neurotransmitter release (Paoletti et al., 2013).

They have unique properties that include voltage-dependent block by Mg^{2+}, a high permeability to Ca^{2+} and unusually slow 'activation/deactivation' kinetics. Magnesium block, in particular, is removed by the activation of AMPA ionotropic glutamate receptors.

NMDARs display sensitivity to an array of endogenous ligands and modulators present near the synapse: their activation requires the presence not only of glutamate but also of a co-agonist, glycine or D-serine, whereas physiological levels of protons suppress NMDAR activation. Extracellular Zn^{2+} and polyamines also act on the receptor to modify its behavior. Furthermore, NMDAR subunits interact with various intracellular scaffolding, anchoring and signaling molecules associated with the post-synaptic density (Cull-Candy et al., 2001).

The subunit composition of NMDARs is plastic, resulting in a large number of receptor subtypes. Each receptor subtype displays distinct biophysical, pharmacological and signaling properties that depend on its subunit composition (Paoletti et al., 2013).

To date, seven different subunits have been identified: GluN1 subunit, four distinct GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D), which are encoded by four different genes, and a pair of GluN3 subunits (GluN3A and GluN3B), arising from two genes (Paoletti et al., 2013). NMDAR subunits contain a long extracellular N-terminal domain, three transmembrane segments, a re-entrant pore loop and an intracellular C-terminal domain of variable length. Whereas the N-terminal domain is involved in the subunit assembly and allosteric regulation, the C-terminal domain is involved in receptor trafficking, anchoring and coupling to signaling molecules (Figure 3) (Sanz-Clemente et al., 2013).

The difference in subunits size is mainly due to the length of the intracellular carboxyl-C terminal domain (CTD) that is the most divergent region of the protein when comparing NMDA receptor subunits (Chen et al., 2007).

NMDARs operate as heterotetrameric assemblies that typically consist of two GluN1 subunits complexing two GluN2 subunits or a mixture of GluN2 and GluN3 subunits Paoletti et al., 2013).

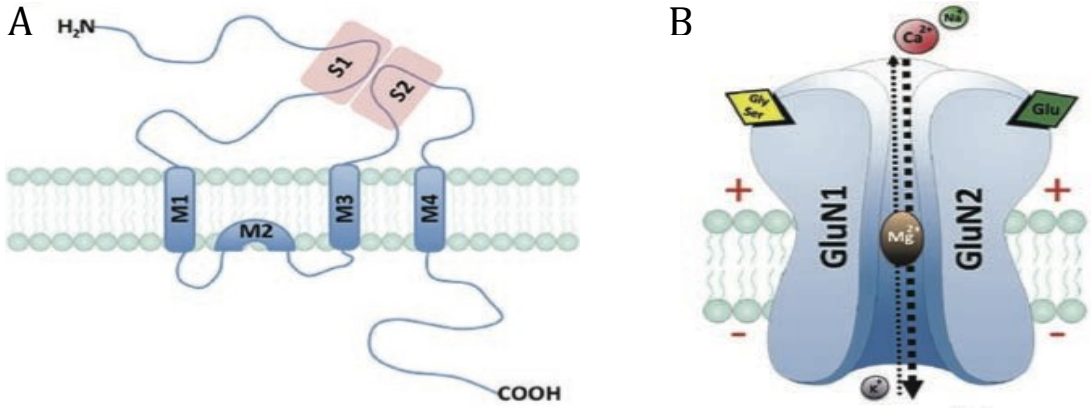


Figure 3. A - Representation of NMDAR subunit composition. B –Structure of NMDA receptor (Sanz-Clemente et al., 2013).

Subunit content determines receptor properties

Each subunit confers to the receptor different biophysical, pharmacological and signaling properties.

The GluN1 subunit is constitutively expressed from embryonic stage to adulthood and exhibits all the classical properties attributed to NMDARs, including glutamate activation, magnesium block, zinc inactivation, glycine activation, interactions with polyamines and pH sensitivity.

The GluN2 subunits instead confer distinct functional properties to the NMDARs by influencing current kinetics and the complement of associated intracellular signaling proteins (Cull-Candy and Leszkiewics, 2004; Gray et al., 2011). In particular, GluN2A and GluN2B subunits have been subject of intense investigation over the past few decades. Both subunits are highly expressed in cortex and hippocampus and play a central role in synaptic function by controlling synaptic plasticity. GluN2A and GluN2B subunits display distinct gating and permeation properties. GluN2A-containing receptors have faster kinetics than GluN2B-containing receptors. Moreover electrophysiological studies showed that GluN1/GluN2A channels have a higher open probability and a faster deactivation time than GluN1/GluN2B ones (Sanz-Clemente et al., 2013, Gray et al., 2011).

GluN2A and GluN2B subunits confer different features to NMDAR mainly due to the C-terminal domain (CTD). This is the most divergent region of the protein when comparing NMDA receptor subunits, consistent with it playing a critical role in the diversity conferred on NMDA receptors by different subunit compositions.

The CTD of NMDAR subunits is a region that is involved in receptor trafficking and couples receptors to signalling cascades and appears to play a critical role in the function of the receptor.

In an elegant study, Martel et al. (2012) demonstrated that the excitotoxicity of NMDARs depends on the identity of the CTD. The author used genetic manipulations to engender chimeric receptors in which GluN2B receptor subunits is C-terminal replaced with the CTD of GluN2A subunit. In this study was demonstrated that excitotoxicity is better promoted by CTD of GluN2B subunit than CTD of GluN2A. The longer tail of GluN2B subunit exhibits stronger coupling to the PSD-95/nNOS pathway, which suppresses pro-survival CREB-mediated gene expression, rendering neurons vulnerable to excitotoxic cell death, through NO production (Martel et al., 2012). NMDAR-dependent activation of CREB-dependent gene expression protects against excitotoxicity (Lee et al., 2005) and can act as a protective response to excitotoxic insults (Mabuchi et al., 2001).

The cytoplasmic C-tail of NMDAR subunits is also a substrate for post-translational modifications that regulate receptor trafficking, localization and signaling. Export from the endoplasmic reticulum and synaptic delivery of NMDARs varies according to GLuN1 C-terminal splicing, a process controlled by neuronal activity. Similarly GluN2A, GluN2B and GluN3A contain distinct motifs that control their intracellular and surface trafficking (Paoletti et al., 2013).

The molecular mechanism underlying the synaptic localization and functional regulation of NMDARs have been subject of extensive studies.

In particular phosphorylation emerges has as a fundamental mechanism that regulates NMDARs trafficking, altering channel properties and receptor localization at the synapses (Lee et al., 2006; Chen et al., 2007).

In CTD of NMDAR subunits have been identified many serine/threonine and tyrosine phosphorylation sites that are phosphorylated by several kinases (Figure 4) (Wang et al., 2014).

Tyrosine phosohorylation is however restricted to GluN2 subunits (Lau and Huganir, 1995). Increased Protein tyrosine kinases (PTKs) activity potentiates NMDA receptor currents and decreased PTKs activity reduces NMDAR transmission (Wang and Salter 1994; Wang 1996; Wang et al., 2013), demonstrating to play a central role in the modulation of NMDAR functionality.

In GluN2A and GluN2B CTD have been identified multiple tyrosine sites phosphorylated by PTKs, especially Src and Fyn (Chen et al., 2007; Wang et al., 2013). In particular, tyrosine phosphorylation of GluN2A was found to potentiate NMDAR currents and de-phosphorylation of Y842 residue may regulate the interaction between NMDAR and AP-2 adaptor, a protein complex that is involved in clathrin-coated endocytic vesicle formation. GluN2A phosphorylation may change the properties and subcellular distribution of GluN2A, leading to an increased number of functional GluN2A-containing NMDARs at the cell surface (Sun et al ., 2017). Whereas the major phosphorylation site of GluN2B CTD is Y1472, localized within a motif that binds directly the AP-2 adaptor (Lavezzari et al., 2003; Chen et al., 2007). Phosphorylation of Y1472 residue of GLuN2B disrupts its binding to AP-2, thereby resulting in inhibition of GluN2B-mediated endocytosis and in an increased NMDAR surface expression (Snyder et al., 2005; Goebel-Goody et al., 2009; Wang et al 2014).

These evidences demonstrate therefore the central role of PTK activity in the modulation of NMDAR activity, specifically regulating surface expression and trafficking of GluN2B- or GluN2A- containing NMDARs.

Another important phosphorylation site on GluN2B CTD is the residue S1480, phosphorylated

by Casein Kinase 2 (CK2) that lead to GluN2B endocytosis and remove the subunit from the synapses.

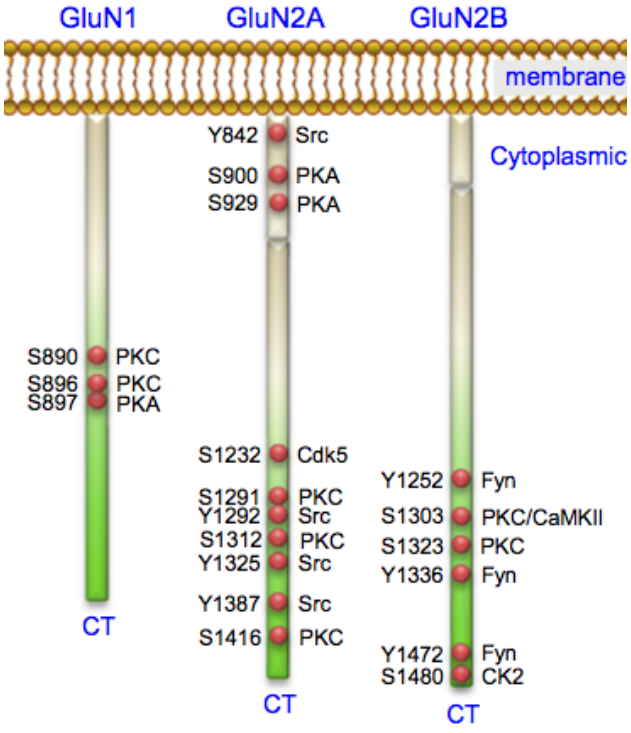


Figure 4. Phosphorilation sites in CT regions of NMDARs (Wang et al., 2014).

Developmental changes in subunit composition

The subunits composition of NMDARs is not static but change during the development in response to neuronal activity or sensory experiences. This plasticity, which was long thought to occur exclusively during development, can also occur at adult synapses. Changes in subunit composition can be rapid and can have profound influences on the functioning of synapses and networks (Paoletti et al., 2013).

Along development, GluN1 subunit is ubiquitously expressed from embryonic stage E14 to adulthood (Watanabe et al., 1992; Monyer et al., 1994), whereas the four GluN2 subunits, which are major determinants of the receptor's functional heterogeneity, show strikingly different spatiotemporal expression profiles. Major changes in the expression patterns of the GluN2 subunits occur during the first 2 postnatal weeks. In the embryonic brain, only GluN2B and GluN2D subunits are expressed. GluN2A expression progressive raises and becomes abundantly only in the adulthood. The GluN2C subunit appears late in development (postnatal day 10), and its expression is mainly confined to the cerebellum and the olfactory bulb (Figure 5) (Paoletti et al., 2013).

GluN3A and GluN3B subunits also display differential ontogenetic profiles. GluN3A expression peaks in early postnatal life and then declines progressively. Conversely, GluN3B amount slowly increases throughout development, and in the adult, it is expressed at high levels in motor neurons and possibly other regions (Paoletti et al., 2013). The specific presence of GluN2B, GluN2D and GluN3A subunits early in development strongly suggests that these subunits are important for synaptogenesis and synaptic maturation (Henson et al., 2010).

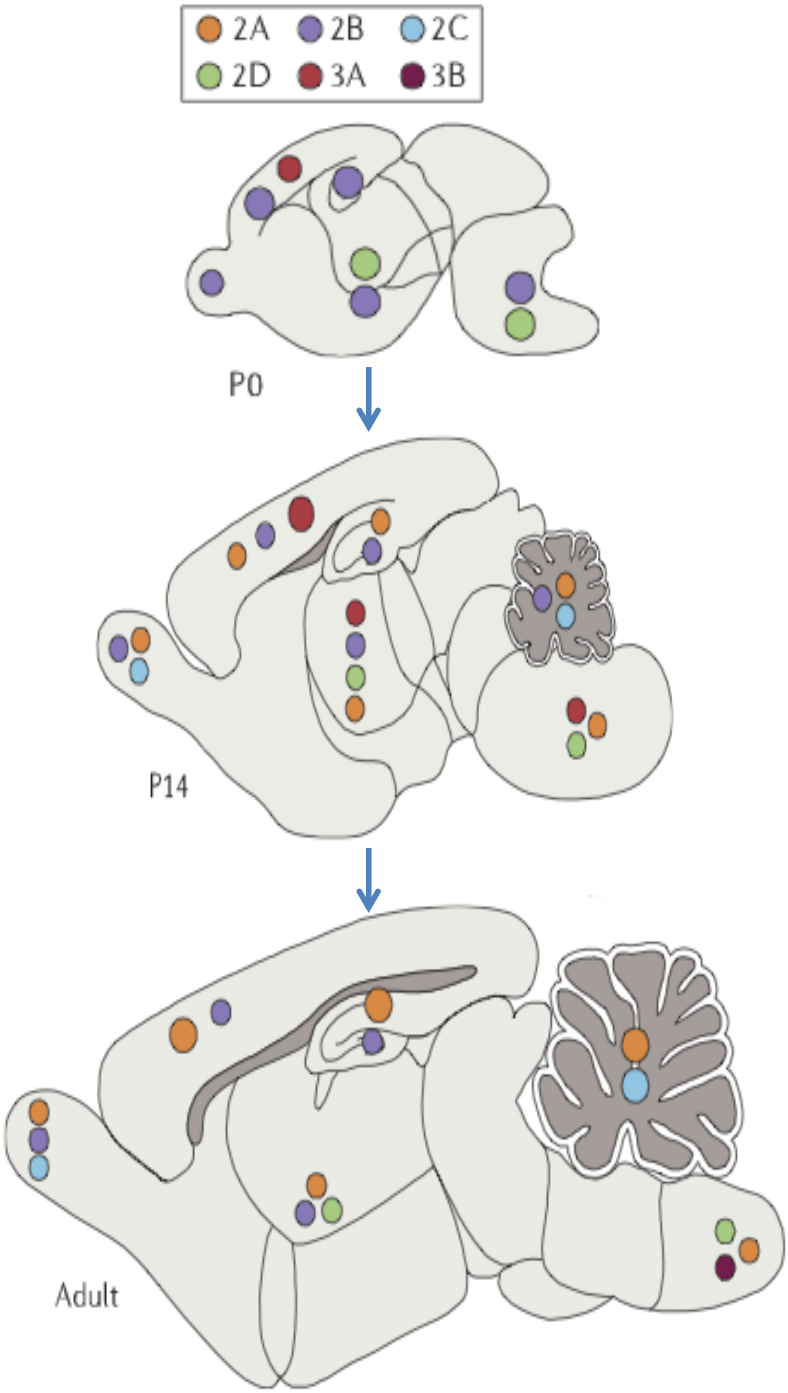


Figure 5. The developmental profile of GluN subunit expression in the mouse brain at day of birth (P0), 2 weeks after birth (P14), and at the adult stages (Paoletti et al., 2013).

During early postnatal development, NMDARs switch their subunit composition from containing primarily GluN2B subunits to a predominance of GluN2A subunits. This subunit exchange is an evolutionarily conserved process observed in frogs, birds, and mammals that occurs in many brain areas, including cortex, hippocampus, amygdala, and cerebellum (Dumas 2005). The timing for the switch varies for each region, but it is coincident with synapse maturation and acquisition of learning abilities, suggesting that this process is important for the refinement and fine-tuning of neuronal circuits (Sanz-Clemente et al., 2012). The increased contribution of GluN2A subunits is obviously accompanied by several distinct changes in NMDAR-mediated synaptic currents, including a marked acceleration in decay time kinetics (Rodenas-Ruano et al., 2012; Paoletti et al., 2013).

GluN2A subunit, in particular has a critical role in LTP and LTD processes. Inhibition of GluN2A subunit results in a block or reduction of LTP in different brain areas. The role in LTD in instead controversial, most studies suggest that the inhibition of GluN2A subunit can prevent LTD, whereas other showed that the inhibition of GluN2A did not affect the appearance of LTD.

In addition several studies show that the GluN2B vs GluN2A developmental switch have a crucial role in regulating regulates AMPAR recruitment to form mature synapse in that GluN2B subunit expression can restrict synaptic incorporation of AMPARs (Hall et al., 2007; Gray et al., 2011).

These findings highlight the importance of maintaining correct GluN2A and GluN2B amount in adults. However, molecular and cellular mechanisms responsible for the long-term developmental switch in NMDAR subunit composition have still to be define (Paoletti et al., 2013).

Experience and environment could influence the developmental swittch in NMDAR subunits affecting synaptogenesis, neural circuitry and higher cognitive functions in which is involved.

In this context emerges the role of epigenetic modification of chromatin as a key regulator of gene expression, a mechanism through which neuronal activity and early experience in life can modify brain development.

Experiences may indeed trigger a chromatine remodeling and transcriptional alteration of genes encoding GluN2A and GluN2B subunits of NMDARs, Grin2b and Grin2a.

In a study showed, for example, a new role for the transcriptional repressor REST in the developmental switch of synaptic NMDARs. REST is activated at a critical window of time and acts via epigenetic remodeling to repress Grin2b expression and alter NMDAR properties at rat hippocampal synapses. Knockdown of REST in vivo prevented the decline in GluN2B and developmental switch in NMDARs (Rodenas-Ruano et al., 2012).

Emerging evidences show that phosphorylation could also have a role in the developmental

switch from GluN2B to GluN2A subunits.

Early in development, the association of GluN2B with MAGUK proteins, such as PSD-95, stabilizes GluN2B at synaptic membranes via phosphorylation of Y1472 by Fyn. Phosphorylation of the Y1472 within the tyrosine-based endocytic motif blocks AP-2 binding. During the critical period for the switch, NMDAR activity induces GluN2B S1480 phosphorylation by CK2, which results in the disruption of NR2B association with MAGUKs. Y1472 residue of GluN2B is now dephosphorylated and AP-2 can bind to the YEKL motif and promote NR2B endocytosis. GluN2A expression increases and GluN2A-containing receptors replace GluN2B-containing NMDARs at synaptic sites (Figure 6) (Sanz-Clemente et al., 2010). The NMDAR subunit switch induced by LTP is blocked in the presence of a CK2 inhibitor (Sanz-Clemente et al., 2010).

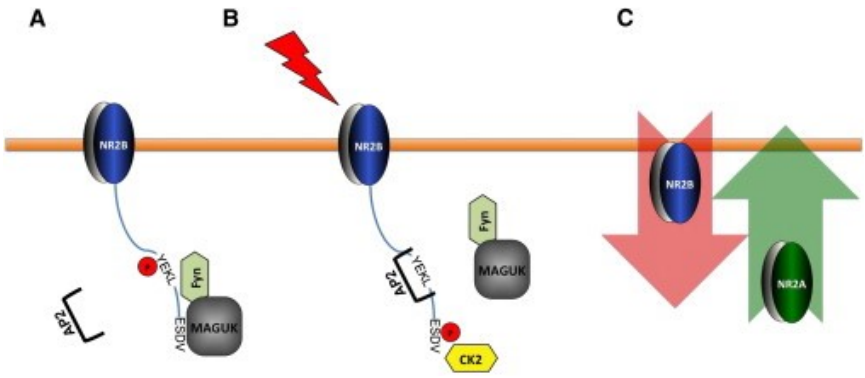


Figure 6. Model of CK2 regulation of synaptic NMDARs (Sanz-Clemente et al., 2010).

(A) Early in development, the association of GluN2B with MAGUKs stabilizes GluN2B at synaptic membranes via phosphorylation of Y1472 by Fyn. Phosphorylation of the Y1472 within the tyrosine-based endocytic motif blocks AP-2 binding. (B) NMDAR activity induces GluN2B S1480 phosphorylation by CK2, which results in the disruption of GluN2B association with MAGUKs. GluN2B Y1472 is dephosphorylated and AP-2 can bind to the YEKL motif and promote GluN2B endocytosis. (C) GluN2A expression increases and GluN2A-containing receptors replace GluN2B-containing NMDARs at synaptic sites.

AMPA receptors

AMPA receptors (AMPARs) are a subtype of the ionotropic glutamate receptors and are essential for excitatory synapse formation, stabilization, synaptic plasticity and neural circuit formation. Physiologically, AMPARs are thought to regulate fast excitation, required to remove magnesium block of nearby NMDARs.

AMPARs are heterotetrameric assemblies of combinations of the subunits GluA1, GluA2, GluA3 and GluA4. Each subunit consists of an extracellular N-terminal domain, three transmembrane domains, one re-entrant loop domain and an intracellular carboxyl-terminal domain.

The subunits composition of AMPARs is crucial for the functional properties of the channel and determines trafficking, conductance and calcium permeability of these receptors. Primarily, these properties depend on the presence or absence of the GluA2 subunit. In the brain the majority of GluA2 mRNA exists in an edited form, resulting in a change of glutamine to arginine at position 607. This alteration of charge, which occurs in the channel pore, blocks the passage of Ca^{2+} ions, prevents the channel from being blocked by intracellular polyamines, reduces the single-channel conductance of the receptor and alters the trafficking properties of GluA2-containing receptors (Henley et al., 2016). Thus, AMPARs either lacking GluA2 or containing unedited GluA2 are calcium permeable, show higher single-channel conductance and are voltage-dependent blocked by intracellular polyamines, but receptors containing edited GluA2 are calcium impermeable and exhibit a lower single-channel conductance (Figure 7).

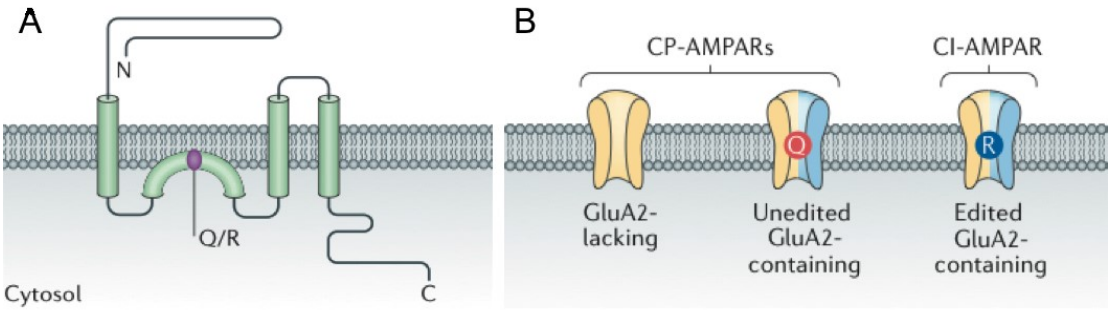


Figure 7. A - Schematic depiction of an AMPAR subunit. B – AMPARs calcium permeable (CP-AMPARs) and impermeable (CI AMPAR) (Henley et al., 2016).

Developmental changes in subunit composition

The profiles of AMPAR subunit expression change markedly during development. Early in development many synapses contain GluA2-lacking AMPARs, which are exchanged for GluA2-containing AMPARs after the second postnatal week (Pellegrini et al., 1992). Soon after birth GluA2 expression is low compared with GluA1 expression, which is consistent with GluA2-lacking AMPARs being important for neonatal synaptic function (Pickard et al., 2000; Henley et al., 2016). GluA1-containing AMPARs are thus calcium permeable, showing higher single-channel conductance. Then the increased expression of GluA2 subunit that occurs during the development reduced calcium influx by forming calcium-impermeable AMPARs where GluA2 forms complexes with GluA1 or GluA3 subunits (Wenthold et al., 1996; Henley et al., 2016). Electron microscopy studies have estimated that GluA3 is present at only 10% of the levels of GluA1 or GluA2 (Sans et al., 2003) and single-cell deletion studies reported that ~80% of synaptic AMPARs in hippocampal neurons comprise GluA1–GluA2 heteromers (Lu et al., 2009). However, other studies of subunit abundance in the rat hippocampus and cortex suggest that AMPARs are mainly heteromers containing GluA1 and GluA2 or GluA2 and GluA3 (Lu et al., 2009; Wenthold et al.,1996; Shi et al., 2001; Henley et al., 2016), with approximately equivalent amounts of each heteromer complex (Kessels et al., 2009; Henley et al., 2016). GluA4, conversely, is tightly developmentally regulated and is diffusely expressed at glutamatergic synapses in principal neurons in the adult brain (Zhu et al., 2000; Heney et al., 2016).

In addition it was demonstrated that the molecular mechanisms regulating synaptic AMPAR content at synapses is strictly related to NMDARs. In mature synapses the incorporation of AMPARs is widely associated with the activation of NMDARs, whereas NMDAR signaling at nascent synapses actually restricts AMPAR currents (Adesnik et al., 2008; Colonnese et al., 2003; Friedman et al., 2000; Tsien et al., 1996; Ultanir et al., 2007; Gray et al., 2011).There is indeed evidence that NMDAR subunits affect AMPARs trafficking in opposite ways, with GluN2A promoting and GluN2B inhibiting surface expression of GluA1 subunits (Martel et al., 2012).

Dendritic spines

Dendritic spines are morphological specializations that protrude from the main shaft of neuronal dendrites and represent the main unitary post-synaptic compartment for excitatory input. The primary function of spines, separated from the dendritic tree only by a neck, is indeed to provide a micro-compartment for segregating post-synaptic chemical responses (Hering and Sheng, 2001).

Since spines are heterogeneous in size and shape and modifiable by activity and experience, they have long been thought to provide a morphological basis for synaptic plasticity.

Based on detailed anatomical studies dendritic spines have been classified by shape as thin, stubby and mushroom-shaped (Chang et al., 1984; Peters et al., 1970; Harris et al., 1992; Hering and Sheng, 2001).

In addition to varying in shape and size, spines also differ in their content of organelles and specific molecules. In general large spines have proportionately larger synapses and contain a greater diversity of organelles. The post-synaptic density (PSD) is an electro-dense thickening of the membrane that is found at synaptic junction, which is usually located at the head of the spine and occupies almost 10% of the surface area of the spine.

The morphology of the spines can influence also kinetics and magnitude of post-synaptic calcium responses. Spines indeed are able to compartmentalize calcium and this function is affected by the morphology of the spines. For example, calcium responses in spines with long necks have a shorter latency and slower decay kinetics than those in short-necked spines (Volfovsky et al., 1999; Korkotian et al., 2000; Majewska et al., 2000a; Majewska et al., 2000b). Moreover spines contain a number of voltage-sensitive calcium channels depending on their size (Sabatini et al., 2001; Sabatini and Svoboda, 2000).

Development of dendritic spines

Spines origin from Filopodia that are widely believed to be the precursors of dendritic spines by drawing the presynaptic contact to the dendrite and leading to the formation of shaft synapses from which mature spines subsequently emerge (Harris et al., 1999).

Filopodia rapidly protrude and retracts from dendritic during early stages of synaptogenesis (Lendvay et al., 2000; Ziv et al., 1996; Fiala et al., 1998). Most abundant in the brain during the first post-natal week in vivo, Filopodia are subsequently replaced by stubby spines. With further development stubby spines decrease in number and synapses on thin and mushroom-shaped spines predominate in the adult rat brain (Figure 8) (Fiala et al., 1998).

Regulated changes in spine morphology and number might reflect mechanisms for converting short-term changes in synaptic activity into lasting alterations in the structure, connectivity and function of synapses. Because spine number and shape probably relate directly to synaptic transmission, there is great interest in the activity-dependent regulation of spine morphology (Yuste et al. 2001). Spine formation and spine density can be affected by activity over both short and long timescales. Changes in spine density have been observed *in vivo,* correlating with environmental factors that affect brain activity (such as visual deprivation, hibernation and the oestrus cycle). In humans, abnormal spine density or shape is associated with many nervous system disorders (for example, mental retardation, Down's syndrome, fragile-x syndrome and epilepsy), indicating at least an indirect link between spine morphogenesis and disease (Swann et al. 2000; Ferrer et al., 1990; Suetsugo et al., 1980; Irwin et al., 2001; Hering and Sheng, 2001). In general, spines seem to be maintained by an 'optimal' level of synaptic activity, with overall spine density increasing when there is insufficient activity, and decreasing when stimulation is excessive.

Activation of NMDARs represents a crucial step for long lasting changes in the strength of excitatory transmission and plays a major role in the rearrangement of synaptic circuits (Adesnik et al., 2008; Vastagh et al., 2012). In hippocampus synaptic NMDARs are involved in the induction of long-term potentiation, which entails a long lasting increase in excitatory post-synaptic transmission and modification of dendritic spine morphology (Lu et al., 2001; Merriam et al., 2011; Vastagh et al., 2012).

GluN2B vs GluN2A subunits balance along development plays also a role in this process (Hamada et al., 2014; Gupta et al., 2015).

Spine morphology is therefore profoundly influenced by the activity of NMDA receptors. NMDA application causes an acute collapse of dendritic spines, and a loss of spine actin in cultured

neurons. It was also demonstrated that the modulation of the levels of GluN2A-containing NMDARs at synaptic site is sufficient to induce a significant alteration of dendritic spine morphology in medium spiny neurons. Moreover treatment with GluN2A antagonist induces morphological modification of dendritic spines (Vastagh et al, 2012).

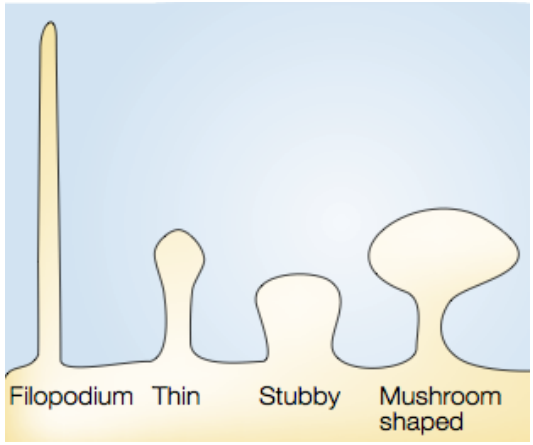


Figure 8. Morphological classification of dendritic spines (Hering and Sheng 2001).

Glutamatergic transmission dysfunction in neurologic disorders

The link between glutamatergic dysfunction and neurodevelopmental diseases is well accepted.

Increasing evidences have suggested a correlation between glutamatergic transmission dysfunctions and the onset of several neurodevelopmental diseases, such as autism spectrum disorders (ASD), epilepsy and schizophrenia (Paoletti et al., 2013, Lepeta et al., 2017; Hardingham et al., 2016; Barker-Haliski et al., 2017).

Clinical and non-clinical studies support the implication of NMDARs in the etiology of ASD. Genetic variants were identified in the genes encoding GluN2A and GluN2B subunits of NMDARs suggesting that differences in subunits composition affect functional properties of NMDARs and the NMDAR-dependent plasticity (Lepeta et al., 2017).

Furthermore human and animal studies demonstrated that NMDAR hypofunction might be a pathogenic trigger for schizophrenia. Treatment of adult rodents with antagonists of NMDAR triggers acute schizophrenia-related behaviours, including deficits in attention and/or vigilance, learning and memory, and sensory gating, broadly mimicking the symptoms of patients with schizophrenia (Hardingham et al., 2016).

In particular, over the last decade an increasing number of reports have demonstrated important roles of GluN2A subunits in pathological processes. Altered expression of GluN2A subunit has been indeed observed in many common human diseases such as cerebral ischemia, seizures disorder, schizophrenia, depression and bipolar disorder (Sun et al., 2017). Mice lacking the GluN2A exhibit several behavioural abnormalities related to schizophrenia and reduced GluN2A expression is correlated with negative symptoms of chronic schizophrenia (Pinacho et al., 2013). The levels of GluN2A subunit are also reduced in different brain regions in prenatally stressed juvenile offspring showing depression like behaviour (Sun et al., 2013).

A decreased expression of GluN2A subunit of NMDA receptors is also observed in prefrontal cortex during schizophrenia (Beneyto et al., 2008) and in perirhinal cortex (Beneyto et al 2007) and hippocampus (McCullumsmith et al, 2007) during bipolar disorders. Other studies link dysregulation of NMDAR trafficking to the behavioural manifestations of schizophrenia and implicate proteins that regulate NMDAR trafficking as potential therapeutic targets for intervention in this mental disorder (Lau and Zukin, 2007).

All these evidences demonstrate the involvement of glutamatergic dysfunction in the progression and maintenance of several central pathologies, including psychiatric and

neurodevelopmental disorders and highlight the importance to understand the glutamatergic synapse and the molecular mechanisms at the basis of its modulation, with the primary aim to improve the knowledge of these pathologies and to define specific NMDA-targeted therapies for neurological disorders.

Pro-inflammatory cytokines and glutamate ionotropic receptors: a step forward understanding in neuro-immune regulation

IL-1β and glutamate ionotropic receptors

Among several cytokines in the CNS, TNF-α and IL-1β are the most studied in the research on the role of the immune system in controlling central nervous system functions and behaviour in health and disease.

Interleukin-1β (IL-1β), a member of the interleukin-1 family, is a potent pro-inflammatory cytokine and plays a crucial role in several inflammatory and autoimmune diseases. IL-1β is produced in the periphery by blood monocytes and tissue macrophages (Dinarello, 1988) during infections, cancers, and trauma (Zhao et al., 2013; Elaraj et al., 2006; Tsai et al., 2017). In addition to peripheral tissue, an immunocytochemical study in rats reported that IL-1β is widely distributed in the brain, particularly in the hippocampus and hypothalamus (Lechan et al., 1990). In the brain, IL-1β is synthesized and released mainly by the microglia and astrocytes (Di Giulian et al., 1986; Davies et al., 1999). In 1998, Schneider et al. first demonstrated that the expression of IL-1β significantly increases during long-term potentiation (LTP) of synaptic transmission, which is a synaptic strengthening process that has been implicated in learning and memory (Schneider et al., 1998). Following animal studies have indicated that physiological levels of IL-1β promote LTP and memory formation (Yirmiya et al., 2002; Tsai et al., 2017). Mice lacking endogenous IL-1β or its receptor show impaired hippocampal-dependent learning and memory, and over-expression of the endogenous IL-1 receptor antagonist impairs LTP and memory in the water maze and fear conditioning paradigms (Goshen et al., 2007; Spulber et al., 2009 a, b; Bilbo et al., 2012). In contrast with this data exaggerated IL-1β within the brain is also strongly associated with memory impairment. The physiological level of IL-1β within the hippocampus is thus tightly regulated during the course of the immune response and is critical important, as too little or too much IL-1β can equally impair learning and memory (Bilbo et al 2012).

Due to the important role in learning processes and cognitive functions, IL-1β has been furthermore implicated in the pathogenesis of various psychiatric disorders.

IL-1β is one of the most studied cytokines in relation to major depressive disorder (MDD) and several evidences support the role of this cytokines in the onset of this pathology. For example, peripheral IL-1β administration produces depressive-like symptoms in rats that can be

attenuated by antidepressant treatment (Connor et al., 1998; Castanon et al., 2001). Moreover increased IL-1β levels in cerebrospinal fluid and serum were found in MDD patients and can be related to the age of MDD onset, the duration of illness and the severity of depression (Levine et al., 1999; Tsai et al., 2017).

IL-1β has been suggested to play a role also in the pathogenesis of schizophrenia and bipolar disorder (BD). Some evidences are changes in peripheral or cerebrospinal fluid IL-1β levels in patients with schizophrenia (Katila et al., 1994; Barak et al., 1995) and higher IL-1β protein and mRNA levels in frontal cortex of patients with BD (Rao et al., 2010).

Several are the data that suggest a link between IL-1β and glutamate toxicity. For example prolonged treatment of human astrocytes with IL-1β has been reported to decrease the expression of the glial glutamate transporter subtype I (GLT-1), leading to a functional decrease in glutamate uptake (Hu et al. 2000). IL-1β treatment of mixed cortical neuron-astrocyte co-cultures results furthermore in increased glutamate export via system xc^- (Fogal et al. 2007). Among these, the ability of cytokines to modulate ionotropic glutamatergic receptors (AMPA and NMDA receptors) is an emergent mechanism that could explain the connection between neuroinflammation and the onset of psychiatric disorders.

Excitatory ionotropic glutamate receptors (AMPA and NMDA receptors) are therefore crucial for the molecular mechanisms disrupted in these disorders, such as cognition and synaptic plasticity (Keifer and Zheng, 2010).

Several in vitro evidences suggest that IL-1β is able to modulate glutamatergic response through the recruitment of glutamatergic ionotropic receptors.

This cytokine exert biological effects primarily through its specific type I IL1 receptor (IL-1RI) that lead to its association with IL-1R accessory protein and the myeloid differentiation primary response protein 88 (MyD88) to form the core of the IL-1/IL-1R signalling complex and activate downstream signalling pathways.

In vitro studies demonstrated that IL-1β in able to modulate ionotropic glutamatergic receptors functionality in primary hippocampal neurons by enhancing NMDA-induced Ca^{2+} increased (Huang et al, 2011; Viviani et al., 2003) and currents (Yang et al., 2005).

The enhancement of NMDA-induced calcium increased is triggered by activation of the src family of kinases (Huang et al., 2011; Viviani et al., 2003) and results in phosphorylation in tyrosine of the GluN2B subunit of NMDAR.

IL-1β interacts specifically with the GluN2B subunit of NMDAR at the post-synaptic site, where IL-1β is enriched together with GluN2B (Gardoni et al., 2011).

The relationship between IL-1RI and GluN2B described in vitro was demonstrated also in a developmental in vivo study, as observed in a model of perinatal stress. A 24h-episode of

maternal separation occurring at postnatal day (PND) 9 redistributes IL-1RI enriching the post-synapses and increases the interaction with the GluN2B subunit of the NMDAR at PND45 (Viviani et al., 2014). This effect specifically occurs in the hippocampus, in male and not female rats, revealing a long-term, sex-dependent modification in IL-1RI receptor organization that might contribute to sensitize hippocampal synapses to the action of IL-1β in the adulthood as a consequence of an early-life stress.

Tumor necrosis factor- α

Tumor necrosis factor-α (TNFα) is a prototypic proinflammatory cytokine produced primarily by monocytes and macrophages in the periphery, and by microglia and neurons in the CNS. It plays a central role in host defence and inflammatory responses, but under certain circumstances it is also involved in cognitive dysfunction, promoting cell death and tissue degeneration (Watters et al., 2011; Mukandala et al., 2016; Probert et al 2015). The role of TNFα in the CNS is still incompletely understood. This cytokine is primarily an innate immune defense molecule, important in the maintenance of homeostasis at the cellular, tissue and organism levels (Vassalli, 1992; probert et al., 2015). Studies in mice showed that in physiologic conditions, TNFα is not necessary for the development and has detrimental effects on memory processes, whereas when homeostasis is alterated it may play a protective role and is required for host defence against pathogens (Probert et al., 2015; Yirmiya et al., 2011). In particular it seems to have a positive effect on the recovery of memory functioning following infections. In a study with mice was demonstrated that after surviving meningitis TNFα-KO mice showed impaired water maze performance compared to surviving wild type controls, suggesting a beneficial role for TNF in memory recovery (Gerber et al., 2004).

Several lines of evidence moreover implicate TNFα in synaptic functioning, in general, and in some forms of synaptic plasticity, in particular. Studies in both hippocampal cultures and slices demonstrated that TNFα selectively secreted by astrocytes enhances synaptic efficacy by increasing surface expression of AMPA receptors. Conversely, blocking TNFα signalling by TNF soluble receptors reduces synaptic strength and decrease AMPA expression (Beattie et al., 2002). However the newly AMPA receptors lack the GLUA2 subunit and they become calcium permeable, contributing to neurotoxicity (Stellwagen et al., 2005).

Aim

The immune system can modulate brain functioning and behavioural processes through its crucial role in remodelling and sculpting the brain. The developing brain, in particular, is exquisitely sensitive to immune system activation that shape the organism's susceptibility to neuroimmune and later in life cognitive and psychiatric diseases.

In this context, the release of cytokines is the link through which the immune system modulates cognitive functions, such as learning and memory processes. IL-1β e TNFα, the most studied cytokines, work therefore as neuromodulator, by controlling neuronal activity both in health and in disease.

The mechanism that explain these effects and their long-term influence on neuronal development is today still unknown; however, several literature data suggest the involvement of the glutamatergic system and its receptors activity.

Previous data from my laboratory prove the ability of the pro-inflammatory cytokine IL-1β to modulate the NMDA receptor response. We thus hypothesized that the ability of IL-1β to specifically interfere with glutamatergic receptors might be an emergent mechanism that could explain the connection between neuroinflammation and the onset of behavioural disorders later in life.

This hypothesis was addressed in vitro in a developing culture of primary hippocampal neurons to evaluate a direct effect of IL-1β avoiding external and internal confounders such as maternal behaviour, hormones and other.

Maturation of glutamatergic neurons is characterized by a shift to NMDAR expressing mainly GluN2B subunits (immature status) to NMDAR sharing mainly GluN2A subunits (full maturity). Our first goal was thus to evaluate the occurrence of this maturational shift in vitro, in order to obtain a valuable model to prove IL-1β effect. For this purpose we used a developmental model of primary hippocampal neurons and we evaluated the expression in both total homogenate and at the post-synapse of the main glutamatergic subunits at different time points.

As a second approach, we exposed primary hippocampal neurons to IL-1β at different stage of maturation: Highly immature (3 days in vitro - DIV), immature (7 DIV) and mature neurons (14 DIV).

IL-1β exposure should mimic a transient inflammatory process during the early development of hippocampal neurons and the aim of this study is to characterize whether this condition can re-program glutamatergic neuronal development.

Hippocampal neurons were exposed to IL-1β 0,05 ng/ml, a concentration demonstrated to interfere with the expression and distribution of NMDAR, for 30 minutes, in order to exposed the neuronal culture to a transient increase of cytokine, as in an inflammatory process.

All the experimental observations, in terms of expression of NMDAR and functional relapse on intracellular calcium modulation, were therefore obtained in mature hippocampal neurons, at DIV 21.

Since the subunits exchange is also important for synaptic AMPARs content regulation, we then observed IL-1β effects on AMPA component of glutamatergic system, through the evaluation of the amount of AMPAR main subunits in homogenate and at the post-synapse at DIV 21.

Our efforts were then directed at a complete understanding of the mechanisms involved. It was evaluated the involvement of SRC-induced tyrosine phosphorylation of the GluN2B subunit, process downstream to IL-1β, and the ability of the cytokine to affect a transcription of specific receptor subunits.

In addition, since biochemical and functional maturation of the glutamatergic spines reflects their morphological features, we have evaluated whether IL-1β intervention could also affect the correct formation of the post-synaptic density and hence the morphology of the dendritic spines.

We have assessed in our model long-term effects of IL-1β on dendritic spine development upon a short exposure to the cytokine analysing spine number and morphology of GFP-transfected neurons by confocal microscopy.

To confirm the specificity of IL-1β effect on NMDARs, hippocampal neurons were finally exposed to another cytokine, TNFα that is known to interfere with AMPARs trafficking.

Methods and Materials

Cell cultures

Primary cultures of hippocampal neurons

Hippocampal neuronal primary cultures were prepared from embryonic day 18–19 (E18-E19). Briefly, brains were removed and freed from meninges, and the hippocampus was isolated (Viviani et al 2003). All procedures were performed in accordance with the current European Law (as indicated in Dlgs N. 26/2014) and are based on a research project approved by the Italian Ministry of Health (n° 475/2015-PR).
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 (Life Technologies, Rockville, MD) supplemented with 1% B-27 (Life Technologies, Rockville, MD) and plated on polyornithine-coated wells.
The cells were seeded at different densities:

- 240000 cells per well in the 6-well plate for western blot analysis and Real-time PCR
- 80000 cells per coverslip in the 24-well plate for [Ca2+]i measurement assay
- 75000 cells per coverslip in the12-well plate for the confocal imaging
- 60000 cells per well in the 24-well plate for MTT test

At DIV4 (day in vitro) 1/3 of the culture medium was changed with fresh medium and Cytosine β-D-arabinofuranoside 1 μM (Sigma-Aldrich) was added in the culture in order to control glial cells proliferation.

Subcellular fractionation

Primary hippocampal neurons were homogenized in a teflon-glass potter in a lysis buffer containing sucrose 0,32M, Hepes 1mM (Sigma Aldrich, St. Louis, MO, USA) $MgCl_2$ 1mM, $NaHCO_3$ 1mM, PMSF 0,1 mM supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Basel, Switzerland) and serin/threonin and tyrosin phosphatase inhibitors (Sigma Aldrich, St. Louis, MO, USA).

Triton-insoluble fractions (TIFs) were isolated from total homogenate as described in Gardoni et al., 1998, 2001.

The total homogenate was centrifuged at 1,000xg for 10 min. The resulting supernatant (S1) was centrifuged at 13,000xg for15 min to obtain a fraction of mitochondria and synaptosomes (P2 fraction). The pellet was resuspended in buffer containing 75 mM KCl and 1% Triton-X 100 and centrifuged at 100,000xg for 1 h at 4°C. The final 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 post-synaptic density (PSD) (Gardoni et al., 1998) due to the limited amount of the starting material.

Western blotting (WB)

Protein concentrations was determined using Bradford method and western blot (WB) samples were prepared in the loading buffer (1M tris-HCl pH 6,5, 6% SDS, 23,8 % Glicerol, Bromophenol Blue and 15% Beta-mercaptoethanol). After denaturation at 95%, 20 ug/sample of total proteins were loaded on 7% SDS-PAGE gels.

Proteins were then blotted onto a 0,45 μM nitrocellulose membrane (Bio-Rad laboratories), blocked in Iblock (Sigma-Aldrich) in TBS-tween 20, and probed with the appropriate primary and HRP-conjugated secondary antibodies. Labelling was visualized by Chemidoc and ImageLab software (Biorad).

WB quantification was performed using ImageLab software.

Measurement of cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$

Primary hippocampal neurons were loaded with 10 μM fura-2 AM (Sigma) for 1 hr at 37°C in their culture medium supplemented with 1% bovine serum albumin, 0.1% pluronic (Sigma). [Ca2+]i measurement was performed in HBSS buffer (NaCl 137 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 4.2 mM, glucosio 5 mM, Hepes 10 mM), pH 7.4, as described in Viviani et al., 2001.

The fura-2AM fluorescence ratio signal in loaded cells was measured in a Perkin- Elmer LS 50 B double-wavelength fluorimeter and calibrated in terms of [Ca2+]i as reported by Grynkievwicz et al. (1985).

Primary hippocampal neurons

For the analysis, primary hippocampal neurons were exposed to NMDA 10 μM and intracellular calcium ([Ca2+]i) was monitored. In order to observe the contribution of each NMDAR subunit on [Ca2+]i enhancement NMDA-induced were then used specific inhibitors of NMDAR subunits. It was measured the amount of [Ca2+]i under the control of NMDAR GluN2A and GluN2B subunits using Ifenprodil 3μM to block NMDARs containing GluN2B subunit, NPV 300 nM to block the NMDARs containing GluN2A subunits and MK 10 μM, an unspecific inhibitor, to block the secondary subunits of NMDAR.

The amount of Ca^{2+} under the control of GluN2B subunit was calculated basing on the arithmetic difference between the [Ca2+]i after NMDA stimuli and the [Ca2+]i obtained after ifenprodil addition. The amount of calcium under the control of GluN2A subunit was the difference between the [Ca2+]i obtained after ifenprodil addition and [Ca2+]i obtained when NVP was added.

RNA isolation and Real-time PCR

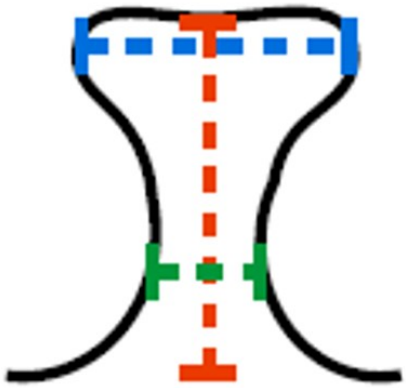
Total RNA was isolated using a commercially available reagent (Tri-reagent, Sigma-Aldrich (S. Louis, MO, USA), following supplier's instructions.

For the synthesis of cDNA, 2.0 μg of total RNA was retrotranscripted using a high-capacity cDNA reverse transcription kit from Applied Biosystems (Foster City, CA, USA), following the supplier's instructions. For real-time PCR analysis, TaqMan-PCR technology was used. For each PCR, 10 ng of total RNA was used. The 18S ribosomal RNA was used as endogenous reference. Quantification of the transcripts was performed by the $2-\Delta\Delta CT$ method (Livak and Schmittgen 2001).

Transfection and Fluorescent immunocytochemistry

Neurons were transfected using calcium-phosphate co-precipitation method with 2–4 µg of plasmid DNA for eGFP, provided by Dr Maria Passafaro (CNR, Milan, Italy).
Cells were then fixed with 4% Paraformaldehyde (PFA)-4% sucrose in PBS solution at 4 °C and washed several times with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature, blocked with 5% BSA in PBS for 30 min at room temperature and then labelled with primary antibody for 1 h at room temperature. Cells were washed with PBS and then incubated with secondary antibody for 1 h at room temperature. Cells were then washed in PBS and mounted on glass slides with Fluoromount mounting medium (Sigma Aldrich).

Confocal imaging and spine morphology



Images were taken using an inverted LSM510 confocal microscope (Zeiss) and the neurons were analysed using ImageJ program. Length of spine, head width and neck width were measured for each spine using straight line function.
Export measurements of spine morphology were finally analysed in a preformed excel file.

Antibodies

The following primary antibodies were used:

- monoclonal antibodies against GluN2B and GluA2 subunits - NeuroMab (Davis, CA, USA);
- polyclonal GluA1 antibody and p-Tyr-1472-GluN2B antibody - Calbiochem (Merck, Darmstadt, Germany);
- monoclonal GluN2A and monoclonal Actin - Sigma-Aldrich (S. Louis, MO, USA)
- polyclonal antibody against IL1RI - Santa-Cruz Biotechnologies (CA, USA)
- Primary antibody for GFP: clone 86/38 (NeuroMab, Davis, CA, USA)

The following secondary antibodies were used:

- Goat peroxidase (HRP)-conjugated anti-mouse antibody - Sigma-Aldrich (S. Louis, MO, USA)
- Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody - BIO-RAD (Hercules, CA, USA);
- Goat AlexaFluor 4988-conjugated anti-mouse antibody - Life Technologies (Monza, Italy)

Statistics

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) or Student's T-test. A significance level of 95% (*$p<0.05$; **$p<0.01$) was accepted.

Treatments

IL-1β treatment

Primary hippocampal neurons were exposed to IL-1β at different time of development: DIV 3, a condition of early development, DIV 7, a condition of intermediate development and DIV 14, a condition of maturity of the glutamatergic system.

For the treatment the neurons were incubated with IL-1β 0,05 ng/l in ACSF (NaCl 125 mM, KCl 2,5 mM, MgCl2 1 mM, CaCl2 2 mM, glucose 33 mM, Hepes 25 mM) for 30 minutes at 37 °C. After the treatment the neurons were washed with ACSF, re-incubated in the original medium and cultured-up to DIV 14 (for mRNA expression analysis), to DIV 18 (for the morphological analysis of dendritic spines) or to DIV 21 (to be evaluated for the expression of NMDAR and AMPAR subunits with WB analysis and for intracellular calcium measurement) (Figure 1).

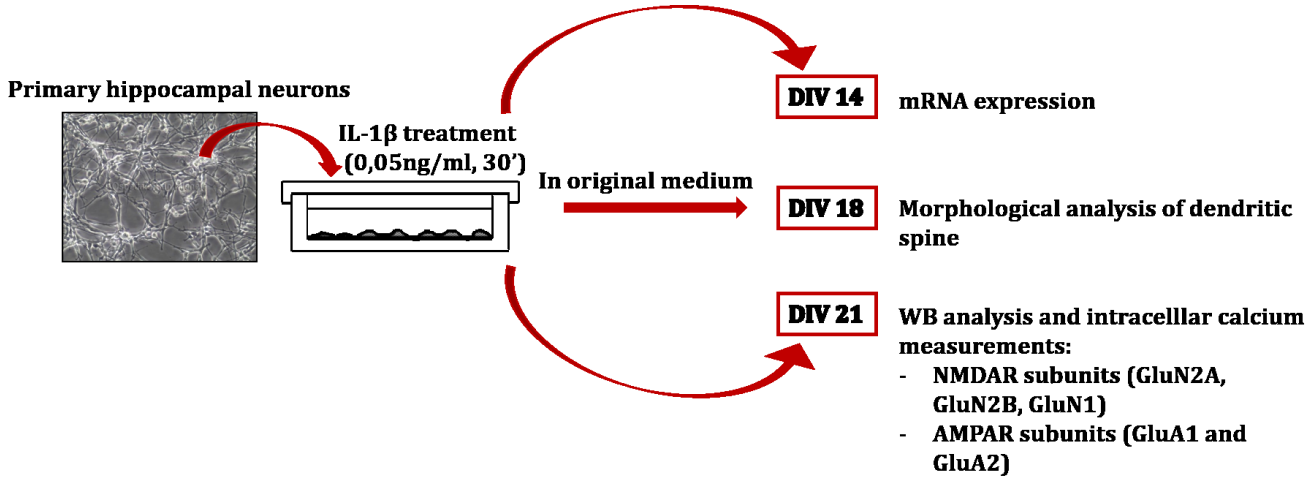


Figure1. Schematic representation of IL-1β treatment in hippocampal neurons and subsequent analysis.

TNFα treatment

Primary hippocampal neurons were exposed at DIV 7 to TNFα 1 ng/l in ACSF (NaCl 125 mM, KCl 2,5 mM, MgCl2 1 mM, CaCl2 2 mM, glucose 33 mM, Hepes 25 mM) for 30 minutes at 37°C. After the treatment the neurons were washed with ACSF, re-incubated in the original medium and cultured-up to DIV 21 (to be evaluated for the expression of NMDAR and AMPAR subunits with WB analysis) (Figure 2).

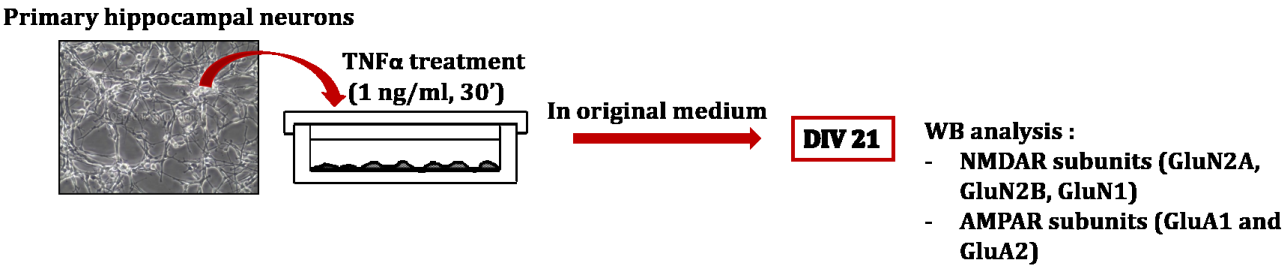


Figure 2. Schematic representation of TNFα treatment in hippocampal neurons and subsequent WB analysis.

IL-1β treatment with PP2 inhibitor

Primary hippocampal neurons were incubated with the Src family tyrosine kinase inhibitor PP2 30 minutes before IL-1β treatment.

At DIV 7 neurons were incubated for 30 minutes with PP2 inhibitor 1μM in ACSF (NaCl 125 mM, KCl 2,5 mM, MgCl2 1 mM, CaCl2 2 mM, glucose 33 mM, Hepes 25 mM) at 37°C. The neurons were then exposed to IL-1β 0,05 ng/l for 30 minutes at 37°C. After the treatment the neurons were washed with ACSF, re-incubated in the original medium and cultured-up to DIV 21 (to be evaluated for the expression of NMDAR and AMPAR subunits with WB analysis and for intracellular calcium measurement) (Figure 3).

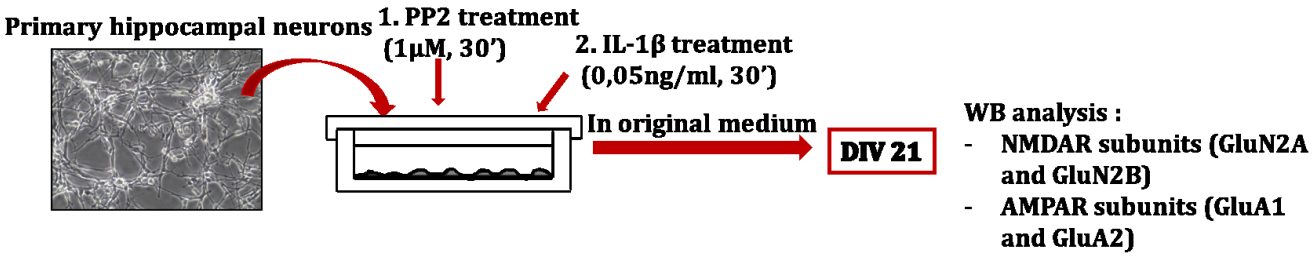


Figure 3. Schematic representation of IL-1β treatment after PP2 inhibitor exposure in hippocampal neurons and subsequent analysis.

Results

Characterization of glutamatergic maturational program in primary hippocampal neurons

Total protein expression and post-synaptic amount of NMDAR and AMPAR subunits in primary hippocampal neurons at different days in vitro (DIV)

The expression of NMDARs subunits (GLUN2A and GluN2B) and AMPAR subunits (GluA1 and GluA2) in primary hippocampal neurons during neuronal maturation was evaluated by WB analysis. Cultivated neurons were homogenized at DIV 7, 14 and 21 and WB analysis performed as described in materials and methods (page 45).

The data show a progressive and significant increase of GluN2A subunit amount during neuronal development (from DIV 7 to DIV 21) coupled to a GluN2B subunit increase during maturation, that reach however a plateau at DIV 14 (Figure 1, A and B). The data were considered also as GluN2B/ GluN2A ratio demonstrating that this tendency lead to a progressive decrease of GluN2B/ GluN2A ratio during the development (Figure 1, C). This differential expression of GluN2A and GluN2B subunits is evident also at the post-synaptic site (TIF) (figure 2, A and B), resulting in a progressive decrease of GluN2B/ GluN2A ratio as well (Figure 2, C).

The results obtained show that the in vivo maturational shift towards a predominance of NMDAR sharing the GluN2A subunit is reproduced in vitro.

GluA1 and GluA2 expression was also monitored.

WB analysis of GluA1 and GluA2 subunits of AMPA receptor, in accordance with in vivo study show a progressive increase expression during the maturation of primary hippocampal neurons (from DIV 7 to DIV 21) both in total homogenate and at the post-synapse (Figure 3, A and B and 4, A and B).

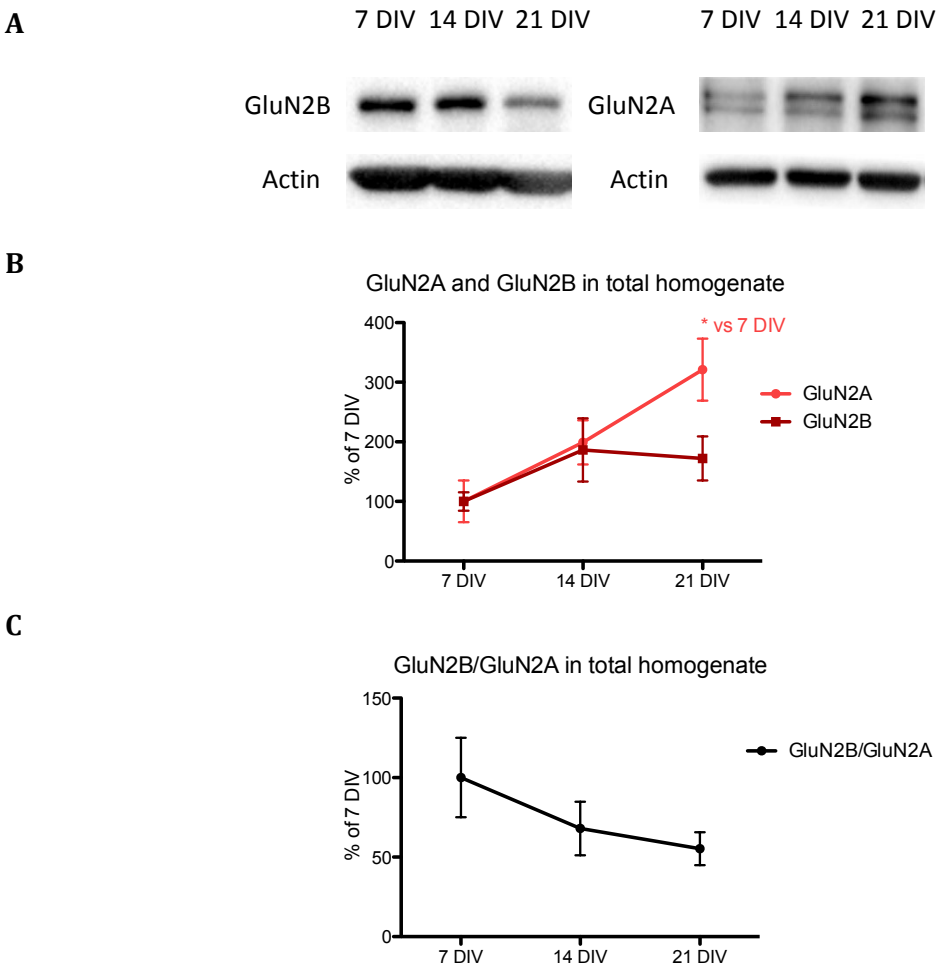


Figure 1. NMDAR subunits expression (GluN2A and GluN2B) and GluN2B/GluN2A ratio in total homogenate of primary hippocampal neurons at DIV 7, 14 and 21

A) Representative western blot analysis of GluN2A and GluN2B subunits in total homogenate obtained from primary hippocampal neurons at DIV 7, 14 and 21. The same amount of proteins was loaded in each lane.

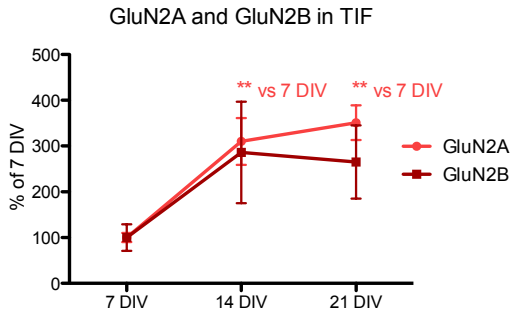
B) GluN2A and GluN2B expression at 7, 14 and 21 DIV expressed as percentage of the Control (7 DIV). GluN2A and GluN2B expression was normalized for the respective actin immunoreactivity. Mean values ± SE (n= 5 of four independent experiments) (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ANOVA followed by Tukey's test).

C) GluN2A/GluN2B ratio at 7, 14, 21 DIV expressed as percentage of the Control (7 DIV). GluN2A and GluN2B expression was normalized for the respective actin immunoreactivity.

Mean values ± SE (n= 5 of four independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

A

B



C

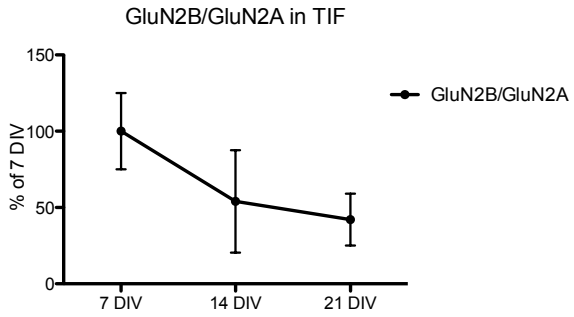


Figure 2. NMDAR subunits expression (GluN2A and GluN2B) and GluN2B/GluN2A ratio in TIF of primary hippocampal neurons at DIV 7, 14 and 21 in TIF

A) Representative western blot analysis of GluN2A and GluN2B subunits in TIF obtained from primary hippocampal neurons at DIV 7, 14 and 21. The same amount of proteins was loaded in each lane.

B) GluN2A and GluN2B expression at 7, 14 and 21 DIV expressed as percentage of the Control (7 DIV). GluN2A and GluN2B expression was normalized for the respective actin immunoreactivity. Mean values ± SE (n= 5 of four independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

C) GluN2A/GluN2B ratio at 7, 14 and 21 DIV expressed as percentage of the Control (7 DIV). GluN2A and GluN2B expression was normalized for the respective actin immunoreactivity.

Mean values ± SE (n= 5 of four independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

A

B

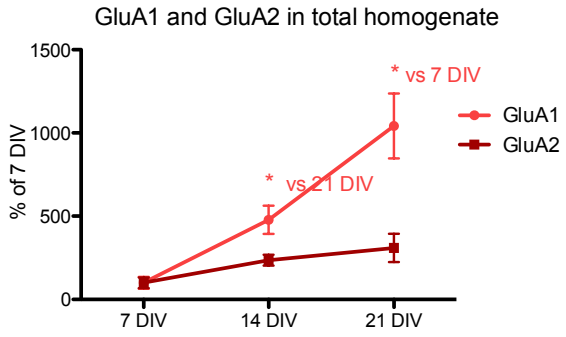
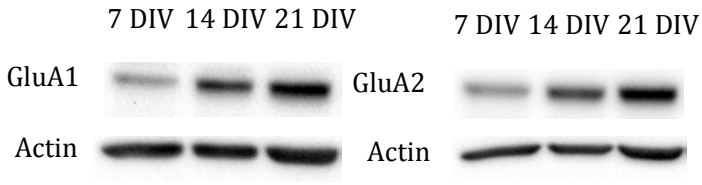


Figure 3. AMPAR subunits expression (GluA1 and GluA2) in total homogenate of primary hippocampal neurons at DIV 7, 14 and 21

A) Representative western blot analysis of GluA1 and GluA2 subunits in total homogenate obtained from primary hippocampal neurons at DIV 7, 14 and 21. The same amount of proteins was loaded in each lane.

B) GluA1 and GluA2 expression at 7, 14 and 21 DIV expressed as percentage of the Control (7 DIV). GluA1 and GluA2 expression was normalized for the respective actin immunoreactivity. Mean values ± SE (n= 5 of four independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

A



B

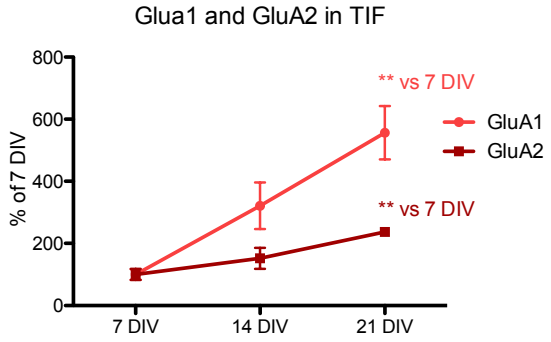


Figure 4. AMPAR subunits expression (GluA1 and GluA2) in TIF of primary hippocampal neurons at DIV 7, 14 and 21

A) Representative western blot analysis of GluA1 and GluA2 subunits in TIF obtained from primary hippocampal neurons at DIV 7, 14 and 21. The same amount of proteins was loaded in each lane.

B) GluA1 and GluA2 expression at 7, 14 and 21 DIV expressed as percentage of the Control (7 DIV). GluA1 and GluA2 expression was normalized for actin immunoreactivity. Mean values ± SE (n= 5 of four independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

Contribution of GluN2A and GluN2B subunits on NMDA-induced Ca^{2+} increase in hippocampal neurons along development

NMDARs are calcium permeable glutamate-gated ion channels whose functionality depends on their subunits composition.

The calcium homeostasis profile during the maturation of the glutamatergic system was therefore observed in primary hippocampal neurons, using a spectrofluorometric technique. Neurons were loaded with a specific dye, Fura-2AM, and at different time of development (DIV 3, 7 14 and 21) intracellular calcium was monitored. Loaded neurons were then challenged with NMDA 10 uM, an agonist of NMDAR. NMDA induces a fast $[Ca^{2+}]_i$ rise that reaches a plateau. The contribution of GluN2B and GluN2A subunits to NMA-induced $[Ca^{2+}]_i$ rise was assessed by sequentially adding (i) Ifenprodil 3 μM (selective inhibitor of NMDAR sharing GluN2B subunit, (ii) NVP 300 nM (selective inhibitor of NMDAR sharing GluN2A subunit) and (iii) MK 10 μM (selective and non-competitive inhibitor of NMDAR). Exposure of loaded neurons to Ifenprodil at the plateau of NMDA response, progressively reduced $[Ca^{2+}]_I$ due to GluN2B blockage (figure 5, A). Further reduction of the response is evident upon NVP addition, due to GluN2A blockage (figure 5, A).

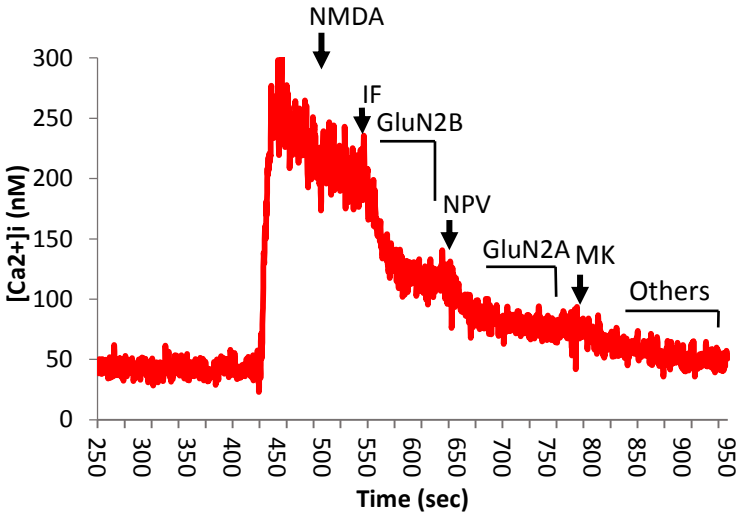
The percentages of GluN2A and GluN2B subunits were calculated as described in Methods and Materials.

Data obtained from this experiment reveal that the level of calcium obtained after NMDA stimulus is higher in neurons at DIV 7 and 14 compared to highly immature neurons (3DIV) (Figure 5, B).

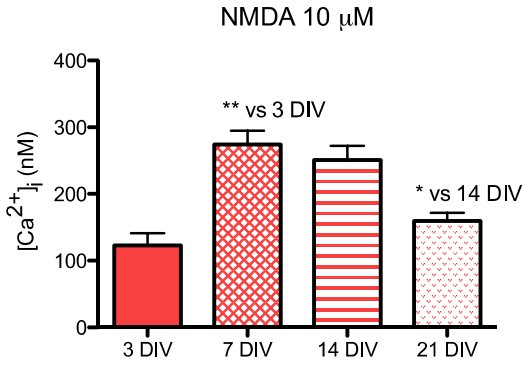
In addition, the graphs show that the contribution of GluN2B subunit on NMDA-induce $[Ca^{2+}]_i$ progressively decreases during the development to reach a significantly lower contribution at 21 DIV compared to 3,7and 14 DIV (Figure 5, C).

On the other hand, the contribution due to NMDAR sharing the GluN2A subunit progressively raises to be significantly higher at 21 DIV compared to 3 and 7 DIV (Figure 5, D).

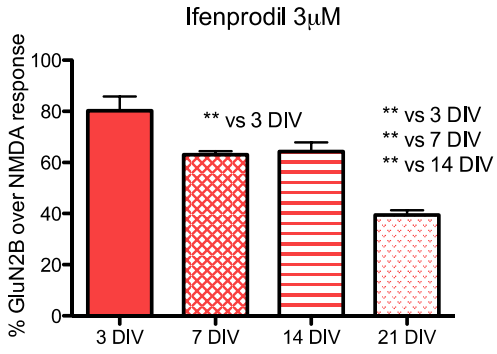
A


NMDA
IF
GluN2B
NPV
GluN2A
MK
Others
[Ca2+]i (nM)
300
250
200
150
100
50
0
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
Time (sec)

B


NMDA 10 μM
[Ca2+]i (nM)
400
300
200
100
0
** vs 3 DIV
* vs 14 DIV
3 DIV
7 DIV
14 DIV
21 DIV

C


Ifenprodil 3μM
% GluN2B over NMDA response
100
80
60
40
20
0
** vs 3 DIV
** vs 3 DIV
** vs 7 DIV
** vs 14 DIV
3 DIV
7 DIV
14 DIV
21 DIV

D

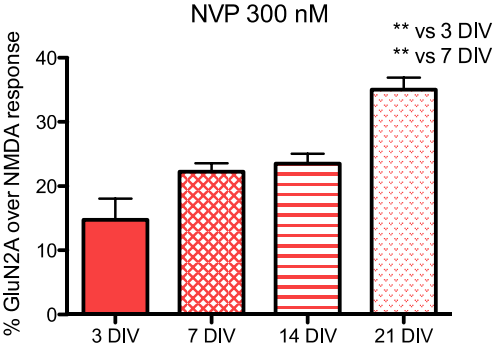

NVP 300 nM
% GluN2A over NMDA response
40
30
20
10
0
** vs 3 DIV
** vs 7 DIV
3 DIV
7 DIV
14 DIV
21 DIV

Figure 5. Intracellular calcium in primary hippocampal neurons exposed to NMDA at DIV 3, 7, 14 and 21 and contribution of GluN2B and GluN2A subunits

A) Representative trace showing the response of primary hippocampal neurons exposed to NMDA 10 μM and the rapid decay in the response of hippocampal neurons when 3 μM Ifenprodil, 300 nM NPV and 10 μM MK-801 were applied after the stabilization of NMDA response, ifenprodil decay and NVP decay respectively. The rate of each decay has been used to calculate the percentage of Ca^{2+} increase dependent on different subunits (see Methods and Materials).

B) Bar graph represents the average peak Ca^{2+} response in hippocampal neurons after stimulation with NMDA 10 μM at DIV 3, 7, 14 and 21. Data are mean values ± SE (n= 5 of two independent experiments for DIV 3 and 14, n=16 of four independent experiments for DIV 7 and n=26 of eleven independent experiments for DIV 21) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

C) Bar graph represents the average Ca^{2+} decay to the application of Ifenprodil expressed as percentage of NMDA-induced Ca^{2+} peak in primary hippocampal neurons at DIV 3, 7, 14 and 21. Data are expressed as percentage of NMDA response. Mean values ± SE (n= 5 of two independent experiments for DIV 3 and 14, n=16 of four independent experiments for DIV 7 and n=26 of eleven independent experiments for DIV 21) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

D) Bar graph represents the average Ca^{2+} decay to the application of NVP expressed as percentage of NMDA-induced Ca^{2+} peak in primary hippocampal neurons at DIV 3,7,14 and 21. Data are expressed as percentage of NMDA response. Mean values ± SE (n= 5 of two independent experiments for DIV 3 and 14, n=16 of four independent experiments for DIV 7 and n=26 of eleven independent experiments for DIV 21) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

Does IL-1β affects GluN2B vs GluN2A shift and thus maturation of glutamatergic neurons?

The in vivo and in vitro observation that IL-1β modulates glutamatergic response through the specific recruitment GluN2B subunit (Viviani et al., 2003), and its role in inducing long-term effects on higher cognitive function regulation when over produced during development, prompted us to evaluate the effect of this cytokine on the expression of GluN2A and GluN2B subunits during the development of primary hippocampal neurons.

In addition, due to the role of NMARs subunits drive AMPARs insertion at the post-synaptic site (Gray et al., 2011), we also evaluated of IL-1β effects treatment on NMDAR and AMPAR subunits expression, with a particular attention at the post-synaptic level.

Primary hippocampal were then exposed as described in methods (page 50), homogenized and processed to obtain the triton insoluble fraction representative of the post-synapse.

Expression of GluN2A, GluN2B and GluN1 subunits of the NMDAR and GluA1 and GluA2 subunits of AMPARs was evaluated both in the total homogenate and in TIF at different stages of maturation.

Subunit composition of NMDA and AMPARs in hippocampal neurons exposed to IL-1β at DIV 3

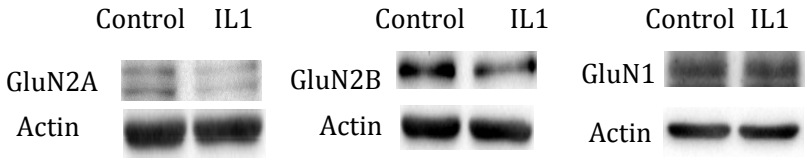
IL-1β 0,05 ng/ml treatment of hippocampal neurons at DIV 3 for 30 minutes does not significantly affect NMDAR subunits expression compared to Controls, at DIV 21.

In the total homogenate the amount of GluN2A, GluN2B and GluN1 subunits do not change upon treatment with IL-1β (Figure 6, A and B).

The WB analysis of AMPAR subunits reveals that the amount of GluA1 subunit of AMPAR is slightly but not significantly lower in IL-1β treated neurons compared to controls and no changes are evident for GluA2 subunit of AMPAR (Figure 7, A and B).

This data are confirm in the TIF fraction for all the analysed subunits (Figure 8, A and B and 9, A and B).

A



B

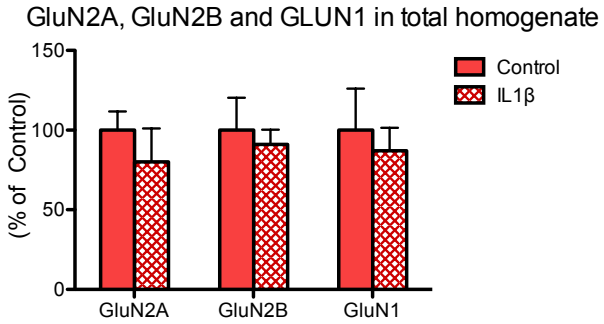
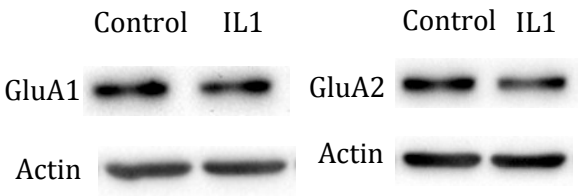


Figure 6. NMDAR subunits expression (GluN2A, GluN2B and GluN1) in total homogenate of primary hippocampal neurons exposed to IL-1β at DIV 3

A) Representative western blot analysis of GluN2A, GluN2B and GluN1 subunits at 21 DIV obtained from total homogenate of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 3. The same amount of proteins was loaded in each lane.

B) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 4 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A



B

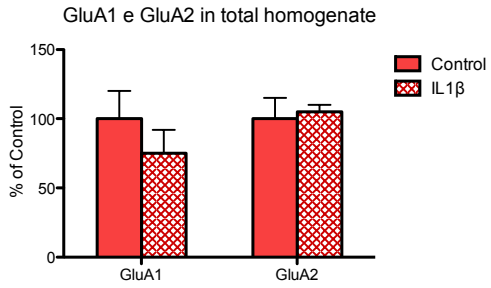


Figure 7. AMPAR subunits expression (GluA1 and GluA2) in total homogenate of primary hippocampal neurons exposed to IL-1β at DIV 3

A) Representative western blot analysis of GluA1 and GluA2 subunits obtained from total homogenate of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 3. The same amount of proteins was loaded in each lane.

B) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 4 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A

B

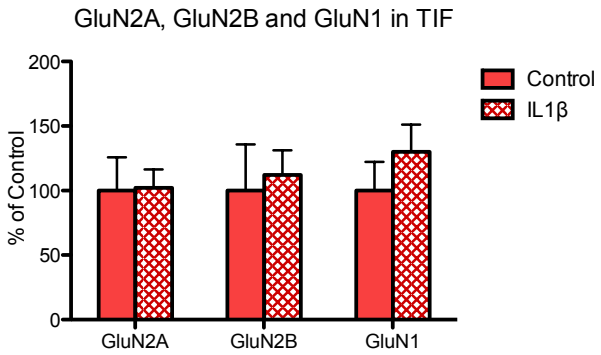
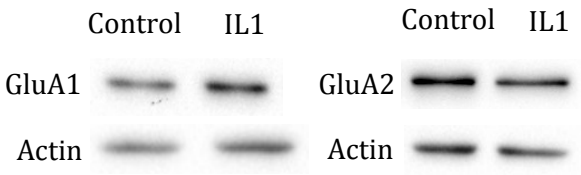


Figure 8. NMDAR subunits expression (GluN2A, GluN2B and GluN1) in TIF of primary hippocampal neurons exposed to IL-1β at DIV 3

A) Representative western blot analysis of GluN2A, GluN2B and GluN1 subunits obtained from TIF of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 3. The same amount of proteins was loaded in each lane.

B) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 4 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A



B

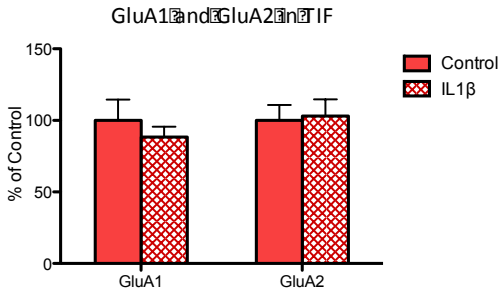


Figure 9. AMPAR subunits expression (GluA1 and GluA2) in TIF of primary hippocampal neurons exposed to IL-1β at DIV 3

A) Representative western blot analysis of GluA1 and GluA2 subunits obtained from TIF of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 3. The same amount of proteins was loaded in each lane.

B) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 4 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

Subunit composition of NMDA and AMPARs in hippocampal neurons exposed to IL-1β at DIV 7

Differently from what observed at DIV 3, IL-1β 0,05 ng/ml treatment of primary hippocampal neurons at DIV 7 alters the composition and the subunits distribution at the post-synapse of NMDAR and AMPAR subunits at DIV 21.

In the total homogenate GluN2A subunit level at DIV 21 is significantly lower in IL-1β treated neurons at 7 DIV compared to Control neurons, while GluN1 and GluN2B subunits expression at 21 DIV do not change (Figure 10, A and B).

We also quantified the levels of GluN2B to GluN2A subunits in homogenate obtained from Control and IL-1β treated neurons as GluN2B/GluN2A ratio. This allowed to evaluate whether IL-1β interferes with the expected shift. Upon IL-1β treatment at DIV 7, the GluN2B/GluN2A ratio at 21 DIV was significantly increased compared to Controls (Figure 10, C).

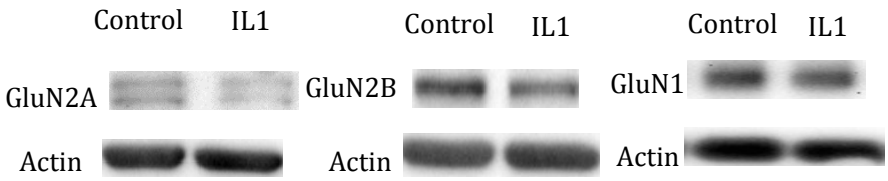
Regarding AMPAR subunits, the amount of GluA1 subunit of AMPAR significantly decreased in IL-1β treated neurons homogenate compared to Controls, whereas no effect was observed for GluA2 subunit (Figure 11, A and B).

Similar to what observed in the homogenate IL-1β at DIV 7 induces a significant decrease of GluN2A subunit expression without changing in the expression of GluN2B and GluN1 subunits of NMDAR (Figure 12, A and B)

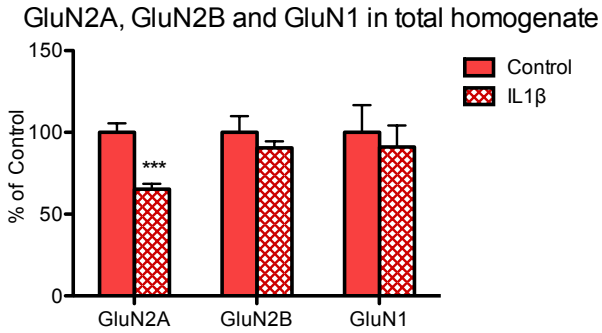
GluN2B and GluN2A subunits in TIF obtained from Controls and IL-1β treated neurons were quantified as previously observed in total homogenate as GluN2B/GluN2A ratio. In accordance with protein expression assessed in total homogenate, also in TIF, the lower GluN2A expression in neurons treated with the cytokine leads to a higher GluN2B/GluN2A subunits ratio. (Figure 12, C).

No changes in both GluA1 and GluA2 expression at DIV 21 were evident at the post-synaptic fraction upon IL-1β treatment (Figure 13, A and B).

A



B



C

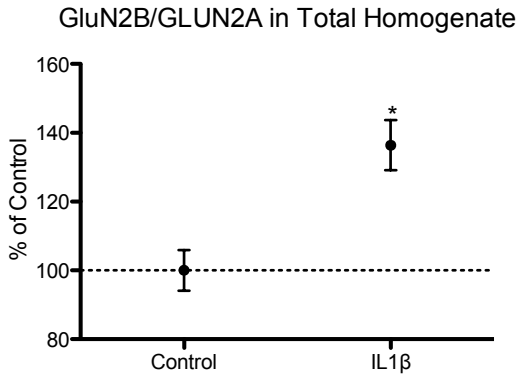
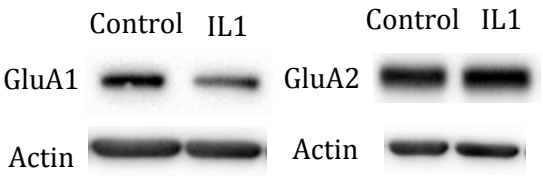


Figure 10. NMDAR subunits expression (GluN2A, GluN2B and GluN1) and GluN2B/ GluN2A subunits ratio in total homogenate of primary hippocampal neurons exposed to IL-1β at DIV 7

A) Representative western blot analysis of GluN2A, GluN2B and GluN1 subunits obtained from total homogenate of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 7. The same amount of proteins was loaded in each lane.

B and C) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 18 of seven independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A



B

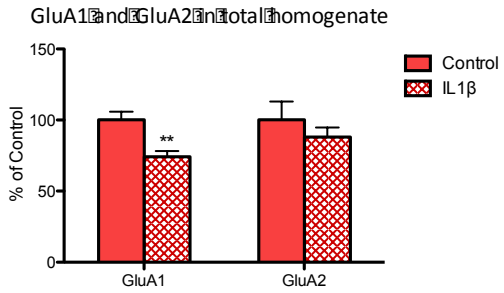
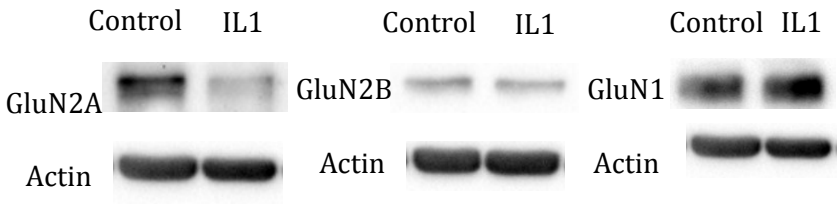


Figure 11. AMPAR subunits expression (GluA1 and GluA2) in total homogenate of primary hippocampal neurons exposed to IL-1β at DIV 7

A) Representative western blot analysis of GluA1 and GluA2 subunits obtained from total homogenate of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 7. The same amount of proteins was loaded in each lane.

B) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 12 of five independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A



B

C

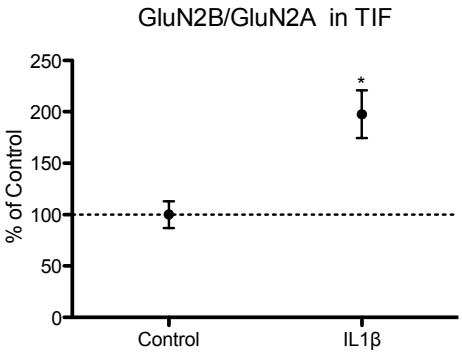
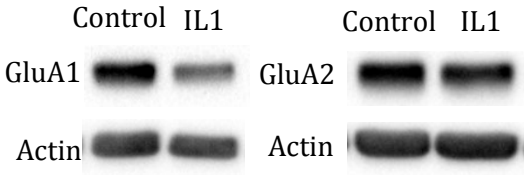


Figure 12. NMDAR subunits expression (GluN2A, GluN2B and GluN1) and GluN2B/ GluN2A subunits ratio in TIF of primary hippocampal neurons exposed to IL-1β at DIV 7

A) Representative western blot analysis of GluN2A, GluN2B and GluN1 subunits obtained from TIF of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 7. The same amount of proteins was loaded in each lane.

B and C) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 8 of three independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A



B

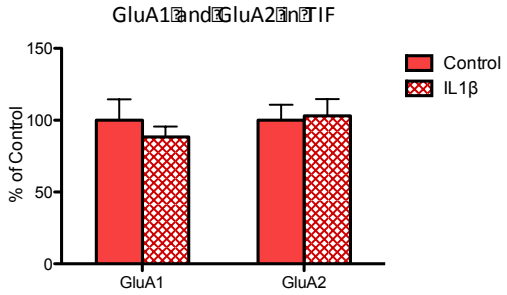


Figure 13. AMPAR subunits expression (GluA1 and GluA2) in TIF of primary hippocampal neurons exposed to IL-1β at DIV 7

A) Representative western blot analysis of GluA1 and GluA2 subunits obtained from TIF of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 7. The same amount of proteins was loaded in each lane.

B) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 8 of three independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

Subunit composition of NMDA and AMPARs in hippocampal neurons exposed to IL-1β at DIV 14

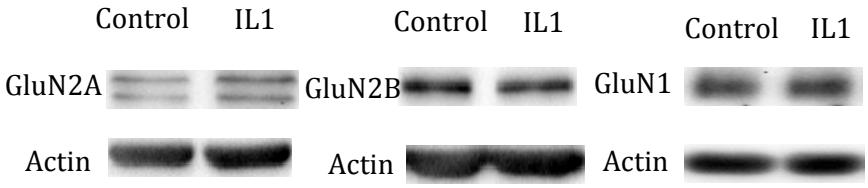
Results from IL-1β treatment of hippocampal neurons at DIV 14 show that the cytokine does not affect the subunits expression at DIV 21.

The amount of GluN2A, GluN2B and GluN1 subunits of NMDAR subunits measured by western blot in total homogenate at DIV 21 after 30 minutes exposure to IL-1β 0,05 ng/ml at DIV 14 does not change compared to Controls (Figure 14, A and B).

Similar results are observed for GluA1 and GluA2 subunits of AMPAR (figure 15, A and B).

These same results are confirm in the TIF fraction obtained from primary hippocampal neurons exposed to IL-1β at DIV 14 for both NMDA ad AMPAR subunits (Figure 16, A and B and 17, A and B).

A



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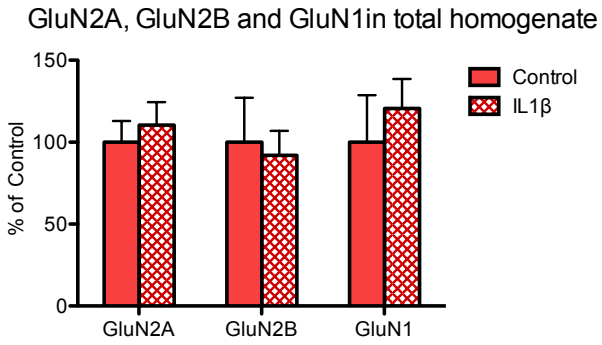
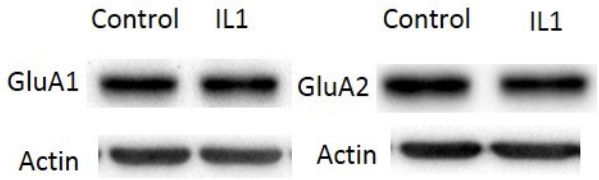


Figure 14. NMDAR subunits expression (GluN2A, GluN2B and GluN1) in total homogenate of primary hippocampal neurons exposed to IL-1β at DIV 14

A) Representative western blot analysis of GluN2A, GluN2B and GluN1 subunits obtained from total homogenate of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 14. The same amount of proteins was loaded in each lane.

B) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 5 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A



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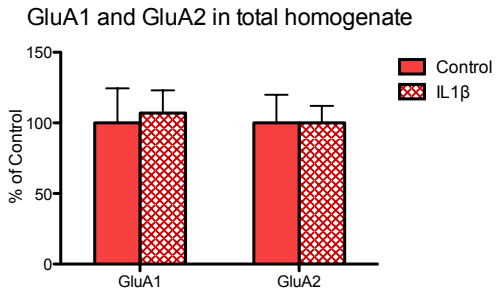
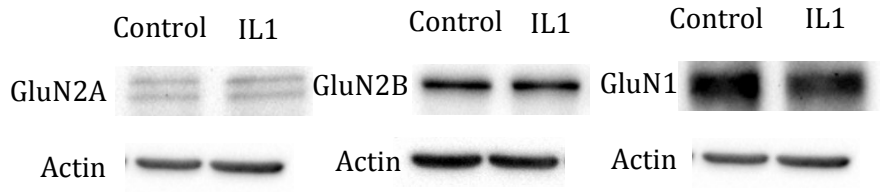


Figure 15. AMPAR subunits expression (GluA1 and GluA2) in total homogenate of primary hippocampal neurons exposed to IL-1β at DIV 14

A) Representative western blot analysis of GluA1 and GluA2 subunits obtained from total homogenate of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 14**.** The same amount of proteins was loaded in each lane.

B) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 5 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A



B

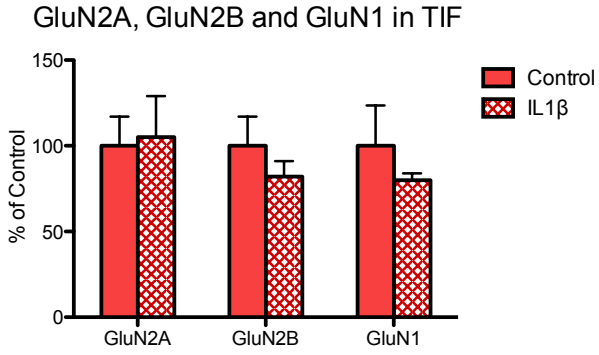


Figure 16. NMDAR subunits expression (GluN2A, GluN2B and GluN1) in TIF of primary hippocampal neurons exposed to IL-1β at DIV 14

A) Representative western blot analysis of GluN2A, GluN2B and GluN1 subunits obtained from TIF of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 14. The same amount of proteins was loaded in each lane.

B) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 5 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A

B

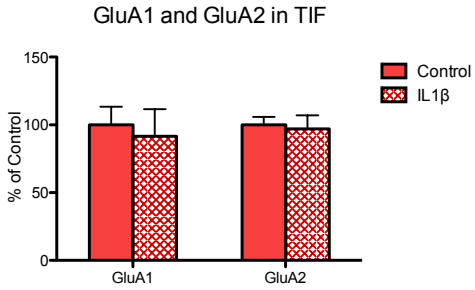


Figure 17. AMPAR subunits expression (GluA1 and GluA2) in TIF of primary hippocampal neurons exposed to IL-1β at DIV 14

A) Representative western blot analysis of GluA1 and GluA2 subunits obtained from TIF of primary hippocampal neurons treated with IL-1β 0,05 ng/ml for 30' at DIV 14. The same amount of proteins was loaded in each lane.

B) Expression of each subunit, expressed as percentage of the Control, was normalized over the corresponding actin. Mean values ± SE (n= 5 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

Intracellular calcium measurement in primary hippocampal neurons exposed to IL-1β

The subunits composition of NMDARs markedly influences receptor's functionality altering NMDAR-mediated synaptic currents and kinetics.

The different NMDAR composition and distribution observed after IL-1β treatment (0,05 ng/ml, 30') at DIV 7 could have a possible functional relapse on calcium homeostasis in primary hippocampal neurons. Intracellular calcium concentration was therefore monitored in mature primary hippocampal neurons (at DIV 21) previously treated with IL-1 β at different time of development (DIV 3, 7 and 14) and the contribution of GluN2B and GluN2A subunit evaluated.

Neurons were incubated in ACSF and exposed or not to IL-1β 0,05 ng/ml at DIV 3, 7 or 14. After the treatment, neurons were washed, incubated in the original medium and cultured until DIV 21 for intracellular calcium measurement. Using the same protocol adopted for calcium assessment during maturation, neurons were loaded with a specific dye, Fura-2AM, and then stimulated with NMDA 10 μM. The contribution of each NMDAR subunit on NMDA-induced calcium peak was calculated using 3 μM Ifenprodil, inhibitor of NMDARs containing GluN2B subunit, 300 nM NVP, inhibitor of NMDARs containing GluN2A subunit and MK-801, selective and non-competitive inhibitor of NMDARs, as previous reported in figure 5, A. The percentages of GluN2A and GluN2B subunits were calculated as described in Methods and Materials.

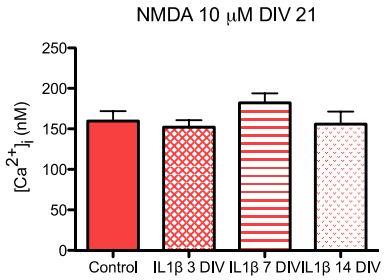
Data obtained prove differences between IL-1β treated neurons and Control neurons (Figure 18).

$[Ca^{2+}]_I$ induced by 10 μM NMDA as not affected by 30 minutes pre-treatment with IL-1β 0,05 ηg/ml at DIV 3, 7 and 14 (Figure 18, A). Nevertheless exposure of primary hippocampal neurons at DIV 7 to 0,05 ng/ml IL-1β for 30' resulted in a higher proportion of $[Ca^{2+}]_I$ under the control of NMDAR sharing GluN2B subunit compared to Controls at DIV 21 (Figure 18, B). Accordingly, the proportion of $[Ca^{2+}]_I$ under the control of the GluN2A subunit is lower compared to Controls at 21 DIV (Figure 18, C).

The persistence of a highest GluN2B contribution to NMDA-induced calcium increase at DIV 21 upon short exposure to IL-1β does not occur when primary hippocampal neurons are pre-treated with IL-1β 0,05 ng/ml at DIV 3 and 14 (Figure 18, B).

This indicated that delay of GluN2B vs GluN2A shift in terms of $[Ca^{2+}]_I$ response induced by IL-1β occurs only when neurons are exposed to the cytokine at DIV 7.

A



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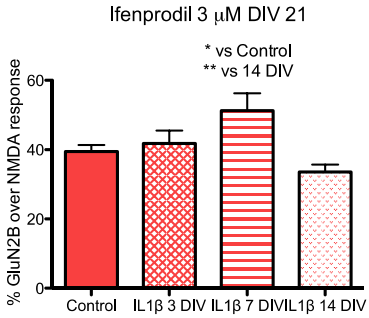

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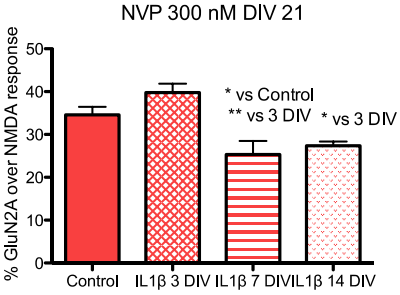


Figure 18. Intracellular calcium in primary hippocampal neurons exposed to NMDA at DIV 21 after IL-1β treatment at different time of development (DIV 3, 7 and 14)

A) Bar graph represents the average peak Ca^{2+}response to the application of NMDA 10 μM in hippocampal neurons at DIV 21, pre-exposed or not to 0,05 ng/ml IL-1β at DIV 3, 7 or 14. Mean values ± SE (n= 17 of 6 independent experiments for DIV 3 treated-neurons, n=10 of 6 independent experiments for DIV 7 treated-neurons and n=12 of 3 independent experiments for DIV 14 treated-neurons) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

B) Bar graph represents the average Ca^{2+} decay to the application of 3 μM Ifenprodil expressed as percentage of NMDA-induced Ca^{2} peak in primary hippocampal neurons at DIV 21previously treated with IL-1β 0,05 ng/ml at DIV 3, 7 and 14. Data are expressed as percentage of NMDA response. Mean values ± SE (n= 17 of 6 independent experiments for DIV 3 treated-neurons, n=10 of 6 independent experiments for DIV 7 treated-neurons and n=12 of 3 independent

experiments for DIV 14 treated-neurons) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

C) Bar graph represents the average Ca^{2+} decay to the application of 300 nM NVP expressed as percentage of NMDA-induced Ca^{2} peak in primary hippocampal neurons at DIV 21previously treated with IL-1β 0,05 ng/ml at DIV 3, 7 and 14. Data are expressed as percentage of NMDA response. Mean values ± SE (n= 17 of 6 independent experiments for DIV 3 treated-neurons, n=10 of 6 independent experiments for DIV 7 treated-neurons and n=12 of 3 independent experiments for DIV 14 treated-neurons) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's test).

mRNA levels of GluN2A and GluN2B subunits of NMDAR in hippocampal neurons exposed to IL-1β at DIV 7

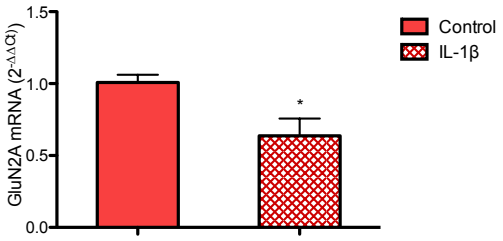
In vivo and in vitro studies demonstrated that the transcriptional profile of NMDAR subunits is in accordance with the protein's maturational program observed in WB analysis. With increasing time in the culture, it has indeed been demonstrated an up-regulation of GluN2A mRNA expression (Hoffmann et al., 2000).

In order to identify the mechanism underlying the effects of IL-1β on NMDAR subunits expression a possible transcriptional effect was investigated.

Thus GluN2B and GluN2A mRNA expression was evaluated at DIV 14 by RT-PCR in primary hippocampal neurons exposed to IL-1β 0,05 ng/ml for 30 minutes at DIV 7.

IL-1β induces significant reduction of mRNA level of GluN2A subunit in neurons treated compared to controls, whereas no significant changes were observed for mRNA level of GluN2B subunit (Figure 19, A and B).

A



B

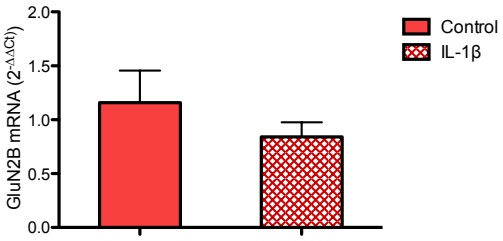


Figure 19. mRNA levels of NMDAR GluN2A and GluN2B subunits at DIV 14 in primary hippocampal neurons exposed to IL-1β at DIV 7

A) Histograms show GluN2A mRNA expression calculated by means of the comparative cycle threshold (Ct) methods in primary hippocampal neurons at DIV 14 that were pre-treated with IL- 1β at DIV 7. Data were normalized for ribosomal unit 18s, used as housekeeping gene. Mean values ± SE (n= 6 of two independent experiments conducted in duplicate) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

B) Histograms show GluN2B mRNA expression calculated by means of the comparative cycle threshold (Ct) methods in primary hippocampal neurons at DIV 14 previously treated with IL-1β at DIV 7. Data were normalized for ribosomal unit 18s, used as housekeeping gene. Mean values ± SE (n= 6 of two independent experiments conducted in duplicate) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

Role of Src kinase in IL-1β effects

The role of Src kinase in the functional regulation of NMDA has been extensively studied and it was demonstrated that NMDA activity is modulated by phosphorylation by several kinases, among which Src kinase (Lee et al., 2006; Paoletti et al., 2013; Wang et al., 2014, Salter et al., 2004). Furthermore we previously demonstrated that IL-1β modulates the activity of NMDAR by increasing the phosphorylation of Tyr 1472 at the GluN2B subunits through the activation of the Scr family of Tyrosine kinases (Viviani et al., 2003).

We therefore investigated the involvement of this kinase in the interference mediated by IL-1β on the physiological GluN2B vs GluN2A switch occurring during hippocampal neurons maturation. For this purpose the Src family tyrosine kinase inhibitor PP2 was used.

Hippocampal neurons at DIV 7 were pre-incubated in ACSF and exposed or not to PP2 (1 μM) 30 minutes before the addition of IL-1β 0,05 ng/ml for other 30 minutes. At the end of the treatment, neurons were washed and grown up to DIV 21 to be then evaluated with WB analysis for the expression of GluN2A and GluN2B subunits.

Since the exposure of primary hippocampal neurons to 0,05 ng/ml IL-1β for 30 minutes at DIV 7 results in a decreased expression of GluA1 subunit of the AMPAR in total homogenate the expression of both AMPAR subunits after inhibition of Src by PP2 were evaluated as well.

Subunit composition of NMDARs and AMPARs at DIV 21 in hippocampal neurons pre-exposed to PP2 inhibitor and then to IL-1β at DIV 7

Neurons exposed at DIV 7 to IL-1β, in the presence of PP2, show a significant reduction of GluN2A subunit expression at DIV 21 (Figure 20, A and B). The expression of GluN2B subunit of NMDAR (Figure 20, A and C) does not change, confirming the results obtained in the previous experiments. These effects lead to a significant increase of GluN2B/GluN2A ratio, calculated as previously described, at DIV 21 in hippocampal neurons compared to controls (Figure 20, D).

Pre-incubation with the Src kinases inhibitor PP2, before IL-1β treatment, prevents the effects of the cytokine on the reduction of GluN2A subunit (Figure 20, A and B) without influencing the expression of GluN2B subunit at DIV 21 (Figure 20, A and C).

This results in a recovery of GluN2B/GluN2A ratio to values comparable to DIV 21 fully mature neurons (Figure 20, D).

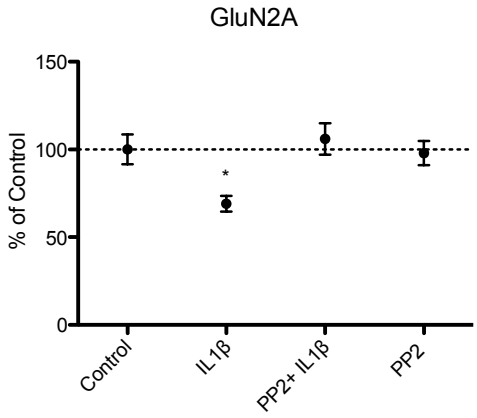
As previously described, 0,05 ng/ml IL-1β pre-treatment of DIV 7 primary hippocampal neurons results in a reduced expression of GluA1 subunit of the AMPAR at DIV 21 (Figure 21, A and B) and no effect on GluA2 subunit (Figure 21, A and C). Again, treatment of DIV 7

hippocampal neurons with 1μM PP2 prevented IL-1β-induced reduction of GluA1 expression at DIV 21(Figure 21, A and B).

Neurons exposed to PP2 alone express same NMDAR and AMPAR subunits composition as Controls at DIV 21, excluding the possibility of a PP2 effect on the glutamatergic receptors subunits expression (Figure 20 and 21).

A

B



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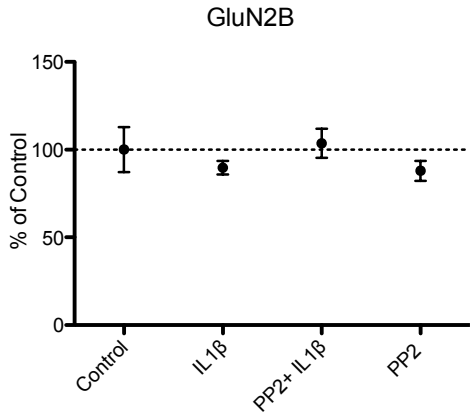


D

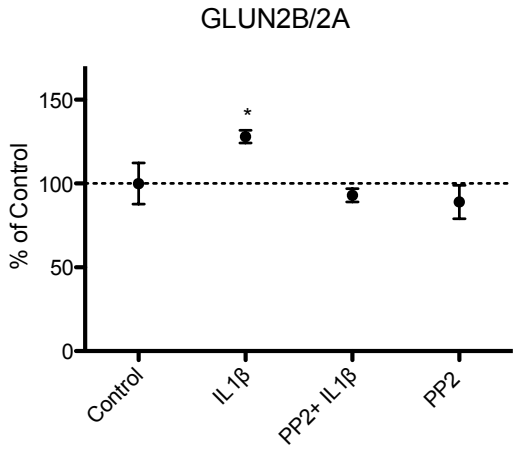


Figure 20. NMDAR subunits expression (GluN2A, GluN2B) and GluN2B/ GluN2A subunits ratio in total homogenate of primary hippocampal neurons at DIV 21, pre-exposed to PP2 inhibitor and then to IL-1β at DIV 7

A) Representative western blot analysis of GluN2A and GluN2B subunits obtained from total homogenate of primary hippocampal neurons pre-treated with 1 μM PP2 inhibitor and 0,05 ng/ml IL-1β at DIV 7. The same amount of proteins was loaded in each lane.

B) Graph showing the average expression of GluN2A as percentage of Control. Data were normalized on actin immunoreactivity. Mean values ± SE (n=6 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's).

C) Graph showing the average expression of GluN2B as percentage of Control. Data were normalized on actin immunoreactivity. Mean values ± SE (n=6 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's).

D) Graph showing the average expression of GluN2B/GluN2A ratio as percentage of Control. Data were normalized on actin immunoreactivity. Mean values ± SE (n=6 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's).

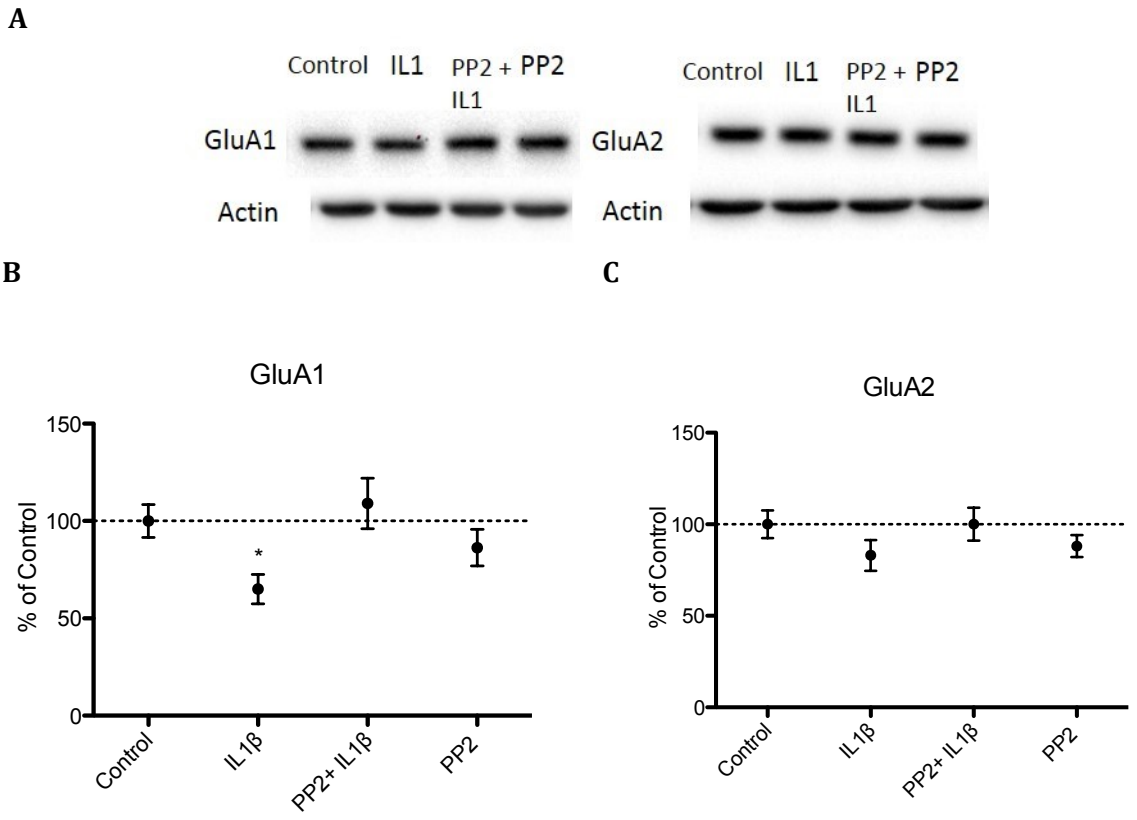


Figure 21. AMPAR subunits expression (GluA1 and GluA2) in total homogenate of primary hippocampal neurons at DIV 21, pre-exposed to PP2 inhibitor and then to IL-1β at DIV 7

A) Representative western blot analysis of GluA1 and GluA2 subunits obtained from total homogenate of primary hippocampal neurons pre-treated with 1 μM PP2 inhibitor and 0,05 ng/ml IL-1β at DIV 7. The same amount of proteins was loaded in each lane.

B) Graph showing the average expression of GluA1 as percentage of Control. Data were normalized on actin immunoreactivity. Mean values ± SE (n=6 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's).

C) Graph showing the average expression of GluA1 as percentage of Control. Data were normalized on actin immunoreactivity. Mean values ± SE (n=6 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's).

Does TNF-α affects the maturation of glutamatergic system in primary hippocampal neurons?

An inflammatory event generally involves the production of several cytokines. Among the main pro-inflammatory cytokines produced in the CNS, TNF-α and IL-1β are the most studied in the research for the immune system role in modulating learning and memory processes. Literature studies suggest that IL-1β preferentially affects NMDAR, while TNF-α has specific interactions with AMPAR mediated by TNF-R1 (Beattie et al., 2002; Ogoshi et al., 2005; Stellwagen et al., 2005; Ferguson et al., 2008).

Beside IL-1β, the effect of TNF-α on expression of both NMDAR and AMPAR subunits along primary hippocampal neurons development was investigated to acquire information on the specificity of action exerted by different pro-inflammatory cytokines.

Subunit composition of NMDARs and AMPARs at DIV 21 in hippocampal neurons exposed to TNF-α at DIV 7

At DIV 7 primary neurons were exposed to TNF-α 1 ng/ml for 30 minutes, as described in methods (page 51). The adopted concentration of TNF-α was chosen based on its ability to alter the expression of AMPAR subunits as reported in literature (Beattie et al., 2002). Neurons were then grown in culture medium until DIV 21 to be evaluated for the expression of GluN2A and GluN2B subunits and GluA1 and GluA2 subunits in total homogenate.

TNF-α 1 ng/ml at DIV 7 does not significantly affects the expression of GluN2A and GluN2B subunits of NMDAR compares to Controls at DIV 21 (Figure 22, A and B).

Although in the absence of an effect on GluN2A expression, and on GluN2B/GluN2A ratio, a brief exposure of DIV 7 primary hippocampal neurons results in a significant decrease of GluA1 subunit expression in treated neurons compared to Control samples and a slightly reduction of the amount of GluA2 subunit in TNF-α treated neurons (Figure 23, A and B).

A

B

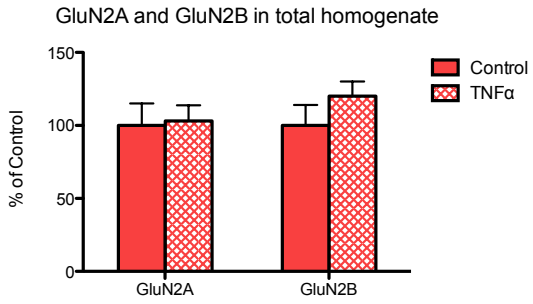


Figure 22. NMDAR subunits expression (GluN2A and GluN2B) in total homogenate of primary hippocampal neurons exposed to TNF-α at DIV 7.

A) Representative western blot analysis of GluN2A and GluN2B subunits obtained from total homogenate of primary hippocampal neurons treated with 1ng/ml TNF-α at DIV 7. The same amount of proteins was loaded in each lane.

B) Graph showing the average expression of GluN2A and GluN2B subunits as percentage of Control. Data were normalized over the corresponding actin. Mean values ± SE (n=6 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's).

A

B

Figure 23. AMPAR subunits expression (GluA1 and GluA2) in total homogenate of primary hippocampal neurons exposed to TNF-α at DIV 7.

A) Representative western blot analysis of GluA1 and GluA2 subunits obtained from total homogenate of primary hippocampal neurons treated with 1 ng/ml TNF-α at DIV 7. The same amount of proteins was loaded in each lane.

B) Graph showing the average expression of GluA1 and GluA2 as percentage of Control. Data were normalized over the corresponding actin. Mean values ± SE (n=6 of two independent experiments) (*p<0.05; **p<0.01; ***p<0.001; ANOVA followed by Tukey's).

Dendritic spines morphology of primary hippocampal neurons exposed to IL-1β

The morphology and functionality of dendritic spines are profoundly influenced by the activity of NMDA receptors. We thus investigated whether exposure of 7 DIV primary hippocampal neurons to IL-1β, which significantly influences GluN2A expression and GluN2B/GluN2A ratio at 21 DIV, might result in an altered dendritic spine maturation.

By means of confocal microscopy, we analyzed density and morphology of dendritic spines at DIV 21 in primary hippocampal neurons exposed to IL-1β 0,05 ng/ml for 30 minutes at DIV 7. For this purpose treated and untreated neurons were transfected with GFP at DIV 10 and cultured until DIV 18. Neurons were then fixed and mounted on glass slides to be evaluated for number and shape of dendritic spines by confocal microscopy.

The analysis of neurons was performed using ImageJ software and for each spine was measured the total length of the spine, the width of the neck and the width of the head.

The measurements of the ratio of head length and width, and the ratio of head and neck width allow to objectively classifying the different spines.

Overall were measured 100 spines for each neuron, considering 10 neurons for each condition (Control samples and IL-1β treated neurons), obtained from three independent experiment.

Three types of spine were considered: stubby spines, short and wide, thin spines, long and thin and mushroom-shaped spines, long and characterized by a tighter neck than the head.

Results show that IL-1β treatment does not affect both spine density, considered as the number of spines in 10 μm (Figure 24, B) and spines percentage of total protrusions (that include Filopodia, precursors of dendritic spines) (Figure 24, C).

The IL-1β treatment reduces, however, the total length of measured spines (Figure 25, A and B).

It has then calculated the percentage of each spine type in both experimental conditions (Control and IL-1β-treated neurons).

In hippocampal neurons shortly exposed to IL-1β at DIV 7 the percentage of mushroom spines, at DIV 21, significantly decreases compared to controls, and this reduction is coupled to a consistent increase of the percentage of stubby spine. The percentage of thin spine does not change upon IL-1β treatment compared to Controls (Figure 26, A).

Finally was assessed the quality of each spine type in IL-1β treated and control neurons in order to identify the presence of structural differences, due to the treatment. Each spine

typology was then measured for spine length and head width in treated and controls neurons. No structural differences were observed between Control and IL-1β-treated neurons for none of spine type investigated (Figure 27, A, B and C).

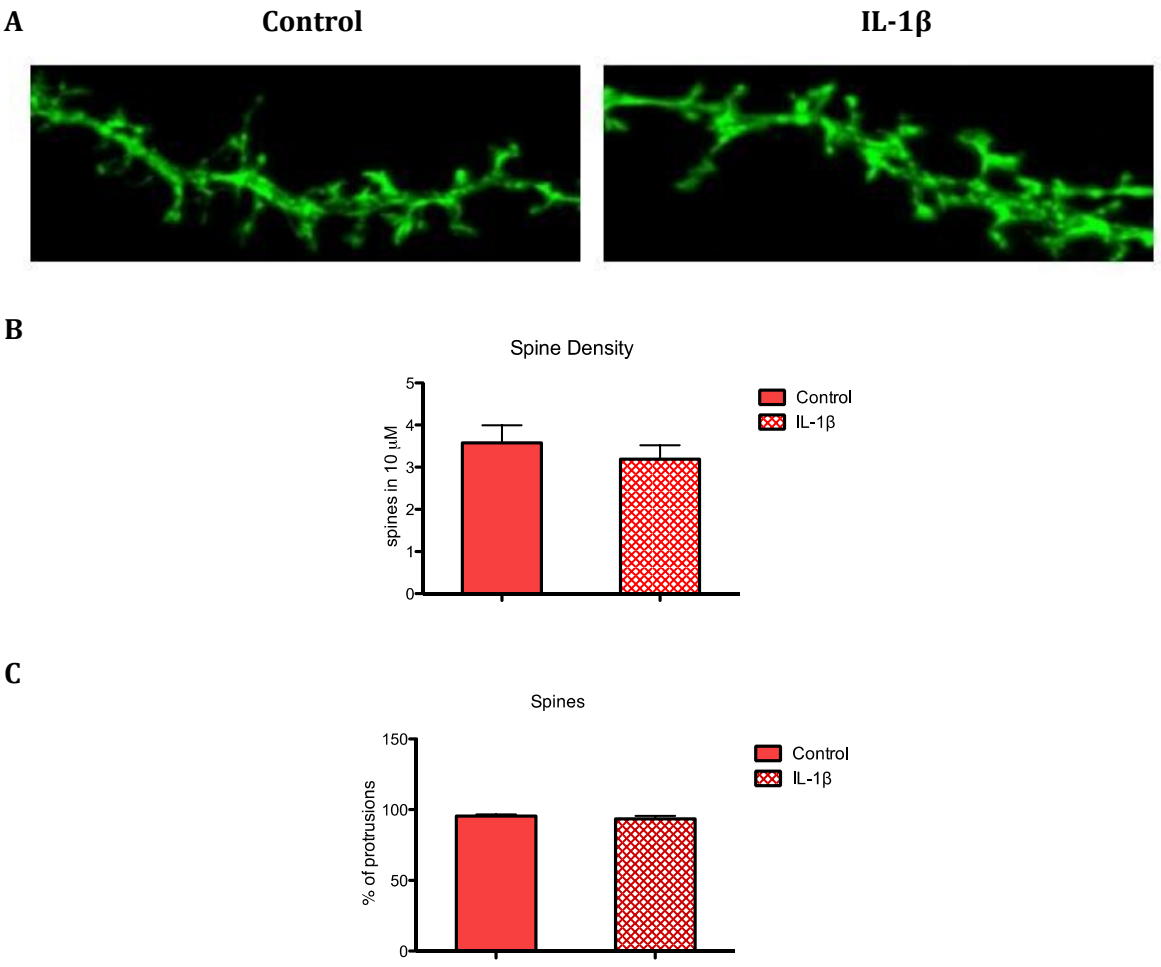


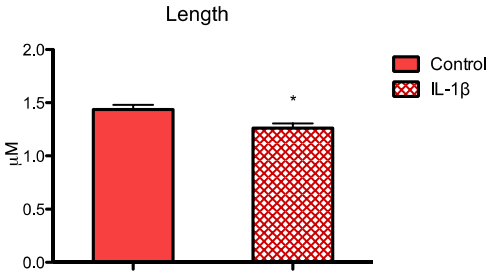
Figure 24. Dendritic spine analysis in primary hippocampal neurons at DIV 18, previously exposed to IL-1β at DIV 7

A) Representative images of hippocampal dendritic spines obtained using confocal microscopy analysis after transfection with GFP of neurons at DIV 10. Neurons were analysed at DIV 18, after IL-1β treatment at DIV 7, and results were compared to control neurons.

B) Spine density of primary hippocampal neurons at DIV 18 previously treated with IL-1β at DIV 7. Data are expressed as number of spines in dendritic segment of 10 µm. Mean values ± SE (n=10 of three independent experiment) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

C) Number of spines of primary hippocampal neurons at DIV 18 previously treated with IL-1β at DIV 7. Data are expressed as percentage of dendritic spines on the total of protrusion analysed (Filopodia included) in Control and IL-1β treated neurons. Mean values ± SE (n=10 of three independent experiment) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A



B

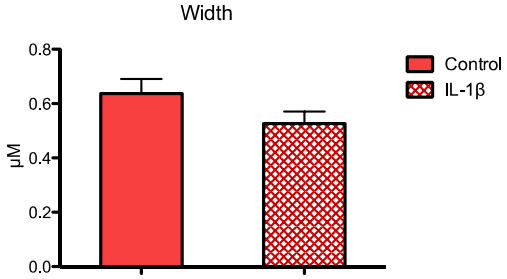


Figura 25. Analysis of the head width and the total length of dendritic spine in primary hippocampal neurons at DIV 18, previously treated with IL-1β at DIV 7

Percentage of different spine types (mushroom, stubby and thin) on total number of spines in primary hippocampal neurons at DIV 18 previously treated or not with IL-1β at DIV 7. Data are expressed as number of spines in dendritic segment of 10 µm. Mean values ± SE (n=10 of three independent experiment) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

For the analysis were measured the length of dendritic spines measured **(A)** and the width of their head **(B)** of primary hippocampal neurons at DIV 18 previously treated or not with IL-1β at DIV 7. Data are expressed as total length and head width values of spines, considering Control samples and IL-1β treated neurons. Mean values ± SE (n=10 of three independent experiment) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A

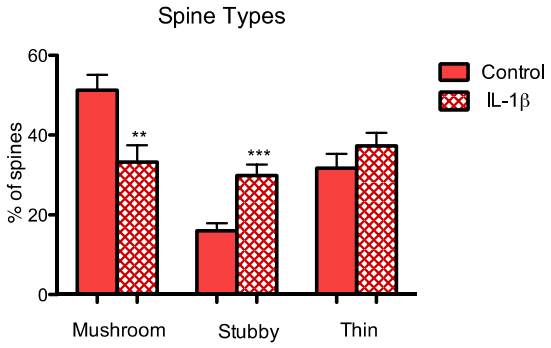
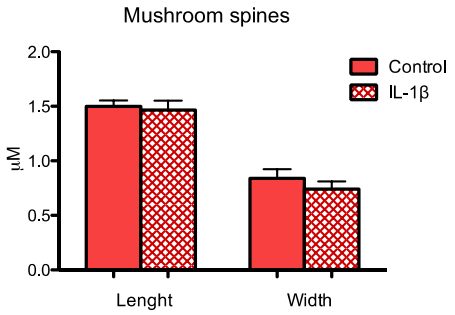


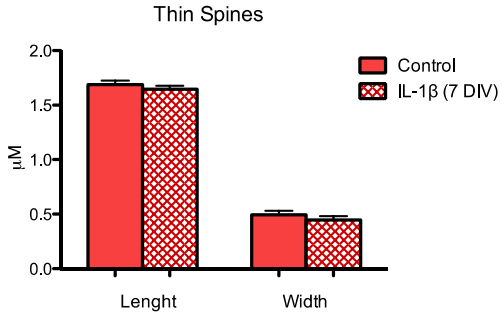
Figure 26. Analysis of dendritic spine types in primary hippocampal neurons at DIV 18, previously exposed to IL-1β at DIV 7

A) Percentage of different spine types (mushroom, stubby and thin) on total number of spines in primary hippocampal neurons at DIV 18 previously treated or not with IL-1β at DIV 7. Data are expressed as number of spines in dendritic segment of 10 μm. Mean values ± SE (n=10 of three independent experiment) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

A



B



C

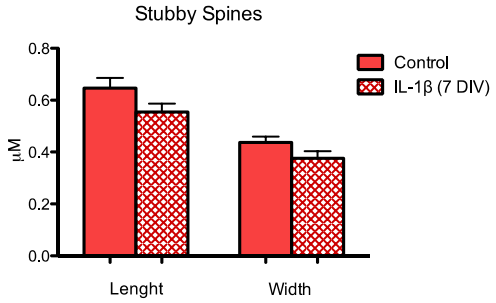


Figure 27. Analysis of dendritic spine for each spine type (mushroom, stubby and thin) in primary hippocampal neurons at DIV 18, previously treated with IL-1β at DIV 7

Percentage of different spine types (mushroom, stubby and thin) on total number of spines in primary hippocampal neurons at DIV 18 previously treated or not with IL-1β at DIV 7. Data are expressed as number of spines in a 10 µm dendritic segment. Mean values ± SE (n=10 of three independent experiment) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

For the analysis were measured the length of dendritic spines and the width of their head of primary hippocampal neurons at DIV 18 previously treated or not with IL-1β at DIV 7. Data are expressed as total length and head width values of spines, considering separately each spine types, *stubby* (**A**), *thin* (**B**) e *mushroom* (**C**). Mean values ± SE (n=10 of three independent experiment) (*p<0.05; **p<0.01; ***p<0.001; Student's t test).

Discussion

Several studies have revealed the unique and distinct plasticity of the developing brain to environmental challenges. Through a mechanism commonly called ”perinatal programming” environmental factors can target the maternal, fetal and/ or neonatal system and induce lasting behavioural and neuroimmune changes in the growing organism that increase organism susceptibility to infections, neuroimmune and neurological diseases.

One important player in the long-term impact of early life challenges is inflammation. New data have indeed highlighted the importance of perinatal inflammation for the etiology of a number of psychiatric and neurodevelopmental disorders, including ASD, schizophrenia, depression and Alzheimer's disease (Spencer S.J. et al., 2017). These finding motivate to study the neuroinflammation process with the aim to clarify the molecular mechanism involved and identify preventive strategies for environmental illness.

In the brain, the immune system modulate cognitive functions through the release of cytokines that act as neuromodulator in the communication between glia cells and neurons both in physiological and pathological conditions. Among the cytokines secreted in the CNS IL-1β is one of the most studied for its important role in cognitive processes (Bilbo et al., 2012).

In physiological conditions this cytokine is important for the development of neural circuits in the CNS. However, when IL1β production in the brain is not strictly regulated and it is secreted in an altered manner can impair learning and memory processes. For this reason, IL1β has been implicated in the pathogenesis of various psychiatric disorders, such as major depressive disorder, Schizophrenia, Alzheimer disease, bipolar disorder as well as in the onset of cognitive disorders.

Glutamatergic receptors (AMPA and NMDA) have a crucial role in the molecular mechanisms disrupted in these disorders since they are essential for excitatory synapses formation, synaptic plasticity mediators and are involved in higher cognitive functions (Paoletti et al., 2013).

The ability of cytokines to modulate ionotropic glutamatergic receptors is an emergent mechanism that could explain the connection between early-life neuroinflammation and the later in life onset of neurological disorders.

In particular it has been demonstrated that IL1β is able to modulate glutamatergic response through the recruitment of glutamatergic ionotropic receptors, by enhancing NMDAR activity (Viviani et al., 2003) and enriching GluN2B subunit of NMDAR at the post-synaptic site (Viviani et al., 2006).

These argumentations encourage me to study, during the PhD period, the relationship between IL-1β and the glutamatergic system in a developing model of hippocampal neurons, analysing the molecular mechanisms involved in IL-1β and NMDAR interaction.

The early postnatal development of the glutamatergic system has been extensively studied in in vivo models that demonstrate a switch in NMDAR subunit composition, from containing GluN2B subunit to a predominance of GluN2A subunit, and a consequent recruitment of AMPAR at the post-synapses (Gray et al., 2011).

Using primary hippocampal neurons we have developed an in vitro model that allows the study and understanding of the molecular mechanisms implicated in the interaction between glutamatergic receptors and IL-1β. The experimental model, that characterize the maturation of hippocampal neurons from days in vitro (DIV) 7 to DIV 21, reproduces exactly the glutamatergic development observed in vivo. In fact, we were able to observe an increase of NMDAR sharing the GluN2A subunits over NMDAR sharing GluN2B subunits along with the development and ageing of the culture from DIV 7 to DIV 21.

Identified this model as a suitable and appropriate model for assessing the development of the glutamatergic system, focusing on the ionotropic receptor component, we have studied a protocol of exposure that could mimic a transient inflammatory event, initially focusing on IL-1β cytokine.

The protocol requires the exposure of primary hippocampal neurons to IL-1β 0,05 ng/ml for 30 minutes at different time of development, DIV 3, a condition of immaturity, DIV 7, a condition of early development and DIV 14, a condition of initial maturity of the neurons.

Concentration and time of exposure have been chosen based on our previous data that demonstrated a clear action of IL-1β on NMDAR activity and distribution at the post-synapses (Viviani et al., 2003). The procedure was performed in ACSF and after the treatment, the neurons were washed and re-incubated in the original medium to be evaluated “long-term” allowing the completion of neurons maturation, DIV 21. At this time point the effect of IL-1β exposure on glutamatergic system development, at different developmental stages, has been evaluated as protein expression, calcium homeostasis and gene expression of different NMDAR and AMPAR subunits and their ability to control intracellular Ca^{2+} homeostasis.

We thus assessed how this pro-inflammatory stimulus can alter the structure and functionality of glutamate receptors and thus have long-term consequences on neuronal functions.

At DIV 21 were therefore assessed the differences in terms of protein expression of GluN1 and GluN2 subunits of NMDAR and GluA1 and GluA2 subunits of AMPAR among neurons exposed to IL-1β and control neurons (exposed only to ASCF) in total homogenate. The neuronal treatment, with a dose of IL-1β capable of mimicking a pathological state of neuroinfiammation, immediately revealed a specific time window in which the system is more sensitive to an exogenous inflammatory signal. Only the IL-1β treatment performed at 7 DIV affects the development of glutamatergic neurons at DIV 21. IL-1β exposure at this time

induces a decrease of GluN2A subunit levels that leads to an unbalance GluN2B-GluN2A switch. These effects, coupled with a reduction of GluA1 subunit expression, maintain through a direct action on neurons the glutamatergic system in a state of immaturity and confirm a role of IL-1β in this process. The data from the treatments performed at DIV 3 and 14 do not show any change in NMDAR and AMPAR subunits at DIV 21 induced by cytokine.

The inhibition of GluN2B-GluN2A switch could have a great influence on the synaptic activity, since the two subunits have different functional properties. GluN2A subunit confers fast kinetics to NMDA channel, GluN2B subunit is characterized by slower kinetics, allowing a greater calcium input into the cell. Furthermore, GluN2B subunit has a longer cytosolic tale than GluN2A, which is subject to phosphorylation by Src kinase activated by IL-1β causing increased channel conductance. Moreover, the C-terminal domain of GluN2B subunit interacts with a series of signal transduction proteins that may mediate, when over-activated, part of the cytotoxic effects induced by IL-1β hyper-stimulation. These argumentations convinced us to assess the subunits expression also at the post-synaptic site, where we observed again a reduced presence of NMDAR sharing GluN2A subunits with an unbalance of GluN2B/GluN2A ratio upon IL-1β treatment compared to controls at DIV 21.

Subunits composition of NMDARs markedly influences receptor's functionality altering NMDAR-mediated synaptic currents and kinetics.

A functional NMDA receptor consists of two GluN1 subunits that form a complex with two GluN2A or GluN2B subunits.

Since an alteration in GluN2A subunits expression by IL-1β was observed, the study of the effect of IL-1β also on the GluN1 subunit expression was considered relevant. The IL-1β treatment at 3, 7 and 14 DIV does not affect GluN1 subunit expression in hippocampal neurons at DIV 21, suggesting the presence of functional receptor in primary hippocampal neurons characterized by different subunits composition compared to control.

NMDAR is a calcium channel, whose functionality depends on NMDAR subunits composition.

The different NMDAR composition and distribution observed after IL-1β treatment at DIV 7 could have anyway a possible functional relapse on calcium homeostasis in primary hippocampal neurons. Measurement of intracellular calcium concentration in mature primary hippocampal neurons (at DIV 21) previously treated with IL-1β at different time of development (DIV 3, 7 and 14) proves differences between IL-1β treated neurons and Control neurons. Consistently with WB findings, this experiment shows that IL-1β interfere with calcium homeostasis regulation under the control of NMDAR only at DIV 7, with a reinforced GluN2B subunit contribution coupled with a weaker GluN2A subunit contribution compared

to controls at DIV 21. This unbalanced GluN2B - GluN2A contribution is lost when neurons are treated at DIV 3 and 14.

The alterations induced by IL-1β identify a sensitivity time window through which IL-1β can induce" long-term" effect on the development of glutamatergic neurons and could represent a molecular basis of major neuronal dysfunctions.

We have indeed chosen DIV 7, the affected period, as critical period for IL-1β treatment in the elaboration of subsequent experiments.

Evidences show that phosphorylation process has a crucial role also for the developmental shift: phosphorylation of Y1472 by Fyn, a member of Src family of kinases, stabilizes GluN2B at synaptic membranes early in development. Then NMDAR activity induces GluN2B S1480 phosphorylation by CK2 that promote GluN2B endocytosis. GluN2A expression increases and GluN2A-containing receptors replace GluN2B-containing NMDARs at synaptic sites (Sanz-Clemente et al., 2010).

Previous results of my lab demonstrate that in mature neurons IL-1β is able to interact specifically with the GluN2B subunit of NMDARs through activation of SRC family of kinases (Viviani et al., 2003). The involvement of the SRC family of kinases in IL-1β effects on GluN2B/GluN2A shift was therefore investigated in our model. The addition of SRC kinases inhibitor PP2 before IL-1β treatment has revealed that a phosphorylation process mediates IL-1β effects also along development of hippocampal neurons preventing GluN2A subunit drop and restoring GluN2B/GluN2A physiological ratio at DIV 21.

Data obtained reveal that GluN2B-GluN2A switch impairment is due to a drop of GluN2A subunit levels in neurons treated with IL-1β compared to controls. To explain the reduction of GluN2A subunit amount we have investigated a possible transcriptional effect of IL-1β. Total mRNA was extracted at DIV 14, at a time antecedent the observation of protein expression reduction. The results reveal that IL-1β specifically interferes with the increased transcription of GluN2A subunit that physiologically occurs during neuronal maturation. These findings demonstrate a transcriptional basis of IL-1β effects on glutamatergic receptors, suggesting that IL-1β through its specific receptor could trigger intracellular events that induce an inhibition or a reduction of GluN2A subunit gene transcription in a time window of neuronal development in which normally increases.

In the first part of my Phd we focused on the alterations at the expense of the glutamatergic system induced by high levels of IL-1β, that simulate an inflammatory event. However, during an inflammatory event the immune system releases several different cytokines, among which IL-1β e TNFα are those most involved in the pathogenesis of neurologic disorders.

In order to verify whether the observed effects were specific for IL-1β, we have checked TNFα effects on glutamatergic systems maturation. Several in vitro and in vivo studies suggest a direct action of TNFα on AMPA component of glutamatergic system (Beattie et al., 2002; Ferguson et al., 2008), without any effects on NMDAR component.

Using our experimental conditions hippocampal neurons were exposed at DIV 7 to TNFα 1 ng/ml, the concentration that interfere with AMPARs expression, and protein expression of NMDAR and AMPAR main subunits was evaluated.

Data show that a short exposure of primary hippocampal neurons to TNFα at DIV 7 is able to reduce GluA1 subunit amount at DIV 21 demonstrating a direct effect on AMPA component of the glutamatergic system, without any effects on NMDAR subunits. We hypothesized that TNFα recruits different mechanisms due to the lack effect on GluN2B/GluN2A ratio. This hypothesis needs further investigation.

Literature studies demonstrate indeed that glutamatergic activity plays a crucial role in the development and maintenance of dendritic spines, influencing dendritic spine morphology. (Hering and Sheng, 2001).

Since functional maturation of the glutamatergic spines due to a proper distribution of ionotropic receptor subunits is reflected in their morphological features, we wondered if IL-1β, affecting the normal development of the glutamatergic system, has also an effect on the correct formation of post-synaptic spines and hence on the morphology of the dendritic spines. We have assessed long-term effects of IL-1β on dendritic spine development in our model of simulated immune activation.

Hippocampal neurons were treated with IL-1β 0,05 ng/ml at DIV 7, washed and re-incubated in original medium. At DIV 10, neurons were exposed to a transfection with GFP (Green Fluorescent Protein), and cultured up to DIV 18 to be evaluated for spine number, density and morphology analysis with confocal microscopy. A specific software allowed to classify the different dendritic spines types.

In control neurons, that represent a condition of maturity, was assessed a predominance of mushroom-shaped and thin spine, with only a small number of stubby spines.

These data are in accordance with literature that reports a predominance of mushroom-shaped and thin spines in the adult rat brain (Fiala et al., 1998).

In neurons exposed to IL-1β, compared to controls, the presence of stubby spines significantly raises compared to controls while the number of mushroom-shaped spines decreases, suggesting the presence of an immature phenotype.

However, neither the number nor the density of dendritic spines are altered by IL-1β that acts, probably, after spines formation, impeding the normal evolution of them.

The experiments performed during the three years of PhD period demonstrate that an inflammatory process characterized by IL-1β production can affect the maturation of glutamatergic neurons in several aspects.

IL-1β interferes with the glutamatergic maturational program blocking the physiological switch GluN2B to GluN2A. This effect is due to a reduced GluN2A subunit gene expression that lead to an altered expression and distribution of this subunit at the post-synapses. These structural aspects determine an altered NMDAR functionality with consequences also at cellular level, with a modified development of dendritic spine morphology and the generation of an immature phenotype.

Although an inflammatory state is characterized by the production of several cytokines, the effects described in this study are specifically induced by IL-1β.

Consistently with in vivo findings, our data demonstrate that an immune activation during the early postnatal development causes lasting structural and functional changes in the brain that could potentially predispose to functional disorders later in life, due to a direct effect of IL-1β on neurons.

Furthermore, in agreement with literature findings our results demonstrate the important roles of GluN2A subunits in pathological processes. Altered expression of GluN2A subunit has been indeed observed in human developmental diseases such as infant seizures disorder and schizophrenia (Sun et al., 2017).

Since glutamatergic activity and dendritic spine morphology are strictly and profoundly linked to the development of neuronal network we can also speculate that their malfunction could cause “long-term” cognitive dysfunctions.

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