Role of IL-1 Signaling in Controlling Synaptic Function

Thesis

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Role Of IL-1 Signaling In Controlling Synaptic Function

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Cristina
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LIST OF ABBREVIATIONS

IL-1  Interleukin-1
IL-1R Interleukin-1 Receptor
CNS  Central Nervous System
PNS  Peripheral Nervous System
LTP  Long-Term Potentiation
BBB Blood-Brain Barrier
TLR  Toll-Like Receptor
TIR  Toll – IL-1 Receptor
DAMP Damage-Associated Molecular Pattern
PAMP Pathogen-Associated Molecular Pattern
IL-1RAcP Interleukin-1 Receptor Accessory Protein
ST2  Growth Stimulating-Expressed Gene 2
IL-1Rrp Interleukin-1 Receptor-Related Protein
TIR8  Toll/Interleukin-1 Receptor 8
SIGIRR Single Ig IL-1-Related Receptor
TIGIRR Three Ig IL-1-Related Receptor
IL-1RAPL Interleukin-1 Receptor Accessory Protein Like
NF-κB Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
mRNA messenger Ribonucleic Acid
MYD88 Myeloid Differentiation Primary Response Gene 88
IRAK Interleukin-1 Receptor–Activated Protein Kinase
TRAF Tumour Necrosis Factor–Associated Factor
JNK c-Jun N-Terminal Kinase
p38 MAPK Mitogen-Activated Protein Kinase p38
CXCL  C-X-C Motif Chemokine Ligand
CCL  C-C Motif Chemokine Ligand
PTGS Prostaglandin-Endoperoxide Synthase
IκBα Inhibitor of kappa-B Alpha
MKP Mitogen-Activated Protein Kinase Phosphatase
PRR  Pattern Recognition Receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLRP3</td>
<td>Nucleotide-Binding Oligomerisation Domain-Like Receptor, Pyrin-Containing 3</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>P2X7</td>
<td>Purinoreceptor 2X7</td>
</tr>
<tr>
<td>IκBβ</td>
<td>IkB Kinase Beta</td>
</tr>
<tr>
<td>Rab</td>
<td>RAS-ASSOCIATED BINDING PROTEIN</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine Triphosphate Hydrolase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>IL-1R KO</td>
<td>Interleukin-1 Receptor Knock-Out</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>PTPσ</td>
<td>Presynaptic Protein Tyrosine Phosphatase-σ</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular Zone</td>
</tr>
<tr>
<td>P2Y</td>
<td>Purinoreceptor 2Y</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>C1q</td>
<td>Complement Component 1q</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement Receptor 3 (also known as CD11b/CD18)</td>
</tr>
<tr>
<td>RGC</td>
<td>Retinal Ganglion Cell</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-Phenylindole</td>
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</tbody>
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ABSTRACT

Although several studies demonstrate that inflammation contributes to neurodegenerative and psychiatric disorders, the physiological functions of immune molecules in synapse formation and functionality are not completely known. Interleukin-1β (IL-1β) has been described as one of the main pro-inflammatory cytokines involved in several neurological disorders. IL-1β is produced by many types of cells, including immune cells in the periphery, as well as glia and neurons within the brain [Dinarello CA, 1996]. The ability of IL-1β to influence cellular functions depends on the expression of the appropriate receptor and the activation of specific intracellular signaling pathways. Two receptors for IL-1β have been identified, but only the type 1 receptor (IL-1R1) is signal transducing [Sims JE et al., 1993]. IL-1R1 is expressed on central nervous system (CNS) glia (astrocytes and microglia), particularly after injury [Friedman WJ, 2001], and also on specific populations of CNS neurons, including hippocampal pyramidal neurons [Yabuuchi K et al., 1994; Ericsson A et al., 1995; Friedman WJ, 2001]. IL-1β elicits distinct functional effects in these CNS cell types, regulating the production of inflammatory cytokines in glia and influencing synaptic function of hippocampal neurons, affecting long-term potentiation (LTP) [Katsuki H et al., 1990; Bellinger FP et al., 1993; Murray CA and Lynch MA, 1998; Kelly A et al., 2001].

Previous studies reported that impaired Interleukin-1 signalling is associated with deficits in hippocampal memory processes and neural plasticity [Avital A et al., 2003] and that IL-1R is essential for the activation of microglia and the induction of proinflammatory mediators in response to brain injury [Basu A et al., 2002].

Despite the well-established role of this cytokine in different brain diseases, its putative physiological function during normal brain development is not completely known. The aim of this study is to clarify if, and how, Interleukin-1 signalling participates in the maintenance of synapse homeostasis in the CNS. In order to study how endogenous IL-1β signaling may alter normal brain development and functionality, we took advantage of an IL-1 type 1 receptor (IL-1R1) knock-out mouse model, where the IL-1 signaling is genetically blocked.

Our results show that lack of IL-1R affects excitatory inputs in vivo, leading to a significant increase in the expression of excitatory synaptic markers in both cortex and hippocampus. In line with this evidence, Golgi-cox staining of fixed brain slices revealed a significantly higher spine density in CA1 pyramidal neurons of IL-1R-/- hippocampi.
Consistently, electrophysiological recordings from hippocampal acute slices demonstrated enhanced frequency and amplitude of miniature excitatory postsynaptic currents in IL-1R^− mice, confirming the functional potentiation of the excitatory signalling in the hippocampus of knock-out mice. We also observed a transiently increased number of parvalbumin-positive GABAergic interneurons in the hippocampal CA1 region of P30 IL-1R-deficient mice (although, this increased number is completely rescued in older mice), suggesting a possible role of IL-1 signalling in the control of the GABAergic system during early stages in life. These data demonstrate a key role of the IL-1 receptor in controlling excitatory and inhibitory network refinement and strengthening during brain development. However, this process was not observed in primary culture of hippocampal neurons established from IL-1R^− mice, thus suggesting a non-cell-autonomous effect. In line with this observation, we observed a transient increase in microglia number in IL-1R^− mice during the first weeks of postnatal life (a critical time point for synapse maturation and elimination), although completely recovered in older mice. Moreover, using mixed co-cultures, we found that IL-1R^− microglia cells were less effective in promoting synaptic elimination when compared to WT microglia cells.

In conclusion, IL-1R signaling seems to play a key role in modulating glutamatergic and GABAergic synapses and its absence leads to a general and long-lasting potentiation of excitatory inputs. Since microglia contribute to synaptic pruning and network refinement in the first postnatal weeks, the alteration in microglia-mediated synapse pruning in IL-1R^− mice may be involved in this process, eventually leading to short-term effects on postnatal synaptic maturation and long-term effects on adult brain networks.
The inflammatory response is a crucial aspect of the tissues’ responses to deleterious injuries or microbial infections and has the physiological purpose of restoring tissue homeostasis. Inflammation can either resolve or progresses to acute or chronic. Chronic inflammation is not only typical of classical inflammatory diseases but also an underlying feature of a variety of human conditions not previously thought to have an inflammatory component, including Alzheimer’s disease, atherosclerosis, cardiovascular disease, and cancer. Inflammation involves a sequence of organized, dynamic responses including both vascular and cellular events with specific humoral secretions. The occurrence of vascular events is rapid as they appear in a few minutes following tissue injury or microbial infection in the presence of other inflammatory stimuli, and they eventually lead to vascular dilation, enhanced permeability of capillaries, increased blood flow and recruitment of inflammatory mediators and leukocytes (such as macrophages, neutrophils, monocytes and lymphocytes) from the circulatory system at the inflamed site, producing interstitial edema. In particular, polymorphonuclear neutrophils are among the first leukocyte responders to accumulate in the inflamed site. These cells are crucial as the first line of defense of the innate immune system because of their phagocytotic and microbicidal functions. Next, mononuclear cells (monocytes and macrophages) enter the inflammatory site and clear cellular debris and apoptotic polymorphonuclear neutrophils by phagocytosis without prolonging inflammation. Leukocytes also release specialized substances including vasoactive amines and peptides, eicosanoids, proinflammatory cytokines and acute-phase proteins, which mediate the inflammatory process by preventing further tissue damage and ultimately resulting in healing and restoration of tissue function. Among these pro-inflammatory mediators, pro-inflammatory cytokines (such as interleukin-1 beta, interleukin-6, tumor necrosis factor alpha) specifically control the initiation of inflammation and its maintenance and regulate its amplitude and the duration of the response. Failure to resolve the inflammatory response, or continuous activation of the responses, become harmful to the tissue and consequently develop into the chronic lesions that are associated with inflammatory diseases [Freire MO and Van Dyke TE, 2013; Sugimoto M et al., 2016; Abdulkhaleq LA et al., 2018].
The communication between the cells of the immune system is mediated by small soluble proteins called cytokines. These proteins have pleiotropic local (paracrine and autocrine) and systemic (endocrine) actions, and they are predominantly produced in substantial amount in response to inflammatory conditions. Inflammation is a non-specific systemic response designed to maintain the integrity of the organism (either localized or diffuse). Once inflammation is triggered, a reciprocal communication between the central nervous system (CNS) and the periphery is required for mounting an efficient and effective defensive response. Thus, systemic inflammation has a neurological component, and the activation of peripheral inflammatory responses is required for neuroinflammation to become effective [for review see Dinarello CA et al., 1996 and Johnson RW et al., 2002]. During inflammation, cytokine actions largely drive, in any organ, both destructive and reparatory processes. For many years, the brain has been considered immune-privileged because of its apparent inability to initiate a classical response to exogenous antigens and the presence of the highly selective blood-brain barrier (BBB). However, it is now well known and largely accepted that the brain can mount a response to injury which is carried out by invading leukocytes and other brain-specific cells, using the same soluble mediators as the systemic immune response. This response is known as neuroinflammation. Cytokines also mediate signalling to the brain when there is the need for a systemic response to protect the organism. First discovered due to their massively increased release in pathological conditions, they were also shown to have positive effects at physiological concentrations on neural activity and were suggested to be essential during this development of the nervous system [Deverman BE and Patterson PH, 2009; Stolp HB, 2013; Mousa A and Bakhiet M, 2013; Ratnayake U et al., 2013]. Cytokine effects were found to differ depending on their levels of expression, the target organ, and, very importantly, the developmental window. Interleukin-1 (IL-1) is one of the most representative cytokines with pleiotropic effects, as it appears to affect almost every cell type in the mammalian organism. In the literature, there is now a great deal of evidence providing irrefutable support for its role in inflammation-driven pathologies, although this cytokine also plays central roles in various physiological processes [Basu A et al., 2004; Fogal B and Hewett SJ, 2008; Shaftel SS et al., 2008; Mills KH and Dunne A, 2009; Gabay C et al., 2010; Dinarello CA et al., 2012; Pozzi D et al., 2018; Mendiola AS and Cardona AE, 2018].

### 1.1 The IL-1 Family of Cytokines and The Innate Immune System

Initially considered the prototypical proinflammatory cytokine, IL-1 has several other effects that are far from promoting inflammation. Some of its pleiotropic central actions
comprise fever [Dinarello and Wolf, 1982], appetite control [Mrosovsky et al., 1989], bone metabolism [Bajayo et al., 2005], central pain modulation [Wolf et al., 2003], stress response by modulating hypothalamic-pituitary-adrenal (HPA) axis function [Goshen et al., 2003] and memory modulation [Schneider et al., 1998].

More than 95% of living organisms use innate immunity for survival whereas less than 5% depend on T- and B-cell functions. More than any other cytokine family, the IL-1 family is primarily associated with innate immune responses, which manifest by inflammatory processes that can be beneficial when functioning as a mechanism of host defence, but also detrimental to survival when uncontrolled.

The IL-1 family of cytokines and receptors is considered to be unique in immunology because of the similar functions shared by the IL-1 and Toll-like receptor (TLR) families. In fact, each member of the IL-1 receptor and TLR family contains the cytoplasmic Toll-IL-1-Receptor (TIR) domain. However, while TLRs trigger inflammation by bacteria, microbial products, viruses, nucleic acids, and damage-associated molecular patterns (DAMPs), innate inflammation via IL-1 family of receptors is triggered by IL-1 family cytokine members. Although the inflammatory properties of the IL-1 family members can also play a role in acquired immunity, they mainly control innate immunity.

What characterizes innate immunity is the lack of specificity towards a certain antigen. Indeed, the numerous biological properties of the IL-1 family are nonspecific. IL-1 family non-specifically augment antigen recognition and activate lymphocyte function. IL-1β is the most studied member of the IL-1 family due to its role in mediating autoinflammatory diseases (see Table 2) [Dinarello CA, 2018].

### 1.1.1 IL-1 And IL-1R Family Members

Although originally the IL-1 family comprised only IL-1α and IL-1β, it has expanded considerably in the last decades. The IL-1 family of ligands now includes 7 molecules with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, β, and γ), three receptor antagonists (IL-1Ra, IL-36Ra and IL-38), and an anti-inflammatory cytokine (IL-37) (Table 1).
The IL-1 family can be divided into subfamilies according to the length of the precursor (the inactive form of the protein) and the length of the propiece (the portion that originates the active form of a protein after cleavage of the precursor), as well as to the primary ligand binding receptor (Figure 1).

Except for the IL-1 receptor antagonist (IL-1Ra), all other members lack a signal peptide and are primarily intracellular precursors. The IL-1 subfamily is comprised of IL-1α, IL-1β and IL-33. This subfamily has the longest proteins with the longest propieces. While the propiece of IL-1β needs to be cleaved intracellularly by caspase-1 to be secreted as a mature cytokine, in the case of the IL-1α precursor, the cleavage is performed by either the membrane protease calpain or the extracellular neutrophil proteases. Extracellular neutrophil proteases also account for the cleavage of the propiece of IL-33. IL-18 and IL-37 are members of the IL-18 subfamily. IL-18 requires the cleavage of its propiece by caspase-1 in order to become active. It is unclear how the propiece of IL-37 is removed. The IL-36 subfamily, which has the shortest propiece, comprises of IL-36α, β and γ, as well as IL-36Ra. In addition, because of its binding to the IL-36R, IL-38 likely belongs to this family [Dinarello CA, 2013, 2018]. A–X–D is a consensus sequence found in all members of the IL-1 family, where A is an aliphatic amino acid such as isoleucine, methionine or leucine, X is any amino acid and D is aspartic acid. The A–X–D motif is conserved in the IL-1 family where it plays a pivotal role in three-dimensional structure of the active cytokine. The actual N-terminus is often located 9 amino acids before the A–X–D site. By eliminating the amino acids before the N-terminus, the first β-sheet structure common to all members of the IL-1 family can form [Carta S et al., 2013; Garlanda C et al., 2013; Boraschi D and Tagliabue A, 2013; Dinarello CA, 2013; Palomo J et al., 2015; Boraschi D et al., 2018].
There are several members of the IL-1 family of receptors. A simplified nomenclature for IL-1R members is proposed here: IL-1R1 (IL-1RI), IL-1R2 (IL-1RII), IL-1R3 (IL-1RAcP), IL-1R4 (ST2), IL-1R5 (IL-18Ra), IL-1R6 (IL-1Rrp2, IL-36R), IL-1R7 (IL-18Rb), IL-1R8 (TIR8, also known as SIGIRR), IL-1R9 (TIGIRR-2), IL-1R10 (TIGIRR-1) (Figure 2).

The receptor chains are generally characterized by an extracellular portion consisting of three immunoglobulin (Ig)-like domains. Notable exceptions are the IL-18 binding protein (IL-18BP) and TIR8, which have a single Ig domain. The intracellular domains of the IL-1Rs share some homology with those of Toll-like receptors (TLR), known as Toll-like/IL-1R (TIR) domains and are essential for starting the signalling recruiting intracellular adaptors. IL-1R2 is the only IL-1R family member lacking a TIR domain.

IL-1R1 binds IL-1α, IL-1β, and IL-1Ra. IL-1R3 (formerly IL-1R accessory protein) is the coreceptor for forming a trimeric signalling complex with IL-1α or IL-1β. In the resting state,
IL-1R1 and IL-1R3 are present on the cell membrane. Once IL-1 (either IL-1α or IL-1β) binds to IL-1R1, a structural change occurs that allows IL-1R3 to bind to IL-1R1. There is no direct contact of the ligand(s) to the IL-1R3 coreceptor. The trimeric complex allows for the proximity of the TIR domains of each receptor chain with subsequent recruitment of intracellular components that triggers a cascade of kinases that produce a strong pro-inflammatory signal leading to the activation of NF-κB. IL-1R3 also exists as a soluble receptor form. A common mechanism for forming soluble receptors is the cleavage from the cell surface as a secreted protein; however, the soluble form of IL-1R3 is also produced by the liver. IL-1R2, a decoy receptor for IL-1β, lacks a cytoplasmic domain, does not signal, but rather sequesters IL-1β. IL-1R2 exists as an integral membrane protein but also as a soluble form. Although soluble IL-1R2 binds IL-1β in the extracellular space, neutralization of IL-1β activity is greatly enhanced by the formation of a complex with soluble IL-1R3. IL-1R2 associates intracellularly with the IL-1α precursor and prevents the release and subsequent extracellular processing of the precursor by calpain. IL-1R3 is also the coreceptor for IL-33, IL-36α, IL-36β, or IL-36γ. The ligand binding receptor for IL-33 is IL-1R4 (formerly, ST2). The IL-36 receptor (IL-1R6) binds IL-36α, IL-36β, or IL-36γ but also IL-38. Importantly, the IL-1 family of receptors also contains anti-inflammatory receptors. These are IL-1R8 (formerly SIGIRR, TIR8), IL-1R9 (formerly TIGIRR-2, IL-1RAPL1), and IL-1R10 (formerly TIGIRR-1). It has been shown that mice deficient in IL-1R8 develop spontaneous inflammation and, when challenged with inflammatory agents, these mice display more severe disease. The IL-18 binding protein (IL-18BP) is not a classic receptor because it is a secreted protein; nevertheless, IL-18BP contains a single IgG domain similar to the extracellular IgG-like domains of the IL-18Rα but no cytoplasmic domain. The IL-18BP functions similarly to other soluble receptors in the IL-1 receptor family, binding and neutralizing the cognate ligand [Carta S et al., 2013; Garlanda C et al., 2013; Boraschi D and Tagliabue A, 2013; Dinarello CA, 2013; Palomo J et al., 2015; Boraschi D et al., 2018].
1.1.2 IL-1 Pathway

The main function of IL-1–type cytokines is to control proinflammatory reactions in response to tissue injury by pathogen-associated molecular patterns (PAMPs, such as bacterial or viral products) or damage- or danger-associated molecular patterns released from damaged cells (DAMPs, such as uric acid crystals or adenosine 5’-triphosphate) [Gaestel M et al., 2009; Dinarello CA, 2009; Martinon F et al., 2009]. While IL-1α is primarily membrane anchored and acts through autocrine or juxtracrine mechanisms [Keller M et al., 2008], IL-1β is secreted by an unconventional protein secretion pathway and can signal in a paracrine manner or systemically [Dinarello CA, 2005]. IL-1α and IL-1β rapidly induce mRNA expression of hundreds of genes in several different cell types, such as monocytes or macrophages [Allantaz F et al., 2007; Jura J et al., 2008], epithelial cells [Hoffmann E et al., 2005], endothelial cells [Bandman O et al., 2002], chondrocytes [Vincenti MP and Brinckerhoff CE, 2001; Joos H et al., 2009] and fibroblasts [Holzberg D et al., 2003; Jeong JG et al., 2004]. In parallel, they also induce expression of
their own genes, allowing a positive-feedback loop that amplifies the IL-1 response in an autocrine or paracrine manner [Dinarello CA et al., 1987; Granowitz EV et al., 1992; Dinarello CA, 1996; Hoffmann E et al., 2005; Gaestel M et al., 2009]. Stimulation of transcription occurs within 30 min of exposure to IL-1α or IL-1β and can be sustained for many hours [Krause A et al., 1998; Hoffmann E et al., 2005; Wolter S et al., 2008]. The gene-regulatory actions of IL-1 are transmitted by a conserved signalling system that relies on the rapid, transient, and reversible assembly of multiprotein complexes that comprise both non-enzymatic adaptor proteins and enzymes [Weber A et al., 2010].

IL-1α and IL-1β independently bind the type I IL-1 receptor (IL-1R1), which is ubiquitously expressed. A third specific ligand, the IL-1 receptor antagonist (IL-1RA), binds the IL-1R1 with similar specificity and affinity but does not activate the receptor with consequent downstream signalling [Eisenberg SP et al., 1990; Vigers GP et al., 1997; Schreuder H et al., 1997; Dunn E et al., 2001]. The IL-1 receptor accessory protein (IL-1RAcP) serves as a co-receptor that is required for signal transduction of IL-1/IL-1R1 complexes, and this co-receptor is also necessary for activation of IL-1R1 by other IL-1 family members, in particular IL-18 and IL-33 [Wesche H et al., 1997; Dunn E et al., 2001; Arend WP et al., 2008].

The initial step in IL-1 signal transduction is a ligand-induced conformational change in the first extracellular domain of the IL-1R1 that facilitates recruitment of IL-1RAcP [Casadio R et al., 2001]. Through the conserved cytosolic regions TIR domains [Radons J et al., 2003], the trimeric complex rapidly assembles two intracellular signalling proteins: the myeloid differentiation primary response gene 88 (MYD88) and the interleukin-1 receptor–activated protein kinase (IRAK) 4 [Li S et al., 2002; Brikos C et al., 2007; De Nardo D et al., 2018]. IL-1, IL-1RI, IL-RAcP, MYD88, and IRAK4 form a stable IL-1–induced first signalling module [Brikos C et al., 2007]. This occurs in parallel to the (auto)phosphorylation of IRAK4, which subsequently phosphorylates IRAK1 and IRAK2, and then this is followed by the recruitment and oligomerization of tumour necrosis factor–associated factor (TRAF) 6 [Cao Z et al., 1996; Kawagoe T et al., 2008]. In response to ligand binding of the receptor and the initial receptor-proximal signalling, a complex cytosolic sequence of combinatorial phosphorylation and ubiquitination events results in activation of nuclear factor B signalling (NF-κB,) and the JNK and p38 mitogen-activated protein kinase (MAPK) pathways, which, cooperatively, induce the expression of canonical IL-1 target genes (such as IL-6, CXCL8, CCL2, PTGS2, IkBα, IL-1α, IL-1β, MKP-1) by transcriptional and posttranscriptional mechanisms [Weber A et al., 2010] (Figure 3). Specific signaling pathways mediate IL-1β actions in different cell types. For instance, IL-1β activates the p38/CREB signaling pathway in hippocampal neurons, in contrast to the activation of NF-κB in glial cells, demonstrating cell type-specific signaling responses to IL-1 in the brain and yielding distinct functional responses [Srinivasan D et al., 2004].
Figure 3. The central signaling module of the IL-1R complexes. Ligand-induced formation of the trimeric IL-1R complex (or other IL-1 family receptor complexes) leads to the intracellular formation of the TIR:TIR scaffold. The recruitment of several MyD88 molecules recruitment to the scaffold via homotypic TIR domain interaction allows further recruitment of IRAK4, first, and then of IRAK1 and/or IRAK2 (not depicted here). IRAK4 subsequently phosphorylates IRAK1 and IRAK2, and then the recruitment and oligomerization of tumour necrosis factor–associated factor (TRAF) 6 follows. This is followed by a complex cytosolic sequence of combinatorial phosphorylation and ubiquitination events resulting in the activation of nuclear factor B signalling (NF-κB) and the JNK and p38 mitogen-activated protein kinase pathways (Adapted from: Boraschi et al., Immunological Reviews 2018).

1.1.3 IL-1β Synthesis, Processing and Release

IL-1β is first synthesized as biologically inactive 31 kDa precursor peptide (pro-IL-1β), which can be cleaved by caspase-1 to generate a 17 kDa mature and biologically active form (mIL-1β) that is subsequently released into the extracellular milieu [Mosley B et al., 1987; Black RA et al., 1988; Eder C, 2009].
This cytokine lacks a leader peptide and is therefore secreted via an unconventional pathway, independent of the endoplasmic reticulum and Golgi apparatus. Production of proinflammatory (pro)–IL-1β is induced by Toll-like receptor (TLR)-mediated NF-κB activation [Gabay C et al., 2010]. IL-1β is processed and released from cells by a mechanism involving caspase 1 [Thornberry NA et al., 1992]. Activation of caspase 1 is regulated by a multimeric cytosolic protein complex, called the inflammasome [Fettelschoss A et al., 2011]. Inflammasomes are large (~1 μm) intracellular protein complexes that comprise a pattern recognition receptor (PRR), an adapter molecule and the enzyme caspase-1 [Schroder K and Tschopp J, 2010]. The best characterised inflammasome is known as the NLRP3 inflammasome, so-called because the PRR molecule is nucleotide-binding oligomerisation domain (NOD)-like receptor, pyrin-containing 3 (NLRP3) [Daniels MJ and Brough D, 2017].

Following caspase-1-dependent processing of pro-IL-1β [Brough D and Rothwell NJ, 2007], pro-IL-1β remains cell-associated until a second stimulus (a further PAMP or DAMP) triggers processing and secretion of the mature cytokine. However, how IL-1β is secreted from the cell is not clear. Multiple observations and proposals have been made that do not converge on a single unified mechanism of secretion, but rather suggest that there may be multiple mechanisms. These of course may not be mutually exclusive but may each make a specific contribution to IL-1β-dependent inflammation [Lopez-Castejon G and Brough D, 2011].

Perhaps the best characterised mode of release is secretion tightly coupled to pyroptotic cell death. Pyroptosis is a form of inflammatory cell death characterised by the concomitant release of IL-1β and IL-18 [Bergsbaken T et al., 2009] and can be induced by NLRP3-activating stimuli such as ATP binding to the extracellular ATP receptor P2X7 [Perregaux D and Gabel CA, 1994; Ferrari D et al., 1994]. This pathway of cell death is uniquely dependent on caspase-1, which is not involved in apoptotic cell death, and caspase-1-deficient cells respond normally to most apoptotic signals. Unlike apoptosis, which has an anti-inflammatory outcome, cell death by pyroptosis results in plasma-membrane rupture and the release of damage-associated molecular pattern (DAMP) molecules into the extracellular milieu, including cytokines that recruit more immune cells and further perpetuate the inflammatory cascade in the tissue [Fink SL and Cookson BT, 2005]. However, recent studies demonstrated that IL-1β can be secreted both in an active mechanism independent of cell death but also in a manner that absolutely requires loss of membrane integrity [Daniels MJ and Brough D, 2017] (Figure 4).
Figure 4. Steps in the processing and release of IL-1 induced by IL-1. (1) Mature IL-1β or the IL-1α precursor activates IL-1 intracellular signalling, which starts with the formation of the IL-1 receptor complex heterodimer comprising IL-1RI and IL-1RAcP. (2) This leads to the proximity of the intracellular TIR domains. (3) Recruitment of MyD88 and phosphorylation of IL-1R–associated kinases (IRAKs) and inhibitor of NFB kinase β (IKKβ). (4) Transcription of IL-1β. (5) Translation of IL-1β mRNA into IL-1β takes place on polysomes. IL-1β mRNA is not bound to actin microfilaments but rather intermediate filaments. (6) ATP released from the activated monocyte/macrophage accumulates extracellularly. (7) Activation of the P2X7 receptor by ATP. (8) Efflux of potassium from the cell after ATP binding to P2X7 receptor. (9) Fall in intracellular levels of potassium, which triggers (10) the assembly of the components of the caspase-1 inflammasome with the conversion of procaspase-1 to active caspase-1. (11) Caspase-1 is found in the secretory lysosome together with the IL-1β precursor and lysosomal enzymes. Active caspase-1 cleaves the IL-1β precursor in the secretory lysosome, generating the active, carboxyl-terminal mature IL-1β. (12) An influx of calcium with an increase in intracellular calcium levels. The rise in intracellular calcium activates phosphatidylcholine-specific phospholipase C and calcium-dependent phospholipase A. (13) The release of mature IL-1β, the IL-1β
precursor, and the contents of the secretory lysosomes by exocytosis in the absence of cell death. (14) Processing of the IL-1β precursor in the cytosol. Rab39a, a member of the GTPase family, contributes to the secretion of IL-1β by helping traffic from the cytosol into a vesicle compartment. Exocytosis is another mechanism described in mouse macrophages. (15) Mature IL-1β exits the cells via loss in membrane integrity, associated with the release of lactic dehydrogenase or microvesicles. (Adapted from: Charles A. Dinarello, Blood 2010)

Two signals have been proposed to be required for IL-1β release from primary macrophages: first, activation of Toll-like receptors (TLRs), resulting in transcription and translation of pro-IL-1β; and second, NLR-induced IL-1β processing and release through a caspase-1-dependent mechanism involving P2X7 receptor activation by extracellular ATP [Arend WP et al., 2008]. Different models for a non-classical secretory pathway to release IL-1β have been recently described for different cell types (e.g. monocytes, macrophages, dendritic cells and brain microglia). For instance, five different release mechanisms have been suggested by Claudia Eder and colleagues in 2009: (1) exocytosis of IL-1β-containing secretory lysosomes, (2) release of IL-1β from shed plasma membrane microvesicles, (3) fusion of multivesicular bodies with the plasma membrane and subsequent release of IL-1β-containing exosomes, (4) export of IL-1β through the plasma membrane using specific membrane transporters, and (5) release of IL-1β upon cell lysis [Eder C, 2009].

In 2011, Gloria Lopez-Castejon and David Brough [Lopez-Castejon G and Brough D, 2011] proposed three categories of secretion mechanism: Rescue and redirect, protected release and terminal release. The rescue and redirect hypothesis is based on the observed fraction of cellular IL-1β localised to vesicles of endolysosomal nature that is targeted for degradation, but that can be redirected to the extracellular space following an appropriate secretion stimulus [Andrei C et al., 1999, 2004]. The protected release mechanism is based upon the observation that bioactive IL-1β can be found in shed microvesicles originated from the plasma membrane [MacKenzie A et al., 2001; Bianco F et al., 2005] and secreted exosomes [Qu Y et al., 2007]. Since it is known that IL-1β has a very short half-life in plasma, it is conceivable that the protected cytokine is destined to reach sites that are distant from the inflammatory lesion. Lastly, terminal release has been proposed to occur under conditions of extreme inflammatory stress and involves a commitment to cell death. Indeed, this mechanism allows a rapid release of large quantities of active IL-1β directly across a disintegrating plasma membrane. However, given the diversity of secretion stimuli, culture
conditions and cell types that secrete IL-1β, elucidating the precise mechanisms by which IL-1β is secreted remains a considerable challenge. It might be possible that all mechanisms contribute simultaneously to the secretion of IL-1β [Lopez-Castejon G and Brough D, 2011].

It has been reported that in activated monocytes a microvesicle shedding from plasma membrane is the major secretory pathway for rapid IL-1β release [MacKenzie A et al., 2001]. Another fraction of cellular IL-1β is released by the regulated secretion of late endosomes and early lysosomes, and this may represent a cellular compartment where caspase-1 processing of pro-IL-1β takes place. In a first step, pro-caspase-1 and endotoxin-induced pro-IL-1β are targeted in part to specialized secretory lysosomes, where they colocalize with other lysosomal proteins. Externalization of mature IL-1β and caspase-1 together with lysosomal proteins is then facilitated by extracellular ATP which triggers the efflux of K⁺ from the cell, followed by Ca²⁺ influx and activation of three phospholipases: phosphatidylcholine-specific phospholipase C (PC-PLC) and calcium-independent (iPLA₂) and -dependent phospholipase A₂ (cPLA₂). Whereas calcium-independent phospholipase A₂ is involved in processing, phosphatidylcholine-specific phospholipase C and calcium-dependent phospholipase A₂ are required for secretion. In particular, the ATP-mediated K⁺ efflux is responsible for phosphatidylcholine-specific phospholipase C induction, which in turn allows the rise in intracellular free calcium concentration required for activation of phospholipase A₂. This activation is ultimately responsible for lysosome exocytosis and IL-1β secretion [Andrei C et al., 2004]. Although it has been already proposed that iPLA₂ is required to facilitate passage of caspase-1 to pro-IL-1β and, consequently, pro-IL-1β processing [Walev I et al., 2000], the molecular nature of a lysosomal transporter for IL-1β – should one exist – is unknown.

1.1.4 IL-1β and Inflammation

More than any other cytokine family, the IL-1 family of ligands and receptors is primarily associated with acute and chronic inflammatory diseases (Table 2).
IL-1 was initially described for its ability to affect various and different biological properties but, for many years, it remained uncertain whether a single polypeptide was indeed capable of such a range of distinctly different biological properties. It was a highly contentious issue that a single molecule could cause fever, induce hepatic acute-phase proteins, activate lymphocytes, and upregulate prostanoid synthesis. The cloning of IL-1 cDNAs [Lomedico PT et al., 1984; Auron PE et al., 2007] and, soon thereafter, the introduction of recombinant IL-1 showed that this protein did in fact possess such a wide spectrum of biological activities and was indeed pyrogenic [Dinarello CA et al., 1986]. In 1984 it was predicted that IL-1 was responsible for many of the acute-phase responses to infection and inflammation [Dinarello CA, 1997]. In addition, recombinant IL-1 was found to mediate many components of the acute-phase response. This evidence demonstrated that IL-1 causes a wide variety of biological effects associated with infection, inflammation, and autoimmune processes [Dinarello CA et al., 1986]. In many ways, the biology of IL-1 played a major role in the new field of cytokines [Dinarello CA, 2006]. IL-1β has emerged as a therapeutic target for an

Table 2 Interleukin-1 in the pathogenesis and treatment of acute and chronic inflammatory diseases

<table>
<thead>
<tr>
<th>Classic autoinflammatory diseases</th>
<th>Probable autoinflammatory diseases</th>
<th>Common diseases mediated by IL-1β</th>
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<tbody>
<tr>
<td>Familial Mediterranean fever (FMF)</td>
<td>Recurrent idiopathic pericarditis</td>
<td>Rheumatoid arthritis‡</td>
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<tr>
<td>Pyogenic arthritis, pyoderma gangrenosum, acne (PAPA)†</td>
<td>Macrophage activation syndrome (MAS)</td>
<td>Periodic fever, aphthous stomatitis, pharyngitis, adenitis syndrome (PFAPA)</td>
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<td>Cryopyrin-associated periodic syndromes (CAPS)</td>
<td>Urticarial vasculitis</td>
<td>Urate crystal arthritis (gout)</td>
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<td>Hyper IgD syndrome (HiDS)</td>
<td>Antisynthetase syndrome</td>
<td>Type 2 diabetes</td>
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<tr>
<td>Adult and juvenile Still disease</td>
<td>Relapsing chondritis</td>
<td>Smoldering multiple myeloma</td>
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<tr>
<td>Schnitzler syndrome</td>
<td>Behçet disease</td>
<td>Postmyocardial infarction heart failure</td>
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<td>TNF receptor-associated periodic syndrome (TRAPS)</td>
<td>Erdheim-Chester syndrome (histiocytosis)</td>
<td>Osteoarthritis</td>
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<tr>
<td>Blau syndrome; Sweet syndrome</td>
<td>Synovitis, acne, pustulosis, hyperostosis, osteitis (SAPHO)</td>
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<td>Deficiency in IL-1 receptor antagonist (DIRA)</td>
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expanding number of local and systemic inflammatory conditions called autoinflammatory diseases. Autoinflammatory syndromes are distinct from autoimmune diseases. In autoimmune diseases, dysfunctional T cell are associated with the pathogenesis of the disease as they drive inflammation. Immunosuppressive therapies targeting T-cell function, as well as antibodies that deplete T and B cells, are effective in treating autoimmune diseases. In contrast, in autoinflammatory diseases, the dysfunctional cells which directly promote inflammation are monocytes-macrophages. Autoinflammatory conditions are characterized by recurrent bouts of fever with debilitating local and systemic inflammation. In general, these diseases are poorly controlled with immunosuppressive therapies, but they are often responsive to pharmacological therapies based upon IL-1β inhibition (Table 2). Although some autoinflammatory diseases are due to gain-of-function mutations for caspase-1 activity, common diseases such as gout, type 2 diabetes, heart failure, recurrent pericarditis, rheumatoid arthritis, and smoldering myeloma also are responsive to IL-1β neutralization, which results in a rapid and sustained reduction in disease severity [Dinarello CA, 2018].

1.2 IL-1 in the Brain

A cytokine, in order to have an effect in the brain, needs to fulfill two conditions: (1) it must be detectable in its biological active form in the extracellular milieu and (2) the target cells must express the appropriate receptors on their plasma membrane. Previous studies demonstrated constitutive expression of mRNA for IL-1α and IL-1β in cerebellar Purkinje cells, hypothalamic neurons and in the hippocampus, mainly in the granule cells of the dentate gyrus [Yabuuchi et al., 1993]. It is now well established that IL-1RI is expressed throughout the brain, with the highest levels found in cerebral cortex and hippocampus [Farrar WL et al., 1987; Takao T et al., 1990; Ban EM, 1994; Gabellec MM et al., 1995; French RA et al., 1999; Eriksson C et al., 2000]. All members of the IL-1 family (i.e., IL-1, IL-1Ra, IL-1RI, IL-1RII, and IL-1RAcP) are expressed in the healthy CNS. Low levels of IL-1β immunoreactivity have been detected throughout the brain of rodents in normal conditions, with highest expression occurring in the hippocampus, hypothalamus, and basal forebrain [Breder CD et al., 1988; Lechan RM et al., 1990; Molenaar GJ et al., 1993; Hammond EA et al., 1999]. The cell types capable of synthesizing IL-1 - microglia [Giulian D et al., 1986; Hetier E et al., 1988; Yao J et al., 1992], astrocytes [Lieberman AP et al., 1989; Tomozawa Y et al., 1995; Knerlich F et al., 1999; Zhang W et al., 2000], oligodendrocytes [Blasi F et al., 1999], and neurons [Lechan RM et al., 1990; Takao T et al., 1990; Watt JA and Hobbs NK, 2000] - also express the signalling receptor [Hammond
EA et al., 1999; Friedman WJ, 2001; Pintaux E et al., 2002; Wang XF et al., 2006]. Finally, IL-1RaCp - the protein necessary for signal transduction via IL-1RI to occur - is also expressed in the rat brain under normal physiological conditions, with particularly elevated levels in the hypothalamus, cortex, hippocampus, and cerebellum [Liu C et al., 1996; Ilyin SE et al., 1998]. Expression of IL-1α and β, IL-1Ra, and IL-1 receptors has also been demonstrated in postmortem human brain [Sheng JC et al., 1998; Huitinga I et al., 2000; Toyooka K et al., 2003]. Although cell type-specific expression of the IL-1 family in human tissue has not been investigated thoroughly, IL-1 and IL-1Ra RNA can be detected in cultured human microglia [Walker DG et al., 1995] and IL-1 immunoreactivity is present in human neurons [Huitinga I et al., 2000; Fogal B and Hewett SJ, 2008].

1.2.1 IL-1 in Brain Development

IL-1 is detectable by immunohistochemistry in the developing mammalian brain as early as the time of formation of the cortical plate [Dziegielewksa KM et al., 2000], but important amounts are not detectable before the end of the period of intense cortical neurogenesis [Caviness VS et al., 2008]. The observation of a peak in IL-1β expression, described in the late prenatal and early postnatal periods [Giulian D et al., 1988], suggests an involvement of this cytokine in synaptogenesis and neuronal network reorganisation, with significant impact on the development of behavioural patterns [Kaffman A and Meaney MJ, 2007]. The delayed effects of prenatal exposure to IL-1 emphasizes the putative role played in the developing brain. Indeed, exposure to IL-1 in mice around E15-17 results in schizophrenia-like behavior late in adulthood [Zaharia MD et al., 1996; Ozawa K et al., 2006]. Therefore, although IL-1 is required for normal brain development [Giulian D et al., 1988], exposure to excessively high levels becomes detrimental [Zuckeman L and Weiner I, 2005; Ozawa K et al., 2006]. This late consequence might be due to the effect of IL-1 exposure to the developing brain cells that know to be associated with schizophrenia (i.e. pyramidal cells, medium spiny neurons (MSNs) and certain interneurons, and to a smaller extent, embryonic, progenitor or glial cells) [Skene NG et al., 2018]. Moreover, previous studies specifically focusing on the dopaminergic system gave a clear evidence concerning the implication of IL-1 in dopaminergic neuron development in the CNS. In fact, it has been demonstrated that IL-1 is necessary to induce the proliferation of mesencephalic dopaminergic progenitors and promote their differentiation into adult dopamine producing neurons from long-term cultures of human fetal mesencephalic precursor cells [Ling ZD et al., 1998; Storch A et al., 2001].
1.2.2 IL-1 and Behaviour

Several behavioural elements are known to be modulated and influenced by cytokines: modulation of pain [Wolf G et al., 2003], anxiety [Anisman H and Merali Z, 1999], learning and memory [Pickering M and O'Connor JJ, 2007; reviewed in Pozzi D et al., 2018], and sickness behaviour [Konsman JP et al., 2002].

IL-1β is among the main pro-inflammatory cytokines involved in sickness syndrome, which is a normal, very well organized, non-specific adaptive response to infection that it is characterized by endocrine, autonomic and behavioural changes and is triggered by soluble mediators that are produced at the site of infection by activated accessory immune cells. These pro-inflammatory mediators, including IL-1α, IL-1β, TNF-α and IL-6, coordinate the local and systemic inflammatory response to microbial pathogens. These peripherally produced cytokines also act as humoral messengers conveying the signals from the periphery to the brain to cause the typical behavioral symptoms of sickness, including lethargy, depression, anxiety, malaise, loss of appetite, sleepiness, hyperalgesia, failure to concentrate, usually accompanied by fever [for review see Johnson RW, 2002; Dantzer R and Kelley KW, 2007]. Two mechanisms mediating the behavioral effects of peripherally released cytokines on the brain have been proposed: (1) a fast neural route represented by the primary afferent neurons that innervate the body site where the infectious process takes place and (2) a slower humoral pathway that involves the production of proinflammatory cytokines by phagocytic cells in response to circulating PAMPs or cytokines, followed by the propagation of these immune signals into the brain parenchyma [Danzer R, 2009].

Besides the well-established contribution of IL-1β to sickness, it has been widely elucidated also its involvement a variety of diseases. There is now a growing body of evidence pointing toward cognition as a shared physiological process hit by this cytokine. This concept originally emerged from a series of pioneering experiment showing that the intraperitoneal or intrahippocampal injection of this cytokine results in learning and memory defects. Many studies based on exogenous administration of IL-1β reported long-term memory deficits, although some of them pointed out the possibility that the memory deficits may be the consequence of the sickness syndrome induced by IL-1β rather than being attributed to any direct effect on memory consolidation [Cunningham C and Sanderson DJ, 2008]. Nevertheless, in vitro data support a direct effect on hippocampal LTP, which may be obscured by the more prominent symptoms of the sickness syndrome [O'Connor JJ and Coogan AN, 1999].

The detrimental effects of IL-1β on cognition were later confirmed using transgenic mice expressing the cytokine in an inducible manner or upon endogenous IL-1β elevations evoked by infections, with the memory deficits being prevented, in the latter case, by intra-
ventricular infusion of the naturally occurring interleukin-1 receptor antagonist (IL-1Ra).

Previous reports also demonstrated that brain IL-1 facilitates learning of a water maze spatial memory task in young mice and that, on the other hand, impaired Interleukin-1 signalling is associated with deficits in hippocampal memory processes [Avital A et al., 2003; Takemiya T et al., 2017]. In particular, it has been reported that the IL-1R antagonist (IL-1Ra) impaired memory in the water maze and passive avoidance paradigms, whereas IL-1β administration facilitated memory in the passive avoidance test [Yirmiya R et al., 2002]. IL-1 receptor type I knockout (IL-1R KO) [Labow M et al., 1997; Glaccum MB et al., 1997] mice have been used to further examine the hypothesis that IL-1 signalling pathways are involved in the physiological mechanisms that control learning, memory, and neural plasticity. For instance, IL-1R KO mice were studied in several paradigms of memory function and hippocampal plasticity. In the spatial version of the water maze test, IL-1R KO mice displayed significantly longer latency to reach a hidden platform, compared with wild-type controls; furthermore, IL-1R KO exhibited diminished contextual fear conditioning, but were similar to control animals in hippocampal-independent memory tasks [Avital A et al., 2003].

1.2.3 IL-1β, Neuroinflammation and Neurodegeneration

Inflammation is generally a beneficial response of an organism to infection but, when prolonged or inappropriate, it can be detrimental. Neuronal loss in acute (e.g. stroke and head injury) and chronic (e.g. multiple sclerosis and Alzheimer’s disease) central nervous system diseases has been associated with inflammatory processes systemically and in the brain. Within the nervous tissue, neuroinflammation is conventionally considered as the ability of the central (CNS) and peripheral (PNS) nervous system to mount an innate immune response during a pathological event. The tissue resident microglia and astroglia are considered the hallmark effector cells involved in mounting inflammatory responses to injury [Allan SM et al., 2005]. However, the fine balance of governing neuroinflammation within the mammalian CNS is a challenging feat which is often associated with a bystander effect leading to exacerbated tissue damage.

Brain inflammation induces rapid expression and release of key inflammatory mediators – cytokines, chemokines, prostaglandins and complement proteins – which in turn upregulate adhesion molecules, increase permeability of the blood–brain barrier facilitating the invasion of peripheral immune cells, induce release of potentially toxic molecules and compromise brain cells. Cytokines are primary mediators of the inflammatory response and special attention has been given to the balance between proinflammatory (e.g. IL-1, IL-6,
TNF-α, IL-18) and anti-inflammatory (IL-4, IL-10, TGF-β) cytokines. As mentioned above, a central role of IL-1β in mediating neuroinflammation has been recognized in most CNS-related diseases including stroke, traumatic brain injury, Alzheimer’s disease and multiple sclerosis, and diseases that affect the ocular system including diabetic retinopathy [Mendiola AS and Cardona AE, 2017].

Microglia represent the main source of IL-1 in the brain upon injury and in neuroinflammation [Yabuuchi K et al., 1993; Eriksson C et al., 1999], with induction occurring within an hour of the onset [Giulian D and Lachman LB, 1985; Yabuuchi K et al., 1993; Eriksson C et al., 1998; Eriksson C et al., 1999], linking IL-1 to the neurodegenerative process. Interestingly, the endogenous antagonist (IL-1Ra) is induced in the same cell types that express IL-1R agonist, but with a certain delay [Eriksson C et al., 1998; Eriksson C et al., 2000]. The lower severity of brain injury and improved recovery upon blocking IL-1 signalling [Tehranian R et al., 2002] further support the implication of IL-1 in neurodegeneration.

1.2.4 IL-1β, Microglia and Neuroinflammation

Simultaneous increased expression of IL-1 and microglia activation in the CNS are frequently associated with neuroinflammation and considered indicators of pathogenesis in CNS diseases. Indeed, in numerous studies, elevated levels of IL-1 and changes in microglial morphology have been used as hallmarks of CNS inflammation. Based on the notion that IL-1 stimulates monocytes/macrophages (the peripheral counterparts of microglia) to amplify inflammation in the peripheral immune system, it has been proposed a central role for CNS IL-1 and microglia in influencing functions of the nervous system. Although the central nervous system exhibits immunological privileges that restrict amplification of inflammation to stringent control, it is now clear that increased CNS IL-1 expression together with microglial activation amplifies neuroinflammation contributing to neuropathology. However, microglia participate in many neural functions that are not related to immunological activities, and several studies evaluate the contribution of immunological and non-immunological aspects of microglia/IL-1 interaction in the CNS and how these aspects might affect health and disease in the nervous tissue [for review see Liu X and Quan N, 2018].

CNS glia (microglial cells and astrocytes) produce and release high amount of IL-1β under conditions of damage, stress, and disease and this cytokine induces production of additional pro-inflammatory cytokines and growth factors, thereby promoting inflammatory activity in the brain [Benveniste EN, 1992; Merrill JE and Benveniste EN, 1996]. The main source of IL-1 is microglial cells, which have also been suggested as targets for its action.
Microglia are resident cells of the brain involved in regulatory processes critical for development, maintenance of the neural environment, response to injury, and subsequent repair. Microglia sense pathological events in the CNS and serve as brain immune cells to orchestrate innate immune responses. They are the resident mononuclear phagocytes of the central nervous system and share phenotypic characteristics and innate immunological functions with other mononuclear phagocytes. Similar to macrophages, M1 polarized microglia can produce pro-inflammatory cytokines and mediators such as interleukin IL-1β, IL-6, tumor necrosis factor-α, CC-chemokine ligand 2, nitric oxide, and reactive oxygen species, suggesting that these molecules contribute to dysfunction of neural network in the CNS. On the other hand, M2 polarized microglia can produce anti-inflammatory cytokines, such as IL-10, and express several receptors that are implicated in inhibiting inflammation and restoring homeostasis. Given the diversity, plasticity, and immunoregulatory functions of M1 and M2 microglia in neurodegenerative and neurological disorders, it has been proposed that an imbalance between M1 and M2 polarization of microglia contributes to these pathological conditions [Nakagawa Y and Chiba K, 2014] (Figure 6).

**Figure 5. M1/M2 polarization of microglia and their immunoregulatory functions.** Resting microglial cells are stimulated with PAMPS or DAMPS via TLR or ATP receptors. In the presence of LPS and IFN-γ, microglial cells polarize to M1 phenotype and produce pro-inflammatory cytokines/mediators including IL-1β, IL-6, TNF-α, CCL2, ROS, and NO. In contrast, IL-4 and IL-13 induce alternative activation of microglia to M2 (‘M2a’) phenotype which down-regulates M1 functions by anti-inflammatory cytokine, IL-10. (Adapted from: Yutaka Nakagawa and Kenji Chiba, Pharmaceuticals (Basel) 2014)
1.2.5 IL-1β and Synapses

The hippocampus is one of several brain regions that together comprise the hippocampal formation. The hippocampal formation is a prominent C-shaped structure bulging in the floor of the temporal horn of the lateral ventricle and consisting of four cytoarchitectonically distinct regions: the entorhinal cortex, the dentate gyrus, the subicular complex and the hippocampus proper. The hippocampus proper consists of three major subfields (CA1, CA2 and CA3). The hippocampus is an anatomical component of the limbic system and has extensive connections with the sensory, as well as with the motor cortices. Based on its extrinsic connectivity, the hippocampal formation receives a vast amount of highly processed multimodal sensory information that is funneled into the hippocampal formation mainly by the entorhinal cortex, which is connected to associational neocortical areas in a reciprocal manner. Extensive hippocampal integration of sensory information is established by a largely unidirectional chain of intrinsic hippocampal projections. Indeed, the hippocampus features a unique unidirectional excitatory projection pattern which starts in the entorhinal cortex that projects to the dentate gyrus via the perforant pathway. The granule cells of the dentate gyrus project to the CA3 subfield of the hippocampus proper via the mossy fibres. The CA3 pyramidal neurons give rise to association fibres terminating in the contralateral CA3 as well as to projections further to the CA1 subfield via the Schaffer collaterals. The subicular complex is the target for the main output pathway of the CA1 pyramidal cells. Even if the current knowledge on hippocampal connectivity and function is largely based on studies of rodents and monkeys and it still remains to be determined to which extent such neuroanatomical data of experimental animals is applicable to the human hippocampal formation, there is a compelling evidence for a key role of the hippocampal formation in memory and learning.

The prominent effect of IL-1 in such behavioural processes indicates that the cytokine may directly affect neuronal and synaptic function. Neurons in the hippocampus also express the type 1 IL-1 receptor, indicating that this cytokine can influence neuronal function directly, also regulating synaptic function of hippocampal neurons with consequences on the modulation of long-term potentiation (LTP) [Katsuki H et al., 1990; Bellinger FP et al., 1993; Murray CA and Lynch MA, 1998; Kelly A et al., 2001; Srinivasan D et al., 2004]. In line with the induction of cognitive defects, excessive IL-1β affects long-term potentiation (LTP), the synaptic process which underlies learning and memory. Indeed, it has been demonstrated that high concentrations of IL-1 act upon neurons to inhibit synaptic strength and LTP [Katsuki H et al., 1990; Bellinger FP et al., 1993; Murray CA and Lynch MA, 1998] in several regions of the hippocampus, including CA1 [Bellinger FP et al., 1993; Ross FM et al., 2003], CA3 [Katsuki H et al., 1990], and dentate gyrus [Murray CA and Lynch MA,
Of note, the synaptic potentiation processes are not only affected in different pathological scenarios, but also during aging, when the overproduction of IL-1β and/or a synapse-specific IL-1 receptor subunit reconfiguration may produce specific deficits in consolidation of hippocampus-dependent memory [Patterson SL, 2015; Prieto GA et al., 2015] and also during stress-related conditions, a particular pathophysiological state which might deeply affect immature brain [Barron H et al., 2017; Depino AM, 2017; Schiavone S and Trabace L, 2017]. In contrast, physiological levels of IL-1 promote LTP and memory formation [Yirmiya R et al., 2002]. The distinct functions of IL-1 are mediated through the same type 1 IL-1 receptor (IL-1R1), which is expressed and regulated by IL-1 in both hippocampal neurons and glial cells [Friedman WJ, 2001]. However, the signalling pathways for IL-1 in these cell types differ [Srinivasan D et al., 2004]: the pathway involving activation of the three MAPKs (ERK, JNK, and P38) is present only in neurons [Huang Y et al., 2011].

IL-1β and its receptors have important roles in a wide range of processes, including neurogenesis and synapse formation and plasticity, from early prenatal CNS development to postnatal development and adulthood [de la Mano A et al., 2007; Yoshida T et al., 2011; Valnegri P et al., 2011; Yoshida T et al., 2012]. IL-1β exerts its biological functions by activating the IL-1R1 and then recruiting a signalling core complex consisting of the myeloid differentiation primary response protein 88 (MyD88) and the IL-1R accessory protein (IL-1RAcP). Although this pathway has been clearly described in the peripheral immune system, very limited information about the molecular composition and distribution of its members in neuronal cells are available. Recent findings showed that IL-1R1, as well as its accessory proteins MyD88 and IL-1RAcP, are differently distributed in the hippocampus and in the subcellular compartments of primary hippocampal neurons in primary cultures. In particular, IL-1R1 and IL-1RAcP are enriched at synaptic sites, where they co-localise with and bind to the GluN2B subunit of NMDA receptors (Figure 5) [Gardoni F et al., 2011].
Figure 6. Synaptic modulation by IL-1β, its receptors and related proteins. Interleukin-1β (IL-1β) exerts distinct effects at the synapse. Binding of IL-1β to IL-1 receptor type 1 (IL-1R1) recruits the IL-1 receptor accessory protein (IL-1RAcP), which increases NMDAR signaling. Unbound IL-1RAcP acts as a trans-synaptic adhesion molecule through its interactions with presynaptic protein tyrosine phosphatase-σ (PTPσ), PTPδ and leukocyte common antigen related (LAR). Another member of the IL-1 receptor family, IL-1R accessory protein-like receptor 1 (IL-1RAPL1), also acts as a synaptic organizer by binding to presynaptic PTPδ. These trans-synaptic interactions exert multiple effects on synapse formation and plasticity. (Adapted from: Myka L. Estes and A. Kimberley McAllister, Nature Review Neuroscience 2015)

Furthermore, it has been observed that treatment with NMDA increases IL-1R1 interaction with NMDA receptors, as well as the surface expression and localization of IL-1RI at synaptic membranes. IL-1β also increases IL-1R1 levels at synaptic sites, without affecting the total amount of the receptor in the plasma membrane. These results confirmed the existence of a dynamic and functional interaction between IL-1RI systems and NMDA receptors that could provide a molecular basis for IL-1β as a neuromodulator in physiological and pathological conditions relying on NMDA receptor activation. They also suggested a possible involvement and contribution of IL-1β to excitotoxicity, thus opening up new possibilities for targeted inhibition strategies that can be used in IL-1β/glutamate-driven CNS diseases [Gardoni F et al., 2011].

In the last decade, extensive evidence has pointed to the concept that IL-1β directly affects synapse structure and function. Indeed, experiments performed in primary cultured neurons exposed to recombinant IL-1β revealed a significant decline in the levels of the presynaptic vesicle protein synaptophysin [Li Y et al., 2003], most likely through regulation of presynaptic functionalities by retrograde mechanisms from the postsynaptic site, and in the number of synaptic sites [Mishra A et al., 2012]. The occurrence of the harmful cytokine effects on the synapse structure has been reported also in different non-neurological disorders associated to increased IL-1β levels, like sepsis and obesity, where mice show memory impairment and reduced number of hippocampal and cortical excitatory synapses, through a mechanism fully prevented by the addition of IL-1Ra [Erion JR et al., 2014; Moraes CA et al., 2015].

Different mechanisms have been taken into account to clarify the association between excessive IL-1β levels and synaptic alterations, including the modulation of the mitogen-activated protein kinase (MAPK) pathway and the modification of trophic factor production,
such as brain-derived neurotrophic factor (BDNF) [Patterson SL, 2015]. Although it is still unclear whether IL-1β may directly act on neuronal proteins involved in modulating the structure and function of dendritic spines and known to be the molecular targets of synaptopathies, it is now an accepted fact that inflammation is an important contributor to defects in brain function, by affecting in particular cognitive processes [Rachal Pugh C et al., 2001; Huang ZB and Sheng GQ, 2010; Yirmiya, R and Goshen I, 2011] through concentration-dependent detrimental effects induced by the pro-inflammatory cytokine IL-1β. Indeed, recent reports have highlighted the ability of IL-1β to selectively affect cell-to-cell communication in the brain, by targeting specific synaptic pathways [Mishra A et al., 2012; Han Q et al., 2017] which are known to be altered in different synaptopathies.

1.2.6 Microglia and the Process of Synaptic Pruning

Microglia play a central role in the cellular response to pathological lesions and are key players in brain injury and disease [Kreutzberg GW, 1996; Ransohoff RM and Perry VH, 2009; Kettenmann H et al., 2011]. However, they were recently shown to play unexpected functions in normal brain development and adult physiology, suggesting potential roles in postnatal development, adult neuronal plasticity, and circuit function (Figure 7).

**Figure 7. Overview of microglial behavior in the healthy brain.** Highly motile microglial processes continuously remodel their local environment (left), structurally and functionally interact with synaptic elements (middle; dendritic branch and spines, green) through direct contacts and exchanges of molecular signals and contribute to restructuring of neuronal circuits by phagocytosing synaptic elements and newborn cells (right; cellular inclusions, blue and green). Microglial morphology and behavior display variability across CNS regions and stages of the lifespan. (Adapted from: Marie-Ève Tremblay et al., *Journal of Neuroscience* 2011)
Microglial phagocytosis of apoptotic newborn neurons is very important in the process of neurogenesis. In the adult hippocampus, stem and progenitor cells persist in the subgranular zone (SGZ) of the hippocampal dentate gyrus and give rise to newborn granule cells that mature and integrate into the hippocampal circuitry over a period of a month [Kempermann G et al., 2004]. While these newborn cells participate in some forms of learning and memory, mood regulation, and fear conditioning [Kempermann G, 2008; Ming GL and Song H, 2011], the majority are pruned early during their development and undergo apoptosis in the first few days of cell life. In this basal condition, phagocytosis is performed by ramified, unchallenged microglia [Sierra A et al., 2010], in contrast to the phagocytosis by amoeboid microglia observed during neurodegeneration [Kettenmann H, 2007]. Microglia are also involved in postnatal developmental synaptic refinement as they phagocytose extranumerary synapses (Figure 8). Indeed, the first 2 weeks of postnatal development are a period of remarkable plasticity with new synapses being actively formed and remodelled. Initially, neurons make far more synaptic connections than are maintained in the mature brain. During an activity-dependent developmental process termed synaptic pruning, a large number of these immature synapses are permanently eliminated while a subset of synapses is maintained and strengthened [Katz LC and Shatz CJ, 1996; Hua JY and Smith SJ, 2004; Huberman A et al., 2008]. Furthermore, a recent study also suggested that microglia participate in slower, experience-dependent remodelling and elimination of synapses in the mature healthy brain [Tremblay MÈ et al., 2010] (Figure 8), contributing to the reorganization of neuronal circuits. Microglial cells are also involved in adult circuit plasticity and function. Adenosine triphosphate (ATP) is a likely candidate mediating activity-dependent recruitment of microglia [Fontainhas AM et al., 2011; Li Y et al., 2012]. ATP has been revealed as a clear “find me” signal, with surveillant microglia strongly attracted to the source of ATP, and activation toward a more phagocytic phenotype occurs via a signaling pathway involving P2Y purinoceptors [Davalos D et al., 2005; Koizumi S et al., 2007; Ohsawa K and Kohsaka S, 2011]. Glutamate has also been considered a potential candidate “find-me” signal mediating activity-dependent microglia migration, although its ability to attract microglia may only hold for a more activated phenotype [Fontainhas AM et al., 2011]. Once microglia are recruited to synapses via a “find-me” signal, one of the potential consequences can be phagocytosis in response to an “eat-me” signal. Several molecules with homology to the peripheral immune system have been shown to play some role in triggering phagocytosis of synapses. These include the major histocompatibility complex (MHC-1) and the complement cascade proteins, including C1q and C3 (Figure 8). Indeed, recent studies showed that the immune complement molecules C1q, C3 and the receptor CR3 (CD11b/CD18) are key molecules contributing to microglial phagocytosis of neurites and synapses [Stevens B et al., 2007; Schafer DP et al., 2012].
C1q is the initiating protein of the classical complement cascade, which is part of the innate immune system. When C1q binds to and coats (opsonizes) synapses, it triggers a protease cascade which leads to the deposition of the downstream complement protein. Opsonization with activated C3 fragments (C3b and iC3b) leads to synapse pruning directly activating C3 receptors on macrophages or microglia, thereby triggering elimination by phagocytosis [Stevens B et al., 2007]. For instance, retinal ganglion cell (RGC) neurons express C1q, the upstream signalling molecule of C3, at P5 [Stevens B et al., 2007]. The receptor molecule for C3, CR3 is expressed in microglia [Schafer DP et al., 2012]. Genetic deletion of either the C3 ligand, or the CR3 receptor, reduced inclusions of presynaptic terminals in microglia, indicating a decreased microglial synapse phagocytosis [Schafer DP et al., 2012].

![Postnatal Development](image1)

**Postnatal Development**

![Healthy Adult Brain](image2)

**Healthy Adult Brain**

Figure 8. Microglia actively survey synapses and refine neuronal circuits in the healthy brain. During early postnatal development (left), microglia (illustrated in green) help refine excessive synaptic connections (illustrated in blue). Insert highlights microglial engulfment of synaptic elements. Microglia-related proteins including complement proteins and fractalkine have been suggested to mediate this process. In the healthy adult brain (right), microglial processes are dynamic and continuously survey surrounding synapses. (Adapted from: Soyon Hong et al., *Current Opinion in Neurobiology* 2016)

Together, these findings indicate that microglia not only orchestrate brain inflammation (see 1.2.4 - IL-1β, Microglia and Neuroinflammation) but also operate in the healthy brain, thus contributing to normal brain physiology. A role of microglia as immunological and non-
immunological key player in brain thus emerges. While IL-1 primarily contributes to the microglia immunological role, it is still to be clarified whether and in which extent this cytokine also influences the non-immunological of microglia roles (Figure 9).

**Figure 9. IL-1 and microglial activation.** Interleukin-1 activity and microglial activation may influence CNS pathogenesis via dysregulation of either their immunological or nonimmunological functions. (Adapted from: Xiaoyu Liu and Ning Quan – *Frontiers in Neurology 2018*)
AIM OF THE PROJECT

The general aim of this project is to obtain a better understanding of how the proinflammatory molecule interleukin-1 beta (IL-1β) contributes to the physiological development of central neuronal circuits. Indeed, in the past decade or so, it has been shown the bidirectional communication between the central nervous system and the immune system in brain diseases, highlighting how immune molecules, such as the proinflammatory cytokine IL-1β, affect several brain functions participating in the pathogenic mechanisms of many neurological illnesses. There is now abundant evidence suggesting that IL-1β plays a detrimental role in hippocampal synaptic function [Avital A et al., 2003], and that the IL-1 type I receptor (IL-1RI) is essential for an efficient activation of microglia and the induction of multiple proinflammatory mediators in response to brain injury [Basu A et al., 2002]. Hence, the possibility that IL-1β may trigger some of the detrimental changes observed in certain neurodegenerative diseases is currently being assessed. However, despite most of these studies investigated the role of IL-1β in inflammatory-associated pathologies, its physiological role in synapse formation and function in the healthy brain is not completely known. Moreover, the finding that IL-1RI was highly expressed even in healthy states in the hippocampus, an area of the brain which plays a pivotal role in memory and learning, supports a possible physiological role for IL-1β in brain development and function.

The aim of the project is to investigate the contribution of IL-1β to brain homeostasis using an IL-1R−/− mouse model, in order to clarify whether and how IL-1 signalling may alter the normal brain development and functionality. By taking advantage of this model, we will evaluate whether IL-1β signalling affects excitatory and inhibitory synaptic transmissions, mainly focusing on the hippocampal excitatory network. By western blot and immunofluorescence, we will first analyse the expression levels of both excitatory and inhibitory synaptic markers in brain homogenates and then, to assess whether possible changes in their expression have consequences on neurotransmission, we will perform electrophysiological recordings of post-synaptic currents in hippocampal neurons from acute brain slices from WT and knock-out mice. Golgi-staining of brain slices for the detection of dendritic spine density in hippocampal neurons will help in further assessing the role of IL-1 signalling in the formation and maintenance of the excitatory synapses during development. We will also analyse whether possible functional changes in the excitatory network result from neuron-autonomous processes or from an altered impact of non-
neuronal cells on synapse development. To achieve this, we will characterize cell-specific properties in either neuronal or glial primary cultures established from WT and knock-out mice. With regards to neurons, we will analyse both expression levels of excitatory synaptic markers by western blot and strength of excitatory neurotransmission by patch-clamp recordings in hippocampal neuronal cultures. Given the importance of microglia cells in normal brain development, we will investigate the role played by IL-1 in shaping the function of these immune-resident brain cells by first evaluating possible changes in their functional state in both primary cultures and brain slices. Taking advantage of neurons-microglia co-cultures, we will then further investigate the possible interplay between microglial cells, IL-1β and synaptic contact refinement. Indeed, microglial cells are known to be the main key players in synaptic pruning. The subsequent elucidation of the molecular mechanism by which IL-1β alters synaptic transmission through the involvement of microglial cells will represent an important and emerging field of research in neuroscience. To finally assess whether possible altered enhanced excitatory neurotransmission and increased density of dendritic spines are associated to behavioural defects in knock-out mice, we will perform behavioural tests aimed to evaluate both cognitive and social capabilities.
MATERIAL AND METHODS

Animals

IL-1R knock-out C57BL/6 mice were obtained from Istituto Clinico Humanitas IRCCS, Milan, Italy. Primary hippocampal cultures were obtained from E17 embryos, while primary glial cultures were obtained from brains of neonatal mice (P0-P4). Tissues for WB analysis were taken from 9-day, 1-month and 7-month old male animals. For behavioural tests, 4-month old male mice were used. Based on the evidence that gender-related differences, determined by genetic and hormonal influences, affect brain anatomy, function and behaviour, only male mice were used for experiments [Ngun TC et al., 2011; Jäncke L, 2018; Choleris E et al., 2018]. No power calculations were done to estimate the adequate animal group size. All the experimental procedures followed the guidelines established by the European Legislation (Directive 2010/63/EU), and the Italian Legislation (L.D. no 26/2014).

Primary Cell Cultures

Neuronal Cultures

Mouse hippocampal neurons were obtained from the hippocampi of embryonic stage E17-18 fetal mice. The dissociated cells were plated onto 24 mm glass coverslips coated with poly-L-lysine at a density of 80,000 cells per coverslip. Cells were maintained in neuronal medium containing Neurobasal (Invitrogen, San Diego, CA) with 2% B27 supplement, 1% antibiotic (penicillin-streptomycin), 1% glutamax, and incubated in a humidified at 37°C incubator supplemented with 5% CO₂.

Glial Cultures

Glial cultures were prepared from the brains of 0-3-day old WT and IL-1R knock-out mice. Briefly, cells from whole brains were collected in glial medium containing Eagle’s Minimum Essential Medium (EMEM - Life Technologies) with 20% heat-inactivated fetal bovin serum (FBS, Sigma), 0.6% glucose, 1% penicillin-streptomycin 100X (10,000 units penicillin and 10 mg streptomycin/mL), 1% Na-pyruvate (100 mM), seeded into 75-cm² tissue culture flasks and grown at 37°C in a 5% CO₂ humidified atmosphere. Secondary
pure microglial cultures were harvested from flasks of mixed glial cultures by shaking at 245 revolutions per minute (r.p.m.) for 45 minutes 10-12 days after dissection. Cells were collected by centrifugation (800g for 10 min, RT), and plated in multiwells (150,000 cells per well in 12 well plates) coated with poly-ornithine hydrobromyde (100 μg/ml). Cells were further cultured in EMEM supplemented with 20% FBS for 2 days before experiments. For microglia and neuron co-culture experiments, WT or knock-out microglia were added to mature hippocampal neurons (13-14 days in vitro) at a microglia to neuron ratio of 1:2 for 24 hours.

Electrophysiology

Brain Slice Recordings

To obtain acute hippocampal slices, IL-1Rα−/− and WT mice aged 26-30 days were deeply anesthetized as previously described and decapitated. Brains were removed and placed in ice-cold solution containing the following (in millimolar): 87 NaCl, 21 NaHCO₃, 1.25 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, 2.5 KCl, 25 D-glucose, and 7 sucrose, equilibrated with 95% O₂ and 5% CO₂ (pH 7.4). Coronal slices (300 μm thick) were cut with a VT1000S vibratome from the hippocampus. Slices were incubated at RT for at least 1 h, in the same solution as above, before being transferred to the recording chamber. During experiments, slices were perfused at 2.0 mL/min with artificial cerebrospinal fluid (ACSF) containing the following (in millimolar): 135 NaCl, 21 NaHCO₃, 1.6 CaCl₂, 3 KCl, 1.25 NaH₂PO₄, 1.8 MgSO₄, and 10 D-glucose, aerated with 95% O₂ and 5% CO₂ (pH 7.4). Cells were examined with a BX51WI upright microscope (Olympus) equipped with a water immersion differential interference contrast (DIC) objective and an infrared (IR) camera (XM10r Olympus). Neurons were voltage clamped at a holding potential of -70 mV with a Multiclamp 700B patch-clamp amplifier (Molecular Devices, Union City, CA) at RT. Signals were amplified, sampled at 10 kHz, filtered to 2 or 3 KHz, recorded using the pClamp 10 data acquisition and analysed with either pClampfit 10 (Axon Instruments, Foster City, CA) or MiniAnalysis (Synaptosoft, Decatur, GA). Low-resistance micropipettes (2–3 MΩ) were pulled from borosilicate glass. Series resistance ranged from 10 to 20 MΩ and was monitored for consistency during recordings. Cells in culture with leak currents > 100 pA were excluded from the analysis. Miniature events smaller than 8 pA were not included in the analysis. Recordings of mEPSCs from hippocampal CA1 pyramidal neurons of WT and KO brain slices were always performed in the same experimental sessions. For miniature excitatory post-synaptic current (mEPSC) recordings, 1 μM tetrodotoxin (TTX), 20 μM bicuculline and 50 μM amino-5-phosphonopentanoic acid (AP5) were added to the ACSF. Pipettes
contained (in millimolar): 135 K+-gluconate, 1 EGTA, 10 HEPES, 2 MgCl2, 4 Mg-ATP, and 0.3 Tris-GTP (pH 7.4). Investigators were not blinded to the genotype of the mice during both electrophysiological recording and data analysis.

**Cell Culture Recordings**

Whole cell voltage-clamp recordings were performed on WT and transgenic embryonic hippocampal neurons maintained in culture for 13-15 DIV. During recordings, cells were bathed in a standard external solution containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2 CaCl2, 6 glucose, and 25 HEPES-NaOH, pH 7.4. Recording pipettes (resistances of 3-5 MΩ) were filled with a standard intracellular solution containing (in mM): 135 K+-gluconate, 1 EGTA, 10 HEPES, 2 MgCl2, 4 Mg-ATP and 0.3 Tris-GTP, (pH 7.4). For mEPSC recordings, 1 µM tetrodotoxin, 20 µM Bicuculline and 50 µM AP5 were added to the standard extracellular solution to block spontaneous action potential generation by GABA-A and NMDA receptors. Recordings were performed in voltage clamp mode at a holding potential of -70 mV using a Multiclamp 700B amplifier (Axon Instruments, Foster City, CA). Signals were amplified, sampled at 10 kHz, filtered to 2 or 3 KHz, recorded using the pClamp 10 data acquisition and analysed with either pClampfit 10 (Axon Instruments, Foster City, CA) or MiniAnalysis (Synaptosoft, Decatur, GA). Series resistance ranged from 10 to 20 MΩ and was monitored for consistency during recordings. Cells in culture with leak currents > 100 pA were excluded from the analysis. Miniature events smaller than 8 pA were not included in the analysis. Electrophysiological mEPSC recordings from WT and KO neurons were always performed in the same experimental sessions. Investigators were not blinded to the genotype of the mouse cultures during both electrophysiological recording and data analysis.

**Immunofluorescent Staining on Free-Floating Sections**

Male mice were deeply anesthetized with intra-peritoneal injections of ketamine/xylazine (ketamine: 100 mg/kg; xylazine 10 mg/kg) and perfused intracardially with 4% paraformaldehyde (PFA). Immunofluorescent staining was carried out on free-floating sections as described in [Menna et al., 2013]. Free-floating sections at the level of dorsal hippocampus were processed with specific primary antibodies at 4°C over-night, followed by incubation with the secondary antibodies for 2 hours at room temperature, counterstained with DAPI and mounted in Fluorsave (Calbiochem, San Diego, CA, USA). Sections were examined by means of an Olympus FV1000 confocal microscope (Olympus, Japan). Images were acquired in the stratum pyramidale or stratum radiatum of the CA1 subfield of the hippocampus (as indicated) using a x40 oil immersion lens with additional
electronic zoom factor of up to 3, maintaining the parameters of acquisition (laser power, pinhole, gain, offset) constant among groups.

**Microglia Count and Morphological Analysis**

For microglial counts and morphologic analysis, confocal images for the selected marker Iba-1 were modified as 8-bit and Z-stack projection images. Iba-1 and DAPI+ cells were counted per HPF. The resulting images were smooth processed, binarized and skeletonized, using the Skeletonize Plugin for ImageJ [Arganda-Carreras I et al., 2010]. Before quantification, a mask using the particle analysis function was created to subtract from skeletonized images. The resulting images were processed by choosing the Analyze Skeleton 2D 3D option in the Skeletonize Plugin, and the number of branches and junctions per cell were obtained from the Results tables.

**Golgi Staining**

Mice (P60) were anesthetized and perfused intracardially as described above (immunofluorescent staining section). The brains were quickly but carefully removed and then transferred into individual Falcon tubes with Golgi-Cox solution and stored at room temperature in darkness for 6 days. After this period of impregnation, brains were moved to a sucrose solution (30%) for at least 2 days. Sectioning of the brains in a 6% sucrose solution followed. Coronal sections of 100 µm thickness were obtained using a vibratome (VT1000S, Leica, Wetzlar, Germany). These sections were placed onto gelatinated coverslips, washed in dH$_2$O and processed with ammonium hydroxide for 30 min, followed by 10 min in Kodak Film Fixer, and were finally rinsed with distilled water, ethanol and xilene and finally mounted with a xylene-based medium. Spine density was estimated on branches of both apical and basal dendrites of pyramidal neurons located in the CA1 subfield of the hippocampus.

**Transfection and Dendritic Spine Analysis**

Mouse hippocampal neurons were transfected with GFP-plasmids using Lipofectamine 2000 (Invitrogen) 2-3 days before experiments or microscope image acquisition, at 11-12 days in vitro (DIV). Neurons were incubated with a pre-warmed DNA-Lipofectamine solution in a humidified 37°C incubator supplemented with 5% CO$_2$ for 30 minutes. Cultures were analysed at DIV15 after fixation in 4% PFA and 4% sucrose for 10
minutes at RT. Images of primary hippocampal cultures were acquired with a 60× oil immersion lens (1,024 × 1,024 pixels) using a confocal microscope (FV1000 Olympus confocal microscope). GFP signal was acquired using an argon laser line, exciting at 488 nm and collecting with a band-pass filter at 505-530 nm. Each image consisted of a stack of images taken through the z-plane of the cell. Confocal microscope parameters were kept constant for all scans in each experiment. Dendritic spine density was analysed using ImageJ software. For spine morphological analysis, spines were classified into the following categories: 1) Thin: spines with a long neck and a visible small head; 2) Mushroom: big spines with a well-defined neck and a very voluminous head.

**Western Blot Analysis**

Samples were collected in a lysis buffer either from primary cultures or tissue homogenates. Protein were resolved in 8-12% sodium dodecyl sulphate-polyacrylamide gels under reducing conditions. After over-night transfer onto polyvinylidene difluoride membranes at 80 mA, blots were blocked for 1 h at room temperature in a 5% milk or 5% BSA solution in Tris-buffered saline with 0.1% Tween-20 (TBS-T) at pH 7.4 and then incubated at 4°C overnight with primary antibodies diluted in 5% milk or 5% BSA TBS-T solution. Subsequently, membranes were washed and incubated for 1 h at room temperature in 5% milk or 5% BSA TBS-T solution with the secondary antibodies. Western blotting was performed by means of Chemi-Doc system + Image Lab software (Bio-Rad). Photographic development was by chemiluminescence (ECL, Amersham Bioscience or Immobilon substrate, Millipore). Western blot bands were quantified by the Image Lab software.

**Real-Time PCR**

Total RNA was extracted from WT and IL-1R<sup>+</sup> hippocampus using TRIzol (Invitrogen). 1 μg RNA was reverse transcribed into first-strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™ – Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer’s protocol. Quantitative PCR was performed using the TaqMan™ Fast Universal PCR Master Mix (2X) (Thermo Fisher Scientific). The following primers were used: 18s, AIF-1, TREM2, C1qA, C1qB, C1qC and TGF-β. All primer sets were purchased from Thermo Fisher Scientific.
**ELISA Assay**

Concentrations of cytokines that are typically secreted by microglia (pro-inflammatory cytokines IL-6, IL-1β, TNF-α, and anti-inflammatory cytokines IL-10, IL-4) were assessed in the cell culture medium using ELISA kits (Peprotech) following manufacturers’ instructions. The absorption values were measured at a wavelength of 450 nm with a reference wavelength of 630 nm. Optical densities (OD) were plotted on a standard curve and expressed as pg/ml.

**Behaviour**

**Self-Grooming**

WT and IL-1R knock-out mice were scored for spontaneous grooming behaviors as described earlier [Silverman JL et al., 2010] with slight modifications. Each mouse was placed individually into a clean mouse cage with a thin (0.5 cm) layer of bedding reducing neophobia, while preventing digging, a potentially competing behavior. After 5 minutes acclimatization, each mouse was scored with a stopwatch for 10 min for cumulative time spent grooming all body regions by the observer, who was not blinded to the genotype of the mice during the experimental procedure.

**Sociability and Social Recognition**

Sociability and social novelty tasks were performed on WT and IL-1R knock-out mice as previously described [Corradini I et al., 2014]. The apparatus consists of a 3-compartment transparent polycarbonate box (20x40x22(h) cm each), with two sliding doors (5x8(h)cm), opening on the central compartment, that can be closed to confine the animal. The proband mouse was habituated in the central compartment for 5 min. For sociability assessment, an unfamiliar adult C57Bl/6 male mouse was placed in one side compartment whereas the opposite contained an empty wire cage. Immediately after the sociability task, the social novelty test was carried out in the same apparatus without any cleaning, placing a new unfamiliar mouse in the wire cage that had been empty during the prior 10-min session. Familiar and unfamiliar animals from different home cages had never been in physical contact with the subject mice or with each other. For both tests, the time spent in each chamber was recorded for 10 min. The observer was not blinded to the genotype of the mice during the experimental procedure.
**Open Field**

General motor activity was tested in an open field task in WT and IL-1R$^{-}$ mice at 4 months of age. Open field test was performed as previously described [Seibenhener ML and Wooten MC, 2015]. Videotracking-based methods (Ethovision, Noldus Systems, Leesburg, Virginia) were used during single 10-min trials to record the distance moved and time spent in the arena under dim lighting, along with a delineated ‘periphery zone’, a delineated ‘non-periphery zone’, and a delineated ‘centre zone’. General locomotion and anxiety-like behaviour parameters were then analysed and compared between WT and IL-1R$^{-}$ mice. The observer was not blinded to the genotype of the mice during the experimental procedure.

**Novel Object Recognition**

A Novel Object Recognition (NOR) task was used to assess learning and long-term memory in WT and IL-1R$^{-}$ mice. Two identical objects were placed into the arena during a 10-minute sample phase. Subsequently, one of the objects was exchanged by a new object and memory was assessed by comparing the time spent exploring the novel object as compared with the time spent exploring the familiar object during a 10-minute test phase [Leger M et al., 2013; Lueptow L, 2017]. The test was conducted in an open plastic arena (60x50x30 cm) in two phases. One week before the NOR experiments, the animals experienced handling by the experimenter and habituation to the arena for 5 and 2 consecutive days, respectively (mouse handling reduces anxiety). During the first phase, lasting two days, mice were habituated to the test arena for 10 minutes. On the third day, mice were subjected to familiarization (T1) and novel object recognition (T2). During familiarization, two identical objects were placed in the arena. Each animal was placed in the centre of the arena, equidistant from the objects, and was left for a maximum of 10 minutes, then it was returned to its home cage. 120 minutes later the mouse was introduced in the same arena with one of the two objects replaced with a new one for a maximum of 10 minutes. Scoring was performed in the same manner as during familiarization. The objects used consisted of coloured plastic falcons and flasks. The arena and the objects were cleaned with 70% ethanol after each trial to remove olfactory cues. A discrimination index was calculated to evaluate the performance of each mouse as $(T_n-T_f)/(T_n+T_f)$, where $n =$ time spent exploring the object in the new location during T2, and $f =$ time spent exploring the object in the familiar location during T2. Mice who did not move in the arena were excluded from the analysis. The observer was not blinded to the genotype of the mice during the experimental procedure.
**Statistical Analysis**

Data are presented as mean ± standard error (SE) or as cumulative distribution from the indicated number of experiments. Statistical analysis and data graphing were performed using GraphPad PRISM 6 software (GraphPad, Software Inc., San Diego, CA, USA). After testing whether data were normally distributed or not, the appropriate statistical parametric or non-parametric test was used. The Kolmogorov–Smirnov test was used to determine significance in cumulative distributions of mEPSC amplitudes and some integrated density values. Differences between multiple groups were analysed by one-way ANOVA with a Bonferroni’s or Tukey’s post hoc test. Comparisons between two groups with a normal distribution were analysed using an unpaired t-test (two-tail distribution) or a Mann-Whitney test. Statistical analysis was performed using GraphPad Prism Software. The differences were considered to be significant if P≤0.05 and are indicated by (*); those at P≤0.01 are indicated by (**); those at P≤0.001 are indicated by (***); those at P ≤ 0.0001 by four (****).
**RESULTS**

*IL-1R<sup>-/-</sup> Mice Display Increased Synapse Density and Enhanced Excitatory Neurotransmission*

We used IL-1 receptor-deficient mice [Labow M et al., 1997; Glaccum MB et al., 1997] to examine the possible role of this cytokine in the development and function of glutamatergic synapses. To this aim, we firstly performed a morphological analysis of glutamatergic synaptic terminals through immunohistochemical analysis conducted at various stages of development (1-7 months), using antibodies against the vesicular glutamate transporter 1 (VGlut1) (*Figure 10B*) and the postsynaptic density protein 95 (PSD95) (*Figure 10D*) in the CA1 hippocampal region (stratum radiatum). The analysis of confocal images revealed that IL-1R deficiency induces a significant increase of the immunopositive area of these excitatory synaptic markers in the CA1 hippocampal region, in both young (P30) and adult (7 months) knock-out mice (*Figure 10C* and *10E*). These data indicate that the excitatory synaptic network is affected by IL-1 signalling perturbation.
Figure 10. Enhanced Immunostaining for the Excitatory Synaptic Markers VGlut1 and PSD95 in the Hippocampus of IL-1R−/− Mice. Representative fields (B and D) and relative quantification (C and E) of VGlut1 (B and C) and PSD95 (D and E) immunostaining in the CA1 hippocampal region of WT and IL-1R−/− mice at different ages (1 month and 7 months). A shows a cartoon depicting hippocampal architecture; in red the stratum radiatum of CA1. Scale bar, 50 µm. **p < 0.01 ***p < 0.001, ****p < 0.0001, unpaired t test. For immunostaining: at 1 month, WT N = 2 mice (n = 6 slices, n = 48 fields), IL-1R−/− N = 2 mice (n = 6 slices, n = 56 fields); at 7 months, WT N = 2 mice (n = 6 slices, n = 53 fields), IL-1R−/− N = 2 mice (n = 6 slices, n = 54 fields).

To evaluate whether the increased staining of glutamatergic synaptic terminals was paralleled by higher expression levels of pre- and post-synaptic excitatory proteins, western blot analysis of the vesicular glutamate transporter VGlut1 and the post-synaptic density protein PSD95 was performed from brain lysates obtained from hippocampus and cortex of IL-1R−/− mice and WT at distinct stages of development (1-5-7 months) (Figure 11A and...
Variations of VGlut1 and PSD95 are expressed as a Fold Change, which is calculated by dividing the mutant value by the wild type value. We found a general increase in the expression levels of these synaptic proteins in both the hippocampus and cortex of IL-1R<sup>−/−</sup> mice, especially at 7 months of age (Figure 11B and 11D).

Figure 11. Excitatory Synaptic Marker Expression Is Increased in IL-1R<sup>−/−</sup> Brains. Western blot analysis (A and C) and quantification (B and D) of PSD95 and VGlut1 in the whole cortex (A and B) and hippocampus (C and D) of WT and IL-1R<sup>−/−</sup> mice at different ages (1 month, 5 months and 7 months). *p < 0.05, **p < 0.01, unpaired t test. For western blotting: at 1 month, WT N = 4 mice, IL-1R<sup>−/−</sup>
To assess whether the higher expression of glutamatergic synaptic markers was paralleled by an enhancement in excitatory neurotransmission, whole-cell patch-clamp recordings of miniature excitatory postsynaptic currents (mEPSCs) (Figure 12C) were performed from the CA1 pyramidal neurons in acute hippocampal brain slices established from IL-1R$^\sim$ and control mice (P26-P30). Given the well-established role of IL-1β in cognition, we focused our electrophysiological characterization on CA1 hippocampal neurons because it has been reported that, within the hippocampal memory system, they are critically involved in the formation, consolidation, and retrieval of hippocampal-dependent memories [Bartsch T et al., 2011]. In line with the histochemical data, both amplitude and frequency of mEPSCs were enhanced in the hippocampus of knock-out mice (Figure 12D and 12E). WT and knock-out hippocampal pyramidal neurons showed no differences in their passive membrane properties (membrane capacitance, Cm, and membrane resistance, Rm, Figure 12A and 12B, respectively). This data confirms that the increased excitatory transmission observed in IL-1R$^\sim$ hippocampal pyramidal neurons depends on changes in synaptic activity rather than on alterations in the basic passive membrane properties of the neurons.
**Figure 12. Glutamatergic Transmission is Increased in the Hippocampus of IL-1R$^−/−$ Mice.** Passive membrane properties of WT and knock-out hippocampal pyramidal neurons are shown in A, membrane capacitance (Cm) and B, membrane resistance (Rm). Representative traces (C) of mEPSC recordings in CA1 pyramidal neurons from WT and IL-1R$^−/−$ hippocampi. Summary graph of mEPSC frequency (D) and amplitude (E). mEPSC frequency: *p < 0.05  **p < 0.01, unpaired t test. Image scale bar 10 μm. Voltage traces: voltage scale bar, 50 pA; time scale bar, 10 s. Membrane capacitance: WT 74.23 ± 8.49, n = 13 cells; IL-1R$^−/−$ 68.64 ± 9.31, n = 11 cells. Membrane resistance: WT 87.85 ± 8.2, n = 13 cells; IL-1R$^−/−$ 87.55 ± 10.67, n = 11 cells. mEPSC frequency: WT 1.47 ± 0.14, N = 4 mice (n = 14 cells); IL-1R$^−/−$ 1.96 ± 0.16, N = 4 mice (n = 13 cells). mEPSC amplitude: WT 12.34 ± 0.36; IL-1R$^−/−$ 15.07 ± 0.57. Data obtained with the contribution of Dr Raffaella Morini.

In line with these data, analysis of spine density performed by Golgi staining (Figure 13A) in P60 CA1 pyramidal neurons revealed a significant increase in the number of dendritic protrusions/dendrite length in IL-1R$^−/−$ mice when compared to WT (Figure 13B and C). Given the evidence that the majority of excitatory synapses are formed on the surface of dendritic spines [Amaral MD and Pozzo-Miller L, 2009], all together these data indicate that the lack of IL-1 receptor results in an elevated number of glutamatergic synapses in the adult brain, thus suggesting a key role of the IL-1 signalling in regulating the formation or maintenance of excitatory network during brain development.
**Figure 13.** IL-1R<sup>−/−</sup> Mice Display Increased Dendritic Spine Density.

Representative images (A) of hippocampus (upper panels) and secondary branches of dendrites (lower panels) of WT and IL-1R<sup>−/−</sup> CA1 neurons (P60) stained by the Golgi-Cox method and relative quantitation in apical (B) and basal (C) dendrites. Number of spines/mm: **p < 0.01, ****p < 0.001, unpaired t test. Scale bar, 250 µm; scale bar zoomed-in images, 25 µm. Apical dendritic spine density quantitation: WT 1.0 ± 0.04, N = 4 mice (n = 20 neurons); IL-1R<sup>−/−</sup> 1.24 ± 0.06, N = 4 mice (n = 16 neurons). Basal dendritic spine density quantitation: WT 0.9 ± 0.03, N = 4 mice (n = 20 neurons); IL-1R<sup>−/−</sup> 1.19 ± 0.06, N = 4 mice (n = 16 neurons). Data obtained with the contribution of Dr Antonella Borreca.

The structural and functional enhancement of the excitatory synaptic network observed in IL-1R<sup>−/−</sup> brain could result from the alteration of processes intrinsic to neuronal cells (neuron-autonomous processes) or could result from an altered impact of non-neuronal cells on synapse development. In order to discriminate between these two possibilities, primary neuronal cultures were established from hippocampi of WT or IL-1R<sup>−/−</sup> embryos (E17). Mature primary hippocampal cultures (days in vitro 14, DIV14) are known to contain a moderate amount of astrocytes and to be completely devoid of microglia [Seibenhener ML and Wooten MW, 2012].
Previous works evaluated the relationship between age and maturity of a culture, as displayed by connections among neurons or spiking activity [Ben-Ari Y, 2001; Biffi E et al., 2012]. Regardless of the tissue origin of the culture, at 7 Days In Vitro (DIV), neuronal cultures generally show a lower synaptic density and less neuronal cell connectivity with respect to older stages, with a peak at 14 DIV [Ichikawa M et al., 1993], which reflects the maturation of the network paralleled by that of the electrophysiological properties. Indeed, at 7 DIV the electrical activity is characterized by only single spikes whereas at 14 DIV networks exhibit an increase in firing rate, a rich and stable burst pattern (i.e. episodes of high frequency spiking) and highly synchronized periods of high frequency activity, encompassing simultaneously different network sites [Chiappalone M et al., 2006; Wagenaar DA et al., 2006]. Since DIV14 is considered a stage in culture where the level of neuronal maturation reflects the one that can be observed in-vivo in adult mice, we performed patch-clamp recordings on primary cultures of WT and knock-out DIV14 hippocampal neurons (Figure 14C) revealed no changes in their passive membrane properties (membrane capacitance, Cm, and membrane resistance, Rm, Figure 14A and 14B, respectively) and, interestingly, similar mEPSCs frequency and amplitude in the two groups (Figure 14D and 14E).

**Figure 14. IL-1R Deficiency Does Not Affect Neuronal Activity in Primary Cultures of Hippocampal Neurons.** Passive membrane properties of WT and knock-out neurons are shown in A (membrane capacitance) and B (membrane resistance). Representative traces of mEPSCs (C) recorded from WT and IL-1R⁻⁄⁻ cultured hippocampal neurons in vitro (ad 14 days in vitro, DIV14) and quantification of frequency (D) and amplitude (E) of mEPSCs. Scale bars, 50 pA, 10 s. Mann-Whitney unpaired t test. Membrane capacitance: WT 111.4 ± 5.27, n = 50 cells; IL-1R⁻⁄⁻ 109.5 ± 3.36, n = 55 cells. Membrane resistance: WT 174.8 ± 8.94, n = 50 cells; IL-1R⁻⁄⁻ 175.1 ± 8.43, n = 55 cells. mEPSCs frequency: WT 0.93 ± 0.11, n = 47 cells; IL-1R⁻⁄⁻ 0.94 ± 0.11, n = 50 cells. mEPSCs amplitude:
Consistently, western blot analysis of WT and IL-1R−/− cultured hippocampal neurons (Figure 15A) confirmed similar levels of expression of the excitatory synaptic markers PSD95 and VGlut1 at 14 days in vitro (DIV14) (Figure 15B and 15C).

Figure 15. IL-1R Deficiency Does Not Affect Excitatory Synaptic Marker Expression in Primary Cultures of Hippocampal Neurons. Western blot analysis of primary WT and IL-1R−/− hippocampal neurons at DIV14 (A) and quantification of PSD95 (B) and VGlut1 (C) expression. Unpaired t test. Bar graphs represent mean ± SEM of 3 independent experiments.

These data, revealing the absence of the synaptic phenotype in IL-1R−/− neurons when isolated from mice brain and grown in primary cultures, represent a strong indication that the enhanced development of the glutamatergic network is not an intrinsic feature of neurons, but instead a process that depends on the brain environment and that is likely to involve the participation of non-neuronal cells.

**IL-1R Deficiency Also Affects the Development of the Inhibitory Synaptic Network**

Having assessed that the genetic lack of IL-1 receptors results in the potentiation of excitatory neurotransmission, we aimed to evaluate whether its absence also impacts the development of the inhibitory network. To this aim we quantified the expression of the inhibitory pre-synaptic protein vesicular GABA transporter, VGAT, in the hippocampi of mice at 1 or 7 months of age (Figure 16A and 16B). Immunostaining of WT and IL-1R−/− brain slices for VGAT revealed an increase in the expression of this protein in the CA1 hippocampal region (stratum radiatum) of 1-month-old knock-out mice (Figure 16C and
However, a different result was observed in 7-month-old mice, where VGAT expression was slightly lower in knock-out mice relative to WT (Figure 16E and 16F). This result suggests the occurrence of age-dependent variations in the density of inhibitory GABAergic synapses in the hippocampi of IL-1R⁻/⁻ mice.

At least 23 classes of GABAergic neurons have been described in the hippocampus. We decided to focus on the major subpopulation of GABAergic neurons, the parvalbumin-immunopositive (PV⁺) cells, by immunofluorescence analysis (Figure 17A and 17B). We found that the number of PV-interneurons in the hippocampus of IL-1R⁻/⁻ mice is higher at 1 month but not at 7 months of age (Figure 17C and 17D) when compared to WT, consistent with the changes in the expression levels of VGAT. In particular, when comparing the parvalbumin-positive neurons distribution within each hippocampal sub-area (dentate gyrus DG, CA1, CA3) between WT and knock-out mice at 1 month of age (Figure 17G), we
observed that the increased number of these interneurons was statistically significant only in the CA1 region. Instead, their distribution did not differ between the hippocampal regions of 7-month old WT and knock-out mice (Figure 17H). This correlation between VGAT expression and the number of PV-interneurons may suggest that PV-interneurons are one of the subtypes of GABAergic neurons involved in the changes in VGAT expression.

Figure 17. Age-Dependent Effects of IL-1R Deficiency on Parvalbumin Interneuron Density. Representative mosaic reconstruction (A and B) of WT and IL-1R⁻/⁻ hippocampi labelled for parvalbumin (PV⁺) interneurons from mice at 1 month of age. Quantification (C and D) of parvalbumin-interneuron density in WT and IL-1R⁻/⁻ hippocampi at 1 month (C) and 7 months (D). Confocal images showing parvalbumin-immunopositive (PV⁺) cells in the CA1 hippocampal region (E and F) of WT and IL-1R⁻/⁻ mice at 1 month (E) and 7 months (F) of age and relative quantification (G and H) of parvalbumin interneurons distribution in the hippocampal regions (DG, CA1, CA3) at 1 month (G) and 7 months (H). Scale bar, 50 µm. **p < 0.01, ***p < 0.001, unpaired t test. For immunostaining: at 1 month, WT N = 2 mice (n = 6 brain slices), IL-1R⁻/⁻ N = 2 mice (n = 6 brain slices); at 7 months, WT N = 2 mice (n = 6 brain slices), IL-1R⁻/⁻ N = 2 mice (n = 6 brain slices).
Identification of The Downstream Pathway Associated to The Increased Excitatory Synaptic Network

Dendritic spines, highly specialized actin-based protrusions, are the primary site of excitatory synapses and are thought to function as the basic unit of synaptic integration [Yuste R and Denk W, 1995; Matus A et al., 2000; Bourne JN and Harris KM, 2008; Sheng M and Kim E, 2011]. Dendritic arborization and spine formation are critical for the functioning of neurons. Many proteins have recently been identified as regulators of dendritic morphogenesis, as well as several intracellular signaling pathways that control these processes. It is now well recognized the involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR and ERK pathways in the formation of functional excitatory synapses and in the control of synaptic plasticity [Sweatt JD, 2001; Kumar V et al., 2005; Howlett E et al., 2008; Cohen-Matsliah SI et al., 2008; Lee CC et al., 2011; Silingardi D et al., 2011]. Based on this evidence and on the demonstration that the intracellular pathway associated with IL-1R activation includes, among the other components, the Akt-mTOR pathway [reviewed in Riva F et al., 2012] and the phosphorylation of the extracellular signal–regulated kinases (ERK) [Kracht M and Saklatvala J, 2002; Fan Z et al., 2007] (Figure 18A), we focused our attention on these two pathways in order to identify the possible downstream signalling pathway associated to the increase in glutamatergic synapses found in the hippocampi of IL-1R−/− mice. Western blot analysis of pAKT and pERK in hippocampus homogenates obtained from 7-month old WT and knock-out mice (Figure 18B) revealed that the absence of IL-1R does not apparently affect any of these pathways (Figure 18C and 18D).

Figure 18. IL-1R Effects on Excitatory Synapses In Vivo Do Not Seem to Be Mediated By AKT/ERK Pathways. Representation of the IL-1 intracellular signalling pathway (A). Western blot analysis (B) and quantification (C and D) of
pAKT and pERK expression in the hippocampus of 7-month old WT and IL-1R\textsuperscript{−/−} mice. Unpaired t test. WT N = 5 mice, IL-1R\textsuperscript{−/−} N = 5 mice.

The recent demonstration that enhanced IL-1β signalling in the brain increases the expression level of MeCP2 in neurons, which negatively impacts synapse function, points to this factor as a potential molecular player linking inflammation and synaptic damage [Tomasoni R et al., 2017] (Figure 19A). To investigate whether IL-1R\textsuperscript{−/−} mice show a change in MeCP2 level, Western blot analysis was employed to assess MeCP2 expression levels in the hippocampus of WT and knock-out mice at 1 month and 7 months of age (Figure 19B). Consistent with the increase of the MeCP2 levels in response to enhanced IL-1β, we found a significant decrease of MeCP2 expression in knock-out mice relative to WT (Figure 19C).

**Figure 19. Decreased MeCP2 Levels are Detectable in the Hippocampi of IL-1R Deficient Mice.** Schematic representation summarizing the functional effects that follow activation of IL-1 receptor induced by IL-1β, which regulates dendritic spine morphology by upregulating the transcription factor MeCP2 in an mTOR-dependent manner [Tomasoni R et al., 2017] (A). Western blot analysis (B) and quantification (C) of MeCP2 expression in the hippocampus of WT and IL-1R\textsuperscript{−/−} mice at 1 month and 7 months of age. *p < 0.05, unpaired t test. For western blotting: at 1 month, WT N = 4 mice, IL-1R\textsuperscript{−/−} N = 4 mice; at 7 months, WT N = 5 mice, IL-1R\textsuperscript{−/−} N = 5 mice.
Given the statistically significant reduction in MeCP2 protein levels observed by western blot in knock-out mice at both 1 and 7 months of age, we decided to perform immunohistochemical analysis of MeCP2 expression in the CA1 region of 7-month old WT and IL-1R\(^{\text{−/−}}\) mice (Figure 20B). We focused on 7 months of age because we assumed it could be a time point when possible compensatory mechanisms are already balanced. Brain slices were immunostained for NeuN, which is a specific marker that labels neuronal nuclei, in order to assess whether the MeCP2 reduction occurs specifically in neurons rather than in other brain cells. The quantification of the cumulative distributions of MeCP2 integrated density values and the MeCP2 mean integrated density in neuronal cells in the two genotypes did not reveal any difference in knock-out versus WT neurons (Figure 20C and 20D). This result suggests the possibility that other brain cells other than neurons might be responsible for the reduced MeCP2 expression in IL-1R\(^{\text{−/−}}\) brain tissue.

Figure 20. Lack of Difference in the MeCP2 Expression Levels in Pyramidal Hippocampal Neurons of WT and IL-1R Deficient Mice. Representation of hippocampal regions (A); in red the stratum of CA1 pyramidal neurons.
Representative images of brain sections (CA1 hippocampus) of WT and IL-1R$^{-/-}$ mice (7 months old) stained for MeCP2 and DAPI, as indicated (B). Graphs show the cumulative distributions of MeCP2 integrated density values (C) and the MeCP2 mean integrated density in CA1 hippocampal neurons (D). Scale bar, 50 µm. Number of analysed mice and neurons: WT N = 2 mice (n = 6 brain slices, n = 48 fields), hippocampal neurons = 483; IL-1R$^{-/-}$ N = 2 mice (n = 6 brain slices, n = 48 fields), hippocampal neurons = 494. Statistical comparison: unpaired t test for D and Kolmogorov-Smirnov test for C (D value 0.07740 with a corresponding p = 0.1072).

Since it has been reported that the transcription factor MeCP2 is also important for microglial function, we investigated whether the reduced MeCP2 expression levels in KO brain was due to this cell type. To this aim, we performed MeCP2 immunocytochemical staining of microglia in the CA1 hippocampal region of 7-month old WT and IL-1R$^{-/-}$ mice (Figure 21A). Microglia were identified by the positive staining for CD39, a cell-surface ATPase highly expressed in this cell type [Braun N et al., 2000; Färber K et al., 2008; Matyash M et al., 2017]. We found a significant decrease in MeCP2 immunopositivity in microglial cells of knock-out mice compared to WT (Figure 21B and 21C). A decrease of MeCP2 expression levels was also detectable by western blotting in cultured knock-out microglial cells (Figure 21D), although not significant, suggesting that the phenotype could be maintained also in ex-vivo preparations (Figure 21E).
Figure 21. Decreased MeCP2 Levels in Hippocampal Microglia of IL-1R Deficient Mice. Representative images of brain sections (CA1 hippocampus) of WT and IL-1R−/− mice (7 months old) stained for MeCP2, CD39 and DAPI, as indicated (A). Graphs show quantitation of the MeCP2 mean integrated density (B) and the cumulative distributions of MeCP2 integrated density values (C) in CA1 hippocampal microglia. Western blot analysis (D) and quantification (E) of MeCP2 levels in WT and IL-1R−/− cultured microglia. ****p < 0.001, unpaired t test or Kolmogorov-Smirnov test. Scale bar, 10 µm. Number of analysed mice and microglial cells (A-C): WT N = 2 mice (n = 6 brain slices, n= 30 fields), hippocampal microglia = 190; IL-1R−/− N = 2 mice (n = 6 brain slices, n= 30 fields), hippocampal microglia = 192. Statistical comparison: unpaired t test for B and E; Kolmogorov-Smirnov test for C (D value 0.2438 with a corresponding p < 0.0001). Bar graph represent mean ± SEM of 2 independent experiments (E).

Lack of IL-1R Affects Microglia Number but not Microglia Morphology or Activation State During the Early Stages of Brain Development

As the lack of IL-1 receptor in microglia cells is associated with lower MeCP2 levels, we hypothesized that the synaptic defects observed in knock-out mice could be ascribed to a specific microglial dysfunction.

In order to assess whether IL-1R deficiency alters microglial phenotype, we first analysed the morphological features of microglia in IL-1R−/− and WT mice brain. Microglia density and morphology in the hippocampus was investigated at postnatal day 9 (P9), a timepoint critical for synaptic refinement [Paolicelli RC et al., 2011]. Microglia were identified through immunostaining with the myeloid cell marker ionized calcium-binding adapter molecule 1 (Iba-1) (Figure 22A and 22B). A significant increase in microglial cell density was detected in the CA1 hippocampal region of IL-1R−/− brains compared to WT, a difference which was fully compensated in older mice (Figure 22C). Similar results were obtained in the somatosensory cortex of knock-out mice (Figure 22D). Interestingly, an increase in the number of VGlut1 positive puncta was observed in the hippocampus of IL-1R−/− mice at the
same developmental stage (postnatal day 9) where the temporary higher microglia density was observed (Figure 22E-G).

Figure 22. IL-1R Deficiency Affects Microglia Number During the Early Stages of Brain Development. Confocal images of CA1 hippocampal and cortical microglia labelled for Iba-1 in brain slices obtained from WT or IL-1R−/− mice at postnatal day 9 (P9) (scale bar, 50 µm) (A and B). Quantification of microglia density (number of Iba-1+ cells per field) in the CA1 hippocampal region (C) and somatosensory cortex (D) of WT and knock-out brain at different ages (P9, 1 month, 7 months). Representative fields (F) and relative quantification (G) of VGlut1 fluorescent signal in the CA1 hippocampal region (E, representation of hippocampal regions; in red the stratum radiatum of CA1) of P9 WT and IL-1R−/−
It is known that microglia morphology and function are closely related. For instance, under basal healthy conditions, a resting phenotype characterized by a ramified morphology predominates. These microglia are not actually resting but continuously scanning their environment [Nimmerjahn A et al., 2005; Olah M et al., 2011], pruning synapses and regulating neuronal activity, providing a “fine-tuning” of neural circuits [Paolicelli RC et al., 2011; Schafer DP et al., 2012; Miyamoto A et al., 2013] and neurotransmitter signaling/synaptic transmission [Li Y et al., 2012; Béchade C et al., 2013; Domercq M et al., 2013]. However, in situations of neuroinflammation or after injury, ramified microglia can transform into an “activated state”, characterized by swollen ramified cells with a larger cell body and shorter, thick processes [Davis EJ et al., 1994; Fernández-Arjona MDM et al., 2017].

To evaluate whether IL-1 deficiency affects microglial morphology, we immunostained brain slices obtained from WT and KO mice for the microglial marker Iba1 and confocal images were processed with a morphological skeleton analysis. The morphological analysis of Iba1-positive cells in the hippocampi of P9 mice (Figure 23A and 23B) did not reveal any difference in the ramified phenotype for either genotype (Figure 23D). Furthermore, the average size of the soma, the average length of the branches and the number of branches per cell did not differ between knock-out and WT microglial cells (Figure 23C and 23D). These data show that, in IL-1R− hippocampi, microglia cells are temporarily augmented in number although their morphology does not appear to be different. This may be consistent with the possibility that their activation state is not affected. In line with this result, knock-out cultured microglia did not differ in the expression or release of anti- and pro-inflammatory cytokines, as assessed by ELISA (Figure 23E), supporting the hypothesis that the deficiency of IL-1R does not affect microglia activation.
Figure 23. IL-1R Deficiency Does Not Affect Microglia Morphology and Activation During the Early Stages of Brain Development. Confocal images of CA1 hippocampal microglia labelled for Iba-1 in brain slices obtained from P9 WT or IL-1R⁻/⁻ mice (A and B). Skeletonized reconstruction (A and B) and quantitative analysis of soma size (C), total branches per cell and average branch length (D) in Iba-1+ cells in the CA1 stratum radiatum. Quantification of IL-1β, IL-6, TNF-α, IL-10 and IL-4 release in the medium of cultured microglia by ELISA (E) did not show difference in the production of nor pro-inflammatory neither anti-inflammatory cytokines between WT and KO microglial cells. Scale bar, 50 µm. For skeletonize analysis (A-D): WT N = 2 mice (n = 4 brain slices, n = 24 hippocampal fields), IL-1R⁻/⁻ N = 2 mice (n = 4 brain slices, n = 24 hippocampal fields). Bar graph represent mean ± SEM of 3 independent experiments (E). Unpaired t test.
Lack of Microglial IL-1 Receptor Results in Defective Synapse Elimination In Vitro

Our evidence, indicating that the synaptic phenotype of IL-1R$^{-}$ neurons is not a cell-autonomous process, together with the changes in number and MeCP2 expression detected in microglia of IL-1R$^{-}$ mice, open the possibility that the overall increase of the excitatory synapses observed in IL-1R$^{-}$ mice might be due to a possible deficit in microglia function. In particular, we hypothesized that knock-out microglia may be defective in the process of synaptic pruning, which would lead to long-term effects on synapse refinement.

To test whether IL-1 signaling in microglia contributes to this process, WT hippocampal neurons were co-cultured with either WT or IL-1R$^{-}$ microglial cells (Figure 24A), and the dendritic spine density was analysed in both conditions (Figure 24B). Consistent with the ability of microglia to phagocytose synapses [Ji K et al., 2013; Lui H et al., 2016; Filipello F, Morini R et al., 2018], neurons co-cultured with WT microglia for 24 hours displayed a significant reduction of dendritic spine density (Figure 24C), as indicated by the reduction of mushroom spine (Figure 24D) and show a trend for thin spine density (Figure 24E). On the contrary, neurons cultured in the presence of IL-1R$^{-}$ microglia did show a smaller reduction of total dendritic spine density (Figure 24C). Interestingly, microglia lacking IL-1 receptor seem to partially fail to remove spines, especially mature mushroom spines (Figure 24D), indicating impaired synapse elimination. Although a direct demonstration of lower levels of synaptic markers in phagocytic organelles of IL-1R$^{-}$ microglia in vitro and in vivo [Filipello F, Morini R et al., 2018] is still required, these data suggest that the lack of the cytokine receptor in microglia impairs the synaptic pruning process.
Figure 24. Lack of Microglial IL-1 Receptor affects Spine Elimination In Vitro. Confocal images representative of hippocampal neurons co-cultured with either WT or IL-1R<sup>−/−</sup> microglia (A) and of spine density and morphology in each condition (scale bar, 10 μm) (B). Quantification of total dendritic spine (C), mushroom spine (D) and thin spine density (E) under the conditions described above. Scale bar, 50 μm; scale bar zoomed-in images, 10 μm. ****/#### p < 0.0001, One-way ANOVA followed by Dunn’s multiple comparison test. ▬ vs ▬ : *; ▬ vs ▬ : *. Bar graph represent mean ± SEM of 2 independent experiments. Number of total spines/branch length: hippocampal neurons (HN) (n = 30 neurons) 0.59 ± 0.02; HN + WT microglia (n = 32 neurons) 0.38 ± 0.01; HN + IL-1R<sup>−/−</sup> microglia (n = 40 neurons) 0.49 ± 0.01. Number of mushroom spines/branch length: hippocampal neurons (HN) (n = 30 neurons) 0.27 ± 0.02; HN + WT microglia (n = 32 neurons) 0.13 ± 0.01; HN + IL-1R<sup>−/−</sup> microglia (n = 40 neurons) 0.23 ± 0.01. Number of thin spines/branch length: hippocampal neurons (HN) (n = 30 neurons) 0.32 ± 0.02; HN + WT microglia (n = 32 neurons) 0.26 ± 0.01; HN + IL-1R<sup>−/−</sup> microglia (n = 40 neurons) 0.27 ± 0.01.

Our group has recently shown that an immune protein, the triggering receptor expressed on myeloid cells 2 (TREM2), is essential for microglia-mediated synaptic refinement during early stages of brain development [Filipello F, Morini R et al., 2018]. Hence, we investigated whether this pathway was involved in the deficient synaptic pruning observed in IL-1R<sup>−/−</sup> microglia. Confocal analysis of immunostained brain slices obtained from P9 WT and IL-1R<sup>−/−</sup> mice (Figure 25A and 25B) did not show differences in microglial TREM2 expression (Figure 25C-G). Also, the expression of CD68, a macrophage-specific phagolysosomal marker, did not differ between the two microglial subpopulations (Figure 25D-H).
Figure 25. IL-1R Deficiency Does Not Affect Microglia Expression of TREM2 and CD68. Confocal images of CA1 hippocampal microglia (A and B) labelled for Iba-1, CD-68 and TREM2 in brain slices obtained from P9 WT or IL-1R\(^{-}\) mice. Representative 3D reconstruction of TREM2 and CD-68 puncta within microglial cell (A). Graphs show cumulative distribution of integrate density values and mean integrated density values for TREM2 puncta (C and E) and CD68 puncta (D and F), and number of TREM2 puncta (G) or CD68 puncta (H) per microglial cell. Scale bar, 50 \(\mu\)m; scale bar zoomed-in images, 10 \(\mu\)m. WT N = 2 mice (n = 6 brain slices, n = 24 fields); IL-1R\(^{-}\) N = 2 mice (n = 6 brain slices, n = 24 fields).
Since it has already been reported that TREM2 protein is developmentally regulated in a region- and age-dependent manner in the mouse postnatal brain, we wanted to investigate its expression at a different and later time point [Chertoff M et al., 2013]. We quantified TREM2 mRNA levels in the hippocampi of P19 WT and IL-1R−/− mice by real time PCR and we observed that its mRNA levels did not differ between the two groups (Figure 26), in line with our immunohistochemical analysis at P9.

However, elimination of abnormal synapses during development might occur though different pathways. For instance, fractalkine (CX3CL1)/CX3CR1 and the classical complement systems are considered mediators of microglia interactions with synapses [Paolicelli RC et al., 2011]. In particular, the classical complement proteins C1q and C3 localize to the synapse and mediate synapse elimination by microglia during brain development [Stevens B et al., 2007; Stephan AH et al., 2012]. We therefore evaluated the mRNA levels of C1q subunits (C1qA, C1qB and C1qC) to uncover a possible defective expression of these classical complement cascade proteins. The glia-derived transforming growth factor β (TGF-β), which is known to regulate neuronal C1q expression and developmental synaptic refinement [Bialas AR and Stevens B, 2013], was used as internal control for C1q subunits. We found a strong decrease in the mRNA levels of each C1q subunit and TGF-β in the hippocampus of KO mice at P19 when compared to WT controls (Figure 26). These results indicate that mice lacking IL-1 receptor have normal TREM2 levels but reduced C1q expression in the hippocampus, suggesting an indirect implication of IL-1R in regulating neuronal C1q expression to initiate complement- and microglia-mediated synaptic pruning.

Figure 26. IL-1R−/− Mice Display Defective Expression of Classical Complement Protein C1q. Real Time PCR (RT PCR) performed in the
hippocampus of P19 WT and IL-1R$^-/$ mice. The analysis revealed a significant decrease in mRNA levels of C1q subunits and TGF-β in KO hippocampi. TREM2 mRNA levels were normalized to Iba-1 and 18s; TGF-β and C1q subunits mRNA levels were normalized to GAPDH. WT N = 3 mice, IL-1R$^-/$ N = 4 mice. *p < 0.05, **p < 0.001, unpaired t test.

**IL-1R$^-/$ Mice Display Altered Behavioural Phenotype**

Despite the heterogeneity of the diseases in which IL-1β is involved, a growing body of evidence points toward cognition as a shared physiological process hit by the cytokine. It is now clear that an alteration of the physiological process of neuronal network refinement via synaptic pruning can directly affect social and cognitive behaviour [Zhan Y et al., 2014; Paolicelli RC et al., 2014; Paolicelli RC and Ferretti MT, 2017; Filipello F, Morini R et al., 2018; Calcagno N et al., 2018]. Based on this evidence, we investigated whether IL-1R$^-/$ mice (P60) display an altered behavioural phenotype.

The Open Field (OF) test provides a variety of behavioural information ranging from general ambulatory ability to data regarding the emotionality of the animal [Wilson RC et al., 1976; Seibenhener ML and Wooten MC, 2015]. Normally, mice seek the protection of the periphery rather than the vulnerability of the centre. Mice that are less anxious are expected to spend more time in the centre, while mice that show signs of motor deficits are expected to cover lower horizontal distances and vertical movements [Ojha J et al., 2011]. Based on this, we tested 4-month-old (4mo) WT and KO mice in an open area and several activities were monitored. To assess locomotor ability, we analysed: (I) counts of ambulatory (walking), stereotypic (repetitive activity, e.g. grooming) and vertical (rearing up) movements; (II) total ambulatory distance travelled; (III) ambulatory velocity (Figure 27B). Anxiogenic behaviour was assessed measuring some of these parameters comparing centre versus periphery (Figure 27C). General locomotor activity was not significantly altered in 4mo KO versus WT mice (Figure 27B). Environment exploration was not affected in these KO mice (Figure 27B). Furthermore, no differences were observed in anxiety-related motor paradigms, as shown by comparable time spent in the corners of the arena or close to the walls (Figure 27C). These data suggest that IL-1R$^-/$ mice have no alteration in motor coordination and anxiety related behaviours.
Figure 27. IL-1R$^{-/-}$ Mice Do Not Display Altered Anxiety-like Behavior or Impairments in General Locomotor Activity. Schematic representation of Open Field (OF) test performed in 4-month old WT and IL-1R$^{-/-}$ mice (CZ, central zone; PZ, peripheral zone) (A). Comparison of general locomotion (B) and anxiety-like behaviour (C) parameters did not reveal any difference between WT and IL-1R$^{-/-}$ mice. Unpaired t test not significant. WT N = 8 mice, IL-1R$^{-/-}$ N = 8 mice. Data obtained with the collaboration of Dr Patricia Marcal Alves Correia.

To assess cognitive functions, WT and IL-1R$^{-/-}$ mice were tested using the Novel Object Recognition (NOR) task (Figure 28A), which is based on the spontaneous tendency of mice to spend more time exploring a novel object than a familiar one. This test normally relies on three sessions: one habituation session, one training session, and one test session. Training involves visual exploration of two identical objects, while the test session involves replacing one of the previously explored objects with a novel one. The choice to explore the novel object reflects the use of learning and recognition memory. The time between training and testing can be altered to assess any changes in short- and long-term memory. It has been shown that the right hippocampus is active during the delay interval of a short-term memory task specifically when associations between an object and its location have to be held online. These findings confirm and extend recent human lesion evidence [Olson IR et al., 2006] by showing that the spatial association held online specifically drives hippocampal contribution to short-term memory maintenance [Piekema C et al., 2006]. For this reason, we decided to investigate performances linked to short-term memory in WT and KO mice.
in a novel object recognition paradigm. Results for this test were expressed as a discrimination index (DI) between objects during the test session, calculated as the difference between the time spent exploring the novel object (Tn) and the familiar object (Tf) divided by the total time exploring both objects (DI = (Tn–Tf)/(Tn+Tf)). The analysis revealed that 4-month old IL-1R⁺ mice do not display defects in the Novel Object Recognition task when compared to WT (Figure 28B).

**Figure 28. IL-1R⁻/+ Mice Do Not Display Impairments in a Memory Task Paradigm.** Novel Object Recognition (NOR) task performed in 4-month old WT and IL-1R⁻/+ mice (A). The discrimination index calculated to evaluate the performance of each mouse did not reveal any defects in the memory task between WT and KO mice (B). p = 0.4334, unpaired t test not significant. WT 0.18 ± 0.09 N = 8 mice, IL-1R⁻/+ 0.28 ± 0.09 N = 8 mice. Data obtained with the collaboration of Dr Patricia Marcal Alves Correia.

Of note, and consistent with the evidence that defects in synapse phagocytosis during development are associated with social behavioural defects typical of autism spectrum disorder (ASD) [Filipello F, Morini R et al., 2018; Calcagno N et al., 2018], several impairments of social behaviour were detected in IL-1R⁻/+ mice. Indeed, by using a sociability test (Figure 29A), we found that in contrast to WT mice, which spent longer time exploring the chamber with a stranger mouse than the empty compartment, IL-1R⁻/+ mice spent a comparable amount of time in the two chambers (Figure 29B). No defects were detected in the social recognition task (Figure 29A), where IL-1R⁻/+ mice performed similarly to controls (Figure 29C). These data suggest that IL-1R⁻/+ mice show significant deficits in social behaviour but not in memory recognition tasks. Furthermore, these mice displayed a strong increase in self-grooming behaviour (Figure 29D), a measure of repetitive behaviour typically associated with autism spectrum disorder [McFarlane HG et al., 2008]. Altogether, these data indicate that lack of IL-1 receptor expression during development leads to behavioural defects reminiscent of ASD.
Figure 29. IL-1R<sup>−/−</sup> Mice Display Impairments in Repetitive and Social Behaviors. Schematic representation of Sociability and social novelty test (A). Sociability test (B) performed in a social choice paradigm, social memory test (C) performed in a social novelty paradigm and self-grooming test (D) in P60 WT and IL-1R<sup>−/−</sup> mice. *p < 0.05, unpaired t test. WT N = 8 mice, IL-1R<sup>−/−</sup> N = 8 mice. Data obtained with the collaboration of Dr Antonella Borreca.
DISCUSSION

Inflammation is increasingly recognized as a key factor influencing many neurological disorders occurring in adulthood, when brain development is completed [Skaper SD et al., 2018]. However, a growing body of evidence is now highlighting a central role played by inflammation at early stages of neuronal development, even at prenatal stages, when the immature brain, upon exposure to viral or bacterial infective agents, may undergo an inflammatory-mediated alteration of development [Hagberg H et al., 2015]. Indeed, extensive research is providing evidence that inflammation has long-term consequences on physiological brain development and could speculatively affect the risk and/or severity of a variety of brain diseases, including autism spectrum disorders (ASDs), schizophrenia, and intellectual disabilities (IDs), which represent recognized synaptopathies [Fan X et al., 2007; Dantzer R et al., 2008; Najjar S et al., 2013]. Pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor alpha (TNF-α), appear to be at the forefront in the communication between the immune and the nervous system, playing dual roles in either mediating physiological and neuroprotective roles in normal brain function [Parish CL et al., 2002; Nakanishi M et al., 2007; Heese K, 2017] or being detrimental and associated with brain diseases, especially when present at elevated concentrations [Ashwood P et al., 2011; Suzuki K et al., 2011; Wei H et al., 2011; Erta M et al., 2012; Chase KA et al., 2016; O’Keeffe GW, 2017].

IL-1β has been one of the most studied cytokines involved in both pathological and non-pathological processes in the CNS. Several recent studies have addressed some of the issues relating to the role of IL-1β in the brain, specifically in the hippocampus, under pathological conditions, and showed a detrimental role of the cytokine in processes such as neural plasticity and memory [Katsuki H et al., 1990; Bellinger FP et al., 1993; Ross FM et al., 2003; Goshen I et al., 2007; Barrientos R et al., 2002; Hein AM et al., 2010]. Conversely, IL-1β physiological role in synapse formation and function in the healthy brain is not completely known. Few recent evidences suggest that, at least under some circumstances, IL-1 may actually be required for a physiological regulation of hippocampal plasticity and learning processes. As a support to this possibility, IL-1 expression in the hippocampus is substantially increased during LTP, both in vitro and in vivo, while blocking IL-1 receptors with IL-1Ra (the interleukin-1 receptor antagonist) impairs the maintenance of LTP [Schneider H et al., 1998; Coogan AN and O’Connor JJ, 1999]. Moreover, IL-1Ra also reduces memory in the water maze and passive avoidance paradigms, whereas IL-1β
administration facilitates memory in the passive avoidance test [Yirmiya R et al., 2002]. Together, these data seem to indicate that the effects of the cytokine can be described by a typical U-shaped curve, with optimal cytokine concentration being required for allowing the occurrence of physiological processes, and “too low” or “too high” concentrations leading to neuronal dysfunctions.

IL-1 receptor type I knockout (IL-1R KO) mice were previously used to evaluate the implication of IL-1 signalling pathways in the physiological mechanisms underlying learning, memory, and neural plasticity. IL-1R KO mice were found to exhibit enhanced paired-pulse inhibition in response to perforant path stimulation and no LTP in the dentate gyrus. Moreover, decreased paired-pulse responses, as well as a complete absence of LTP, were observed in vitro in the CA1 region of hippocampal slices taken from IL-1R KO mice compared to WT controls [Avital A et al., 2003]. These results suggest that IL-1 contributes to the regulation of memory processes as well as short- and long-term plasticity within the hippocampus. These findings have important implications for several conditions in humans, which are associated with long-term defects in IL-1 signaling, such as mutations in the IL-1 receptor accessory protein-like gene, which are involved in a frequent form of X-linked mental retardation [Avital A et al., 2003]. Despite this evidence, the underlying molecular mechanisms are still unknown.

**Lack of IL-1 Signaling Leads to a General Enhancement of The Excitatory Neurotransmission And an Increase in Dendritic Spines Density**

Our study aimed to clarify how IL-1β contributes to brain homeostasis and to identify the process by which the lack of the IL-1 signaling may alter learning and memory processes. It has already been reported that IL-1 signaling affects neuronal synaptic activity by regulating synaptic scaffolding protein levels in the hippocampus. In particular, it was found that the absence of IL-1 receptor increases the expression of both the excitatory scaffolding protein postsynaptic density-95 (PSD95) and the inhibitory scaffolding protein gephyrin in the hippocampus of IL-1R deficient mice [Avdic U et al., 2015]. In line with these findings, we found that mice genetically devoid of IL-1R show an abnormal enhancement of excitatory transmission. Through western blot of brain homogenates and immunofluorescence analysis of sections from young or adult IL-1R KO and WT mice we indeed observed an increased expression of excitatory pre- and post-synaptic markers (VGlut1 and PSD95, respectively) in the KO mice, which is maintained throughout the entire life span of mice. Consistently, electrophysiological recordings of hippocampal CA1 pyramidal neurons highlighted a significant increase in the frequency and amplitude of
excitatory miniature post-synaptic currents, pointing towards a potentiation of the excitatory network. A further confirmation of the structural potentiation of the excitatory network in IL-1R KO mice was obtained by the analysis of Golgi-stained brain slices of KO mice, which showed a significant increase in spine density in both apical and basal dendrites of CA1 hippocampal neurons.

Avdic and colleagues previously showed an increased expression of the inhibitory scaffolding protein gephyrin in the hippocampus of IL-1R<sup>−/−</sup> mice at 4-5 months of age. However, when we immunostained brain slices obtained from young (1 month) and adult (7 months) WT and KO mice for the inhibitory pre-synaptic marker VGAT, we observed opposite results at the two different time points examined. While VGAT expression resulted significantly increased in the CA1 hippocampal region of 1-month old KO brain when compared to WT, no differences were detectable in older mice. Different alterations of excitatory and inhibitory transmission seem therefore to occur in the hippocampus of knockout mice at distinct ages, with specific age-dependent impacts to the excitatory/inhibitory balance. In young KO mice, we found a general potentiation of both excitatory and inhibitory transmission, possibly suggesting a transient compensatory elevation of inhibitory transmission aimed at controlling the increase in the excitatory transmission, which is instead long-lasting, in order to restore balance and maintain network homeostasis. Similar compensatory processes, including homeostatic plasticity mechanisms, have been previously described in a variety of invertebrate [Davis GW, 2006; Marder E and Goaillard JM, 2006] and vertebrate [Pozo K and Goda Y, 2010; Turrigiano G, 2011; Turrigiano GG and Nelson SB, 2004] model systems. However, in older mice, the selective increase of excitatory neurotransmission results in alterations of the excitatory/inhibitory (E/I) balance which favours excitation. In the adulthood, the inhibitory neurotransmission fails in balancing the excess of excitation and this fact opens the possibility that different processes may be at the basis of the synaptic potentiation occurring at the two stages. The effects of IL-1R lack on excitatory and inhibitory networks and their important implications on the E/I balance, which is known to be crucial for preserving circuit function, may be responsible for the behavioural deficits observed in IL-1R deficient mice. Indeed, it is now recognized that excitation/inhibition imbalance is implicated in neuropsychiatric disorders (autism spectrum disorders, schizophrenia, epilepsy) [Fritschy JM, 2008; Žiburkus J et al., 2013; Gao R and Penzes P, 2015; Nelson SB and Valakh V, 2015; Lee E et al., 2017; Selten M et al., 2018].
Neuronal network function depends markedly on the inhibitory system mediated by GABAergic interneurons. These cells form a wide and heterogeneous population which can be classified into several cell types, based on physiological properties, neurochemical marker content, somatodendritic location and most notably, location of their axon terminals [Freund TF and Buzáki G, 1996]. Among the various interneuron types, perisomatic inhibitory interneurons (PIIs) have attracted greatest attention because they comprise one of the largest interneuron sub-population in cortical circuits [Freund TF and Buzáki G, 1996]. Although PIIs show defined morphological properties, they can also be classified on the basis of their neurochemical content into two major types, the parvalbumin (PV)- and the cholecystokinin (CCK)-expressing PIIs. In particular, PV+ basket cells form synapses on the cell body and axonal initial segment of pyramidal cells, regulating their output and coordinating the activity of neuronal assemblies. Changes in their number may have therefore severe functional consequences. For example, it is known that a hallmark of the pathology of schizophrenia is an alteration of the GABAergic system in prefrontal cortex (PFC) [Lewis DA et al., 2005] and hippocampus [Zhang ZJ and Reynolds GP, 2002], and that the loss of hippocampal interneurons, by shifting the excitation-inhibition balance, may be at the basis of the onset of temporal lobe epilepsy (TLE) [Sayin U et al. 2003; Wyeth MS et al. 2010]. Alterations in the number and/or function of parvalbumin-expressing (PV+) GABAergic interneurons in the cortex and in the hippocampus may therefore result in dysfunctions typical of different neurological disorders. We observed an enhancement in
VGAT expression and VGAT-positive presynaptic terminals, which was in line with the increase in PV+ interneurons in the hippocampus, in particular in the CA1 region, of young KO mice. Of note, the number of PV neurons was slightly decreased in older KO mice. Our observation opens therefore the possibility that IL-1 signalling may play a role in the control of GABAergic system. Further studies are needed in order to elucidate the involvement of this cytokine in the maturation and development of PV+ neurons in the adult mice.

**Adult IL-1R−/− Mice Display Behavioural Phenotypes Typical of Autism Spectrum Disorders**

Autism-spectrum disorders (ASD) and schizophrenia, neurological diseases characterized by marked disruptions in information processing and cognition, are recognized to result from altered synaptic connectivity and plasticity in the brains of affected individuals. They are, in fact, called “synaptopathies”, i.e. “diseases of the synapse”. Accordingly, several studies have demonstrated that alterations in the number of synapses during development occurs in these pathologies, as shown by the age-dependent analysis of dendritic spines, which represent the structural correlate of postsynaptic compartment.

![Dendritic spine formation and synaptic elimination](Modified from: Dendritic spine pathology in neuropsychiatric disorders – Peter Penzes et al., *Nature Neuroscience* 2011)

Indeed, Golgi stained post-mortem ASD human brain tissue revealed an increase in spine density on apical dendrites of cortical pyramidal neurons. Of note, spine density was inversely correlated with cognitive functions [Penzes P et al., 2011]. These findings are consistent with the emerging hypothesis that the brains of individuals with ASD are
characterized by hyper connectivity in local circuits and hypoconnectivity between brain regions [Geschwind DH and Levitt P, 2007]. Similar to typical autism, the fragile X brain is characterized by elevated spine density, with elongated and tortuous spine morphologies, indicative of altered function. It is possible that spine dysmorphism contributes to abnormalities in specific circuits, which in turn may underlie the behavioural impairments characteristic of these disorders [Penzes P et al., 2011].

We found that IL-1R$^{-/-}$ mice, beside the increased density of dendritic spines demonstrated by Golgi staining, display some altered behavioural phenotypes typically found in mouse models of ASD. These include an increased self-grooming and alterations of the social behaviour. Conversely, no impairments in other cognitive paradigms such as novel object recognition task or social novelty test were detected. The established concept that autism spectrum disorders is characterized by an increase in spine density [Penzes P et al., 2011], imbalance in the ratio between excitation and inhibition [Rubenstein JL and Merzenich MM, 2003], as well as repetitive behaviours and social defects [Ravizza SM et al., 2013] suggests that the lack of IL-1 receptor expression during development leads to behavioural defects reminiscent of ASD. It is conceivable that an increase in synaptic connections and a consequent alteration of synaptic strength may reshape the neuronal wiring patterns and eventually cause atypical brain functions. This possibility is also supported by the results from Fang et colleagues, who demonstrated that overproduction of neocortical excitatory neurons leads to autism-like features in mice, through the perturbation of dendrites and spines formation in excitatory neurons which leads to dysregulated E/I balance. Importantly, these mice exhibit behavioural abnormalities resembling those of human autism [Fang WQ et al., 2014]. The brain phenotype that we found in IL-1R$^{-/-}$ mice resembles this condition: in fact, we demonstrated a significant enhancement of excitatory transmission and an increase in spine density in the hippocampus of these mice, with an excitatory/inhibitory balance shifted towards excitation in old mice, resulting in defects in behavioural phenotypes typical of autism. However, in our mice we did not observe any sign of excitotoxicity, and this might be due to the fact that the threshold level of excitatory transmission required to trigger neuronal excitotoxic damages and death is not reached.

**Autism-like behaviors**

- Deficits in social interaction
- Compulsive and repetitive behaviors
- More self-grooming
- Buried more marbles

(Modified from: Overproduction of Upper-Layer Neurons in the Neocortex Leads to Autism-like Features in Mice - Wei-Qun Fang et al., Cell Report 2014)
In our case, however, we didn’t find any apparent increase in the number of excitatory neurons. What could then be the reason of the increase in spine and excitatory synapse density detected in IL-1R$^{-}$ brain? The first indication came from our observation that the excessive excitatory neurotransmission is not detectable in primary cultures of IL-1R$^{-}$ hippocampal neurons. These data suggested that the genetic lack of IL-1 receptor does not induce alterations in synaptic transmission through a neuron-autonomous process, pointing instead to a key contribution of non-neuronal cells in driving the process.

**IL-1 Signaling and Microglia-Mediated Synaptic Pruning**

The most likely candidate to be involved in this process is microglia, the immune component present in the brain. One of the most studied function of microglia is their significant contribution to synaptic stripping, a process by which microglia monitor synaptic function and contribute to synapse stabilization or elimination in the first weeks of postnatal life. The process of synaptic pruning is central during brain development, when the excess number of synaptic contacts is subsequently eliminated. Correct synaptic pruning is necessary for the strengthening of remaining synapses and is critical for normal brain development [Riccomagno MM and Kolodkin AL, 2015], leading to short-term effects on postnatal synaptic maturation and long-term effects on neural circuit formation. Microglial cells play major roles in the process of synaptic pruning, participating in synapse remodelling and plasticity [Kettenmann H et al., 2013; Paolicelli RC et al., 2011; Schafer DP et al., 2012; Tremblay MÈ et al., 2010]. Microglial phagocytosis relies on cell surface receptors, coupled to downstream signaling pathways that control actin remodelling and particle engulfment. Previous studies have demonstrated that the microglial engulfment and removal of dendritic spines during development occurs via signaling of the neuronal chemokine, fractalkine (CX3CL1), to its receptor on microglia (CX3CR1). Using a mouse model lacking this receptor, Paolicelli and colleagues observed a significant reduction in microglia density, which correlated to a transient deficit in synaptic pruning and circuit maturation [Paolicelli et al., 2011] and eventually led to impairments in cognitive function in the adult mice [Rogers JT et al., 2011].

Differently from CX3CR1 KO mice, IL-1R KO mice display a transient increase in microglial density at postnatal day 9 in both hippocampus and cortex when compared to WT, a difference which was fully recovered in 1-month-old mice. To investigate whether, although being more elevated in their number, microglia could be functionally defective, we cultured KO microglia with WT neurons and showed that microglia lacking of IL-1 receptor prevents the process of synapse elimination in vitro. Our data therefore point to the
possibility that, although temporarily increased in their number, microglia from KO mice may be defective in the process of synapse elimination even in vivo. The microglia increase in vivo might be an event occurring to somehow compensate the deficient microglia-mediated synapse pruning. Although we do not provide a direct demonstration that IL-1 receptor deficiency in microglia directly contributes to the observed effects on excitatory synapses, our results, together with the evidence of reduced synapse elimination when neurons and IL-1RKO microglia are cultured in contact, point to IL-1 signaling being involved in synapse phagocytosis, which is consistent with the higher density of pre- and post-synaptic markers in the hippocampi of knock-out mice, more abundant dendritic spines and enhanced excitatory neurotransmission in acute brain slices.

As a further evidence pointing to microglia as possible major contributors to the brain phenotype found in IL1R KO mice, we observed in these mice defects in a social behaviour typical of autism spectrum disorders that has been also demonstrated in Cx3cr1 KO mice, further confirming the possibility that a reduced synaptic pruning during development in microglia may contribute to circuit-level deficits across neurodevelopmental disorders, including autism [Zhan Y et al., 2014].

Interestingly, ASD development is suggested to involve microglia, through their role in synaptic refinement during development. Kim and colleagues showed that deletion of genes that are vital for microglial autophagy resulted in social behavioural defects and repetitive behaviours [Kim HJ et al., 2017], which recall the IL-1R KO behavioural phenotype. Even more interestingly, recent results from our group have demonstrated that defective synaptic pruning by microglia lacking the TREM2 receptor results in increased density of excitatory terminals and dendritic spines, accompanied by social defects [Filipello F, Morini R et al., 2018], a phenotype which recapitulates the main features of IL-1R KO mice. However, we observed no difference in the microglial expression of this receptor between WT and IL-1RKO hippocampi both at 9 and 19 days of age. Since elimination of abnormal synapses during development might occur though different pathways, it is possible that the unchanged expression of TREM2 in our knock-out microglia does not exclude alterations or impairment in other pathways involving the classical complement proteins C1q and C3 among others. In fact, we found a strong decrease in the mRNA levels of each C1q subunit in the hippocampus of KO mice at P19 if compared to WT controls, suggesting that IL-1R might indirectly regulate neuronal C1q expression to initiate complement- and microglia-mediated synaptic pruning.

The molecular mechanisms by which IL-1 receptor in microglia may control the pruning of supernumerary synapses in the developing brain is still to be defined. We have recently reported that in a mouse model of inflammation lacking IL-1R8, a receptor which
dampens the activation of the Toll-like receptor (TLR) and IL-1 receptor signalling pathways, the IL-1 signaling is hyperactivated [Tomasoni R et al., 2017]. In this context, we demonstrated that the activity of the IL-1 receptor pathway directly affects, in neurons, the levels of expression of the methyl-CpG-binding protein 2 (MeCP2), a protein involved in neurological diseases (Rett syndrome and MeCP2 duplication syndrome) characterized by defective plasticity, impaired cognition and intellectual disability. Our study, by demonstrating that hyperactivation of the IL-1 receptor pathway results in the overexpression of MeCP2, a synaptic factor that controls spine morphogenesis, synaptic transmission and plasticity [Na ES et al., 2013], provides a major breakthrough in the field of the cross talk between nervous and immune systems.

Given the evidence found in IL-1R8 KO mice, characterized by the hyperactivation of IL-1 pathway, we reasoned that in IL-1R KO mice a decreased expression of MeCP2 could be expected. Indeed, western blot analysis of brain homogenates revealed decreased MeCP2 levels in both cortex and hippocampus of IL-1R deficient mice. Unexpectedly however, immunostaining of brain slices for MeCP2 revealed that this decrease was not ascribable to pyramidal neurons in the IL-1R<sup>−/−</sup> hippocampi, but rather to microglial cells. These results indicated that a reduction of MeCP2 in neurons is likely not responsible for the augmented synaptic transmission and synaptic density observed in the hippocampus of IL-1R KO mice. This is also in line with our evidence that the synaptic effects of the genetic lack of IL-1R KO do not result from a neuron-autonomous process. Furthermore, they further confirm microglia as central player in the observed process. It is known that the transcription factor MeCP2 is important for microglial functionality. In fact, MeCP2 regulates microglia and macrophage responsiveness to environmental stimuli to promote homeostasis [Cronk JC et al., 2015]. MeCP2 expression is also strongly related to regulating the activation states of microglia [Jin XR et al., 2017; Zhao D et al., 2017]. Notably, microglia deficient in MeCP2 show a strong reduction in phagocytic activity, which is important also in synapse stripping. Interestingly, the phenotype of MeCP2 knockout mice can be partially rescued by selective expression of MeCP2 in cells of the monocytic lineage, including microglia, further supporting the central role of this cell type [Derecki NC et al., 2012]. At present there is no evidence for a direct correlation between MeCP2 in microglia and synaptic pruning and we currently aim to provide this demonstration. The functional analysis of microglia isolated from heterozygous MeCP2 mice, which express reduced MeCP2 levels and thus more closely reflect the situation we found in IL-1R KO mice, could be advantageous in this context.
Conclusions and Future Perspectives

We found that IL-1R\(^-\) mice display behavioural defects reminiscent of ASD that are associated to an enhanced excitatory neurotransmission and an increased density of dendritic spines. Our data suggest that the behavioral defects observed in adult IL-1R deficient mice might be due to a deficient microglia-mediated synaptic pruning during development rather than to a neuron-autonomous synaptic impairment. To obtain a direct demonstration of an impaired microglia-mediated synaptic pruning. We will evaluate the levels of synaptic markers in phagocytic organelles of IL-1R\(^-\) microglia in vitro and in vivo. The molecular mechanism responsible for these deficits might involve an impaired TGF-\(\beta\) secretion by knock-out microglia which, in turn, leads to a downregulated expression of C1q proteins and to a dysfunctional elimination of synapses. To assess this hypothesis, we will not only quantify secretion of TGF-\(\beta\) from cultured WT and knock-out microglia and its effects on C1q expression in vitro by real-time PCR, but we will also evaluate C1q Levels in WT and knock-out synaptosomes from brain homogenates by western blotting and WT and IL-1R\(^-\) microglia engulfment of synaptosomes from brain tissue by FACS analysis and immunostaining.

It also remains to be determined how the chronic lack of IL-1 receptor in microglia results in MeCP2 decrease. IL-1 receptors are known to be expressed on microglia (specifically IL-1R1, IL-1R2 and IL-1R\(\alpha\)P [Pinteaux E et al., 2002]). From the literature it is known that a transcriptional analysis of purified cultured microglia showed weak expression of IL-1RI and stronger expression of IL-1RII, whereas the treatment with LPS significantly increased expression of IL-1RI, IL-1RII, and IL-1R\(\alpha\)C [Kettenmann H et al., 2011]. Although it is now well known that IL-1R signalling in microglia leads to IL-6 and PGE\(_2\) release, strong activation of ERK1/2, p38 and JNK (p46/p54) and increased NF-\(\kappa\)B activity [Pinteaux E et al., 2002], the specific role of IL-1 receptor on microglial development and function is not completely unravelled. However, it has been observed that the IL-1 type 1 receptor is essential for the efficient activation of microglia and the induction of multiple proinflammatory mediators in response to brain injury [Basu A et al., 2002]. We have previously found that, at least in neurons, the downregulation of MeCP2 expression downstream of IL-1R involves PI3 Kinase and mTOR pathways [Tomasoni R et al., 2017]. We are currently investigating whether the same pathway is also operating in microglial cells. Furthermore, it will be interesting to assess the possibility to recapitulate the observed phenotype by administering pharmacological inhibitors of IL-1 receptor (e.g. anakinra) at specific developmental stages.


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