UNIVERSITA' DEGLI STUDI DI NAPOLI
FEDERICO II

Dottorato di Ricerca in Organismi Modello nella Ricerca Biomedica e Veterinaria ciclo XXVIII

Behavioural, Cellular and Molecular Insights into Hyperexcitability using zebrafish as model system

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ANNO ACCADEMICO 2013/2016

Abstract

Neurons communicate through the release and uptake of excitatory or inhibitory neurotransmitters across their synapses. The equilibrium between neuronal synaptic neurotransmission and ion concentrations define the functionality of neuronal network and the ongoing brain homeostasis. As consequence, the alteration of this equilibrium can modify the normal neuronal activity and result in a condition of hyperexcitability in the brain. This condition may lead to seizures due to excessive and synchronous neuronal activity that cause the interruption of normal behaviour and consciousness. While seizures represent the transient manifestation of altered brain functions, epilepsy is a chronic neurologic disorder, characterized by two or more unprovoked or recurrent seizures caused by genetically predetermined process or by an initial insult. Acute and chronic seizures may damage the neuronal cell physiology thus prompting a rapid neuroinflammatory response. Studies suggest that seizures develop in children with significant higher incidence than in adults, indicating that the immature brain is more prone to develop an excessive neuronal activity. Although the threshold for seizure generation is lower in the immature brain than in the adult brain, due to differences in intrinsic neuronal properties, the developing neurons are less vulnerable in terms of neuronal damage and cell loss than mature neurons. However, prolonged seizures in immature brain could contribute to acute and long-term deleterious effects. Both mature and immature rodents have been extensively used to study the cellular and molecular alterations linked to seizures. Recently, the contribution of inflammatory reactions to seizure induction and progression in epilepsy has been formulated, with a particular focus on interleukin 1 beta signalling (IL-1β), suggesting proconvulsant properties of IL-1β in acute seizure activity. Unlike these evidences, other works are consistent with an acute anticonvulsive function of IL-1β in seizure generation. Moreover, several other aspects of the molecular and cellular machineries that promote and define the seizure process, and that derive from them, are not clearly understood. As rodents are among the most frequently used model organisms for seizure and epilepsy studies, other non-mammalian organisms are proposed to tackle these phenomena. Among these,

zebrafish is an emerging model system in developmental neurobiology and drug discovery. Recent studies have described that both larval and adult zebrafish develop seizures when exposed to chemoconvulsant agents, and they present similar profiles of responsiveness to anticonvulsant compounds, enlightening the potential of zebrafish models for the *in vivo* study of brain functions and dysfunction. The aim of my PhD project is to characterize the induction of seizures in the embryonic brain of zebrafish, using a known proconvulsant agent, pentylenetetrazole. First, I have described the transcriptional, morphological and behavioural responses associated with neuronal hyperactivity during the period of structural and physiological maturation of the brain. Then, I have investigated the eventual contribution of inflammatory reactions in seizure induction in the zebrafish immature brain, with a particular regard to gaining novel insights on the role of IL-1β signalling in seizure induction in fish. Altogether, my doctoral work has shown that, in line with other animal models, seizures in the immature brain of zebrafish larvae are rapidly associated with a neuronal active state and with the induction of a neuroprotective mechanism. As far as the inflammatory response is concerned, acute seizures cause activation of non-neuronal cells such as astrocytes and, unexpectedly, selective IL-1β release and not of other cytokines. This is an unprecedented finding in zebrafish, and it is corroborated by genetic manipulation and pharmacological treatments that strongly support the hypothesis of a direct role of IL-1β in the maintenance of an active state at the neuronal level.

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CHAPTER 1

Introduction

1.1. Alteration of Brain Homeostasis: Seizures and Epilepsy

Neurons in the brain communicate which each other through the release and uptake of neurotransmitters. These small molecules are released at the synaptic level by exocytosis from the pre-synaptic neuron, and bind specific transmembrane receptors on the surface of the post-synaptic neuron. Ligand-receptor binding leads to a reversible conformational change in the receptor, that could mediate a passage of ions flow through it, or activate a cascade of second messengers able to open other ion channels, which in turn allows specific ion flux in or out the receiving neurons. The main neurotransmitters in the brain are glutamate and gamma amino butyric acid (GABA). Glutamate is considered an excitatory neurotransmitter because, upon binding its receptors, triggers a positive flow of ions into the neuronal cytoplasm, causing depolarization of cell membranes and action potentials. On the contrary, GABA receptor binding leads to a chloride influx in the cells that causes membrane hyperpolarization, with no firing of action potential. In some circumstances, disturbance of the normal balance in the distribution of sodium, potassium, chloride or calcium ions across neuronal plasma membranes may cause loss of inhibitory neurotransmission or aberrant increase in excitatory neurotransmission (Cloix, 2009). As a consequence, the threshold of membrane depolarisation is decreased, facilitating the firing of action potentials in the brain. When this balance is disrupted in a large number of neurons, they depolarize simultaneously giving raise to seizures. The term “seizures” refers to abnormal, synchronized and repetitive burst firing of neuronal populations in the central nervous system (CNS) (McNamara, 1994; Shin & McNamara, 1994). The seizure is a “transient” alteration of normal neuronal activity that affects a restricted area, or that could propagate through the brain. Based on (i) the specific brain compartment interested by the onset of seizures, (ii) the pattern of

propagation and (iii) the degree of maturity of the brain, seizures may generate different behavioural changes and electroencephalograms (Fisher et al., 2005). Electroencephalography is a technique based on recording electrodes that measure the action potentials produced synchronously by clusters of neurons, thus generating an electroencephalogram that illustrates the abnormal neuronal activity. Based on the differences in behaviour and electroencephalogram, seizures are classified in different ways, according to the International League of Epilepsy, a world's preeminent association of professionals that are interested in understanding and diagnosing patients with epilepsy (Fisher et al., 2005; Engel et al., 2001; Berg et al., 2010). Seizures are described as partial or focal if the onset is localized, or generalized if the electrical activity involves the whole brain. Generalized seizures are classified in myoclonic, tonic-clonic and absence. Myoclonic seizures are characterized by violent muscular contraction, while tonic-clonic seizures produce an alternating episode of muscular contraction and relaxation followed by loss of consciousness. Absence, on the contrary, is the loss of consciousness and movement for few seconds, followed by fast recovery of both. Another type of seizure is *Status Epilepticus* (SE), characterized by continuous seizures lasting more than 30 min. SE causes significant mortality and morbidity, including an increased risk to develop future epilepsy, and represents an emergency condition (Reddy et al., 2013).

Epilepsy is a chronic neurologic condition characterized by the presence of recurrent unprovoked seizures. It represents a common health problem, because it affects about 3 million people in the U.S.A. and approximately 65 million people world-wide (Jacob et al., 2009) of all ages and both genders. Epilepsy is a complex disorder with many possible causes. In the majority of cases (50%), it is idiopathic (unknown cause). In the remaining cases, recurrent seizures may result from a variety of secondary conditions including trauma, anoxia, metabolic imbalance, CNS infection, or can occur as the result of genetic alterations primarily involving ion channels (Reddy et al., 2013). The term "epileptogenesis" is used to describe the complex plastic changes that convert the non-epileptic neuronal circuit into a seizure-generating circuit (Vezzani et al., 2007). Epileptogenesis is a slow process that is thought to consist of three stages: (i) the initial precipitating event, (ii) the latent period, and (iii) the chronic period with spontaneous seizures (Figure 1.1.). Yet, little

is known about the exact mechanisms that induce and underlie the development of epilepsy.

The complex context of seizures and epilepsy prompted researchers to study the molecular and cellular mechanisms associated with these pathophysiological conditions, as well as to discover new therapeutic strategies.

Figure 1.1. The pathophysiological stages of epileptogenesis (Reddy et al., 2013).

1.2. Animal Models of Seizures and Epilepsy

A choice of chemical, electrical or genetic tools is available to induce epileptic seizures or to model epilepsy (Figure 1.2). Chemical models consist in the local or systemic administration of agonist or antagonist drugs that mainly target excitatory and inhibitory current receptors (De Deyn, 1992). Among these, the most used molecules are pentylenetetrazole (PTZ), picrotoxin (PX), pilocarpine and kainic acid (KA), which differ about their action mechanism. PTZ and PX are considered non-competitive antagonists of GABA A receptors that reduce the GABA-mediated chloride influx into the neuron, thus preventing neuronal hyperpolarization. KA is a L-glutamate analog, and pilocarpine is a muscarinic acetylcholine receptor agonist (Kandratavicius et al., 2014). Pilocarpine and KA are used to induce the SE in immature and adult rodent models, while PTZ is used to generate acute seizure models, therefore its application does not lead to the generation of animal models of epilepsy. An important aspect to consider when using PTZ is that seizure models are useful for rapid screening of anti-epileptic drug (AED) action, but they do not necessarily result in chronic epilepsy (Loscher, 2011). Electrical stimulation is also used to induce an alteration of brain homeostasis. One example of electrical stimulation is electroshock-induced seizures. This approach features the most studied models for electrical seizure stimulation, because it does not require implantation of electrodes in the brain, but it consists of a single electrical stimulation of whole brain (Landratavicius et al., 2014). In both cases, chemical and electrical tools can be differently used to model acute or chronic epileptic seizure conditions. This difference is fundamental, because an acute model of seizure, in general, is characterized by a single prolonged seizure (SE) in non-epileptic animals such that it does not represent a model of epilepsy, while it is rather useful for studying the seizure *per se* (Losher, 2011). On the other hand, chronic models can lead to epilepsy generation in terms of spontaneous and recurrent seizures, allowing the study of how seizure insults may eventually lead to the development of epilepsy. Kindling is the most common chronic model of epilepsy, and it consists of the administration of repetitive subconvulsant doses of chemical or electrical stimulation in brain region as amygdala or hippocampus that are susceptible to seizure activity. Although the

kindling model elicits spontaneous seizures and it is a useful model of epilepsy, it is laborious to obtain and time-consuming. Mutant or transgenic animal models that carry alterations in genes involved in epilepsy are generally used to study epilepsy and to develop therapeutic strategies.

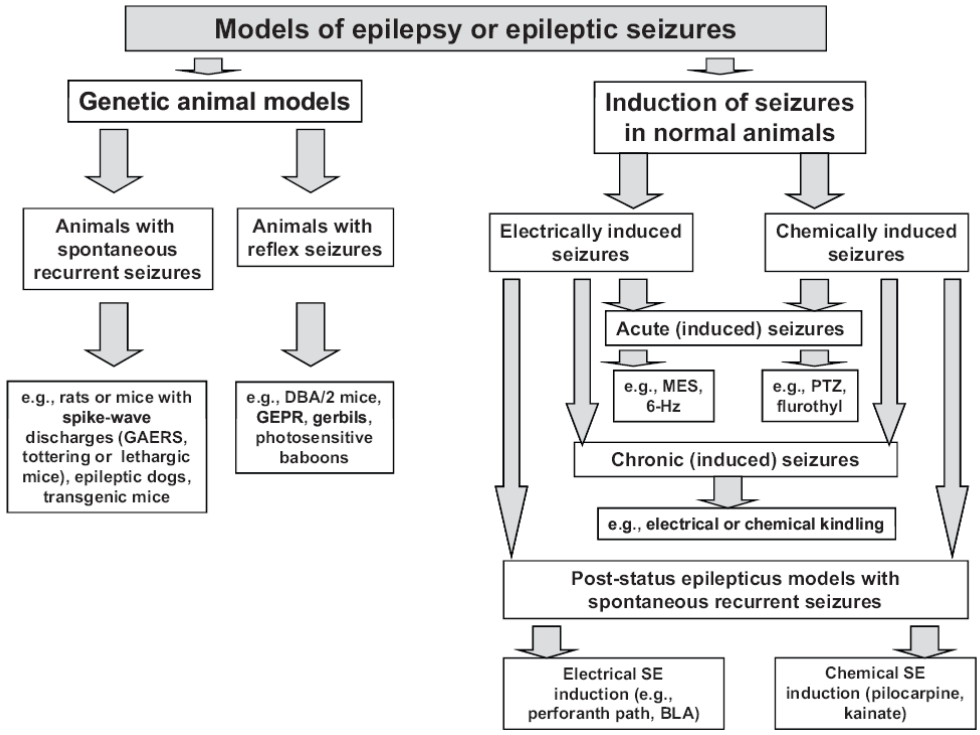


Figure 1.2. Animal models of epilepsy and epileptic seizures (Loscher, 2011).

1.3. New Concepts in Inflammation and Seizure Activity in the Brain

In epilepsy research, adult rodents are extensively studied to characterize the cellular and molecular mechanisms underlying the complex process of epileptogenesis. In the mature brain, prolonged seizures or SE are often used to generate the inciting event and to model some forms of human epilepsy. The consequence of this stimulation is an evident loss of vulnerable neurons within the brain region, followed by reactive formation of new synapses and abnormal reorganization of neuronal circuits (Pitkainen et al., 2002). As neuronal cell loss is considered the triggering event, it has been proposed that other factors contribute to the generation of epilepsy. In addition to cell death, in fact, the induction of seizures and SE in the rodent brain stimulates an extensive inflammatory reaction, that consists in the increased levels of both IL-1β mRNA and protein and of related cytokines (Plata-Salamàn et al., 2000; Minami et al., 1990). The majority of studies in epilepsy research has supported the crucial role of cytokines and inflammation in neuronal injury associated with seizure activity and in the mechanism of epileptogenesis. More emphasis has been devoted to the IL-1β cytokine. IL-1β mRNA and protein expression rapidly increase in various brain areas after experimentally induced seizures (Vezzani et al., 2002). At the same time, IL-1β expression is also increased in the cortex and hippocampus of chronic animal models (Minami et al., 1990; Vezzani et al., 1999; Eriksson et al., 2000; Rizzi et al., 2003), and it is first detected in activated microglia and astrocytes. In the rodent epilepsy model, intrahippocampal injection of IL-1β leads to prolonged seizure activity, as demonstrated by both electrical and behavioural analyses (Vezzani et al., 2000), suggesting the proconvulsant role of this cytokine. In support of this observation, injection of its natural antagonist IL-1Ra reduces behavioural convulsion in a rodent seizure model (Vezzani et al., 2002). In order to verify the proconvulsant effect of IL-1β, several studies suggest that excess of IL-1β can augment nitric oxide formation and increase neuronal excitability in different ways. IL-1β could increase neuronal hyperexcitability by inhibiting GABA A receptor, by increasing NMDA receptor function and by inhibiting K^+ efflux (Viviani et al., 2003; Miller et al., 1991; Zhu et al., 2006). However, another group of studies, such as those involving intraventricular injection of IL-1β, indicates that IL-1β may have an

anticonvulsant activity (Sayyah et al., 2005). In addition, *in vitro* studies showed that IL-1β causes GABA-increase in chloride permeability, and also that IL-1β may increase K^+-evoked GABA release without affecting K^+-evoked glutamate release, acting in the potentiation of GABAergic transmission (Zhu et al., 2006). IL-1β involvement in seizure and epilepsy has been reported also in human studies. While *IL-1β* gene polymorphism is associated with an increased susceptibility to seizures and epilepsy (Kanemoto et al., 2003), an increased expression of IL-1β and its receptor is detected in brain samples from patient s surgically treated for a form of refractory epilepsy (Ravizza et al., 2008; reviewed by Li et al., 2011). While understanding of the role of the innate immune system in epilepsy and seizure threshold changes, and in particular of the associated molecules with inflammatory properties, has advanced tremendously over the last decade, yet there are a number of questions that remain open and require further investigation (Wilcox and Vezzani, 2014).

1.4. Inflammation in the Brain

Inflammation refers to the natural body defence reaction to various types of insult able to endanger the integrity of cells and tissues. Inflammatory response triggers can be an aseptic insult, such as tissue damage caused by mechanical or chemical injury, or non aseptic as bacterial or viral invasion. Inflammation consists of a complex cascade of events that occur locally within the injured tissue, and eventually systemically, and it is closely linked to the activation of the immune system. These events include specific signalling mediators, such as cytokines as well as many physiological responses, as fever and behavioural changes (Allan and Rothwell, 2003). The central nervous system (CNS) presents distinctive features and has commonly been considered an immune-privileged site. This so-called phenomenon of "immune privilege" was recognized in the mid-20th century by Sir Peter Medawar who was awarded the Nobel Prize in 1960 together with Sir Frank Macfarlane Burnet for the discovery of acquired immune tolerance (Amor et al., 2010). This privileged status of the CNS is dependent on several elements, such as an efficient natural protection from mechanical aggression by the skull and from biological and chemical attack by the presence of a blood–brain barrier (BBB), the lack of a conventional lymphatic drainage, and an apparently low traffic of monocytes and lymphocytes (Vezzani and Granata, 2005). However, this concept is gradually changing as a result of recent developments in the research field of innate immunity that support the role of CNS-resident cells acting as innate-immune-competent cells (Aronica et al., 2012), and that BBB is not a physical barrier that separate the CNS from the periphery but it can be stimulated to both release and transmit pro-inflammatory mediators and allow leucocyte migration into the brain. This new formulation has led to the introduction of the term neuroinflammation to describe a range of immune responses in the CNS that differ in several ways from the inflammation in the peripheral tissues. The neuroinflammatory response may have beneficial as well detrimental consequences in the CNS, principally in the repair and recovery processes. Excessive and prolonged neuroinflammatory response can result in synaptic impairment and neuronal death, leading to the emerging concept of the central role of neuroinflammation in different acute and chronic brain diseases.

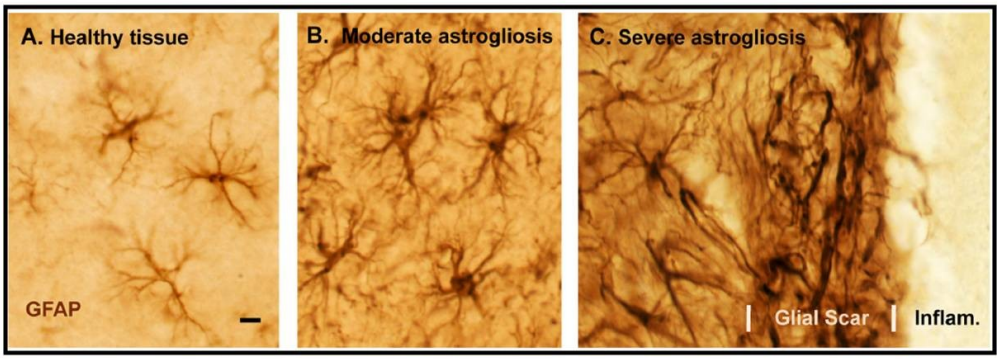
1.5. Components of Neuroinflammation

The microglial cells are considered the resident macrophages of the brain. These are cells of the monocyte/macrophage lineage derived from the embryonic yolk sac that invaded the primitive nervous system, the neuroepithelium, from the early vasculature. On the basis of their nature, they act as a first line of defence in the CNS: they are well equipped to remove debris and apoptotic cells, to respond to infectious and non-infectious danger signals and to regulate oxidative processes. These brain phagocytic cells are prone to respond to such insults by producing both toxic and harmful molecules, particularly cytokines, nitric oxide, growth factor and extracellular matrix components, and by taking on the morphology of activated macrophages (Perry and Teeling, 2013, Graeber et al., 2011). This activation is characterized by alterations in their morphology, such as hypertrophy of the cell soma, increased branching, upregulation or *de novo* synthesis of cell surface or intracellular molecules and proliferation (Perry and Teeling, 2013). The role played by activated microglia depends on several factors such as the type and duration of the activating stimuli, the microenvironment (*e.g.* the presence of pro- or anti-inflammatory cytokines) and the interaction with other immune modulators, such as astrocytes (Chen et al., 2010).

Unlike microglia, astrocytes descend from neuroepithelial stem cells. These complex and highly differentiated cells make a numerous essential contributions to normal functions in the healthy CNS, including regulation of blood flow, provision of energy metabolites to neurons, participation in synaptic function and plasticity, and maintenance of the extracellular balance of ions, fluid and transmitters. In addition, astrocytes respond to all forms of CNS insults such as infection, trauma, ischemia and neurodegenerative disease by a process commonly referred to as reactive astrogliosis (Sofroniew, 2009; Verkhratsky et al., 2013). As reported, the reactive astrogliosis is not an all-or-none response, nor a single uniform process, nor it is ubiquitously synonymous with scar formation (Sofroniew, 2009). The events underlying this process vary with the nature and the severity of insult, in a graded progressive alteration that includes molecular expression, cellular hypertrophy and,

in severe cases, also proliferation and scar formation. It is defined as a mild and moderate form when the reactive astrogliosis exhibits the potential for resolution if the triggering mechanism has resolved, because cells return to an appearance similar to the one observed in healthy tissues. On the other hand, a severe level of activation (in response to tissue damage and inflammation) of reactive astrogliosis involves scar formation with new proliferated cells and with overlapping astrocyte processes, in a manner not seen in healthy tissue (Figure 1.3A). These responses of reactive astrocytes are regulated in a context-specific manner by different signalling events that have the potential to modify the astrocyte activities both through gain- and loss-of-functions that can impact beneficially and/or detrimentally on surrounding neural and non-neural cells (Figure 1.3B).

A



B

Process in astrogliosis	**Implicated factors**
Cytokines & growth factors	Il6, LIF, CNTF, IL1, Il10, TGFβ, TNFα, INFγ, etc. [49, 67]
Mediators of innate immunity	LPS & other Toll-like receptor ligands [51]
Neurotransmitters & modulators	Glutamate, Noradrenalin [89]
Small molecules released by cell injury	ATP [90]
Molecules of oxidative stress	Nitric oxide (NO), reactive oxygen species (ROS) [43]
Ischemia associated	Hypoxia and glucose deprivation [43]
Neurodegeneration associated	Amyloid-beta [91]
Systemic metabolic toxicity	NH_4^+ [92]

Figure 1.3. Reactive Astrocytes. A. Photomicrographs of astrocytes in healthy tissue and with different gradations of reactive astrogliosis and glial scar formation after tissue insults of different types and severity; B. Molecular triggers and modulators of reactive astrogliosis (Sofroniew, 2009).

A large number of studies provide different kinds of evidence *in vivo* and *in vitro* that show that reactive astrocytes can protect CNS cells and tissue in various ways,

including the uptake of potentially excitotoxic glutamate (Rothstein, 1996), by facilitating blood brain barrier repair (Bush et al., 1999), by stabilizing extracellular fluid and ion balance and by reducing seizure threshold (Zador et al., 2009). However, reactive astrocytes may also have a proinflammatory potential, as shown by studies reporting that deletion or knockdown of certain astrocyte-expressed molecules is associated with a reduced inflammation (Okada et al., 2006).

The mechanisms by which both microglia and astrocytes recognize the presence of danger signals involved the expression of an array of germline-encoded pattern-recognition receptors (PRRs) (Kopitar-Jerala, 2015). This family of receptors include principally the membrane-bound toll-like receptors (TLRs) that are able to scan the extracellular milieu and endosomal compartments for pathogens-associated molecular patterns (PAMPs). PAMPs are lipids, glycolipids, lipoproteins, proteins and nucleic acids from a large number of microbial taxa including bacteria, viruses, parasites and fungi. All TLRs elicit conserved inflammatory pathways that culminate in the activation of the NF-kB and activating protein (AP-1) transcription factors that drive pro-inflammatory cytokine/chemokine production (Kawai and Akira, 2006). Intracellular nucleic-acid sensing PRRs cooperate with TLRs to provide cytosolic surveillance, or an “inward looking”, including the RNA-sensing RIG-like helicases (RLHs) and the DNA sensors, DAI and AIM2. A further set of intracellular PRRs are the NOD-like receptors (NLRs) that are able to recognize endogenous cellular products associated with tissue injury or self-danger signals (danger associated molecular patterns DAMPs), such as toxic compounds, defective nucleic acid or presence of normal cell components in atypical extracellular or intracellular locations (reviewed by Schroder and Tschopp, 2010).

1.6. Cytokines in Inflammation: Biology of Interleukin1

Cytokines are a group of signalling proteins produced transiently, like a self-limited event, after activation of several different cell types that act as humoral regulators in a locally autocrine or paracrine manner. The name cytokine derives from Greek *kytos* that means "vessel" and *kinein* that means "to move". Among the cytokines, IL-1 β is considered a major orchestrator of inflammatory and immune response for its well known pleiotropic activities. IL-1β belongs to IL-1 family ligands, together with interleukin 1 alpha (IL-1α), naturally occurring competitive IL-1-receptor antagonist (IL-1RA) and other seven members (Garlanda et al., 2013). IL-1 was the first interleukin to be identified as endogenous pyrogen for its ability to produce fever. Later studies demonstrated that IL-1 endogenous pyrogen did more than cause fever. It was recognized as lymphocyte-activating factor, hemopoietin-1, and osteoclast-activating factor, catabolin, until the final terminology of IL- 1, that now includes all previously described properties. IL-1 consists of two separate ligands, IL-1α and IL-1β (Dinarello, 1996). These proteins have high sequence homology, despite being the product of different genes. They are synthesized as large precursor proteins of 31 KDa. Pro-IL-1α is biologically active and it is cleaved by calpain to generate the smaller mature protein. By contrast, IL-1β is produced in the form of biological inactive pro-cytokine in the cytosol. IL-1β exerts its biological effects by binding the membrane-bound type I IL-1 receptor (IL-1R1). The receptor contains extracellular immunoglobulin domains and a Toll/IL-1 receptor (TIR) domain in the cytoplasmic portion. Binding of the ligand allows the recruitment of a second receptor subunit, the IL-1R accessory protein (IL-1RacP or IL-1R3) to form a complex that activates intracellular signalling. The receptor heterodimer complex induces signalling because the juxtaposition of the two TIR domains enables the recruitment of Myd88, IL-1R associated kinase 4 (IRAK4), TNFR-associated factor 6 (TRAF6) and other signalling intermediates. The ensuing biological response typically involves the activation of the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways that lead to the induction of a large number of pro-inflammatory and immune genes. IL-1R1 can bind the agonist cytokine IL-1β and also IL-1α, both with high affinity (0.1–1.0 nM), and the IL-1Ra with comparable efficiency.

However, the contact mode between IL-1Ra and IL-1R1 only induces a partial wrapping of the receptor around IL-1Ra, with the receptor structure being more extended and open than the one of the IL-1β–IL-1R1complex. There is also a second receptor for IL-1, called type II IL-1 receptor (IL-1R2), with an extracellular domain highly homologous to that of IL-1R1 and being able to bind IL-1β, but unable to initiate signalling because of its very short cytoplasmic portion lacking the TIR domain (reviewed by Boraschi and Tagliabue, 2013). IL-1R2 can perform its role either on the cell surface or as soluble receptor after it is detached from the plasma membrane, acting as molecular trap for IL-1 and functioning as a negative regulator. Thus, IL-1R2 is considered a decoy receptor because of its capability to recognize the ligands with high affinity and specificity, although its structure is not able to activate intracellular signalling. IL-1R2 and IL-1Ra, a polypeptide antagonist, exert a tight control of the IL-1 signalling system during potentially devastating local and systemic inflammatory reactions (Figure 1.4C).

1.7. Activation of IL-1β

Many stimuli can activate the synthesis of the inactive form of IL-1β, including bacterial lipopolysaccharides (LPS), microbial products, cytokines and immune complex. However, a second step is required to produce the active protein. The processing enzyme that cleaves the inactive IL-1β into mature protein is a cystein protease, called IL-1β converting enzyme (ICE or caspase-1). This caspase, like other caspases, is present in the cells as catalytically inactive zymogen and generally undergoes proteolytic processing during activation (Martinon and Tschopp, 2007). The best-studied models for caspase-1 activation are linked to the stimulation of NOD-like receptor 3, NLRP3. This receptor, like other NLRs, is able to recognize a range of compounds that are indicative of injury, including extracellular ATP, metabolic stressors, such as elevated levels of glucose and monosodium urate crystals, environmental irritants, as UVB irradiation. Upon NLRP3 activation, its oligomerization leads to the activation of the adaptor protein ASC that in turn binds the recruit domain CARD of procaspase-1. The effect of this binding is the auto-cleavage and formation of the active caspase-1 (reviewed by Schroder and Tschopp, 2010) (Figure 1.4A).

Since the IL-1β protein lacks a conventional signal peptide, the precise mechanisms of protein secretion are not well known. Typically, proteins are cotranslationally translocated into the endoplasmic reticulum (ER), although some are posttranslationally translocated. The presence of a signal sequence at the N-terminus of the nascent peptide is the first step for its translocation into the ER lumen. Here, the signal peptide is removed and, with the aid of chaperones, assumes a correctly folded state. A complex of proteins facilitate its passage into the Golgi, where it can receive further posttranslational modifications before its packaging and transport to the final destination, that can be another organelle or exocytosis.

This idea arose from different studies in which the routes of secretion employed are dictated by the strength of the inflammatory stimulus and by the extracellular levels of IL-1β required to mount an effective inflammatory response. Three categories of

secretion mechanisms have been proposed (reviewed by Lopez-Castejon and Brough, 2011) (Figure 1.4B).

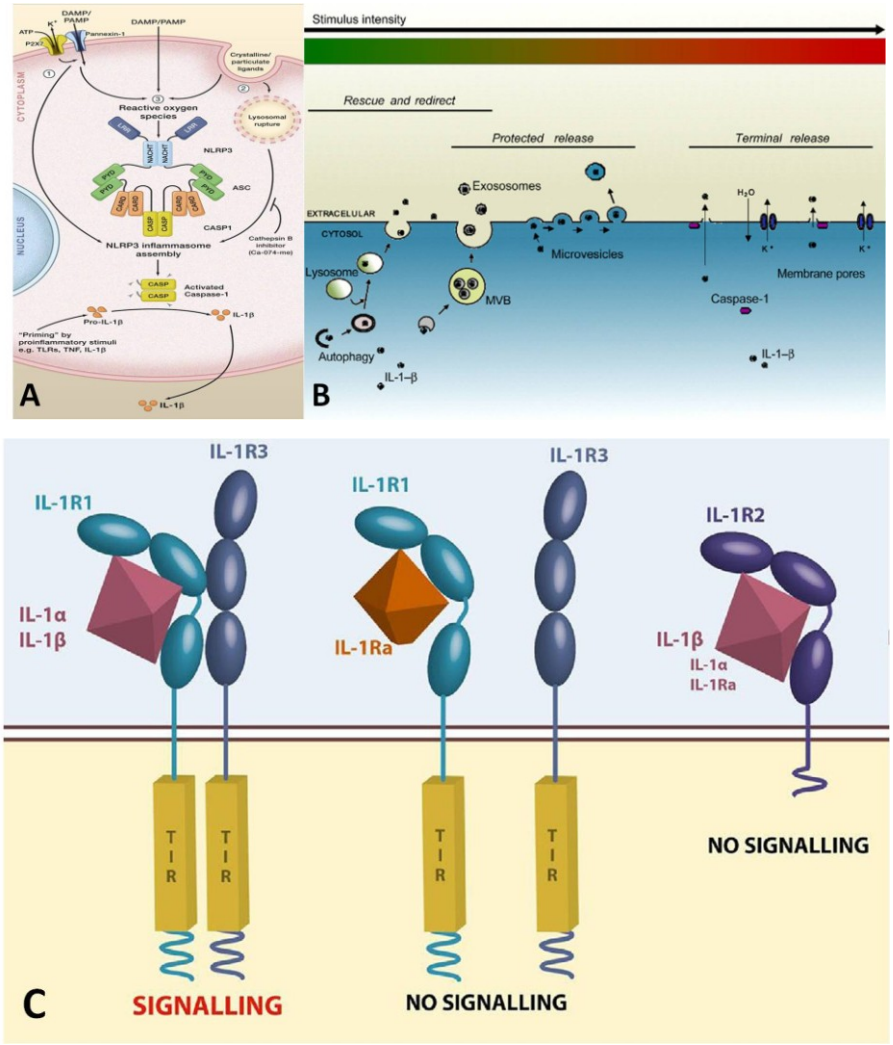


Figure 1.4. IL-1β signalling. A. Mechanisms of activation of inflammasome and active IL-1β production. B. Different routes of Il-1β secretion. C. IL-1 receptor complexes (adapted from, respectively, Schroder and Tschopp, 2010; Lopez-Castejon and Brough, 2011; Boraschi and Tagliabue, 2013).

1.8. IL-1β in Adult Seizure and Epilepsy Models

The inflammatory responses associated to seizure induction have been discussed for long time. Several studies suggest that inflammatory mediators and glial cells are important contributors in the process of epilepsy, with particular regard to IL-1β. First, this cytokine may be released in the brain after seizure induction in some rodent models organism (Plata-Salamàn et al., 2000; Minami et al., 1990; Vezzani et al., 1999; Vezzani et al., 2002; Eriksson et al., 2000; Viviani et al., 2003; Steffensen et al., 1994; Zhu et al., 2006). Second, clinical evidence from human studies suggests that *IL-1β* gene polymorphism is associated with an increased susceptibility to seizures and epilepsy (Kanemoto et al., 2003). These findings define a distinct pro-epileptogenic role for IL-1β (Vezzani et al., 2007). During the process of epileptogenesis, regardless of whether neuronal cell death take place or not, IL-1β promotes the activation of classical signalling pathways that involve the transcriptional responses mediated by MAPK and NF-kB, and a non-conventional rapid effect on neuronal transcription that involved change in ion channels and neurotransmitter receptors (Figure 1.5).

Beyond these experimental evidences, it has also suggested an anti-convulsant effect of IL-1β in seizure generation. The study of IL-1β and IL1R1 null adult mice treated with two different proconvulsants (PTZ and KA) has shown an increased seizure severity and duration (Claycob et al., 2012). Retrospectively, *in vitro* studies support this recent finding, suggesting that IL-1β can act in potentiating the GABAergic transmission and by reducing the hyperexcitability of the brain (Zhu et al., 2006). Thus, the effects of IL-1β on the excitability of neurons could depend on many factors, such as the IL-1β concentration in the brain, the functional state and the type of neurons involved in the seizure, and the neuronal exposure time to this cytokine.

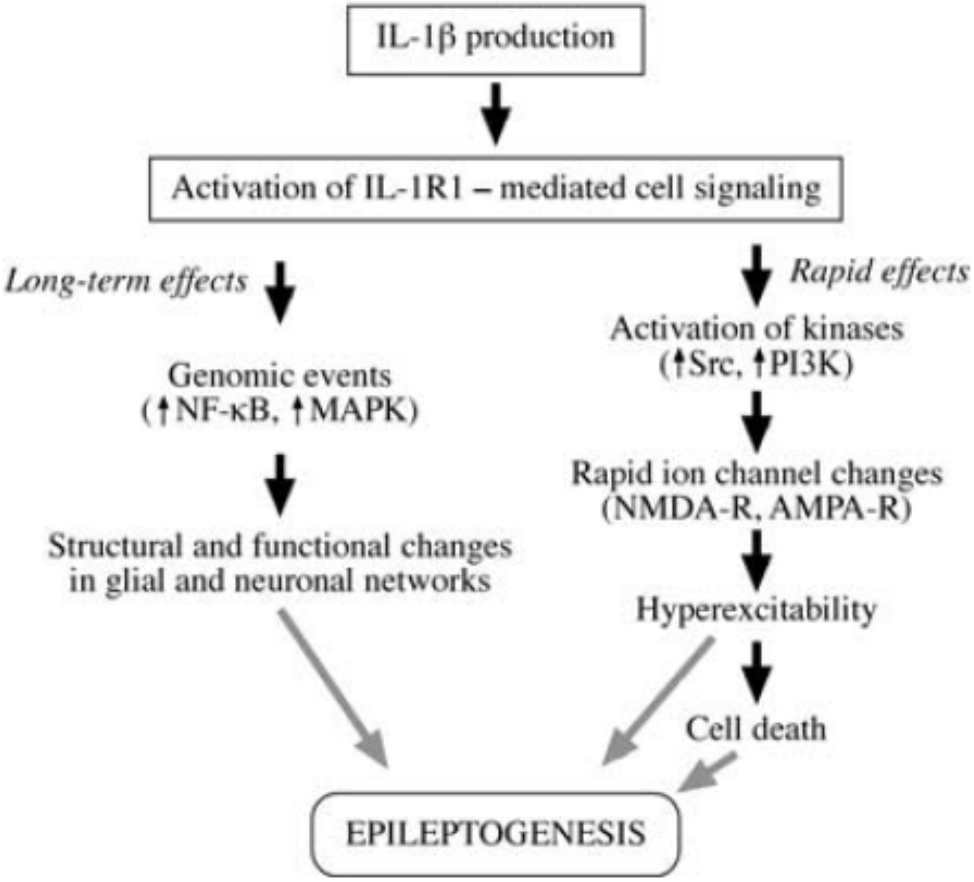


Figure 1.5. Il-1β involvement in epileptogenesis processes (Vezzani et al., 2007).

1.9. IL-1β vs Seizure and Epilepsy in the Immature Brain

During the first months of life, children are exposed to a particularly high risk for seizures, because of varying injuries can occurs such as birth trauma, infections, intracranial haemorrhages, and metabolic disturbances (Holmes and Ben-Ari, 2001). Moreover, fever rarely results in seizures in adults while it causes "febrile" seizures in 3-5% of infants and children in the Western world (with peaks up to 14% in Japan) and constitute the most common seizure type in the developing brain (Hauser, 1994). All together, immature brain is clearly more prone to seizures: this feature is directly linked to the developing context. During development, excitatory and inhibitory receptors are not expressed simultaneously and their functionality is different with respect to the mature brain. In neonate, it has been demonstrated that GABA receptors have depolarizing properties (reviewed by Holmes and Ben-Ari, 2001). Although in children most seizures are benign and do not evolve into epilepsy later in life, experimental studies using animal models suggest that frequent or prolonged seizures in the developing brain can generate long-lasting effects (Ben-Ari and Holmes, 2006). Seizure may, in fact, perturb the developing neurons and affect the proliferation and migration and the establishment of new synapses, a process that is essential for the correct formation and wiring of the brain network and circuitry. For this reason, animal models of seizures in the immature brain provide a unique opportunity to study the enhanced excitability during development.

Rats are commonly used as animal organism in epilepsy research focusing on the developing brain. Rats are born in a premature state relative to human: a 8–10 day old, postnatal (P) rat corresponds to a full-term neonate, a P12–18 rat to an infant/toddler, and a P25–38 rat to a peripubertal child (Haut et al., 2004; reviewed by Holopain, 2008). Mechanisms linked to cell death in the mature brain, such as the inflammatory reactions, do not seem to affect the young rat brain. Rizzi et collegues (2003) demonstrated that the activation of glia and related production of cytokines is age-dependent. Ravizza et collegues (2005) have proposed weak microglia activation in the hippocampus of P9 rats, whereas strongly immunoreactive microglia cells appeared not only in the hippocampus but also in the extrahippocampal areas in older

rats (P15 and P21). Moreover, the expression of GFAP and interleukin IL-1β mRNAs does not increase in P9, is upregulated in P15, and is extensively augmented in P21 along with the expression of all the cytokines studied, *i.e.* interleukin-6 (*IL-6)* and Tumor Necrosis Factor alpha (*TNFα*), with the appearance of degenerating neurons. Age-dependent activation and expression levels of cytokines, and glial reactivity, have been recently confirmed (Javela et al., 2011). The proposed mechanism consists in a rapid expression of inflammatory cytokines, followed by an activation of glial cells in a subacute phase that follows seizure induction in the juvenile brain (P15). In the immature brain (P9), on the contrary, the expression of IL-1β is increased after 24 hour of seizure induction, with no morphological changes in the resting states of microglia. The transient increase of cytokine mRNA expression, and the persistence of glial cell reaction at the subacute phase, suggests the existence of a fulminant and general initial reaction towards a more moderate and precisely targeted response (Javela et al., 2011).

Studies in animal models have made important contributions in the understanding of the effects of seizures and in the identification of related long-term consequences for behavioural and cognitive processes in the epileptic brain. A better comprehension of seizure events "from genes to behaviour" relies on the precise definition of the different levels of time-dependent changes (Holopain, 2008). Indeed, we still know little not only about the entire temporal dynamics of the seizure event in itself, but we also need to gain deeper insights on the relationship between seizures and the specific ontogenetic window in which they occur. To this aim, the use of a simple vertebrate model organism may offer the opportunity to dissect the complex molecular and cellular events associated with seizure and epilepsy. In particular, emerging model systems such as the zebrafish may be also instrumentals to the exploration of the degree of phylogenetic conservation of the seizure-related mechanisms.

1.10. The Zebrafish Model

Zebrafish (*Danio rerio*), a small tropical fish native to Southeast Asia, is now a well-established model organism in biomedical research, because of its characteristics that make it very versatile. Its multiple advantages include small size, the limited dietary requirement and a short generation that allow keeping many fishes in a confined space, with low cost- and space-effectiveness. The great proliferative capacity and the number of progeny (up to 200 embryos per reproductive event) guarantee a huge amount of experimental units within a year. Thanks to the transparency of zebrafish embryo and larvae, including optical access to the central nervous system (CNS), and to the external fertilization, it is possible (i) to observe all stages of embryonic development, (ii) to follow the different cellular fates and (iii) to monitor at each stage the expression of specific genes. Embryonic development is rapid so that it is possible to observe distinct morphological characteristics 24 hours post fertilization (hpf) (Kimmel et al., 1995) (Figure 1.6).

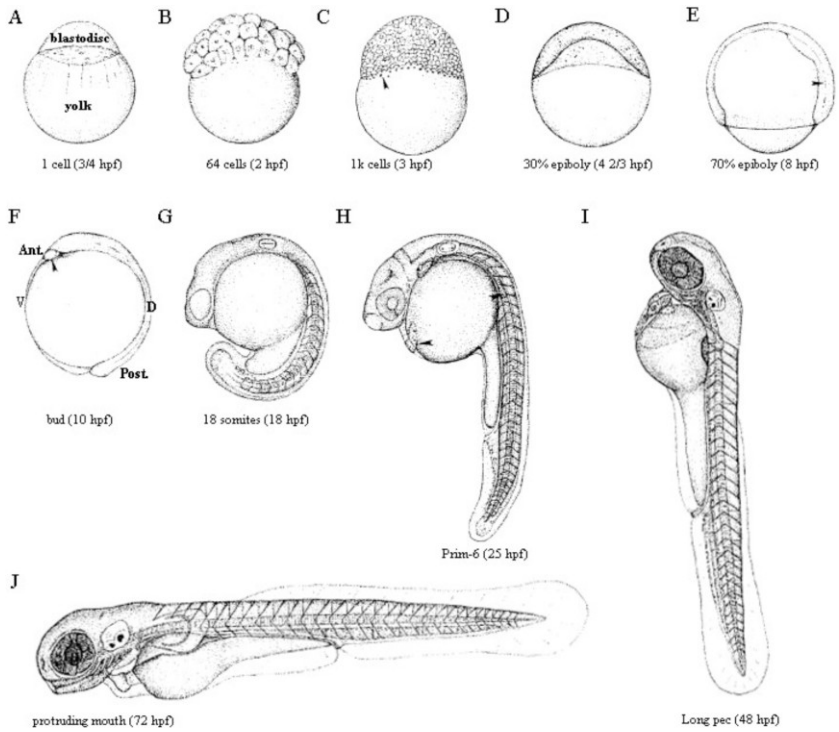


Figure 1.6. Zebrafish developmental stages. A-E The animal pole is to the top for the early stages, (F) anterior is up or (G-L) to the left at later stages (Kimmel et al., 1995).

Other advantages include the ability to easily manipulate eggs and embryos, allowing transplantation, cell ablation or microinjection of nucleic acids (*e.g.* Morpholino antisense oligonucleotides and mRNA) to achieve gene knockdown or gain-of function. Finally, zebrafish possess high physiological and genetic homology to mammals (Howe et al., 2013). Both larval and adult zebrafish are extensively used in neurobiology research also to model and address various brain disorders. Specifically, zebrafish offer the potential for brain imaging, behavioural phenomics and high-throughput screening (HTS). This model organism is also critical for drug discovery and for identifying novel candidate genes implicated in brain disorders, ranging from neoplastic to neurological and neuropsychiatric illnesses (Stewart et al., 2014) (Figure 1.7). Taken together, these evidences indicate that *Danio rerio* is one of the main organisms in translational neuroscience research, complementing both rodent and clinical models of major brain disorders. In fact, also epilepsy, commonly studied in rodents, can be modelled in zebrafish. In this regard, Baraban and colleagues examined for the first time in 2005 the feasibility of using developing zebrafish larvae as an epilepsy model system. They hypothesized that zebrafish larvae at 7 dpf has developed the brain structures that are implicated in the development of complex seizure activity. These authors demonstrated the general usefulness of the zebrafish model, describing changes in behavioural, electrophysiological and *c-fos* gene expression, the latter being typically elevated during seizures in rodents (Baraban et al., 2005). Subsequently, other scientists have used adult zebrafish to model seizures (Mussulini et al., 2013), as well as larvae to test the action of several anti-convulsant agents. These approaches permitted to define zebrafish as a powerful HTS model for testing various pro- and anti-epileptic drugs (Baxendale et al., 2012). Moreover, targeted mutations in zebrafish orthologues of known genes that cause epilepsy in humans and rodents are being produced. One example is the *mind-bomb* mutant, in which alterations of E3 ubiquitin ligase activity and of Notch signalling result in defects in brain development and in spontaneous seizures (Hortopan et al., 2010). Another example is the mutation in the zebrafish orthologue of *SCN1A*, a gene that encodes a voltage-gated sodium channel. In humans, mutations in this gene cause characteristic Dravet syndrome with severe intellectual disability and drug-resistant seizures. At the same

time, *scn1Lab*$^{-/-}$ mutant zebrafish display a spontaneous seizure-like activity, supporting the use of zebrafish for modelling also pediatric epilepsy (Baraban et al., 2013).

As for neuronal activity studies, zebrafish offers many advantages as model for analyzing immune responses associated with human diseases. The zebrafish immune system presents similarities with that of humans, with comparable immune cell types and signalling pathways associated to pathogen infections as well as to mechanical and chemical injuries (Nguyen-Chi et al., 2014; Ogryzko et al., 2014; van Ham et al., 2014). Already one day post fertilization, zebrafish embryos present functional macrophages that are capable of sensing and responding to pathogens. At 2 dpf, the capability to combat infections is increased in concomitance with the appearance of differentiated neutrophils. Both macrophages and neutrophils are able to migrate rapidly to sites of infections or wound-induced inflammation (Figure 1.8B). Between 2 and 3 dpf, primitive macrophages colonize the brain and retina and became early microglia cells, thus starting to express high levels of apolipoprotein-E, with the concomitant downregulation of l-plastin, a common marker of leucocytes (Figure 1.8A) (Meijer and Spaink, 2011). Not only almost all cell types of the mammalian immune system have been identified in zebrafish, but also the receptors signalling molecules and pathways have their fish orthologue counterparts. For example, the TLR genes are expressed during all stages of embryonic development (van der Sar et al., 2006), and also the NLR family has a number of orthologues in all fish species (Angosto and Mulero, 2014; Ogryzko et al., 2014). Among the latter family, NALP3 responds to various stimuli by forming the inflammasome complex that promotes the release of IL-1β (Angosto and Mulero, 2014).

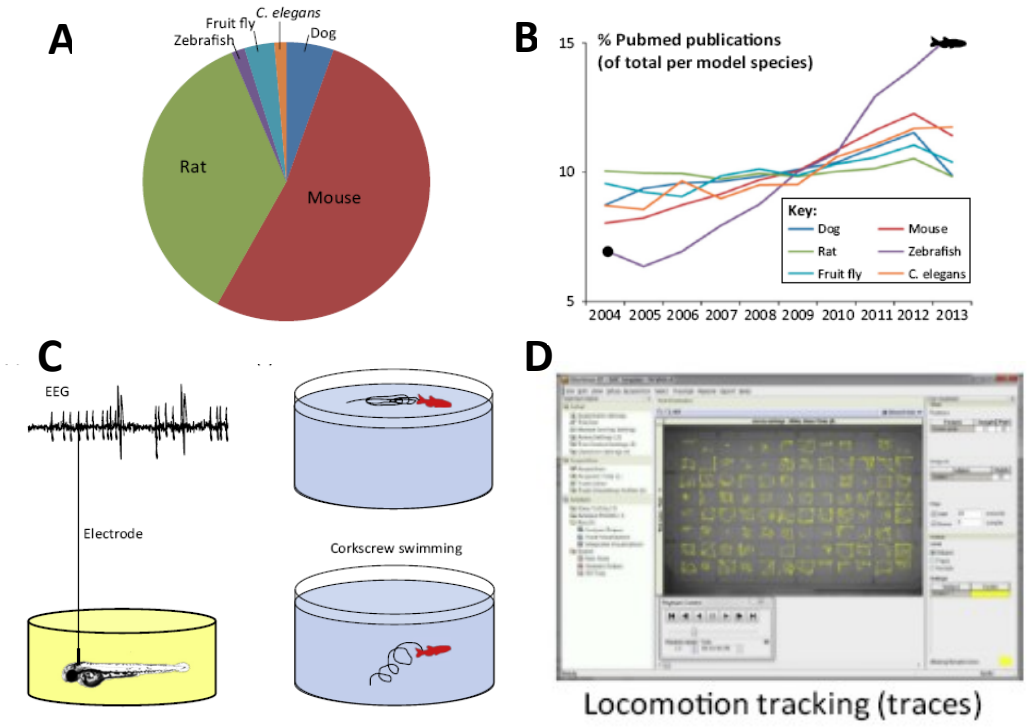


Figure 1.7. Representative advantages in zebrafish biomedical research (modified from Stewart et al., 2013).

With respect to IL-1 biology, early studies in zebrafish had highlighted important differences with respect to the mammalian orthologous gene, including human, such as the lack of a conserved caspase-1 cleavage site in the zebrafish homologue and an amino acid identity of only 27%. However, it was later shown that the C-terminal domain of the mature protein has higher identity with mammals. In fact, the Phyre structural predition server allowed to identify the presence of a β-sheet rich trefoil structure in zebrafish IL-1β sequence that closely matches with the mature human protein (Ogryzko et al., 2014) (Figure 1.9A-B). In addition, the presence of two inflammatory caspases in zebrafish has been reported. These zebrafish inflammatory caspases are able to process IL-1β *in vitro* by using different amino acid residues with respect to the mammalian gene (Figure 1.9C), and are sensitive to mammalian caspase-1 inhibitors *in vivo* (Vojtech et al., 2012; Ogryzko et al., 2014). Together with the increasing evidence of evolutionary and functional aspects in the zebrafish

inflammasome, the power of zebrafish as model to study the IL-1β inflammatory pathways is well established.

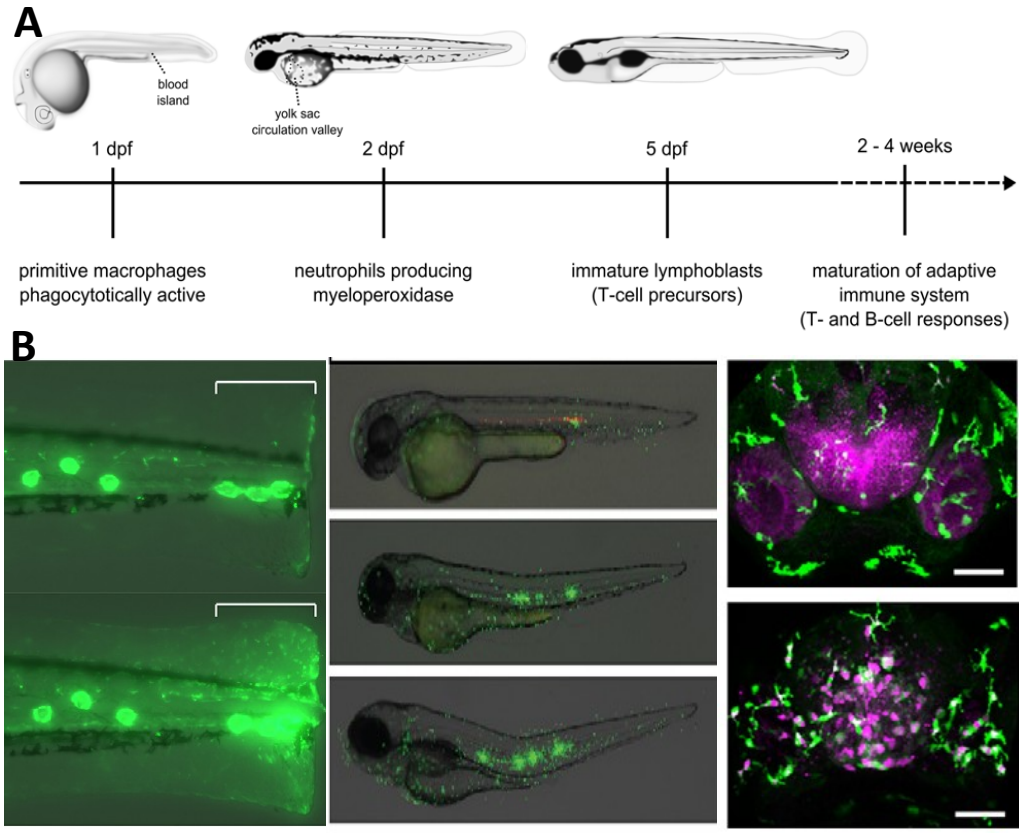


Figure 1.8. Zebrafish Immunity. A. Schematic representation of zebrafish immunity development (Meijer and Spain, 2011). B. Examples of zebrafish model of inflammatory response (Chi et al., 2014; Ogryzko et al., 2014; van Ham et al., 2014).

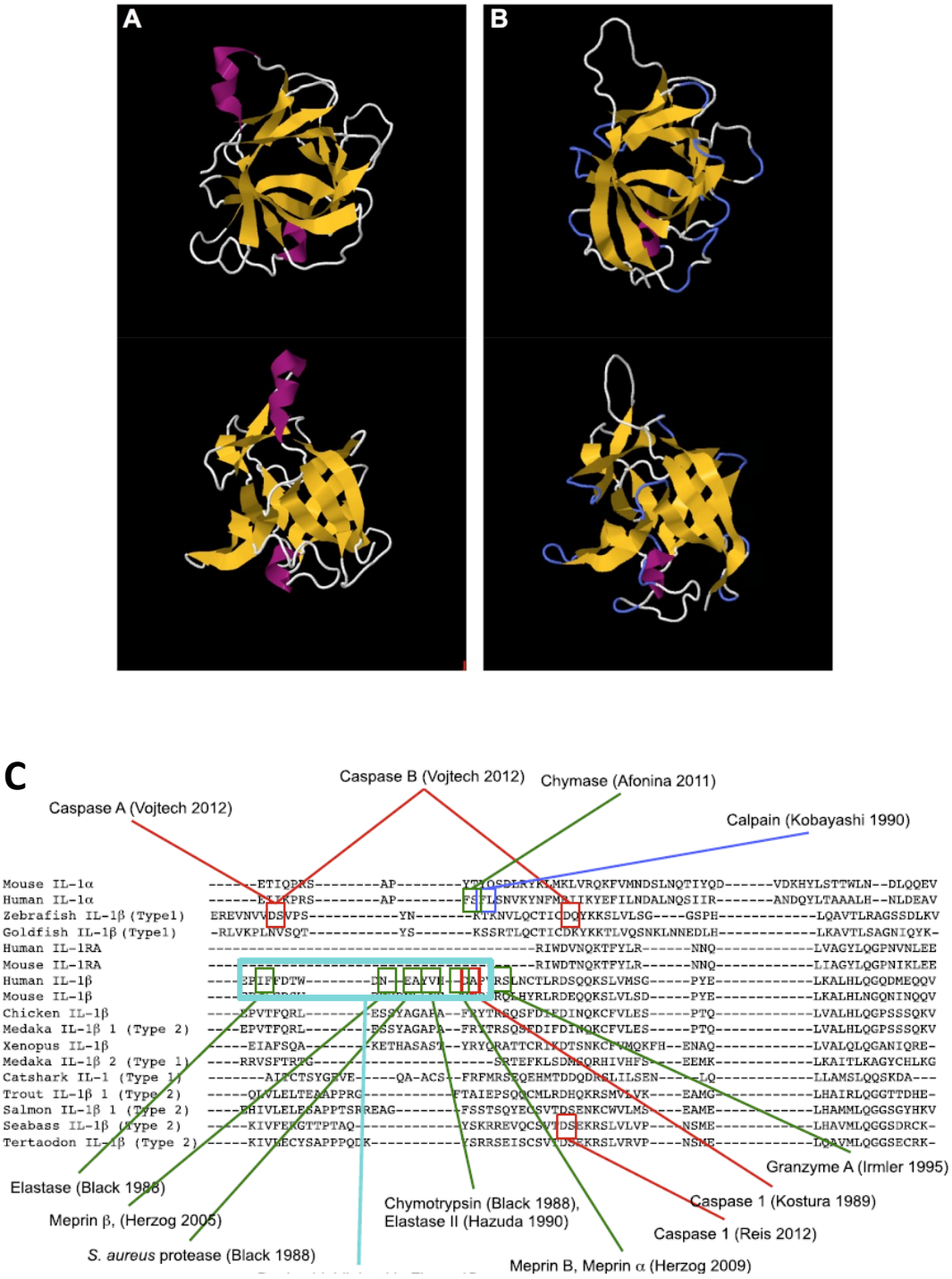


Figure 1.9. IL-1β protein. A. Representation of Human IL-1β. B. Representation of zebrafish IL-1β. C. Cut site of zebrafish caspase-1 orthologues (Ogryzko et al., 2014.)

1.11. Aims of the Project

The aims of my PhD project are to characterize seizures and seizure-related effects in an early phase of functional maturation of zebrafish brain by using PTZ. Current literature demonstrated that PTZ administration induces seizures in zebrafish larvae and adults, associated with alteration in locomotory responses and changes in expression of *c-fos* gene, a marker of neuronal hyperactivity (Baraban et al., 2005; Mussulini et al., 2013). Recently, Baxedale and collegues (2012) have investigated the possibility in developing seizures also in the early phase of zebrafish development (2 dpf), showing that GABA components are still expressed in the embryonic zebrafish brain and, like *c-fos*, other genes are regulated by PTZ-induced seizures, one of which is *bdnf*. Until now, PTZ model of seizure has been used for studying the activation of epilepsy mechanisms and for anti-epileptic drugs discovery. Therefore, we still know little about the temporal regulation of seizures in the PTZ zebrafish model during the entire event from induction to recovery. Starting from the evidence that PTZ can promote seizures in 3 dpf zebrafish larval brain, the first objective of my PhD project is to characterize the locomotory phenotype and the transcriptional responses associated with the hyperexcitability condition. The second objective is to analyse the transcriptional and cellular mechanisms of inflammation involved in PTZ-induced seizures in zebrafish and to verify to which extent inflammatory responses are eventually responsible for the seizure event itself.

In this PhD project, I will address the following questions in the immature brain of zebrafish larvae:

- Which is the temporal regulation of hyperexcitation after seizures?
- Are acute seizures able to activate an inflammatory response?
- Do inflammatory molecules contribute to seizure chronicization?

CHAPTER 2

Material and Methods

2.1. Zebrafish (*Danio rerio*) care

Adult zebrafish are kept in a Tecniplast Stand alone unit (Italy) under standard controlled conditions: 28°C, 400 µS and pH 7.5. The lighting is kept on daily cycle of 14 hours of light and10 hours of dark to the fish to breed. Embryos were staged according to hours post fertilization (hpf) and morphological criteria (Kimmel *et al*., 1995). To perform experiments, embryos were manually dechorionated and anaesthetized in tricaine before fixed overnight, using 4% Paraformaldehyde (PFA) in PBS. Then, they were washed in PBT (PBS + 0.1% Tween-20), dehydrated and stored in methanol at -20°C.

2.2. RNA extraction and RNA quality detection

Anaesthetized control and treated 3 dpf zebrafish larvae (n=15) were collected in 500 µl TRIZOL reagent (Invitrogen), and frozen at -80°C until RNA extraction. The tissue was homogenized using Tissue Lyser for 5 minutes at 25 Hertz (Hz). After the homogenisation, the samples were incubated at room temperature (R.T.) for 5 minutes and then placed on ice while the other samples were processed. When the 5 minute incubation period of the last sample was completed, 100 µl of chloroform (Sigma) were added to the homogenates. Samples were vortexed and incubated at R.T. for 3 minutes. After this short incubation, they were centrifuged at 12000 rpm at 4°C for 15 minutes. The aqueous phase was transferred into a clean tube and the RNA precipitated with 200 µl phenol –chloroform and centrifuged at 12000 rpm at 4°C for 15 minutes. After then, the RNA were precipitated using 2 volume of isopropanol, 1/10 volumes of acetic sodium 3 M pH 5.2, 1 µl of glycogen and

transferred at -20°C for 30 minutes. The tubes were centrifuged for 5 minutes at 13500 rpm, the supernatant was discarded and the pellets were washed in 70% ethanol in DEPC treated water by centrifuging the samples for 5 minutes at 10000 rpm. The supernatant was once again discarded and the pellets were left to air dry, then dissolved in 30 µl DEPC and stored at -80°C.

RNA was analyzed by the 2100 ByoAnalyser machine (Agilent Tecnologies) using the Eukaryote total RNA Nano Series assay. The integrity of RNA was estimated by RNA Integrity Number (RIN) values, calculated by an algorithm that assigns a 1 to 10 RIN score, where level 10 RNA is completely intact value (Figure 2.1).

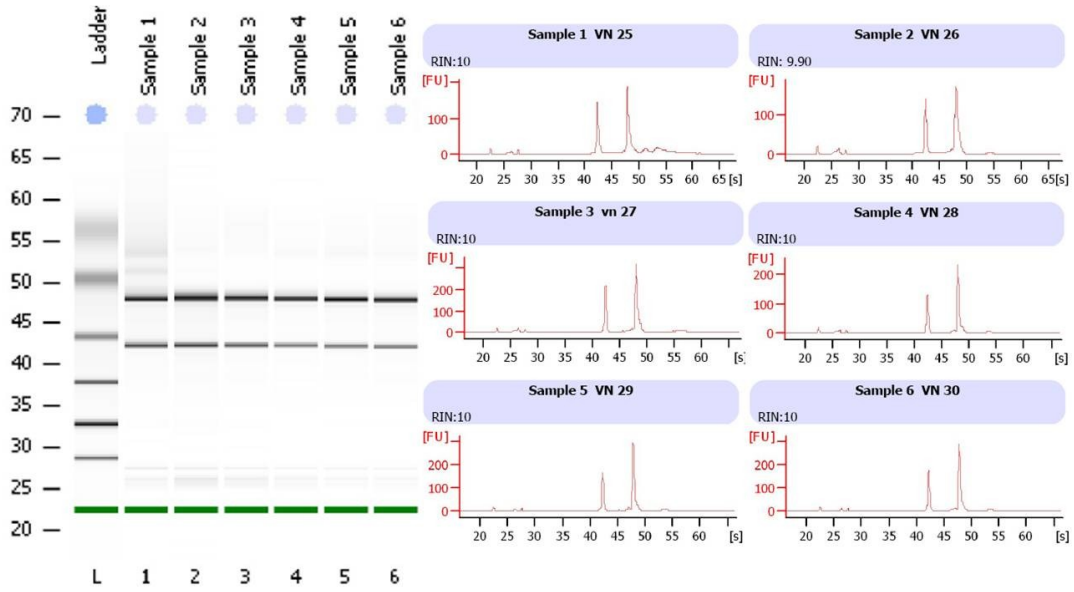


Figure 2.1. RNA quality assay. The right panel shows the electrophoresis gel image of the ladder (first lane) and of 6 RNA samples (samples 1-6) prepared from control and treated larvae. The right panel shows the electropherogram profile summary of the RNA samples (1-6).

2.3. Reverse Transcription

First strand cDNA synthesis from total RNA was obtained by SuperScript VILO™ MasterMix Kit (Invitrogen). This SuperScript® VILO™ Master Mix kit includes SuperScript® III RT, RNaseOUT™ Recombinant Ribonuclease Inhibitor, a proprietary helper protein, random primers, $MgCl_2$, and dNTPs. 1 µg of total RNA was added to 4 µl of SuperScript® VILO™ Master Mix and RNase-free H_2O to obtain a final volume of 20 µl. The reaction mix was incubated at 25°C for 10 minutes, then, at 42°C for 60 minutes and finally stopped by heating at 85°C for 5 minutes. The cDNA was stored at –20°C.

2.4. PCR amplification

The PCR reactions have been performed in a total volume of 50 µl, using cDNA as template, 0.2 mM of dNTP mix (dATP, dTTP, dCTP, dGTP), 1x PCR buffer (Roche), 0.05 U/µl of Taq DNA polymerase (Roche) and forward and reverse suitable oligonucleotides. The PCR amplification program has been set as follows:

- First step (1 cycle). DNA denaturation: 5 minutes at 95°C.
- Second step (repeated for 35 cycles).
  DNA denaturation: 1 minute at 95°C.
  Oligonucleotide annealing: 1 minute at suitable temperature (the temperature used in this step has been set at least 5-8°C below the melting temperature of the oligonucleotides (Table 1 and 2)).
  Polymerization: 72°C for a suitable time, calculated considering the desired amplified fragment length and the Taq DNA Polymerase processivity, that is around 1 Kb/minute.
- Final elongation step: 10 minutes at 72°C.

In order to separate the amplified fragments from the template and from dNTPs and oligonucleotides excess, gel electrophoresis have been performed using, as fragment length marker, 1x GeneRuler™ 1Kb DNA Ladder (Fermentas), according to the expected length of the fragment.

2.5. DNA gel electrophoresis

Preparative and analytic DNA gel electrophoresis has been performed on 1% of agarose gel in 1x TAE buffer (TAE Stock solution 50x: 252 g of Tris base; 57.1 ml glacial acetic acid; 100 ml 0.5 M EDTA; H_20 to 1 liter) and adding 0.5 μg/ml of Ethidium Bromide (EtBr).

2.6. DNA gel extraction

PCR amplified fragments have been extracted from gel cutting them with a sterile sharpen blade, using the GenElute™ Gel Extraction Kit (Sigma-Aldrich), following the manufacturer's instructions. After the extraction, the concentration has been estimated by gel electrophoresis.

2.7. TOPO cloning

PCR amplifications were performed on cDNAs at 3 dpf. The amplicons obtained were cloned in the pCR®II vector (TOPO® TA Cloning Dual Promoter Kit, Invitrogen), following the manufacturer's indications.

2.8. Bacterial cell electroporation

This approach allows transforming bacterial cells with plasmids containing DNA of interest. Briefly, the circular plasmid DNA and competent *E. coli* bacterial cells (prepared by the Molecular Biology Service of the Stazione Zoologica Anton Dohrn of Naples), were placed in a 0.2 cm electrocuvette. The electrocuvette was subjected to an electric pulse at constant 1.7 V using a Bio-Rad Gene Pulser™ electroporation apparatus.

The transformed *E. coli* cells were allowed to recover for one hour at 37ºC in 1ml LB medium (NaCl 10 g/l, bactotryptone 10 g/l, yeast extract 5 g/l,). An aliquot was spread on a pre-warmed LB solid medium (NaCl 10 g/l, bactotryptone 10 g/l, yeast extract 5 g/l, agar 15 g/l) in the presence of specific selective antibiotic and let grow at the same temperature overnight.

2.9. Plasmid DNA Mini-Preparation

A single bacterial colony containing the plasmid DNA of interest was grown in a suitable volume of LB (4-5 ml) in the presence of the appropriate antibiotic and shaking at 37°C overnight. The Sigma-Aldrich Plasmid Purification kit, based on alkaline lyses method, was used to isolate the plasmid DNA from the cells according to the manufacture‘s instruction.

2.10. Sequencing

The DNA sequences have been obtained using the Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) by the Molecular Biology Service of the Stazione Zoologica Anton Dohrn of Naples.

2.11. DNA digestion with restriction endonucleases

Analytic and preparative plasmid DNA digestions have been performed with the appropriate restriction endonucleases in total volumes of at least 20 times more than the enzyme volume used. The digestion reaction has been prepared as follows: the solution contained the desired amount of DNA, suitable restriction enzyme buffer (1/10, New England Biolabs), restriction enzyme (5 units enzyme per 1 µg of DNA) and BSA (1/100, if required). Reaction specific temperatures have been used as suggested by manufacturer's instructions.

2.12. Digested plasmid purification

In order to eliminate protein contaminations and to obtain the template for riboprobe synthesis, the plasmid DNA linearized has been purified with 1 volume of phenol:chloroform:isoamylic alcohol (25:24:1), vortexed vigorously and centrifuged at 13000 rpm for 5 minutes at 4°C. The soluble phase has been recovered and 1 volume of chloroform:isoamylic alcohol (24:1) has been added; the sample has been vortexed vigorously and centrifuged at 13000 rpm for 5 minutes, at 4°C. The aqueous phase has been recovered and the DNA has been precipitated adding 2.2 volumes of ethanol 95%, 1/10 volume of Sodium Acetate 3 M pH 5.2 and 1 µl of glycogen. The sample has been mixed and stored over night at -20°C or 1 hour at -80°C. Then, it has been centrifuged at 13000 rpm for 15 minutes, at 4°C. The precipitated DNA has been washed with ethanol 70% (sterile or DEPC-treated), centrifuging at 13000 rpm for 15 minutes at 4°C. The ethanol has been removed and the sample has been air-dried at R.T. At the end, the DNA has been diluted in a suitable volume of H_2O (sterile or DEPC-treated). Its concentration has been evaluated by gel electrophoresis, using a spectrophotometer (Nanodrop 1000, Thermo SCIENTIFIC). To ascertain the absence of chemical (phenol, ethanol) and protein contamination, the values at the wavelengths of 230, 260 and 280 nm have been read and the ratio between 260/230 nm and 260/280 nm has been calculated.

2.13. Ribonucleic probe preparation

The in vitro transcription was performed using the DIG RNA labeling kit (Roche). 1 µg of purified and linearized DNA has been used as template for the ribonucleic probe synthesis. This template has been added to the following reaction mix: transcription buffer (10X 2 µl); Digoxigenin mix, containing 1 mM of ATP, CTP and GTP, 0.65 mM UTP and 0.35 mM DIG-11-UTP (10X 2 µl); Sp6 or T7 RNA polymerase (20 U/µl); Protector RNase inhibitor (20 U/µl), in H_2O DEPC-treated. The mix has been briefly centrifuged and incubated for 2 hours at 37°C. Then, DNaseI RNase free has been added in order to remove the DNA template. The sample has been incubated for 20 minutes at 37°C. Finally, the reaction has been stopped adding 0.2 M EDTA pH 8.0. The RNA riboprobes have been precipitated by adding LiCl (4 M) and ice cold 100% ethanol at -20°C, and then centrifugated at 4°C. The pellet has been washed with ice cold 70% ethanol and allowed to air-dry. The probes has been dissolved in DEPC water. The ribonucleic probe quality has been checked by gel electrophoresis. Recovered samples have been immediately stored at -80°C until the use.

2.14. Whole mount *in situ* hybridization

Day 1: embryos were rehydrated stepwise in methanol / PBS and finally put back in PBT. Samples were incubated in proteinase K (10 µg/ml in PBT) for a period of 30 minutes for 3 dpf embryos. Reactions were stopped by rinsing in PBT followed by post-fixation in 4% paraformaldehyde in 1x PBS for 20 minutes at R.T. and by rinsing four times in PBT (5 minutes). Zebrafish embryos were pre-hybridized for at least 1 hour at 65°C in hybridization buffer (50% formamide, 1.3x SSC, 5 mM EDTA (pH 8.0), 50 µg/ml yeast RNA, 0.2% Tween 20, 0.5% CHAPS 10%, 100 µg/ml heparin). Embryos were then incubated overnight in the hybridization solution containing the probe at 65 °C (probe was denatured for 10 minutes at 95 °C).

Day 2: probe was removed by 30 minutes step - wise washes in 100, 75, 50 and 25% hybridization buffer and 2x SSC for 5 minutes, in 2x SSC for 10 minutes, in 0.2x SSC for 30 minutes, in 10 mM PIPES and 0.5 M NaCl for 10 minutes, and finally in Maleic Buffer Tween - 20 (MBT). Subsequently, embryos were incubated in 2% Roche Blocking Reagent (Roche Applied Science, code 11 096 176 001) for 2 hours and then left in Fab - alkaline phosphatase (Roche Applied Science, code 11 093 274 910) at a 5000 - fold dilution in fresh MBT plus blocking reagents overnight at 4 °C.

Day 3: After several washes in MBT, embryos were incubated in a staining buffer (100 ml NaCl 5 M; 100 ml Tris HCl 1 M, pH 9.5; 50 ml $MgCl_2$ 1 M; 0.1% Tween - 20) and then in BM Purple (Roche Applied Science), a chromogenic substrate for alkaline phosphatase until staining was sufficiently developed. After stopping the reaction, embryos were post-fixed in 4% paraformaldehyde in 1x PBS for 20 minutes and finally stored in 95% glycerol at 4°C. Embryos were imaged using a Zeiss Axio Imager M1 microscope equipped with Axiocam digital camera (Zeiss). WISH experiments were performed in triplicate.

2.15. Double Immunofluorescence analysis

Day 1. 3 dpf larvae kept in MeOH at -20°C, were rehydrated through a 10 minutes wash in 50:50 MeOH:PBS and then washed 3 times for 5 minutes in PBTr (PBS + 0.01% Triton-100). The larvae were incubated in PBTr containing 10 μg/ml proteinase K for 45 minutes at R.T. Following the proteinase K treatment, the samples were washed 3 more times for 5 minutes with PBtr. Samples were fixed in fish fix for 20 minutes at R.T. and then incubated in PBS-Block (4% Normal goat serum, 1% BSA, 1%DMSO, 0.1% Tween and 0.01% Triton) for at least 1 hours. The larvae were left overnight in PBS-Block (1% NGS, 1% BSA, 0.5% DMSO, 0.1% Tween-20 and 0.1% Triton-100) containing primary antibodies (1:300, GFAP Dako, 1:300 GS Millipore) at 4°C and 1:10000 Topro3 (Life Technologies).

Day 2. The samples were washed for 30 minutes at R.T. with PBSTT (0.1% Tween-20 and 0.1% Triton-100). Then the PBSTT was replaced with PBSTT with anti-rabbit and anti-mouse Alexa Fluor coniugates (Invitrogen).

Day 3. The samples were washed 5 times at R.T. for 30 minutes with PBSTT and then mounted in glycerol 75% for confocal microscope analysis, using the two-photon confocal SP8X Leica.

2.16. Microinjection of morpholino oligos into fertilized eggs

The morpholinos are antisense oligonucleotides with a variable length between 18 and 25 nucleotide bases that are complementary to the sequence of the gene of interest. Their main function is to block mRNA translation and hence protein synthesis. In these oligonucleotides, the deoxyribose is replaced by a ring N-morpholino that gives great stability. In my case, I have used a morpholino for the knockdown the *IL-1β* gene that had been tested and validated in previous studies. The oligonucleotide sequence is supplied as a liophilized product from Gene Tools, LLC:

- IL-1β ssMO: 5'-CCCACAAACTGCAAAATATCAGCTT - 3'

The entire instrumental setting is composed of a stereomicroscope Zeiss, a micromanipulator MN of Narishige and a Picospritzer® III of Parker Instrumentation. The fertilized eggs were collected, cleaned and oriented with the animal pole into the capillary. The needles used for the microinjection were made from tubes capillaries (Microcaps from Drummond Sci. Co., Broomall, PA, USA), appropriately drawn with a specific instrument —microelectrode puller (Model PN - 3, Narishige, Tokyo) choosing suitable conditions of pressure and temperature to the needs of microinjection. Once needles were prepared, they were filled with a solution containing a concentration range of morpholino between 8 µg/µl and 0.1 µg/µl in sterile water, 0.5 % of phenol red (Sigma) as marker, and used for microinjection

into fertilized eggs. The volume injected was calculated measuring the diameter of drop through micrometre under the microscope and, using the formula of sphere volume ($4/3\pi\ r^3$). In order to identify potential endogenous and non-specific effects of morpholino oligonucleotides, as a negative control, a standard control morpholino was injected. In particular, a standard control morpholino from Gene Tools, directed against human β-globin pre-mRNA has been used. The experiments were repeated several times.

2.17. Quantitative Real-time PCR

The quantitative PCR (qPCR) allows to quantify the nucleic acid of a sample in relation to the amount of DNA produced in a PCR reaction, measured evaluating the fluorescence of an intercalating DNA dye, which is monitored at each cycle during the amplification. During the PCR exponential phase the amount of product increases linearly (on a log plot). The number of cycles needed to attain a threshold concentration (Ct) of products is measured in order to compare different samples and determine which among them contains the higher amount of a specific sequence. A qPCR experiment requires a known reference gene as internal control with constant expression in all tested samples and whose expression is not changed by the treatment under study. The number of cycles needed for the standards to reach a specified Ct is used to normalize the Ct for the selected genes. To capture intra-assay variability all RT-qPCR reactions have been carried out in triplicate and the average Ct value was taken in to account for further calculations.

The dye used was Fast Sybr Green Master Mix (Applied Biosystems), which binds to double stranded DNA (dsDNA). SYBR green dye cannot distinguish between the amplicon and contamination products from mispairing or primer-dimer artifacts. To overcome this, not only DNA synthesis is monitored, but also the melting point of the PCR products is measured at the end of the amplification reaction. The melting temperature of a DNA double helix depends on its base composition and its length.

For each gene, qPCR primers have been designed to generate products of 100-300 bp, by using online based "Primer 3, v.0.4.0" software (Table 3).
The efficiency of each pair of primers was calculated according to standard method curves using the equation E=10-1/slope. Five serial dilutions have been set up to determine the Ct value and the efficiency of reaction of all pairs of primers. Standard curves were generated for each oligonucleotides pair using the Ct value versus the logarithm of each dilution factor. Diluted cDNA was used as template in a reaction containing a final concentration of 0.7 pmol/µl for each primer and 1X Fast SYBR Green master mix (total volume of 10 µl).

PCR amplifications have been performed in triplicate in a ViiA7 ABI Applied Biosystems thermal cycler, using the following thermal profile: 95°C for 20", one cycle for cDNA denaturation; 95°C for 1" and 60°C for 20", 40 cycles for amplification; 95°C for 15", 60°C for 1' and 95°C for 15", one cycle for melting curve analysis, to verify the presence of a single product. Each assay included a no-template control for each primer pair. For triplicate samples, Ct is calculated as the average among the replicates. All samples were normalised to the levels of the housekeeping gene *β-actin1* and *elf1a*.

To calculate the effect of PTZ treatment on transcript levels for each target gene, the difference between ΔCt for the control sample and ΔCt for the experimental sample was obtained: *i.e.,* ΔΔCt = ΔCt (experimental) - ΔCt (control), and converted into fold change (FD), assuming FD= $2^{-\Delta\Delta Ct}$.

2.18. Pharmacological Induction of Seizures and Pharmacological Treatment

Pentylenetetrazole (Sigma) was prepared in stock solution of 200 mM and diluted at the final concentration of 15 mM in fresh E3 medium. Larvae were exposed to the drugs then analysed as required. Z-YVAD-FMK (YVAD) and Z-VAD-FMK (pan-caspase) (Enzo Life Sciences) were stored at -20°C and utilized at the final concentration, as required.

2.19. Tracking analysis

The locomotor activity of larvae was recorded using the DanioVision instrument (Noldus). AB larvae at 3 dpf, were transferred to a 96-well plate, one larva per well in 100 to 150µl E3 medium with 15mM PTZ. Controls containing E3 alone were also included. Right after adding compounds into the E3 media, the plate was placed in the observation chamber and the locomotor activity was recorded for 45 minutes period, using a two different protocols. In both was included a period of 5 minutes, the acclimation time, in which the larvae were detected from the instrument. In the first protocol, the light was switch on at maximum intensity of 100% for the entire recording period (45 minutes). In the second protocol, the light was switch on off every 10 minutes (10' 100% light on-10' 0% light off). After the recording, the analysis data was exported and was utilized for the statistical analysis. The experiments were repeated until the number of larvae analysed per treatment was equal or higher than 20.

The treatment with YVAD and pan-caspase drugs was performed in 6-well plate in a total volume of 3 mL E3 medium. A number of 10-15 larvae was then transferred to a 96-well plate and placed in the observation chamber. The locomotory activity was analysed as described before.

2.20. Statistical Analysis

Data are presented as mean values ± standard error of mean (SEM). Statistical analysis was performed using the GraphPad Prism version 6.0 (GraphPad Software, CA, USA). In all analyses, significance level was set at $p \leq 0.05$. Statistical comparisons between two groups were performed using the Mann–Whitney test and Student's *t* test were used to determine the significant differences in relative expression level for the RT-qPCR analyses. Statistical comparisons between three or more groups were performed using one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test.

Tab . 1 Oligonucleotides used for TOPO cloning		
Gene	Oligonucleotide	Sequence (5'-3')
c-fos	*c-fos* Forward	AGCCCATGATCTCCTCTGTG
	c-fos Reverse	CGTCGTTTTCTGGGTAGGTG
Bdnf	*Bdnf* Forward	ATAGTAACGAACAGGATGG
	Bdnf Reverse	GCTGTCACCCACTGGCTAAT

Tab . 2 Oligonucleotides used for RT-PCR		
Gene	Oligonucleotide	Sequence (5'-3')
IL-1β ssMO	*IL-1β* ssMO Forward	TGCCGGTCTCCTTCCTGA
	IL-1β ssMO Reverse	GCAGAGGAACTTAACCAGCT

Tab. 3 Oligonucleotides used for qPCR		
Gene	Oligonucleotide	Sequence (5'-3')
c-fos	*c-fos* Forward	AACTGTCACGGCGATCTCTT
	c-fos Reverse	TTGGAGGTCTTTGCTCCAGT
bdnf	*Bdnf* Forward	TCGAAGGACGTTGACCTGTATG
	Bdnf Reverse	TGGCGGCATCCAGGTAGT
TNFα	*TNFa* Forward	TCGCATTTCACAAGGCAATTT
	TNFa Reverse	GGCCTGGTCCTGGTCATCTC
IL-6	*Il-6* Forward	TCAACTTCTCCAGCGTGATG
	Il-6 Reverse	TCTTTCCCTCTTTTCCTCCTG
IL-1β	*IL-1β* Forward	ATGCGGGCAATATGAAGTCAC
	IL-1β Reverse	GGCCAACTCTAACATGCAGG
gfap	*gfap* Forward	ATTCCAGGTCACAGGTCAGG
	gfap Reverse	ATTCCAGGTCACAGGTCAGG
gs	*gs* Forward	ACTTCGGTGTGGTAGCTTCA
	gs Reverse	CAGTGAGTCGACGAGCATTG
gad 1	*gad 1* Forward	AACTCAGGCGATTGTTGCAT

	gad 1 Reverse	TGAGGACATTTCCAGCCTTC
gabra 1	*gabra 1* Forward	TCAGGCAGAGCTGGAAGGAT
	gabra 1 Reverse	TGCCGTTGTGGAAGAACGT
elf1α	*elf1α* Forward	GTACTTCTCAGGCTGACTGTG
	elf1α Reverse	ACGATCAGCTGTTTCACTCC
βactin-1	*βactin-1* Forward	GCCAACAGAGAGAAGATGACAC
	βactin-1 Reverse	CAGGAAGGAAGGCTGGAAGAG

CHAPTER 3

Results

3.1. Temporal Regulation of the Behavioural and Transcriptional Responses associated to Neuronal Hyperexcitation.

Locomotory analyses

As described in the introduction, Baraban and collegues were the first to use the zebrafish as model organism for studying epileptic seizures. In their research, 7 dpf zebrafish larvae were treated with 15 mM GABAA receptor antagonist PTZ, and seizure induction was validated by recording the swimming behaviour and transcription of the *c-fos* gene and by using electrophysiological analysis to visualize the epileptiform-like activity of the brain.

To describe the processes associated to seizure induction at early stage of zebrafish brain maturation (3 dpf), I have treated zebrafish larvae with 15 mM PTZ for 45 minutes. First, the locomotory activity was analysed during the treatment by measuring the total distance moved and the mean velocity of both control and treated larvae in a condition of continuous light stimuli in a video-recording session using the DanioVision instrument (Noldus). The behavioural analysis shows a statistically significant increase of the two measured parameters in the locomotory responses in the treated larvae (Figure 3.1).

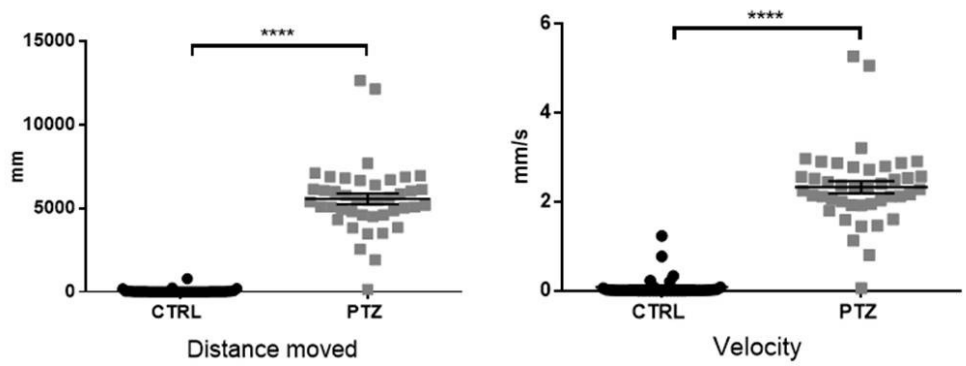


Figure 3.1. Treatment of 3 dpf zebrafish larvae with 15 mM PTZ induces measurable convulsive movements. Zebrafish larvae were treated with 15 mM PTZ in E3 medium and locomotor swimming behaviour was recorded from the time of PTZ administration to the medium until 45 minutes after the onset of treatment. Larvae were analysed using a continuos light stimulus (100% intensity). The graph shows the distance moved and velocity between control and treated zebrafish larvae. Circle and square symbols represent each larvae; the error bars indicate the s.e.m. Asterisks mean statistically significant difference, calculated using a Mann-Whitney test ($p<0.0001$)

Since the basal locomotory activity is very low in 3 dpf zebrafish larvae, the locomotory analysis was performed also under a different protocol in which the light was switched on-off every 10 minutes. Also in this experimental condition, PTZ treated larvae showed a significant alteration in term of distance swam and velocity (Figure 3.2A). These results suggest that treatment of 3 dpf embryos with 15 mM PTZ elicits a very rapid and robust seizure-like behavioural response, as indicated by the locomotory traces that illustrate the first 5 minutes of treatment (Figure 3.2B). Based on these results, the locomotory assay clearly is a useful technique to validate the convulsive response to PTZ treatment.

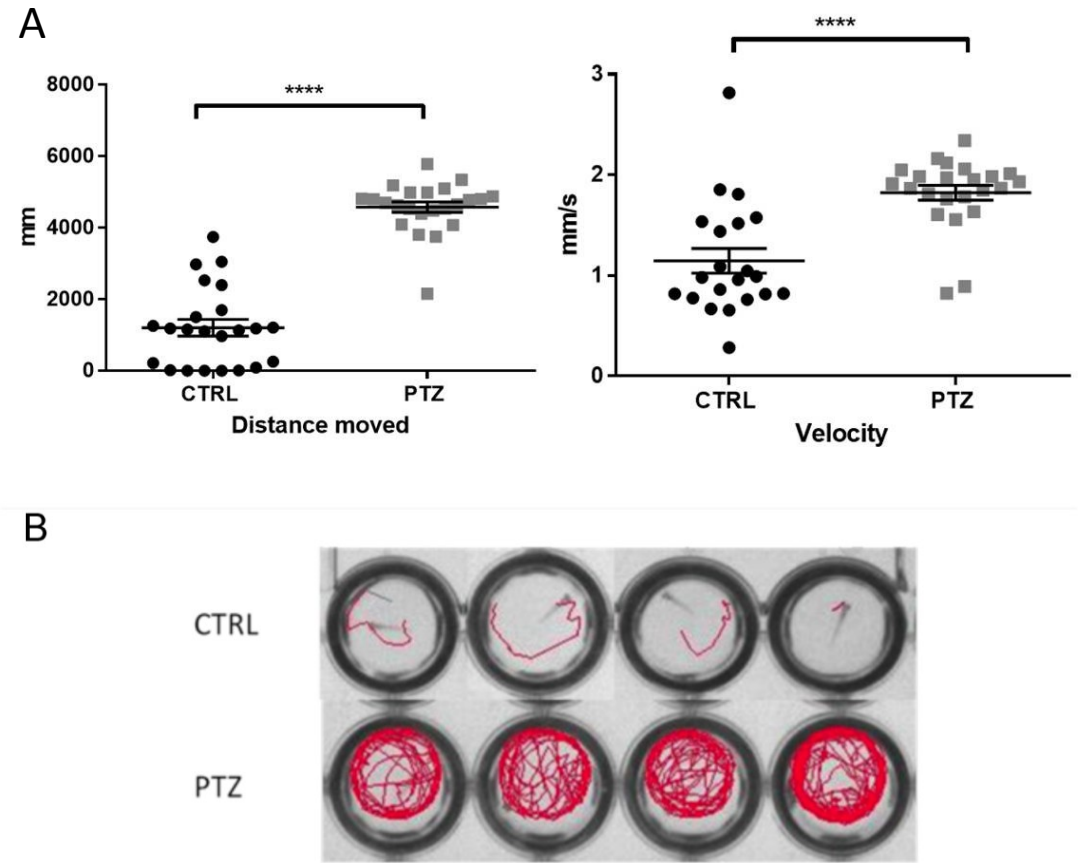


Figure 3.2. Effect of a light-driven protocol on the treatment of 3 dpf zebrafish larvae with 15 mM PTZ. A. The graph represents the distance moved and the velocity between control and PTZ treated zebrafish larvae for a total duration of 45 minutes, from the moment of PTZ administration, using a 10' light on-10' light off stimulus. Circle and square symbols represent each larvae; the error bars indicate the s.e.m. Asterisks refer to significant difference, calculated using a Mann-Whitney test ($p<0.0001$). B. Locomotory traces representing the first 5 minutes of recording.

In order to define the temporal expression and regulation of the locomotory phenotype after PTZ removal, I have performed DanioVision-based analysis at two distinct time point, 2h (short-term effect) and 24h (long-term effect) after PTZ removal. As shown in Figure 3.3, the total distance moved and the velocity were not significant altered 2h as well as 24h after PTZ removal (Figure 3.4).

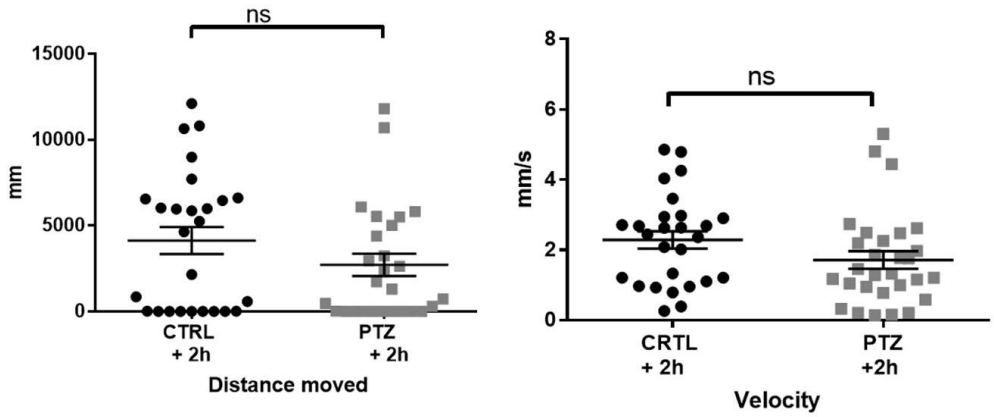


Figure 3. 3. **The behavioural responses of zebrafish larvae 2h after PTZ treatment.** The locomotory parameters of treated larvae were similar to control group, using a 10' light on-10' light off stimulus. The graph represents the comparison of distance moved and velocity between control and PTZ treated zebrafish larvae, for a total duration of 45 minutes recording. Circle and square symbols represent each larvae; the error bars indicate s.e.m. No significant difference, using a Mann-Whitney test ($p > 0.05$).

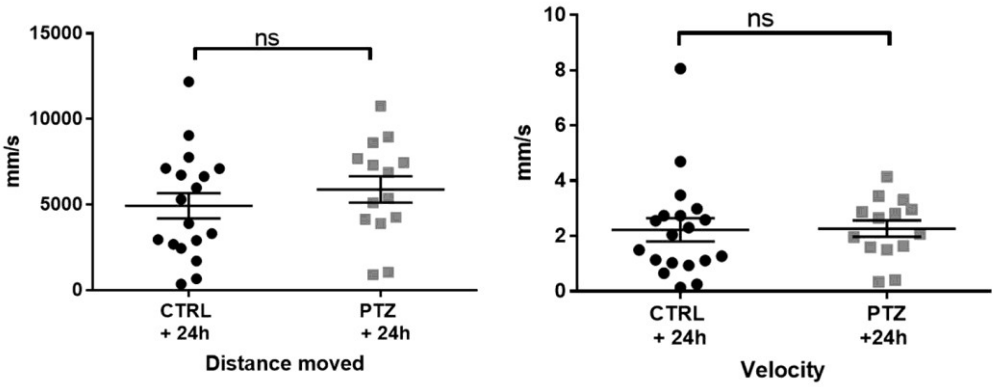


Figure 3.4. The behavioural responses of zebrafish larvae 24h after PTZ treatment. The locomotory activities of treated larvae returned to normal levels 24h after the end of the treatment. The graph represents the comparison of distance moved and velocity between control and PTZ treated zebrafish larvae, for a total duration of 45 minutes of recording, using a 10' light on-10' light off stimulus. Circle and square symbols represent each larvae; the error bars indicate s.e.m. No significant difference, using a Mann-Whitney test ($p > 0.05$).

Transcriptional analyses

To explore the transcriptional responses of genes directly associated to the PTZ treatment, including markers of the active state of neurons as well as markers of GABA signalling, I have used two different molecular approaches, that is whole mount *in situ* hybridization (WISH) and Real Time quantitative PCR (RT-qPCR).

One of the most studied genes linked to neuronal activity is the transcription factor *c-fos*. It is one of the first known "immediate early genes, IEG" that has been identified and it represents the prototypical example of activity-dependent neuronal gene transcription. The upregulation of *c-fos* in neurons of the intact brain was observed in specific brain regions in response to seizures and to a wide range of physiological stimuli (Morgan and Curran, 1987). Several studies have used the induction of the activity dependent transcription factor *c-fos* as a marker of neurons in the active state.

In order to characterise the activation of the *c-fos* gene in response to PTZ at the end of the treatment, I have performed a whole mount *in situ* hybridization (WISH) in 3 dpf zebrafish larvae. In controls, no expression of *c-fos* mRNA was detected; on the contrary, the transcriptional activity of *c-fos* did strongly increase in the brain of PTZ-treated fish. In particular, *c-fos* expression was observed in the forebrain, including the telencephalic area, diencephalon, tectum and hindbrain. A diffuse signal was present in trunk muscles, consistent with the observation of an intense locomotory activity of the larvae (Figure 3.5).

Another well-characterized gene that is regulated by the synaptic activity in mammalians is *BDNF* (brain-derived neurotrophic factor). The *BDNF* gene plays important roles in neuronal growth, survival and synaptogenesis (reviewed by Flavell & Greenberg, 2008) and has been investigated in other models of epilepsy based on the evidence that both BDNF mRNA and protein are upregulated during different types of seizures (reviewed by Koyama and Ikegaya, 2005). Previous results have demonstrated that *bdnf* is overexpressed also in the brain of 4 dpf PTZ-treated

zebrafish larvae (Baxedale et al., 2012). Using WISH, I have observed the expression pattern of *bdnf* after PTZ treatment in 3 dpf zebrafish larvae. As shown in Figure 3.6, the endogenous expression of *bdnf* in control larvae is localized in a small region of the zebrafish brain that includes the diencephalon (De Felice et al., 2014). After 45 minutes of PTZ treatment, the expression level of *bdnf* was strongly upregulated throughout the brain, with an expansion of the expression pattern that is similar to the one found for *c-fos*. Altogether, 3 dpf zebrafish larvae treated with PTZ showed a precise seizure phenotype characterized by robust increase in locomotory activity and up-regulation of synaptic-activity regulated genes.

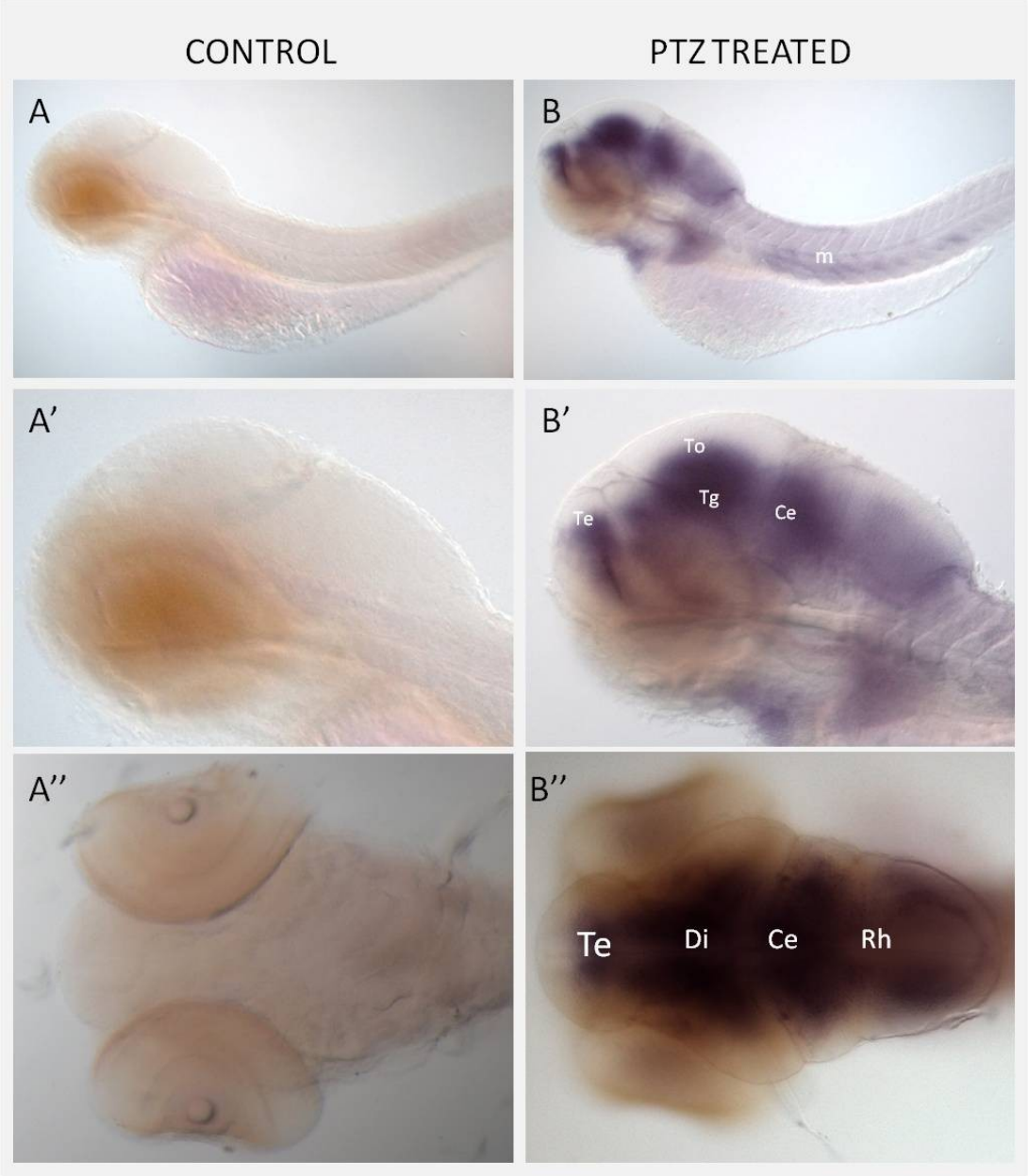


Figure 3.5. Expression of *c-fos* gene after PTZ treatment in 3 dpf zebrafish larvae. Left column shows the control larvae and the right, PTZ treated zebrafish larvae. A, A', B, B'. Lateral view of control (A, A') and treated (B, B') zebrafish larvae. A'', B''. Dorsal view of the head of control and treated zebrafish. B, B', B''. *c-fos* up-regulation in the larval brain. Te, telencephalon; To, optic tectum; tg, tegmentum; Ce, cerebellum; Di, diencephalon; Rh, rhombencephalon; m, muscles.

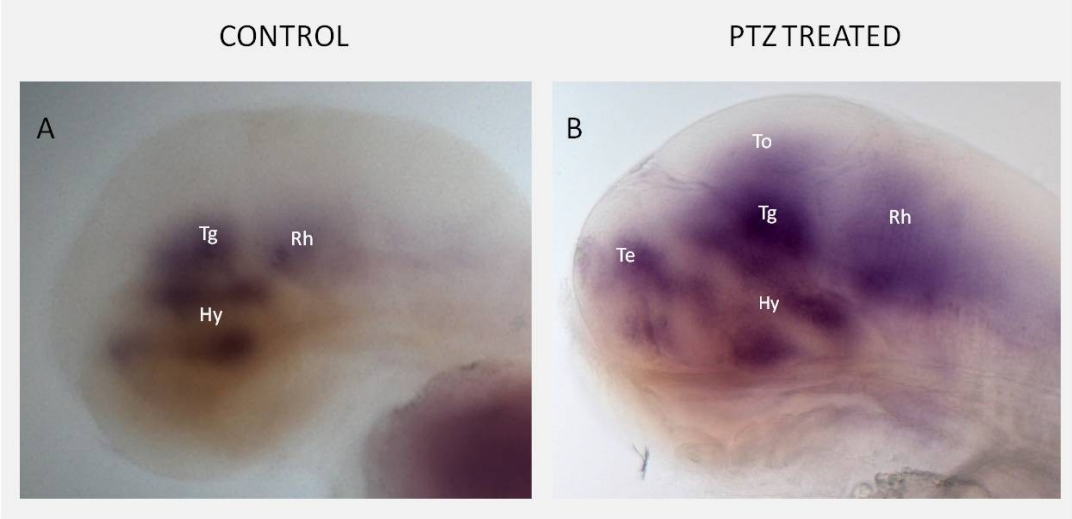


Figure 3.6. Expression of *bdnf* gene after PTZ treatment in 3 dpf zebrafish larvae. A, B. Lateral view of the head of control and treated larvae. WISH analysis shows the increased expression of *bdnf* mRNA in different territories of the treated larvae. Te, telencephalon; To, optic tectum; Tg, tegmentum; Hy, hypothalamus; Rh, rhombencephalon.

In order to define the temporal expression and regulation of these genes after PTZ removal, I have performed WISH analysis at the same time point tested (2 and 24h). As shown in Figure 3.7, the expression of *c-fos* at 2h after PTZ treatment was drastically reduced, suggesting that its activity is required only during PTZ antagonistic activity. Differently, *bdnf* expression remained elevated at 2h, and returned at basal levels only 24h after PTZ withdrawn (Figure 3.8). This dynamics of expression suggest that, unlike for *c-fos*, the effect of PTZ treatment on *bdnf* gene activity lasts longer.

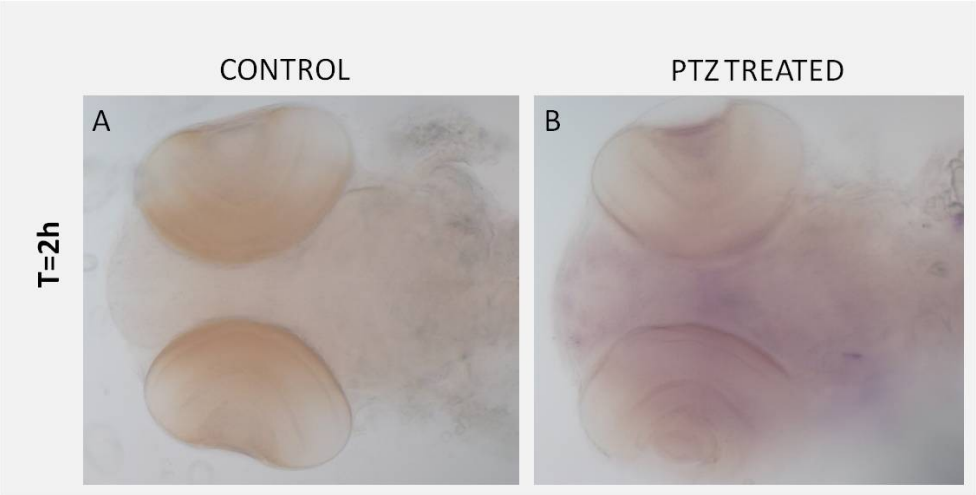


Figure 3.7. Expression of *c-fos* gene after PTZ removal in 3 dpf zebrafish larvae. A, B. Dorsal view of the head of control and treated larvae. WISH analysis showed that *c-fos* expression in treated larvae is similar to control after 2h post treatment.

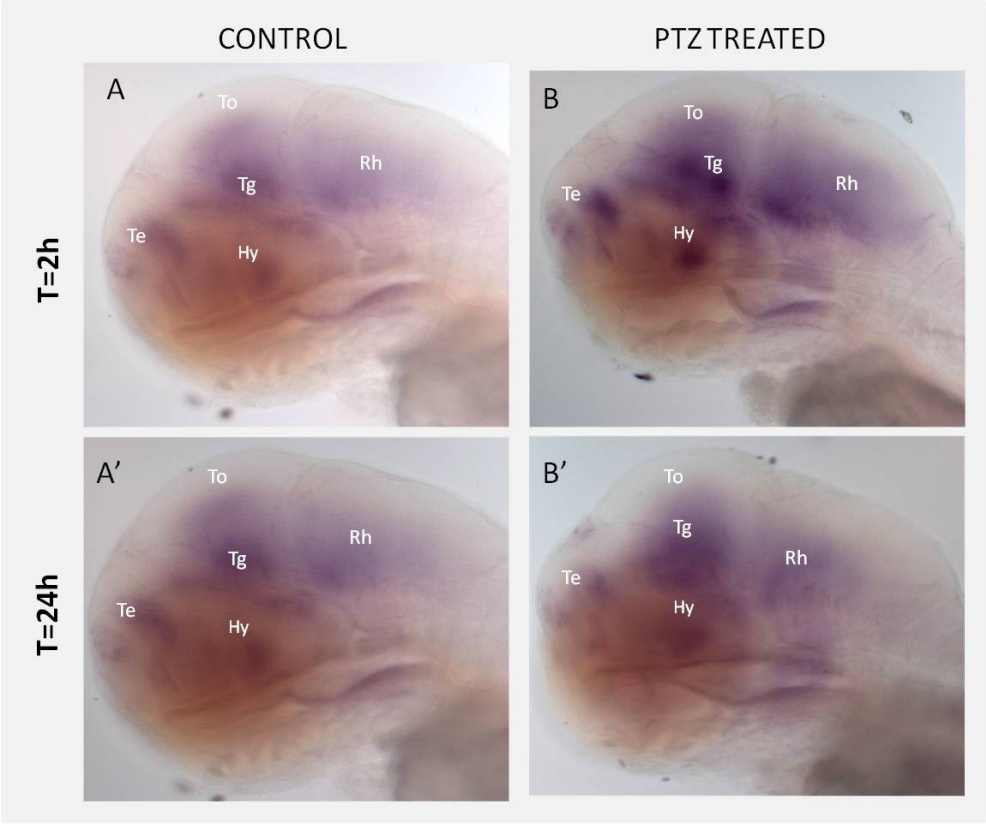


Figure 3.8 *bdnf* gene expression after PTZ removal in 3 dpf zebrafish larvae. The panel shows the lateral view of control and treated zebrafish heads. A, B. WISH analysis shows that the expression of *bdnf* mRNA in different brain territories of the treated larvae is still

present 2h after PTZ removal. A', B'. No difference was observed 24h after PTZ removal. Te, telencephalon; To, optic tectum; Tg, tegumentum, Hy, hypothalamus; Rh, rhombencephalon.

In order to validate *bndf* data by a second independent methodology, I have performed RT-qPCR analysis using the total RNA extracted from zebrafish larvae. As shown in Figure 3.9, RT-qPCR confirms the statistically significant increase of *bdnf* expression relative to control at the three different time points analysed.

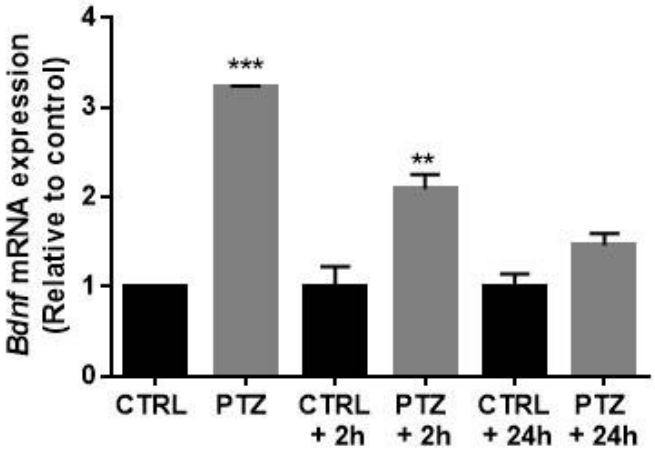


Figure 3.9. RT-qPCR analysis of relative *bdnf* expression. The graph represents the mean of the fold change of the *bdnf* gene relative to the control. The error bars represent the standard deviation. The asterisks represent the statistically significant value (***p 0.0001; **p < 0.001), using Student's t-test.

Since PTZ is an antagonist of GABA A receptors, it has been shown that seizures induced by PTZ administration are associated with an alteration of GABA A receptor subunit mRNA in different brain areas of rodents (Walsh et al., 1999). Therefore, I asked whether PTZ treatment is able to influence genes associated with GABA

signalling also in zebrafish brain. To this aim, I have performed RT-qPCR analysis for *gabra1* gene, that encodes one GABA A receptor subunit. In parallel, I have investigated also the transcriptional responses for *gad1*, another gene involved in GABA signalling. The *gad1* gene is responsible for GABA synthesis and is associated with GABA-mediated synaptic signalling.

As shown in Figure 3.10, the levels of *gabra1* and *gad1* mRNA were not significantly altered at the end of the PTZ treatment. Interestingly, a mild although not statistically significant increase of *gabra1* and *gad1* expression was seen 2h after the treatment. At 24h, both genes presented comparable levels of expression with respect to controls.

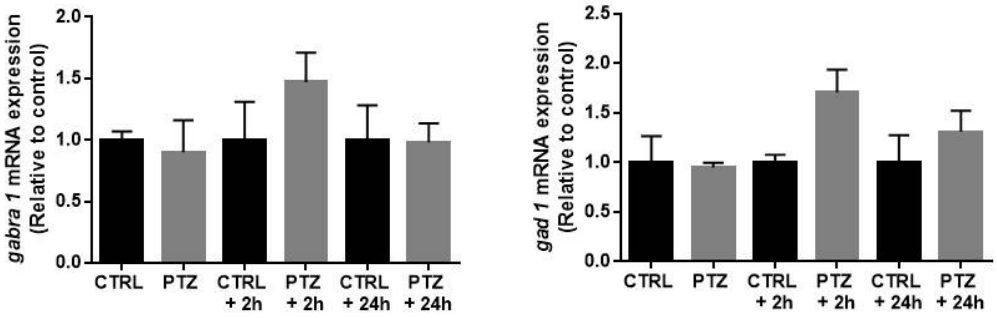


Figure 3.10. RT-qPCR analysis of relative *gabra1 and gad1* expression at different time points. The graphs represent the mean of the fold change relative to the control. The error bars represent the standard deviation. No significant difference, using Student's *t*-test ($p > 0.05$).

To summarize this composite set of data, PTZ treatment in 3 dpf zebrafish larvae is associated to a very significant locomotory phenotype that is highly indicative of seizure occurrence. Moreover, the observed transcriptional responses of *c-fos* and *bdnf* genes are in line with seizure induction. Interestingly, while *gabra1* and *gad1* do not show clear changes of their expression levels at the end of PTZ treatment, a mild *gad1* upregulation is detected 2h after treatment. Lack of statistical significance

in support of the observation of a delayed transcriptional GABA signalling response will be further addressed by additional techniques in order to confirm this interesting observation. As a matter of fact, the analysis of the specific locomotory and transcriptional events that I have analysed in this PhD project reveal that the molecular, cellular and behavioural activities altered by PTZ return to basal level 24h after the end of the insult. This indicates that the immature brain of zebrafish larvae is capable of rapidly recovering its normal physiological condition in a specific temporal window.

3.2. Temporal Regulation of the Cellular and Transcriptional Responses in the Inflammatory Process

Inflammation is a natural physiological reaction to different types of insults and its role is to re-establish the lost homeostasis. In rodent seizure models, inflammation may have a proconvulsive role that may eventually lead to seizure chronicization and epilepsy. Therefore, it is particularly important to provide a contribution to the dissection of the relationships between the seizure phenotype and the cellular and molecular components of the inflammatory response. Remarkably, no data are currently available concerning this fundamental biological process in the zebrafish PTZ models of seizure.

Non-neuronal immune cells

Astrocytes are one of the major types of brain immune cells that are able to respond to various insults by changing their morphological, biochemical and transcriptional profiles (see Introduction). In addition, astrocytic responses to seizures have been described in the brain of various adult rodent models of epilepsy. Differently, seizure induction in immature brains provokes changes in the morphology and function of astrocytes in a age-dependent manner (see Introduction for references).

With these premises, I have first performed immunohystochemical and gene expression studies with the purpose to evaluate the dynamics of astrocyte activity. Markers of mammalian astrocytes are glial fibrillary acidic protein (GFAP), an intermediate filament protein, and glutamine synthetase (GS), the enzyme that converts glutamate in glutamine. In the zebrafish brain, the main GFAP-expressing cells occur in the radial glia. These GFAP-positive cells possess properties attributed to astrocytes as well as radial glia in mammals. While mammalian astrocytes are stellate cells with multiple processes, zebrafish radial glia cell bodies are localized at the brain ventricles with a single long process spanning the brain, whereas they

shared typical properties with mammalian astrocytes include glutamate re-uptake from the synaptic cleft (Oosterhof et al., 2015). Both *gfap* and *gs* genes are expressed in zebrafish and are used in adult zebrafish brain as markers to label astrocytes and to validate the reactive astrocyte states after an insult (Group et al., 2010; Baumgart et al., 2012; Schmidt et al., 2014). Using confocal microscopy, I have analysed the signal of both astrocytic protein in double whole mount immunohistochemistry (WIHC). As shown in Figure 3.11, GS immunoreactivity signal revealed a characteristic increased intensity of fluorescence in the region of tectum and in the hindbrain (arrows) soon after PTZ removal (T=0). At the same time, GFAP protein immunofluorescence showed a localized intensity in the cellular processes present in the hindbrain (arrowheads) of PTZ treated zebrafish brain (T=0).

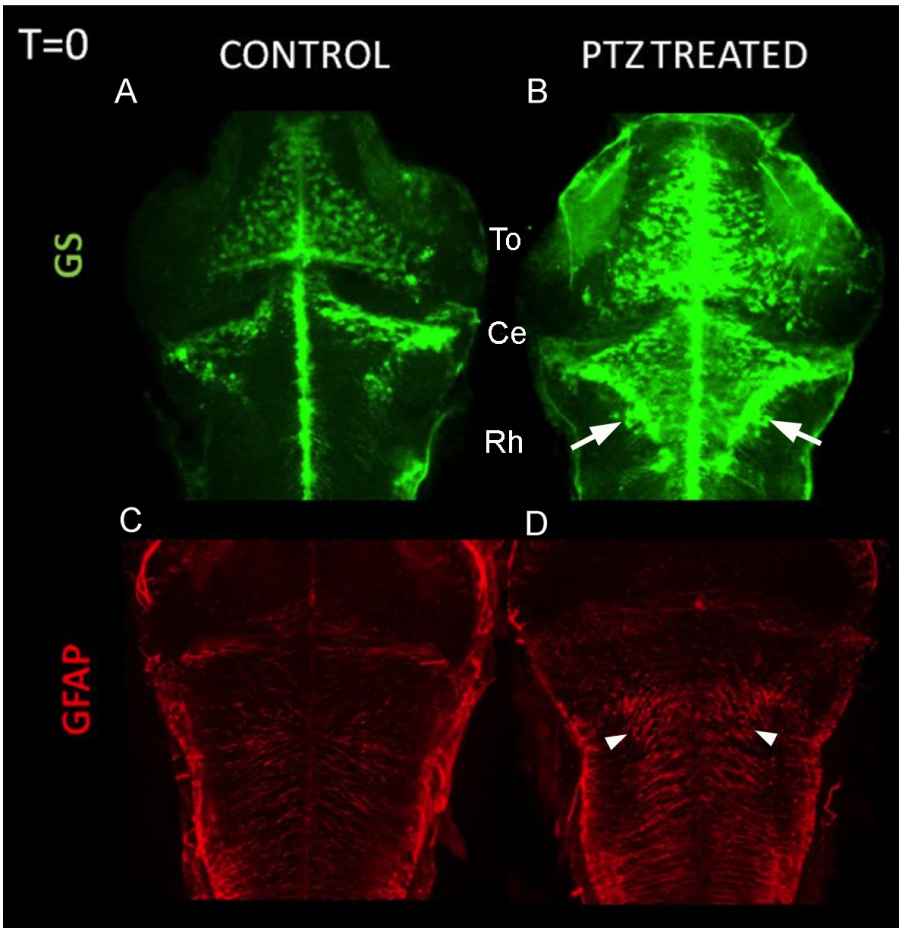


Figure 3.11. GS and GFAP immunoreactivity in control and PTZ treated zebrafish larvae soon after PTZ treatment. The image represents the maximum intensity of the z-stack analysed using ImageJ. White arrows and arrowheads indicate regions of increased

signal. The intensity of the signal is the same for control and treated larvae in each experiment.

Two hours after PTZ removal (T=2h), GS immunoreactivity in the tectum returned to control levels, a change that was observed also in the case of GFAP immunolabelling in the cellular processes of the hindbrain (Figure 3.12).

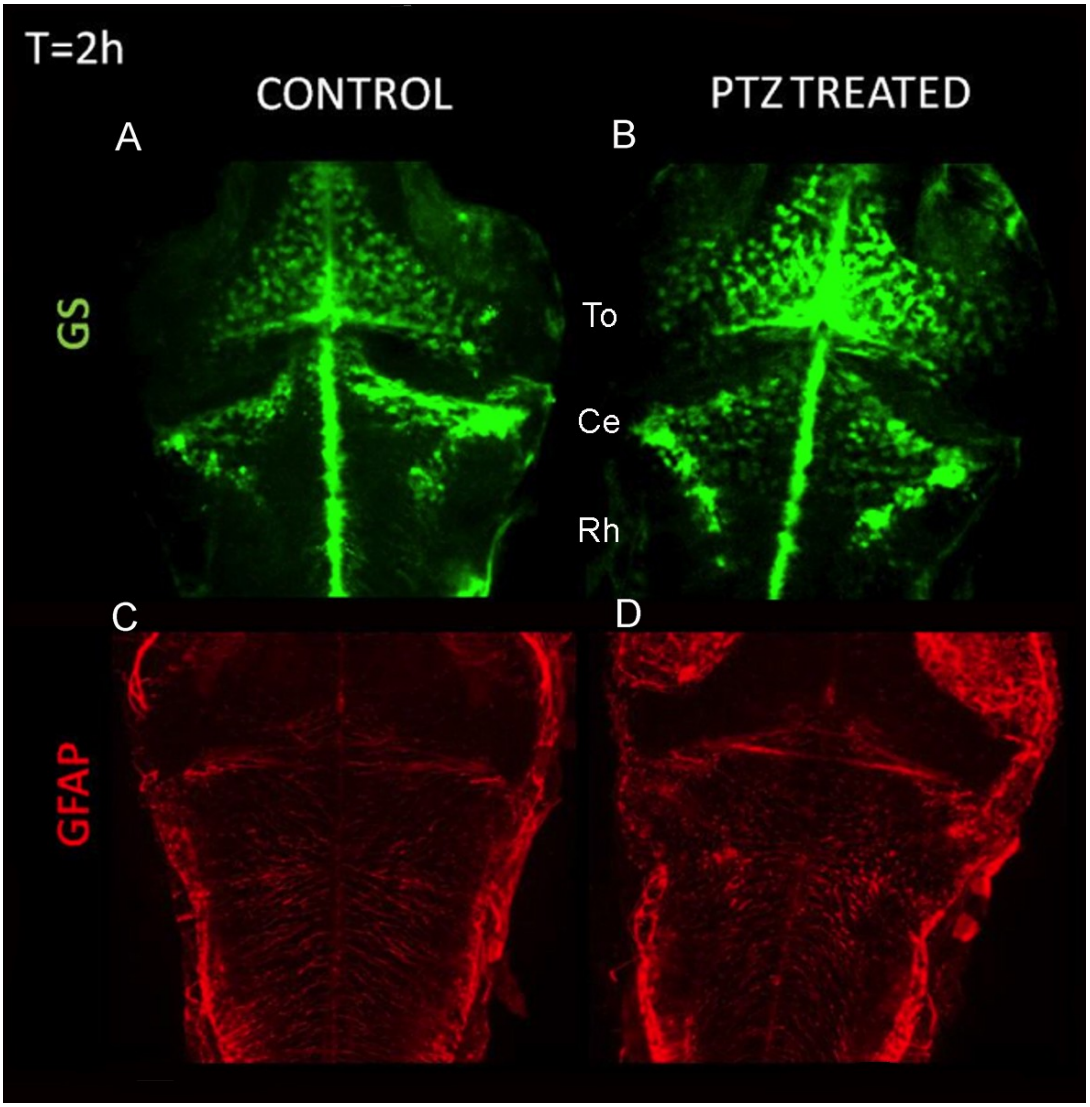


Figure 3.12. GS and GFAP immunoreactivity in control and PTZ treated zebrafish larvae 2h after PTZ treatment (T=2h). The image represents the maximum intensity of the z-stack analysed using ImageJ. The intensity of the signal is the same for control and treated larvae in each experiment.

As well, it was not possible to distinguish any measurable difference between control and PTZ treated larvae 24h post treatment (Figure 3.13).

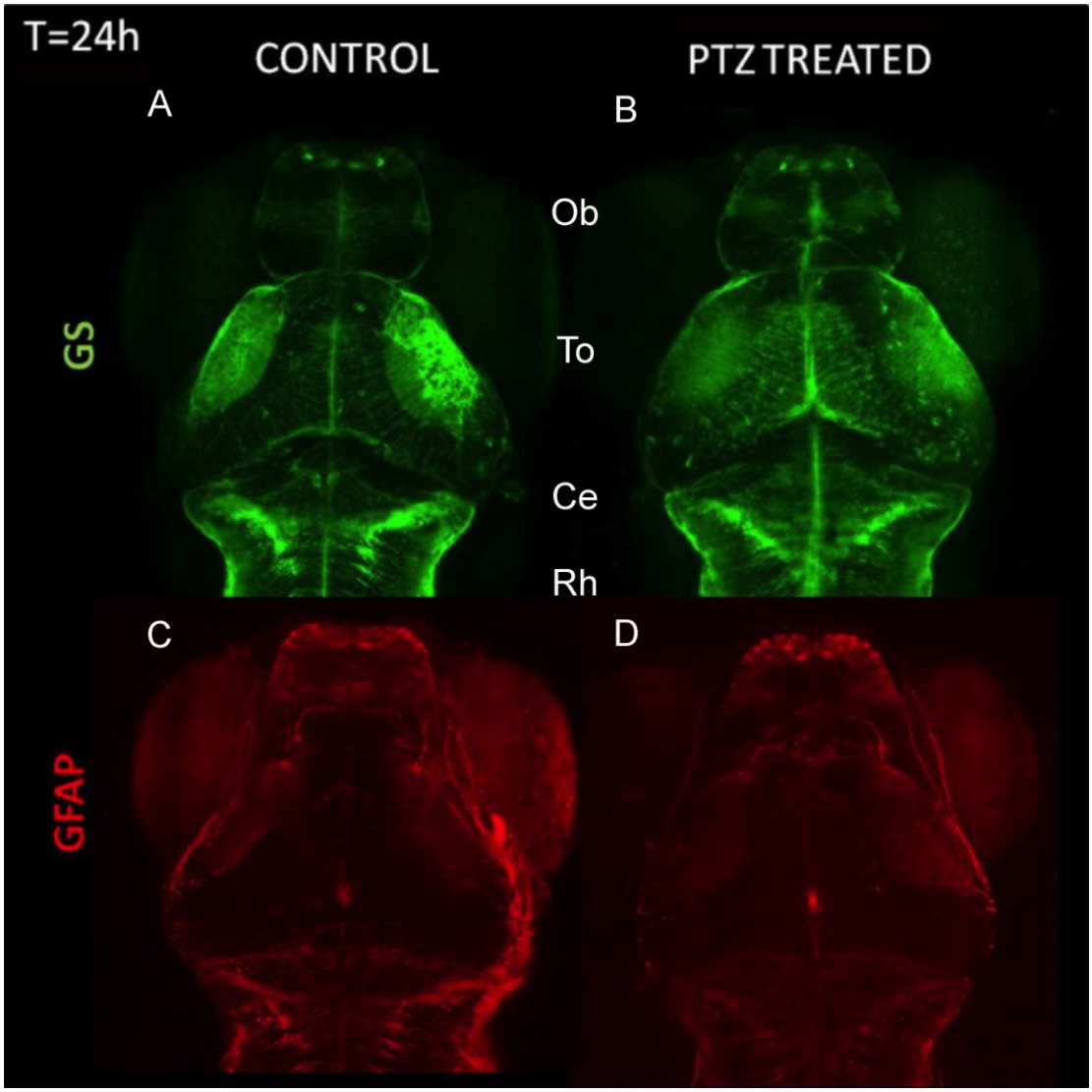


Figure 3.13. GS and GFAP immunoreactivity in control and PTZ treated zebrafish larvae 24h after treatment (T=24h). The image represents the maximum intensity of the z-stack analysed using ImageJ. The intensity of the signal is the same for control and treated larvae in each experiment.

Following, I asked whether the observed immunochemical changes after PTZ treatment were associated with changes in the transcriptional activation of these genes. To address this question I have performed a RT-qPCR analysis for *gfap* and

gs genes using total RNA extracted from 3 dpf zebrafish larvae. Although the qPCR data indicate that there is no statistically significant increase in transcriptional activity as an effect of PTZ treatment, *gfap* expression seem to be affected by PTZ treatment, especially in terms of increase at T=0 and following decrease at T=2h. Remarkably, also *gs* expression shows a mild increase at T=0 (Figure 3.14).

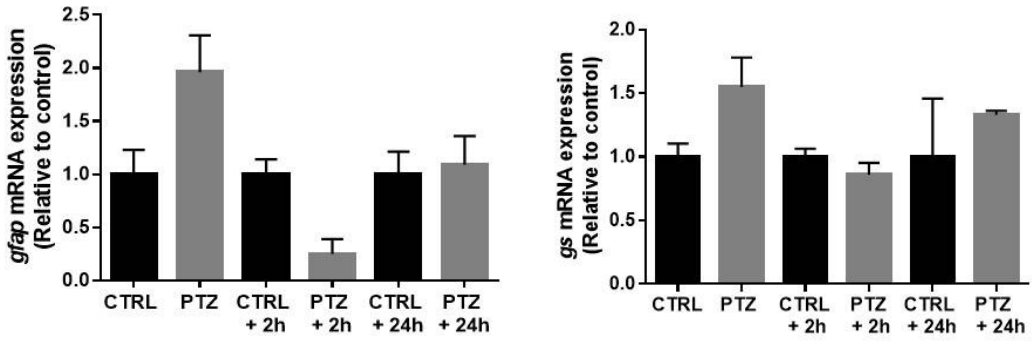


Figure 3.14. RT-qPCR analyses of relative *gfap* and *gs* expression at different time points. The graph represents the mean of the fold change relative to the control. The error bars represent the standard deviation. Not significant difference, using Student's *t*-test ($p > 0.05$).

Inflammatory molecules

To test the hypothesis that also PTZ induced seizures in 3 dpf zebrafish can stimulate *IL-1β*, *IL-6* and *TNFα* expression, I have performed a gene expression analysis by using classical Reverse Transcriptase PCR (RT-PCR) and RT-qPCR. Initially, I have demonstrated an increased expression of *IL-1β* in PTZ-treated zebrafish larvae at T=0 using RT-PCR analysis (Figure 3.15).

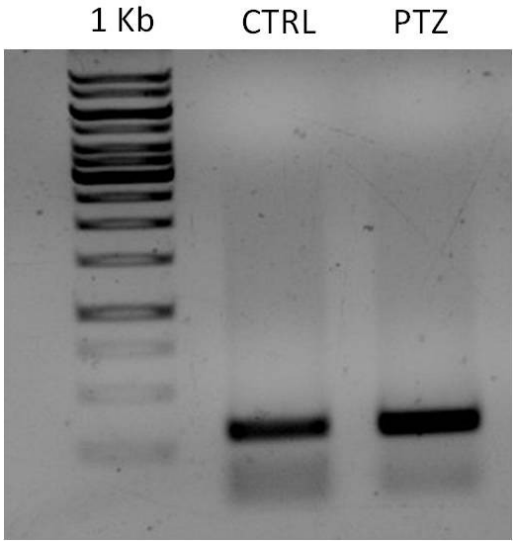


Figure 3.15. Levels of *IL-1β* expression at T=0. Gel electrophoresis showing the difference in expression of *IL-1β* mRNA after 45 minutes of PTZ treatment, using RT-PCR analysis.

To further extend and quantify this result, I have investigated the expression of the *IL-1β* gene at the three time points considered in this study using RT-qPCR. First, I have treated 3 dpf zebrafish larvae with PTZ and extracted the total RNA from the whole larvae. As shown in the graph, the relative level of *IL-1β* expression soon after treatment is significantly increased with respect to control, an increase that is observed also at 2h post treatment. No significant change was detected 24h after PTZ removal (Figure 3.16).

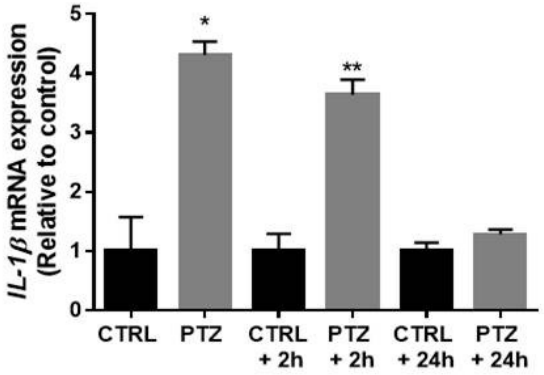


Figure 3.16. RT-qPCR levels of *IL-1β* expression at the three time points. The graph represents the mean of the fold change relative to the control. The error bars represent the standard deviation. Asterisks mean statistically significant difference, using Student's *t*-test (*p< 0.05, **<0.001).

In order to verify if the transient transcriptional activation of the *IL-1β* gene is capable of activating the other cytokines that are classically stimulated by *IL-1β* signalling, I have performed a RT-qPCR analysis for *TNFα* and *IL-6* genes. As shown in Figure 3.17, no significant transcriptional alteration is found, suggesting that the increase in *IL-1β* activity is not sufficient to activate the other cytokines.

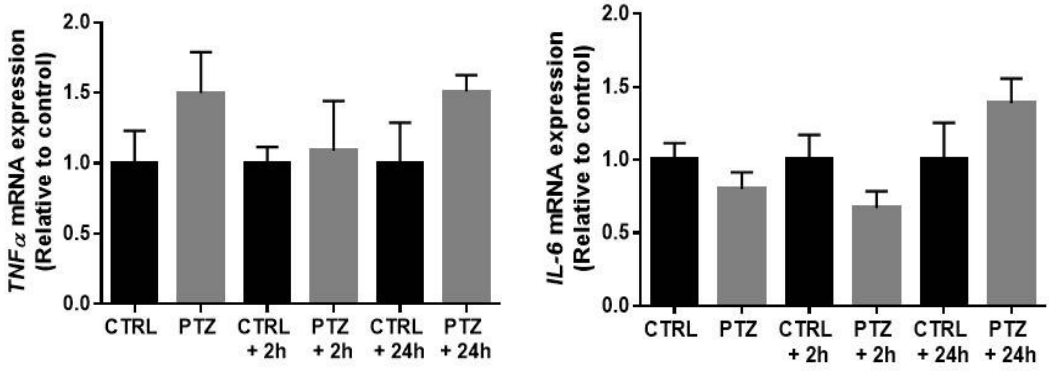


Figure 3.17. Expression level of *TNFα* and *IL-6* genes at the three time points. The graph represents the mean of the fold change relative to the control. The error bars represent the standard deviation. No significant difference, using Student's *t*-test ($p > 0.05$).

3.3. Analysis of IL-1β involvement in PTZ seizures

The transient increase in *IL-1β* mRNA during and after PTZ treatment suggests a possible involvement of *IL-1β* in seizures. To test this hypothesis, I have performed a knockdown experiment by using the morpholino (MO) oligonucleotide-based technology in order to block a splicing site of the wild type *IL-1β* mRNA. The sequence of the MO oligomer has been tested in other scientific works where it was used to study the involvement of *IL-1β* in the inflammatory responses to various stimuli in zebrafish (Lopez-Mugnoz et al., 2012; Ogryzko et al., 2014). The *IL-1β* splice site MO (*IL-1β* ssMO) was microinjected at a concentration of 0.5 mM in the yolk of embryos at one-cell stage of development. As control, I have microinjected a standard control MO (St.CTRL MO). This control morpholino is an oligomer that is not able to recognize any other mRNAs in the embryo transcriptome. This is a usual method to test the validity of the microinjection technique in zebrafish. As described in previous reports, *IL-1β* ssMO recognizes the splice site between exon 2 and exon 3, causing intron retention in morpholino-injected embryos. Integration of this intron created in-frame premature stop codons that resulted in truncated proteins lacking most of the mature carboxy-terminal domain. However, the *IL-1β* morpholino splice site only reduced the expression of wild type mRNA by about 50%, and thus its use does not lead to a full functional ablation of this gene.

Figure 3.18 displays the RT-PCR analysis of the mRNA in control and microinjected (morphant) larvae. *IL-1β* ssMo microinjection induced altered splicing of the *IL-1β* transcript, as demonstrated by the presence of two bands in morphant mRNA, of which the upper one corresponds to the morpholino-induced variant transcript which includes the retention of ~100nt intron 2-3, while the lower band corresponds to the properly spliced mRNA. The single band in standard control injected zebrafish larvae represents the *IL-1β* wild type transcript. As described, no altered locomotion was observed for *IL-1β* morphants.

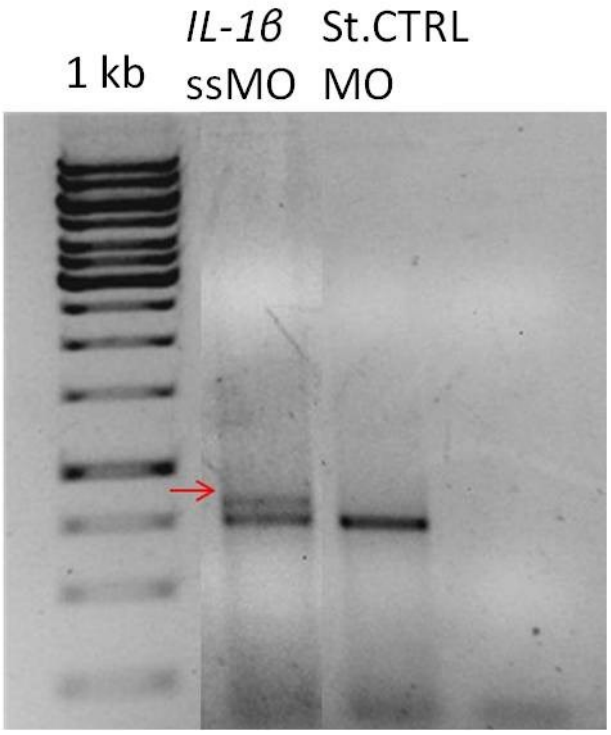


Figure 3.18. *IL-1β* ssMO alters correct splicing of *IL-1β* transcript in 3 dpf zebrafish morphant larvae. Agarose gel showing RT-PCR amplification of cDNA products derived from *IL-1β* transcripts. Amplification product of normal primary transcript expected size: 700 bp (St.CTRL MO). The inclusion of a 100 bp intron in the *IL-1β* mRNA of *IL-1β*ssMO-microinjected larvae is indicated by the red arrow.

The result of RT-PCR analysis shows that *IL-1β* ssMO-microinjected larvae possess both wild type and alternative transcript variants, indicating the occurrence of a partial *IL-1β* knockdown.

At 3 dpf, *IL-1β* morphant and St.CTRL MO control larvae were treated with PTZ and analysed for locomotory profile by measuring the total distance moved and the mean velocity. The protocol utilized is the light on-light off stimuli (see above). Figure 3.19 shows that 45 minutes of PTZ treatment cause a significant decrease of distance moved compared to control St.CTRL MO larvae, but not variation in terms of mean velocity, suggesting that the partial block of the normal synthesis of *IL-1β*

protein may influence, in part, the behavioural responses associated with prolonged PTZ treatment.

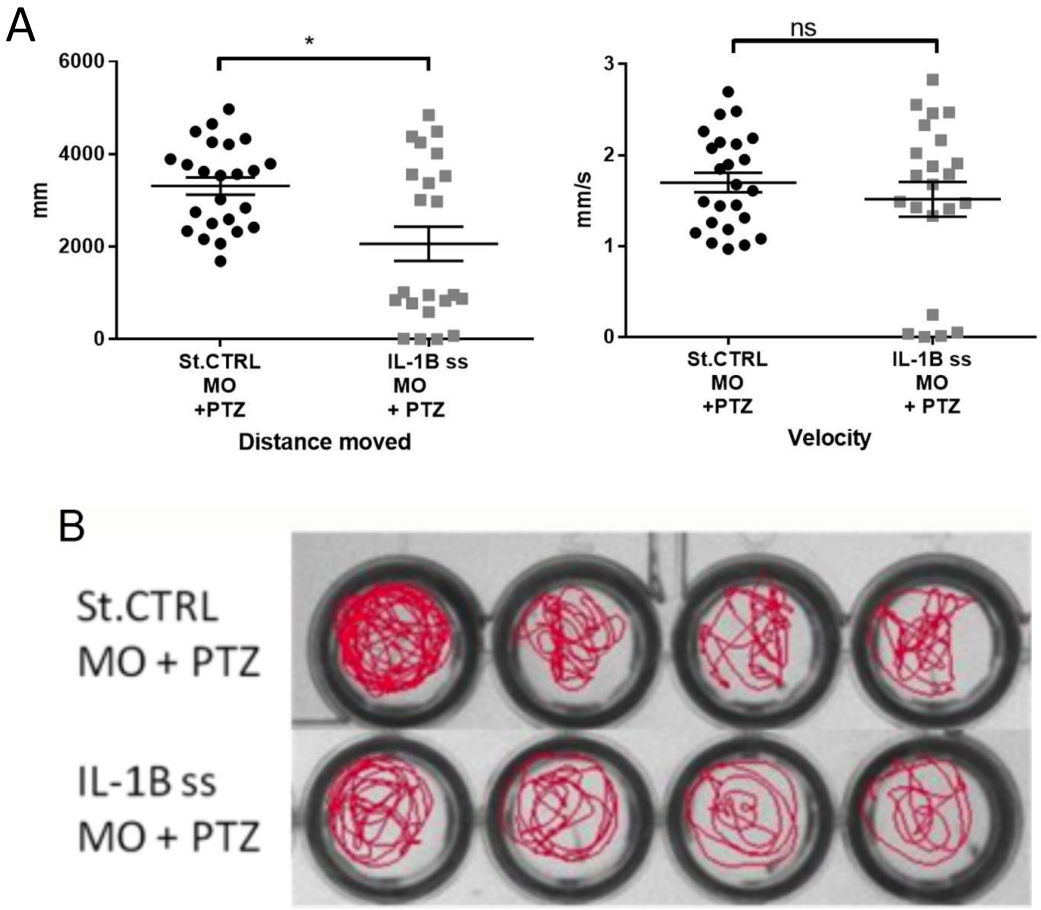


Figure 3.19. Altered locomotory activity of *IL-1β* morphant (*IL-1β* ssMO). A. The graph represents the distance moved and velocity for a total duration of 45 minutes. Circle and square symbols represent each larvae and the error bars indicate s.e.m. Asterisks show statistically significant difference, using a non parametric Mann-Whitney test (* $p < 0.05$). B. Locomotory tracks that represent the first 5 minutes of recording.

To address the role of *IL-1β* in seizure via a different approach, I have tested the effect of caspase-1 inhibition on PTZ treatment in wild type larvae using a known agent, YVAD, that was previously used to block the catalytic activity of caspase 1 enzyme in zebrafish (Ogryzko et al., 2014). This inhibitor was applied 3h before the PTZ treatment, at two different concentrations, 50 μM (as described before) and 25

µM, to estimate a potential dose-response effect. After 3h, PTZ-treated larvae were submitted to the same recording assay. As shown in the graph, the block of caspase-1 generate a more evident decrease of locomotory activity in zebrafish larvae treated with PTZ, respect to control larvae, and this effect is dose-dependent (Figure 3.20).

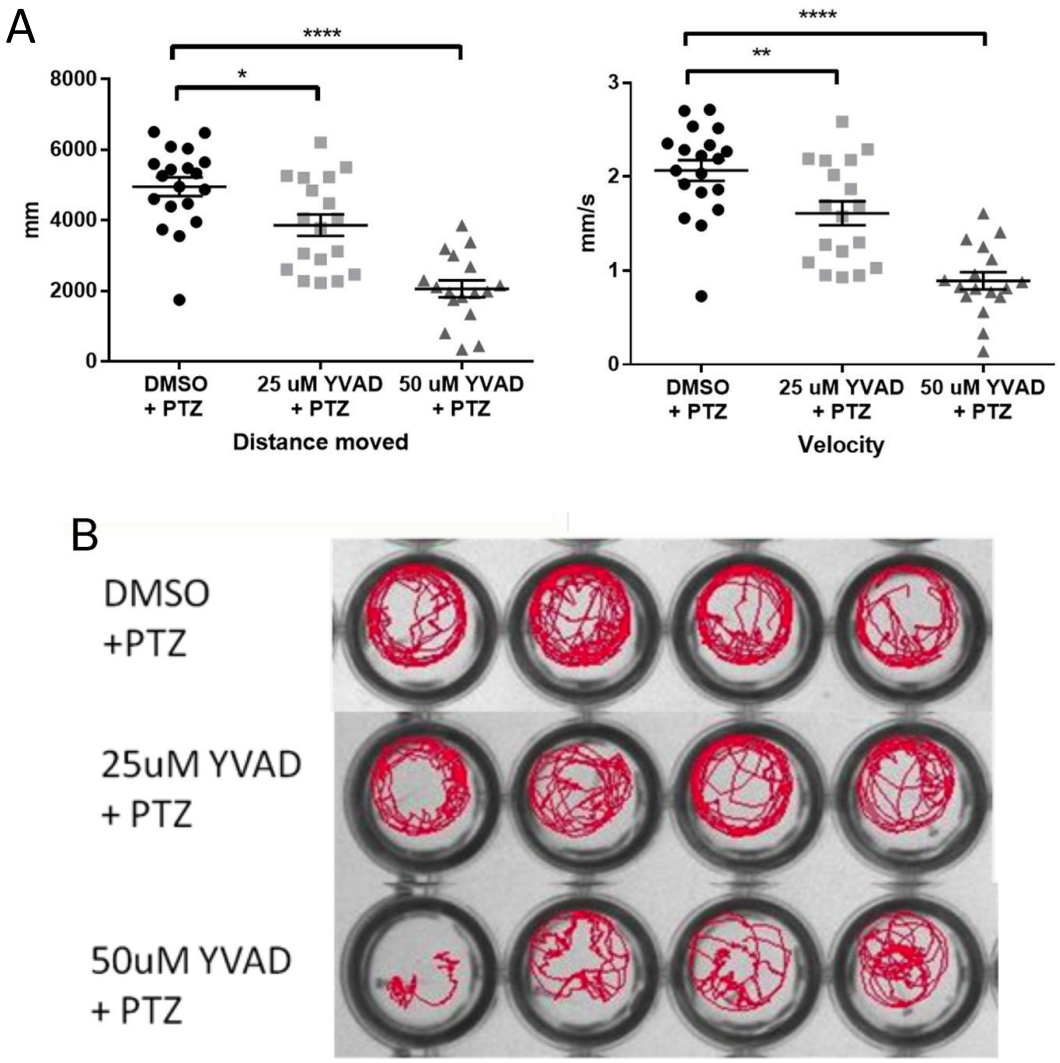


Figure 3.20. The effect of different concentrations of caspase 1 inhibitor (YVAD) on locomotory activities of 3 dpf zebrafish larvae treated with PTZ. A. Circle, square and triangle symbols represent each larvae and the error bars indicate s.e.m. Asterisks show the statistically significant difference, using one-way ANOVA with Dunnett's *post hoc* test (* $p < 0.05$, **** $p < 0.0001$). B. Locomotory track that represent the first 5' of recording.

To validate the results obtained by pharmacological inhibition of caspase 1, I have performed the same experiment by using a non-selective caspase inhibitor. As shown in Figure 3.21, the treatment with a generic pan-caspase inhibitor did not attenuate locomotory activities in zebrafish larvae treated with PTZ.

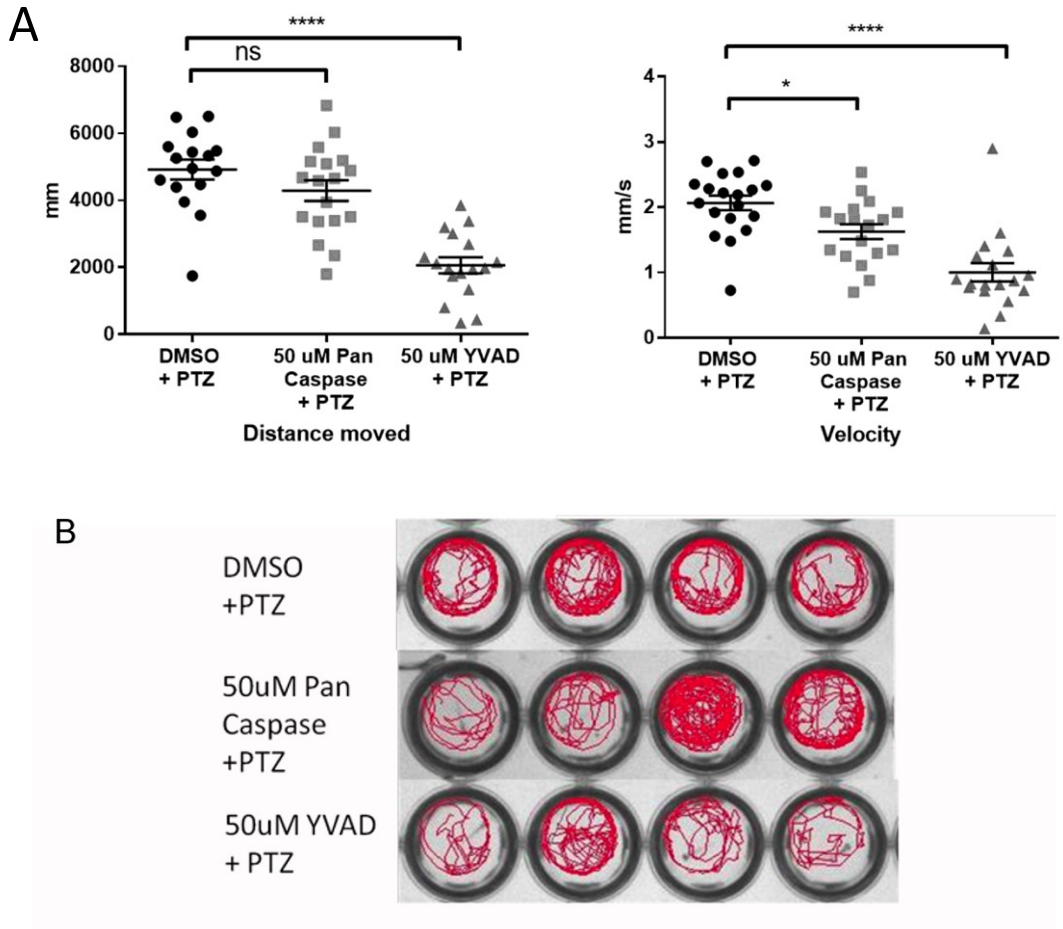


Figure 3.21. The effect of YVAD and pan-caspase on locomotory activities of 3 dpf zebrafish larvae treated with PTZ. A. The graph represents the distance moved and the velocity of PTZ treated larvae after pre-treatment with caspase 1 (50uM) and pan-caspase (50uM) inhibitors. Circle, square and triangle symbols represent each larvae and the error bars indicate s.e.m. Asterisks mean the statistically significant difference, using one-way ANOVA with Dunnett's *post hoc* test (* $p < 0.05$, **** $p < 0.0001$). B. Locomotory track that represent the first 5' of recording.

To verify if the reduced locomotory behaviour generated by IL1β inhibition is associated to an alteration of neuronal activity, I have performed a WISH analysis for the *c-fos* gene, although in a single biological replicate. As shown in Figure 3.22, concomitant treatment of zebrafish larvae with YVAD and PTZ causes a reduction of *c-fos* gene expression, while no difference was observed in pan-caspase-treated larvae respect to PTZ control group.

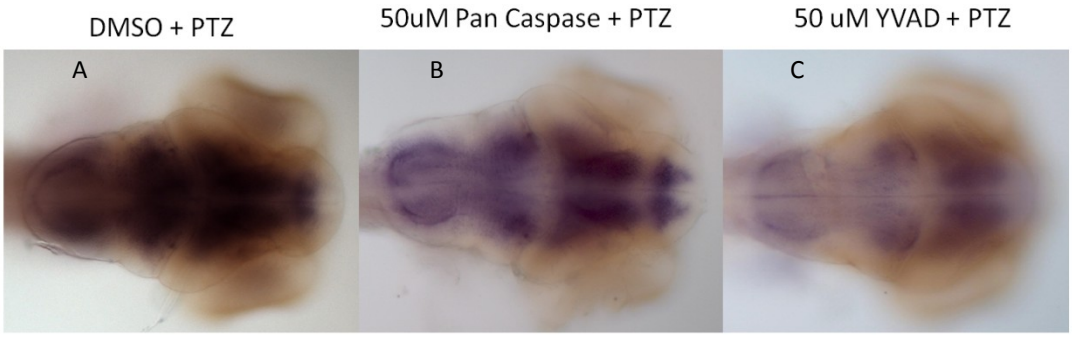


Figure 3.22. Decreased *c-fos* expression in YVAD pre-treated larvae after PTZ. WISH analysis of *c-fos* gene in 3 dpf zebrafish larvae treated with pan-caspase (B), YVAD (C) and control DMSO (A), soon after PTZ treatment, shows similar pattern between pan-caspase and DMSO control treated larvae, while the intensity of *c-fos* signal is decreased in YVAD pre-treated zebrafish larvae. In A, B and C, dorsal view of 3 dpf zebrafish heads.

CHAPTER 4

Discussion

The necessity of studying the brain and its physiology represents the goal in the field of neuroscience and drives the researchers, since long time, to investigate the mechanism of brain function and the processes linked to brain dysfunction. In particular, the comprehension of the mechanisms that underlie the brain pathophysiology is important for the identification of new targets and for the evaluation of therapeutic strategies to various brain diseases and disorders. In this respect, animal models offer an invaluable opportunity in this field. Most research studies in neurobiology are conducted in rodents and with the aim to mimic the human conditions of a particular pathophysiological mechanism. The search for new insights about the processes involved in complex conditions has led scientists to develop animal models that offer the opportunity to combine the study of brain disease mechanisms and to test therapeutic strategies. Zebrafish is a powerful model system for modelling a wide range of human disorders in translational neuroscience (Steward et al., 2013). In recent years, zebrafish larvae and adults have been used to model epilepsy and seizures for CNS drug discovery and to identify novel epileptogenic genes by means of forward genetics (Baraban et al., 2005; Baxedale et al., 2012, Mussulini et al., 2013). The advantages of using zebrafish larvae to study seizures provide novel opportunities in the field of epilepsy research that deserve investigation.

Seizures in the immature brain

The alteration of gene expression or signalling pathways linked to seizure induction and the effect of these changes during embryonic development is one major conundrum that attracts an increasing number of investigators. During embryonic development, the activity and regulation of gene expression patterns are well

orchestrated in order to define the correct maturation and functionality of the organism. Disturbance of these mechanisms at a particular stage of embryonic development can provoke long lasting effects. Seizures may perturb a wide range of developmental phenomena in an activity-dependent manner, including cell division, migration, sequential expression of receptors, as well as formation and probably stabilization of synapses that are essential for the correct formation and wiring of the circuitry, such as the migration of neurons, formation of new synapses (Holmes and Ben-Ari, 2001). Studies of the mature epileptic brain have revealed the contribution of cellular and molecular factors to seizures expression and epilepsy. How these factors alter the functional maturation and development of the immature brain paves the way to the unravelling of the susceptibility to seizures and seizure-related outcomes in different developmental stages. The immature rat brain has been used to study the impact of seizures on the highly regulated developmental processes (Holopain, 2008). This model has been used to investigate neuronal vulnerability in term of cell loss, cellular and synaptic reorganization, acute and sub-acute cellular and molecular alterations, contribution of inflammatory-like reactions and alteration of structure and function of inhibitory GABA A and excitatory glutamate receptors.

The zebrafish immature brain: an instructive tool to study seizures

The objective of my PhD project is to study seizure-related mechanisms in young zebrafish larvae as *in vivo* model of seizure induction in a functional and maturational phase of brain development. One main goal is to assess the validity of studying some contributing processes to seizure in immature brain also in zebrafish. A wider characterization of hyperexcitability, neuroinflammation and other seizure-related processes like cell death is required to improve our understanding of the zebrafish immature brain model of acute seizures. In perspective, validation of the zebrafish immature brain model may represent an instructive tool for a better understanding of the potential meaning of seizure related effects on the outcomes of brain maturation. For this purpose, I have used a 45 minute-long treatment with a known pro-convulsant agent, PTZ, to analyse the behavioural, cellular and

transcriptional responses to seizure induction at 72 hpf, a developmental stage that corresponds to the early maturation of the brain, with a focus on the recovery timing of the normal physiological conditions that includes long term observations. Second, I have tried to contribute to the pro- *vs* anticonvulsive role of the inflammatory response by manipulating inflammatory molecules in order to add unprecedented information in a non-mammalian model of seizures.

Neuronal activation in the PTZ-induced seizure zebrafish larval model

The active state marker *c-fos*

The expression levels of various genes involved in seizures in mammalian models were examined during the development of zebrafish brain. The *c-fos* gene is one of them. It encodes a transcription factor of 56 kDa that is expressed in neuronal and non-neuronal cell types in response to a number of different stimuli. It represents one of the first known "immediate early genes, IEG" that has been identified. The acronym IEG refers to a group of genes with rapid and transient expression with no need of *de novo* protein synthesis (Greer and Greeben, 2008). IEG genes are not constitutively expressed, but when their transcription is induced, they are able to regulate the activation of "late response genes" that give rise to more detailed responses. Upregulation of *c-fos* expression in neurons of the intact brain was observed in specific brain regions in response to seizures and in association with a wide range of physiological stimuli by pioneering studies of Morgan and Curran in 1987. Several studies have demonstrated that the induction of *c-fos* is a useful tool to mark neurons that have been recently activated. The intracellular signalling cascades involved in IEG activation in both physiological and pathological conditions have been extensively investigated in neurons. Neuronal depolarization generates a strong increase of intracellular levels of second messenger cAMP and Ca^{2+} that in turn activate intracellular kinases that converge on the phosphorylation of the transcription factor CREB. This event activates *c-fos* mRNA transcription, followed by translation into *c-fos* protein that is able to act as a transcription factor for

different neuron-specific genes (reviewed by Flavell and Greenberg, 2008). This process occurs rapidly and permits neurons to transform the depolarizing stimuli into a different intracellular long-lasting responses, such as the induction of genes involved in synaptic plasticity, long-term memory and cell death, critical processes for brain development as well as for many cognitive functions in the adult. To better define the effect of PTZ treatment in seizure induction, I have performed expression analysis for the *c-fos* transcription factor. The *c-fos* gene is a marker of neuronal activation that provides a functional mapping of brain activity in response to various acute or chronic challenges (pro-convulsant agents) (Morgan and Curran, 1987), and has been described also in other zebrafish models of seizures (Baraban et al., 2005). The *c-fos* expression is not detected in controls, indicating that this gene is not transcriptionally active in normal condition (absence of stimuli). On the contrary, PTZ treatment caused a robust induction of *c-fos* transcription in the brain, suggesting that the administration of PTZ in the normal medium generates neuronal responses throughout the developing brain. Together with *c-fos* induction in trunk muscle, results indicate generalized seizures caused by PTZ-dependent synaptic depolarization.

The neurotrophin BDNF

There is a subset of genes activated specifically in response to excitatory synaptic transmission that triggers calcium influx into the postsynaptic neuron. Previous studies of the transcriptional consequences of exposing *in vitro* cultured neurons to convulsants have demonstrated that other genetic components are expressed in addition to synaptic activity-regulated genes like *c-fos*. One gene whose expression is specifically induced by neuronal activity in neurons is the brain-derived neurotrophic factor (BDNF). Among other neurotrophins, which are important regulators to CNS function, BDNF is the best characterized (Binder and Scharfman, 2004). The neurotrophin BDNF is central to many facets of CNS function. BDNF acts as a survival- and growth-promoting factor in a variety of neurons (Acheson et al., 1995, Huang and Reichardt, 2001). A multitude of stimuli has been described that alter

BDNF gene expression in both physiological and pathological states (Lindholm et al., 1994). For example, BDNF expression has been shown to increase upon light stimulation in visual cortex (Castrén et al., 1992), osmotic stimulation in the hypothalamus (Castrén et al., 1995; Dias et al., 2003), and physical exercise in the hippocampus (Neeper et al., 1995). BDNF expression is upregulated in epileptic animal models, and the time, extent and duration of transcriptional induction depend on the type and severity of seizure (reviewed by Patterson, 2001). One of the first demonstrations of an activity-dependent transcription of the BDNF gene was supported by detection of its mRNA following intense neuronal activity in kainic acid-induced seizure (Zafra et al., 1990). Induction of BDNF has been detected also in other types of experimentally induced epileptiform activities, as well as electrically and chemically induced seizures, including PTZ (Patterson et al., 2001). This discovery raised the idea that seizure-induced expression of neurotrophic factors may contribute to the alteration of synapsy formation and to the lasting structural and functional changes underlying epileptogenesis and the correlated consequences in brain functionality. In zebrafish, BDNF is expressed in the brain of 3 dpf larvae, suggestive of a functional implication in brain development (De Felice et al., 2014). Like *c-fos*, PTZ treatment induces BDNF upregulation and wider expression across the entire brain, within a neuroanatomical distribution similar to that of *c-fos* expression. These results indicate that both *c-fos* and BDNF gene are transcriptionally regulated during seizures in 3 dpf zebrafish larvae.

The GABA signalling

GABA is the major neurotransmitter in the mature mammalian brain, and several studies have speculated about the changes in the mRNAs levels of its receptor subunit after different types of seizures associated with epilepsy processes. In particular, prolonged seizures (*status epilepticus* or SE) result in altered expression and membrane localization of several GABAR subunits, associated with changes in GABAR-mediated inhibition. Changes in GABAR subunit expression and function in epileptic animals precede the development of epilepsy, suggesting that these

changes contribute to the epileptogenesis process (Brooks-Kayal et al., 2009). In contrast, SE (postnatal day 10) in neonatal rats results in increased GABAR α1 subunit expression and does not lead to the subsequent development of epilepsy. In order to better describe the effect of PTZ treatment, I have verified the transcriptional regulation of genes involved in GABA signalling. To this aim, I have selected the *gad1* gene, responsible of GABA neurotransmitter synthesis, and the *gabra1* gene that encodes for one subunit of GABAA receptor. The somewhat predictable results obtained in my thesis work suggest that PTZ treatment does not influence the normal transcriptional activity of both genes. This suggests that the inhibition of GABA receptors does not influence the normal transcriptional levels of GABA signalling genes.

The locomotory profile

Previous studies in zebrafish larvae have shown that seizures induced by PTZ treatment cause a robust increase in locomotory activity (Baraban et al., 2005). I have analysed the locomotory response associated with PTZ treatment in 3 dpf zebrafish larvae using the DanioVision instrument that allows automatized and simultaneous recording of the locomotory behaviour of up to 96 larvae. Badexale et collegues (2012) have suggested that light exposure is an important parameter for high-troughput screening of anti-epileptic drugs so to discriminate the sedative effect of compounds that were originally identified as anti-epileptic. Using a protocol of continuous light stimuli, I have observed a significant difference in the locomotory parameters analysed (total distance moved and mean velocity) between control and treated zebrafish larvae. Since little spontaneous swimming of 3 dpf larvae hampers the detection and appreciation of the locomotory activity, I have tested the locomotory response using a protocol of 10’ light on- 10’ light off. The effect of switching on and off the light permits to stimulate a basal activity also in control larvae and represents a more precise protocol to describe the locomotory responses to treatment. The results obtained with both protocols indicate that treatment of 3 dpf zebrafish larvae with 15 mM of PTZ elicits a seizure-like phenotype. Together with

the behavioural analysis, my PhD work has produced different molecular, cellular and locomotory data that are altogether consistent with a neuronal hyperexcitation condition caused by acute seizures.

Partial inflammatory response to acute seizures in the zebrafish immature brain

Epilepsy research emphasizes the main role of inflammation and immune mediators in seizure and epilepsy. In particular, most attention is devoted to Il-1β signalling. However, results in rodent models differ in various respects. In the adult brain, the involvement of Il-1β has been studied in epileptogenic models, where this inflammatory molecule may eventually contribute to increased threshold for spontaneous seizures and neuronal cell loss, processes that are associated with alteration of inflammatory factors and immune cells. A double function has been proposed for Il-1β in epileptogenic processes (Vezzani et al., 2007), both associated with a pro-convulsant action: one rapid effect is mediated by direct activation of neurotransmitter receptors; the other is a long term effect generated by the IL-1β activation of a classical inflammatory cascade, associated with transcription of other inflammatory cytokines (TNFα, IL-6) together with morphological and functional changes in glia cells (from resting to active state) that contribute to neuronal injury and increased susceptibility to seizure. On the contrary, some age-dependent differences are found in the context of the immature brain. First, an increase of cytokine levels and glia cell activation are present prominently in P15 and P21 immature rats and the temporal time of alteration is visible after few hours and persist for few days (Rizzi, 2003, Ravizza, 2005). In the postnatal brain, on the contrary, *IL-1β* mRNA is not induced soon after seizures, but the level of expression is increased 24h post seizures. As well, GFAP immunoreactivity and microglia activation are only weakly induced in the brain at this age (Javela et al., 2011).

I have investigated the mRNA levels of three cytokine coding orthologous genes expressed in the mammalian brain, *TNFα*, *IL-1β* and *IL-6*, as well as the activity of astrocytic cells by immunohistochemical and transcriptional assays. Interestingly, I

have found an increased level of *IL-1β* mRNA soon after PTZ treatment. This increase is not accompanied by significant changes in the level of the two other inflammatory cytokines. In parallel, I have observed an increase in the immunoreactivity for GFAP and GS proteins, markers of astrocyte cells. The presence of increased immunoreactive signals suggests that, during PTZ exposure, astrocytes are affected by the alteration of normal brain homeostasis. The transcriptional activity of GFAP and GS genes, in addition, is not altered, suggesting that PTZ treatment could probably generate morphological and enzymatic changes at protein levels and producing self-limiting responses. This finding is more similar to responses associated with seizures in the postnatal immature brain of rats, suggesting that, in the context of immature zebrafish brain, PTZ-induced seizures is not able to generate glial cell reactions at the transcriptional level.

However, the presence of a rapid *IL-1β* activation that is lasting after acute seizures, and the fact that this response is not followed by the classical inflammatory cytokines (*TNFα* and *IL-6*) in PTZ treated zebrafish larvae, evokes the implication of regulatory mechanism for *IL-1β* expression in the zebrafish PTZ seizures. In adult rodent brain, for example, the increase of *IL-1β* mRNA and protein occurs within less of 30 minutes, and decline in time of window 48-72 hours from seizures induction (Vezzani et al., 2002), but is followed by the expression of *TNFα* and *IL-6* cytokines, partially in line with my results. If TNFα and IL-6 inflammatory cytokines are not produced, probably the mechanism activated by PTZ seizures induction in zebrafish brain could be different from the classical response observed in mammalians such that the inflammatory response is limited to the IL-1β molecule.

Rapid post-seizure recovery

To study how basal conditions are regained after the end of the acute seizure event, I have performed same analyses as above in a short (2h) and long (24h) time window after PTZ removal. Interestingly, while *c-fos* expression and locomotory parameters are drastically reduced in the brain already 2h after the end of the PTZ treatment, *bdnf* remains upregulated, though not in a statistically significant manner, also 24h after PTZ removal. Therefore, my results provide new insight into the temporal consequences of seizure induction. I have found that one prolonged seizure associated with 45 minutes treatment with 15 mM PTZ in 3 dpf zebrafish larvae produce a long lasting alteration of the transcriptional activity of the *bdnf* gene in different neuronal cell types. The *bdnf* signal is markedly present in its normal brain distribution, and it is extended also in nearby areas. As neurotrophic factor, BDNF could elicit neuroprotective effects after seizures to buffer the altered brain context and limit neuronal depolarization, as proposed from Greer and Greenberg (2008). Further studies are necessary to identify both the neuronal cell types that express BDNF, to clarify the role of this neurotrophin and to identify the mechanisms involved in the regulation of this gene in seizure. The *gabra1* gene was already found to be altered for at least 24h in PTZ rodent epilepsy models (Walsh et al., 1999), suggesting that variations in GABAA receptor mRNAs after prolonged seizures may be associated with alterations in GABAA receptor function and numbers. In zebrafish, a mild regulation of *gabra1* and *gad1* was detected 2h after treatment. Although not statistically significant, this slight transcriptional increase is indicative of a positive feedback mechanism by which GABA receptor inhibition positively regulates GABA signalling homeostasis. Further investigations are needed to better understand this mechanism. As to inflammatory molecules, *IL-1β* increase persists also 2h after PTZ removal while it becomes negligible after 24h. Instead, *TNFα* and *IL-6* maintain their steady state expression at any time point analysed. Similarly, the effect of seizure on inflammatory cell types such as astrocytes is limited after PTZ removal, because at 24h post treatment no increased signal in GFAP positive cells was found in the zebrafish brain, indicative of a rapid and self-limiting response (see above).

The proconvulsive role of IL-1β

In order to gain functional insights into the role of IL-1β in PTZ-induced seizure in 3 dpf zebrafish larvae, I have performed a genetic and pharmacological analysis. I have found that the partial block of the IL-1β protein caused a moderate attenuation in term of locomotory activity response in 3 dpf zebrafish *IL-1β* morphant larvae and that this effect is partially rescued by pharmacological approaches. In particular, by blocking the production of the IL-1β protein with one specific inhibitor of the enzyme caspase 1, I have observed a more prominent attenuation of this behavioural response, in support of a direct implication of *IL-1β* in eliciting seizure-related processes. On the contrary, *IL-1β* morphant treatment with a generic caspase inhibitor did not significantly attenuate the loss of locomotory activity observed in PTZ treated larvae. Further, I have verified the transcriptional responses of *c-fos* gene after PTZ treatment in larvae pre-treated with these pharmacological inhibitors, using WISH (single biological replicate). As shown in the Results chapter, YVAD pre-treated zebrafish larvae showed a reduction in *c-fos* expression upon PTZ treatment, suggesting that IL-1β signalling positively contributes to PTZ induced seizures in 3 dpf zebrafish larvae. A similar mechanism for IL-1β action has been proposed in other models of epilepsy, suggesting the evolutionary conservation of this mechanism in zebrafish and mammals. However, these results are only preliminary observations and require further investigation.

If confirmed, this result constitutes the first evidence of the proconvulsive role of IL-1β signalling in PTZ-induced seizures in 3 dpf zebrafish larvae. More questions remain open. For example, the short and long term consequences of IL-1β blockade in zebrafish behavioural and transcriptional responses in PTZ-induced seizures, as well the main cell types involved in this mechanism and the principal targets. At the same time, this observation corroborates the value of a new model system to investigate the effect of signalling pathways involved in seizure generation.

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