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Analysis of the transcriptional and behavioural responses to seizure onset in a zebrafish model of epilepsy.

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Abstract

Epilepsy is a common neurological disorder characterised by recurrent epileptic seizures. It affects approximately 0.7% of the worldwide population. Even though many patients respond to the available treatments, around a third of people with epilepsy do not respond to existing anti-epileptic drugs (AEDs). Therefore, there is a need to better understand epilepsy in order to develop new therapeutic strategies for the treatment of this disorder.

In this study, a model of pharmacologically-induced epileptic seizures using young zebrafish larvae was developed and characterised. It was found that the brains of young zebrafish larvae exhibited altered PTZ-sensitivity in response to repeated seizure onset or exposure to stress hormone. In both cases, the severity of the PTZ-evoked locomotor convulsive response was enhanced, and expression of selected PTZ-induced genes was reduced. In order to identify more genes involved in the response to PTZ seizure-induction, and which might be involved in the adaptation of the CNS to seizure induction, a two-colour microarray analysis was carried out and many novel PTZ-responsive genes were identified. The function of a new epilepsy risk factor, *sestrin 3,* was also investigated using the zebrafish PTZ model of epileptic seizures, which revealed that *sesn3* promoted locomotor convulsions and regulated expression of a subset of PTZ-induced genes.

In addition to the studies of seizure mechanisms in the zebrafish, the new transgenic line *NBT:GCaMP3* was created, in which expression of the fluorescent genetically encoded calcium indicator was targeted to the CNS, to visualize *in vivo* and in real time, seizure initiation, propagation and suppression by an antiepileptic compound. In the future, combining NBT:GCaMP3 with the new technologies to create zebrafish mutations in orthologues of genes mutated in human epilepsy, will enable novel experimental studies to investigate the pathogenetic mechanisms underlying epilepsy, and facilitate novel approaches to the discovery of anti-epileptic drugs.

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Chapter 1

1 Introduction

1.1 Epilepsy

Epilepsy is a neurological syndrome characterised by transient, recurrent episodes of uncontrolled, synchronised bursts of neuronal activity in the central nervous system called seizures, which give rise to severe physical symptoms that can include intense muscular contractions and loss of consciousness (Mac et al., 2007; McNamara, 1994; Shin & McNamara, 1994). With a prevalence of 0.7% of the worldwide population, epilepsies are common and because more than 30% of the patients continue to have seizures in spite of being under treatment (Remy & Beck, 2006), there is a significant unmet clinical need for new drugs and therapies.

1.1.1 Causes of epilepsy

Epilepsy can occur as a secondary consequence of a brain injury such as head trauma, problems during development, or cerebrovascular diseases, but the most common type of this syndrome is idiopathic. Idiopathic epilepsies are found in patients with no other neurological disorder and it is believed that they are caused by mutations (Steinlein, 2001). Some of the genes affected in several types of epilepsies have been already identified; however, others still remain unknown, such as the genes involved in juvenile myoclonic epilepsy, and absence epilepsy (Reviewed by Steinlein, 2004).

The common characteristic of all epileptic syndromes is the presence of seizures, and the occurrence of a prolonged or continuous state of persistent seizure is known as status epilepticus (Fisher et al., 2005). The clinical manifestations of epileptic seizures depend on the localisation and spreading of the abnormal electrical discharge across the brain, as well as on the maturity of the brain itself and the symptoms that might be originated from another

disease such as Rett Syndrome or other genetic disorders, and infections like meningitis (Engel, 2013; Glaze, et al., 1998; Qureshi & Mehler, 2010)

1.1.2 Classification of epileptic seizures and epileptic syndromes

Seizures are described as being *partial* or *focal* if the onset is localised, or *generalised* if the seizure is initiated more widely throughout the brain. Seizures can also be classified as *complex*, if the person suffers from loss of awareness or consciousness, or *simple* if there is no such loss (McNamara, 1994; Fisher, 1989; Fisher et al., 2005; WHO). Table 1.1 summarizes the classification of seizures according to the International League Against Epilepsy (Berg et al., 2010).

Table 1.1 Classification of seizures (adapted from Berg et al., 2010).

Classification of seizures
Generalised seizures - Tonic-clonic - Absence - Myoclonic - Clonic - Tonic - Atonic
Focal seizures
Unknown - Epileptic spasms

Some examples of generalised seizures are myoclonic, tonic-clonic (grand mal) and absence (petit mal) seizures. Myoclonic seizures are characterised by sudden, violent muscular contractions. By contrast, tonic seizures produce sustained muscular contractions, whereas tonic-clonic seizures consist of alternating episodes of muscular contraction and relaxation that last up to 60 seconds and are accompanied by loss of consciousness. Absence seizures are more common in children than in adults and are characterised by loss of cognitive abilities and cessation of movement for a few seconds followed by a fast, abrupt recovery of movement and consciousness (McNamara, 1994). The most common examples of focal seizures are

complex partial seizures, which usually originate in the cortex of the temporal lobes (McNamara, 1994).

Epilepsies are complex syndromes and they cannot be classified solely by the type of seizure that is manifested. When several symptoms including type of seizure, age of seizure onset, etiology and clinical manifestations occur frequently together, they are denominated as a distinct type of epileptic or epilepsy syndrome (Berg et al., 2010; Engel, 2001, 2006). A list of the epileptic syndromes known to date can be found on Table 1.2 (Engel, 2006).

Table 1.2 Epileptic syndromes (adapted from Engel, 2006)

Epilepsy syndromes and related conditions	
Benign familial neonatal seizures	Reflex epilepsies
Early myoclonic encephalopathy	Idiopathic photosensitive occipital lobe epilepsy
Ohtahara syndrome	Other visual sensitive epilepsies
[a]Migrating partial seizures of infancy	Primary reading epilepsy
West syndrome	Startle epilepsy
Benign myoclonic epilepsy in infancy	Autosomal dominant nocturnal frontal lobe epilepsy
Benign familial infantile seizures	Familial temporal lobe epilepsies
Benign infantile seizures (non-familial)	[a]Generalized epilepsies with febrile seizures plus
Dravet's syndrome	[a]Familial focal epilepsy with variable foci
HHE syndrome	Symptomatic (or probably symptomatic) focal epilepsies
[a]Myoclonic status in non-progressive encephalopathies	Limbic epilepsies
Benign childhood epilepsy with centrotemporal spikes	Mesial temporal lobe epilepsy with hippocampal sclerosis
Early onset benign childhood occipital epilepsy (Panayiotopoulos type)	Mesial temporal lobe epilepsy defined by specific etiologies
Late onset childhood occipital epilepsy (Gastaut type)	Other types defined by location and etiology
Epilepsy with myoclonic absences	Neocortical epilepsies
Epilepsy with myoclonic-astatic seizures	Rasmussen syndrome
Lennox–Gastaut syndrome	Other types defined by location and etiology
Landau–Kleffner syndrome	Conditions with epileptic seizures that do not require a diagnosis of epilepsy
Epilepsy with continuous spike-and-waves during slow-wave sleep (other than LKS)	Benign neonatal seizures
Childhood absence epilepsy	Febrile seizures
Progressive myoclonus epilepsies	Reflex seizures
Idiopathic generalized epilepsies with variable phenotypes	Alcohol-withdrawal seizures
Juvenile absence epilepsy	Drug or other chemically induced seizures
Juvenile myoclonic epilepsy	Immediate and early post cerebral insult seizures
Epilepsy with generalized tonic-clonic seizures only	Single seizures or isolated clusters of seizures
	Rarely repeated seizures (oligoepilepsy)

The types of epileptic seizures and syndromes are so diverse that it makes the study of epilepsy very challenging. It is by investigating molecular basis of this disorder that we might be able to understand what initially gives rise to a seizure and how these abnormalities develop into complex, recurring, neurological disorders. However, this has not been an easy

task and to facilitate the development of this knowledge-base, various experimental models of epilepsy have been developed over the years (Fisher, 1989; White, 2002).

1.1.3 Molecular basis of epilepsy

At a molecular level, epileptic seizures arise as a consequence of the emergence of a homeostatic imbalance in the distribution of sodium, potassium, chloride or calcium ions across neuronal plasma membranes. Thus, defects in neurotransmitter receptors and ion channel function, which cause loss of inhibitory neurotransmission or aberrantly increase excitatory neurotransmission, can raise neuronal resting potentials, facilitating membrane depolarisation and firing of action potentials. When the balance between inhibitory and excitatory neurotransmission is disrupted in large numbers of neurones, they are depolarised simultaneously and give rise to the massive ictal discharges that characterise seizures (McNamara, 1994a; Shin & McNamara, 1994). Molecular genetic analysis of inherited forms of epilepsy have identified mutations in multi-subunit voltage-gated sodium, potassium, calcium and chloride channels, confirming the central roles of these transmembrane protein complexes in the regulation of synaptic neurotransmission and control of seizure onset (reviewed by Scheffer, 2011).

1.1.3.1 Synaptic transmission

Neurones communicate with one another via the regulated release and uptake of neurotransmitters. At synapses, signal-transmitting, pre-synaptic neurons release small molecule and peptide neurotransmitters by exocytosis into the synaptic cleft, from where they diffuse and bind to specific transmembrane protein receptors on the surface of signal-receiving post-synaptic neurones. Engagement of a neurotransmitter with its receptor typically causes a reversible conformational change in the receptor, allowing ions to flow through it, or activates

a cascade of second messengers that opens other ion channels, allowing specific ions to enter or exit the post-synaptic neuron (Kandel & Siegelbaum, 2000a).

The resulting flux of ions through specific receptors on post-synaptic cells can cause either membrane depolarisation and the firing of action potentials, or hyperpolarisation, which inhibits the likelihood of firing of a post-synaptic action potential (Kandel & Siegelbaum, 2000a).

Relative to the extracellular environment, neuronal cytoplasm has a high concentration of potassium and low concentrations of sodium, chloride and calcium ions. These ionic differences typically cause the intracellular environment of mammalian neurons to have an overall net negative charge, with a resting potential of -65mV (Schwartz & Westbrook, 2000). Amino acid neurotransmitters can be excitatory or inhibitory. Glutamate is said to be excitatory because binding to its receptors on neurons causes cation channels to open, leading to the flow of positively charged ions into the neuronal cytoplasm, causing membrane depolarisation and firing of an action potential. By contrast, inhibitory neurotransmitters such as gamma-amino butyric acid (GABA) cause neuronal hyperpolarisation preventing action potential firing, which in the case of GABA is achieved by the opening of a channel in the $GABA_A$ receptor that allows chloride to move into the cell, as well as by activating signalling pathways via additional receptors (Kandel & Siegelbaum, 2000b).

Variation in the membrane potential of neurons can be recorded by using electrodes. The electrical activity of the brain can be detected by electroencephalography, which is a technique that uses electrodes that are placed on the scalp. These electrodes are able to sense the action potentials produced synchronously by groups of nearby neurons, known as field potentials. Therefore, an electroencephalogram or EEG is a recording of various field potentials, detected by the electrodes that are placed on the scalp and can be used to detect abnormalities on the electrical activity of the brain (Westbrook, 2000).

EEG traces recorded during generalised and partial seizures are different; thus electroencephalograms are used to diagnose the type of epileptic seizures a patient suffers (Figure 1 taken from Rang, et al., 2012).

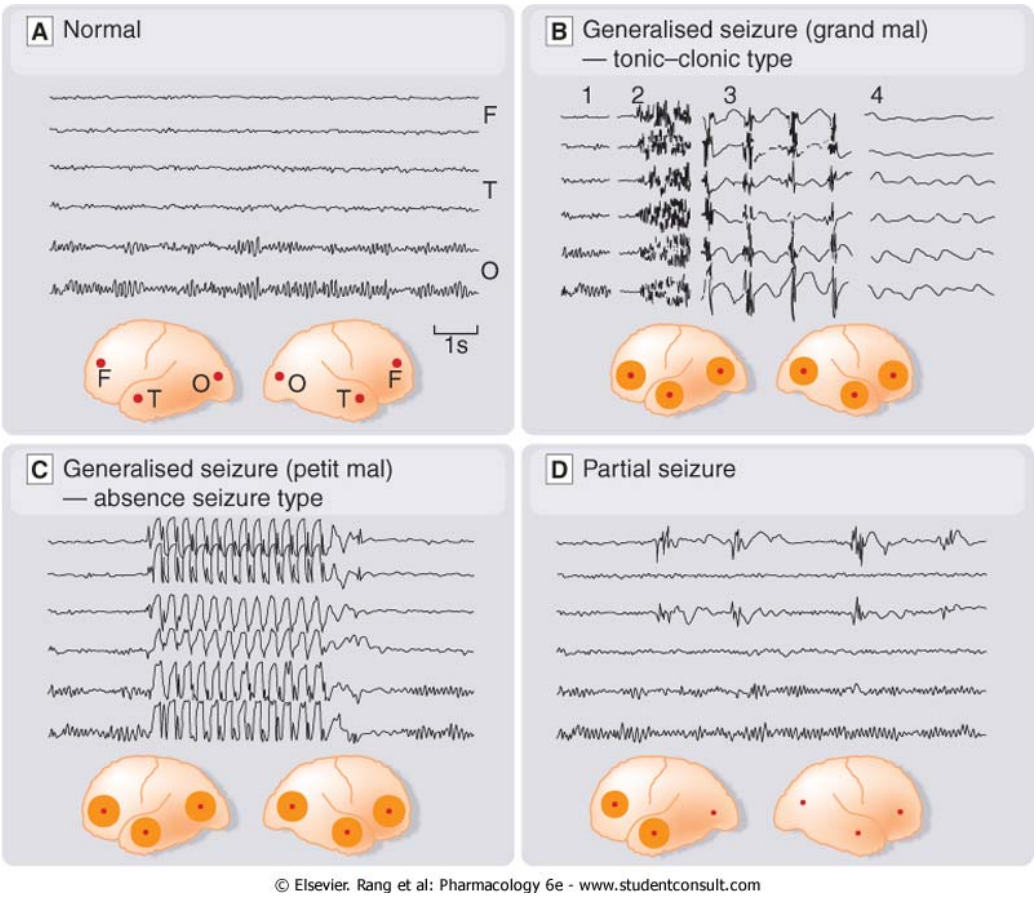


Figure 1.1 Electroencephalography records in epilepsy (taken from Rang et al., 2012). A) EEG in normal conditions (F-frontal lobe, T-temporal lobe, O-occipital lobe). B) EEG recording during a tonic-clonic seizure, 1 represents normal recording, 2 shows the tonic phase, 3 shows the clonic phase and 4 shows the post-convulsive coma. C) EEG recording during an absence seizure. D) EEG recording of a partial seizure, showing abnormal discharges only in the frontal and temporal lobe of the left hemisphere.

Electrophysiological recordings such as EEG are very useful in the study of epilepsy. In addition to its use in clinical diagnosis and monitoring of epilepsies, this technique is also commonly used for the analysis of various animal models of epilepsy. The use of

electrographic recordings in the analysis of animal models of epilepsy will be explained in more detail in a following section of this Chapter.

Changes in the electrical activity of the brain are not the only readout that can be measured when a seizure occurs. In addition to these rapid changes in neuronal excitability, the ion fluxes that characterise synaptic activity directly regulate gene transcription in post-synaptic cells (Greer & Greenberg, 2008).

1.1.3.2 Synaptic activity-induced gene expression

Whilst synaptic activity exerts immediate effects on the excitability of post-synaptic neurones, it also generates powerful signals that are transduced directly to the nuclei of these cells where they promote transcription of specific genes. Excitatory neurotransmitters such as glutamate signal to the nucleus through the calcium-dependent activation of Protein Kinase A, Calcium/Calmodulin-dependent Kinases II and IV, and the Ras/ERK-dependent Kinase RSK, which phosphorylate the CREB transcription factor, thereby, enabling it to activate a specific set of target genes (West et al., 2001; West, Griffith, & Greenberg, 2002). These targets include a group of genes known as immediate-early genes (IEGs). They received this name because they were initially discovered as genes whose transcription was directly induced in fibroblasts treated with growth factors in the presence of a protein synthesis inhibitor such as cycloheximide, which indicated that their transcription was triggered directly by pre-existing transcription factors without the need for new protein synthesis (reviewed by Hughes & Dragunow, 1995). It was soon discovered that some of these genes were also activated in cultured neurones by membrane depolarisation (Morgan & Curran, 1986), implying that these genes might play important roles in neuronal signalling.

Immediate-early genes are not constitutively expressed in cells, but once their expression is induced, they are able to regulate the activation of 'late response' genes which give rise to more delayed responses. The first immediate-early gene to be identified was *c-fos*. This gene

was first isolated from mice osteosarcoma by Finkel, Biskis and Jinkin and was first known as FBJ-virus (Finkel, et al., 1966). Then, it was discovered that this gene is also found in the DNA of normal cells and it was classified as a proto-oncogene or cellular oncogene (reviewed by Hughes & Dragunow, 1995). The gene *c-fos* codes a 56KDa transcription factor which is expressed in neurons in response to calcium influx when they are depolarised (Morgan & Curran, 1986). Neurotrophic factors and neurotransmitters can also induce *c-fos* expression, but normal levels of stimulation do not trigger its expression (reviewed by Kovács, 1998). The c-Fos protein makes a transcriptional factor complex known as Activator Protein-1 (AP-1) when it heterodimerises with proteins of the Jun family (reviewed by Kovács, 1998).

The expression of *c-fos* in the brain has been used as a tool for mapping neuronal activity due to its low basal levels in resting conditions and its inducibility upon different types of neuronal stimulation (Kóvacs, 1998). After *c-fos* was identified as a neuronal activity-regulated gene, many other genes were discovered that are induced in cultured mammalian neurones in response to membrane-depolarising agents. These genes include brain-derived neurotrophic factor (*bdnf*), as well as synaptotagmin 4, *arc* and *homer1a*, which are involved in the formation and/or functional re-modelling of synapses (Hughes, et al., 1993; Vician et al., 1995; Ehrengruber, et al., 2004). In addition, the transcription factor genes *zif268*, *c-Jun*, *npas4*, *MEF2*, *atf3* and *nr4a1* have also been identified as synaptic activity-dependent genes (reviewed by Loebrich & Nedivi, 2009; Lin et al., 2008).

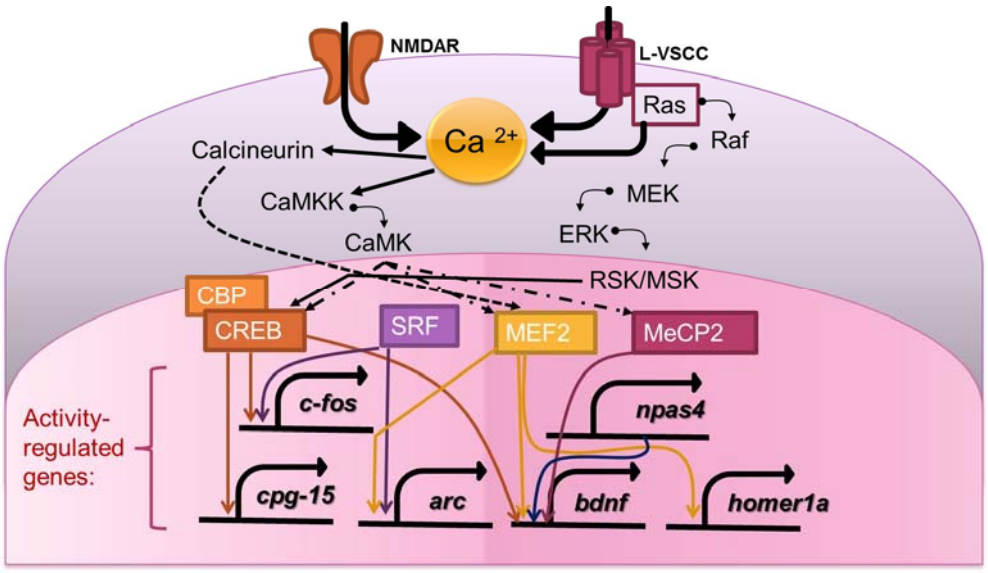


Figure 1.2 Synaptic activity-dependent genes are induced by calcium influx (adapted from Flavell & Greenberg, 2008). Calcium influx via voltage-gated calcium channels and neurotransmitter receptor channels induces the activation of various enzymes responsible of the activation of the expression of activity-dependent or activity-regulated genes like *c-fos*, *bdnf*, *npas4*, among others.

More recently, large scale genome-wide screens in cultured mammalian neurones have begun to identify larger groups of synaptic activity-regulated genes. Gene Ontology analysis indicates that some of these genes can be classified into functionally related sub-groups with predicted or known roles in neurotransmitter release and uptake, synaptic structure, synaptic remodelling, neuronal differentiation and neuronal survival; processes that are important for synaptic plasticity (reviewed by Loebrich & Nedivi, 2009). However, the developmental and physiological functions of most of these genes have not yet been thoroughly investigated. Early on in the functional analysis of *c-fos* and related genes such as *zif268*, it was observed that these IEG genes are strongly induced in neurones of the dentate gyrus of the mouse hippocampus as a direct response to the pharmacological induction of seizures (Dragunow & Robertson, 1987, 1988).

1.2 Animal models of epilepsy

To date, the *in vivo* molecular and cellular mechanisms underlying epileptic seizures have been investigated most extensively in adult mouse and rat. A large body of work has exploited the use of pharmacological tools to induce and resolve seizures and the resulting neurophysiological studies have characterised the functional relationships between ion channels and neurotransmitter receptors in regulating seizure onset and resolution. Chemically-induced models of epilepsy are developed by administrating glutamate receptor agonists or GABA receptor antagonists, or other molecules that affect the activity of neurotransmitter signalling pathways, either systemically or focally in a certain region of the brain. Pentylenetetrazole (PTZ), picrotoxin (PTX), pilocarpine, kainic acid (KA), N-methyl D-Aspartate (NMDA), bicuculline and flurothyl are some of the most used drugs in developing these types of epilepsy models. The mechanisms of action for seizure induction of PTZ, PTX and KA will be explained in Chapter 3.

Along with chemical induction of seizures, electrical stimulation is another broadly used method to disturb brain excitability in animal models of epilepsy; and both chemical and electrical treatments have been used to induce acute and chronic seizures (Fisher, 1989). In acute models of seizure induction, many neurons exhibit epileptiform activity making these seizures very intense. In addition, acute models present a very large defined focus that would not be seen normally in human patients. Moreover, the drugs and their used concentrations to generate acute seizures could cause other effects which might have little to do with epilepsy (Fisher, 1989). On the other hand, chronic models are useful to study the changes that occur in the period between an insult to brain and the development of epilepsy. However, developing a chronic model is a laborious work and takes a long time. Kindling is an example of a chronic model of epilepsy, it refers to a model of epileptogenesis where the animals receive repeated sub-convulsive chemical or electrical stimulation, until the excitability of the brain is destabilised sufficiently to engender the onset of spontaneous seizures (Fisher, 1989; White, 2002). Kindling models will be discussed in more detail in Chapter 5.

More recently, gene targeting studies have shown that genetic inactivation of specific subunits of ion channels or neurotransmitter receptors can be sufficient to induce spontaneous seizures. This has been the premise for the creation of genetic models for the study of epilepsy (Reviewed by Fisher, 1989; White, 2005; Baraban, et al 2005). However, mutations in these genes are responsible for a small proportion of idiopathic cases of epilepsy. Mutations in many other types of genes, such as *LGI1, ARX, EFHC1, STXBP1, PCDH19* and *TBC1D24* can also predispose to this disorder, through mechanisms that are still poorly understood (Poduri & Lowenstein, 2011). The study of both, induced models and genetic models of epilepsy can give different insights into the pathogenesis of epilepsy which can derive in a better understanding of the disorder and the finding of more effective treatments.

Even though rodents are the most used animals for the study of epilepsy, less complex organisms such as worms, flies and fish are also used to study different aspects of epilepsy (Howlett, et al., 2013; Kroll & Tanouye, 2013; Lin & Baines, 2014; Williams, et al., 2004; Baraban, et al., 2005; Baraban, et al., 2013; Baxendale, et al., 2012; Hortopan, et al., 2010). There are three distinct types of phenotype that can be taken into account when studying epilepsy in animal models: a) the electrophysiological responses of the neurons (seizure onset), b) the expression of synaptic activity-dependent genes in activated neurones (caused by the calcium influx during seizures) and c) the resulting behavioural changes such as loss of consciousness or awareness of others, or physical motor convulsions.

1.2.1 Detecting electrical activity of the brain in animal models of epilepsy

The electrophysiological responses of large and small animals can be recorded from a discrete region of the brain or even from a single neuron by using electrodes. Electrographic recordings are used to detect peaks of electrical activity caused by seizures, known as ictal spikes (reviewed by McNamara, 1994). The electrodes can be placed over the skull of surgically operated animals (Lee, et al., 2011), or they may be inserted through the skull of a surgically operated animal to record directly from the cortex (Colas et al., 2008; Reibel et al., 2001), making this a very invasive technique. The techniques to get electrographic recording from

flies (Stilwell, et al., 2006) and zebrafish (Baraban et al., 2005) are also complicated and invasive, as they have to be immobilised and an electrode has to be inserted in their brains.

Two distinctive spikes can be detected in electroencephalograms taken from human patients of epilepsy and experimental animals of epilepsy models. These two patterns of electric activity are known as ictal (seizure-like), and interictal (between seizures) (Fisher, 1989; McNamara, 1994). Interictal spikes are localised to the region where the seizures originate, and they are detected during asymptomatic periods (McNamara, 1994). Many studies have investigated the causes of the transition from interictal to ictal state. A recent *in vitro* study suggested that the interictal state is caused by GABAergic activity and the exhaustion of presynaptic release of GABA and the consequent increase of glutamate activity cause the ictus onset (Zhang et al., 2012). Figure 1.3 shows an example of electrophysiological recordings from the brain of a zebrafish larvae treated with PTZ, illustrating the ictal and interictal spikes (taken from Baraban et al., 2005).

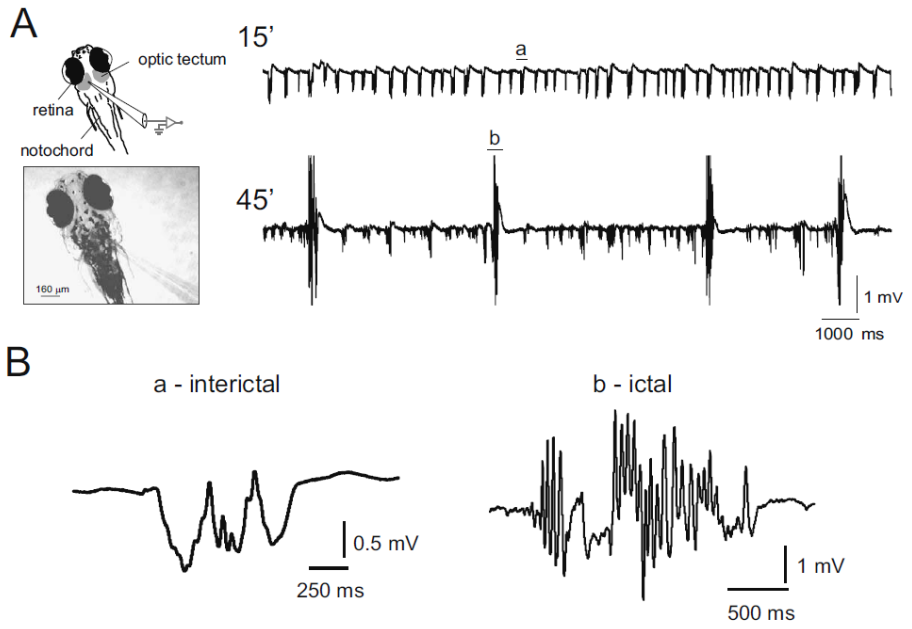


Figure 1.3 Example of electrophysiological recordings from the optic tectum of zebrafish larvae treated with 15mM of the $GABA_A$ receptor antagonist Pentylenetetrazole (PTZ) (taken from Baraban et al., 2005). A) An electrode was placed in the optic tectum of the zebrafish larvae where the electrical activity was recorded after 15 and 45 minutes of treatment with 15mM PTZ. B) Isolated interictal and ictal spikes shown at a faster time resolution.

Recently, a non-invasive way to visualise neuronal activity *in vivo* has been developed using genetically encoded calcium indicators (GECIs) (Hires, et al., 2008; Tian et al., 2009). The most commonly used calcium indicator is GCaMP, which is made from the fusion of green fluorescent protein (GFP), the calcium binding protein calmodulin and the M13 domain of myosin light-chain kinase (Nakai, et al., 2001). In the presence of calcium, calmodulin binds to the calcium ions causing a conformational change in the molecule, increasing the fluorescence of GFP (Figure 1.3). More about the use of GCaMP will be explained in Chapter 7.

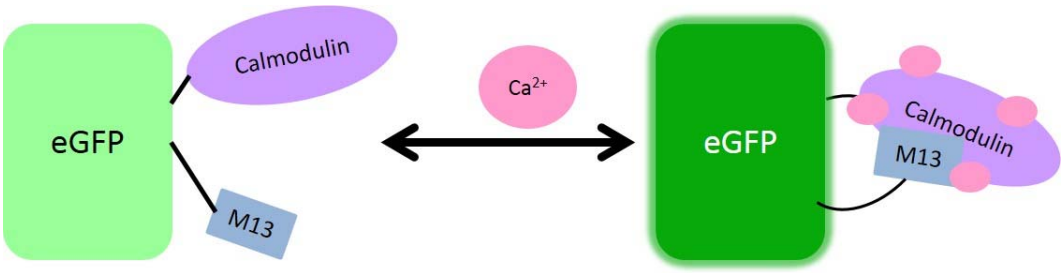


Figure 1.4 Schematic representation of GCaMP sensing mechanism (adapted from Hires et al., 2008). A conformational change in the fused molecule of calmodulin-M13-GFP occurs in the presence of calcium, causing an increase in the fluorescence levels of the green fluorescent molecule.

The electrophysiological readout is a good parameter for studying epileptic seizures and it is used clinically to diagnose epilepsy. However, the presence of spike-like patterns does not necessarily indicate epilepsy, because epileptiform-like activity can be detected in EEGs of non-epileptic patients (Goodin & Aminoff, 1984). Therefore, to diagnose epilepsy, EEG has to be used alongside other clinical observations (Goodin & Aminoff, 1984). Supportive behavioural manifestations such as convulsions are another important piece of evidence that need to be taken into account for a definitive diagnosis of epilepsy (Fisher, 1989).

1.2.2 Identification of convulsive responses in animal models of epilepsy

The study of seizure-associated convulsive motor behaviour in animal models of epilepsy is based on a list of behavioural changes that occur during and after seizure induction, described by Racine in 1972 (Racine, 1972). Racine divided the behavioural response to seizures in 5 stages: 1) mouth and facial movements, 2) head nodding, 3) forelimb clonus, 4) rearing, 5) rearing and falling (Racine, 1972). Since then, the Racine scale has been slightly modified, but the basic characteristics of each stage remain the same (Lüttjohann, et al., 2009).

This scale was first designed to be used in rodent models; however, this scale can be modified and applied to non-mammalian models of epilepsy like *Drosophila* and zebrafish. In response to seizure onset, bang-sensitive (BS) mutant adult flies display leg shaking, contractions of the abdominal muscle, wing-flapping and a short period of paralysis, followed by tonic-clonic-like convulsive spasms (Song & Tanouye, 2009).

The use of zebrafish for epilepsy studies has been accompanied by the adaptation of the stages of the Racine scale to measurable equivalent behaviours for both larvae and adult zebrafish. Table 1.3 shows the scoring system for seizure behaviours in both larvae (Hortopan et al., 2010b) and adult zebrafish (Mussulini et al., 2013).

Table1.3 Scoring system for seizure-associated convulsive behaviours in zebrafish (*Hortopan et al., 2010; +Mussulini et al., 2013)

	Behaviour	
Stage	**Larvae***	**Adults+**
0	Very little swim activity	Short swim mainly in the bottom of the tank
1	Increase in swim activity	Increased swimming activity and high frequency of opercular movement
2	Rapid whirlpool-like circling around the outer edge of the well	Burst swimming, left and right movements, and erratic movements
3	Brief head-to –tail convulsions followed by a loss of posture	Circular movements
4		Clonic seizure-like behaviour (abnormal whole-body rhythmic muscular contractions)
5		Fall to the bottom of the tank, tonic seizure-like behaviour
6		Death

A more detailed description of how the convulsive behaviour analysis of zebrafish larvae is performed can be found in Chapters 2 and 3.

Seizures not only cause dramatic alterations in behaviour, they also elicit transcriptional changes in neurones within synaptically-activated circuits. The expression patterns of synaptic activity-dependent genes in the brain can therefore indicate both the pattern and degree of neuronal excitation.

1.2.3 Synaptic activity-induced gene expression in models of epilepsy

The function of synaptic activity-induced genes in response to seizure induction is not very well understood. The induction of synaptic-activity dependent genes in response to seizure induction has been observed in many animal models of epilepsy (Loebrich & Nedivi, 2009b). However, little is known about the expression of these genes in patients of epilepsy. A microarray study found that the expression of EGR1, EGR2, c-fos and MKP3 was increased in tissue from the neocortex of patients with pharmacoresistant epilepsy. Moreover, the expression of c-fos, EGR1 and EGR2 correlated with several parameters of interictal spikes such as amplitude, frequency and area (Rakhade et al., 2005, 2007). In this study, the average amplitude of interictal spikes was larger in epileptic tissue compared with control tissue of patients with epilepsy. Additionally, the frequency of these spikes was higher in the epileptic zones and the expression of c-fos, EGR1 and EGR2 was found increased in those epileptic areas when the spikes' amplitude and frequency were high (Rakhade et al., 2007).

The vast majority of synaptic activity-induced genes have been identified in cultured neurones and have not yet been functionally characterised *in vivo*. *In vivo* characterisation of this group of genes is likely to provide important new insights into the molecular mechanisms underlying the nervous system response to experimentally-induced seizures, which may be highly relevant to the changes in the brain that are engendered by seizures in patients with epilepsy. Studies to date, indicate that there are multiple distinct types of functions for genes that are

transcriptionally activated in response to seizure. Genes such as *CPG-15*, *Arc* and *Homer1a* are likely to be involved in promoting neuronal differentiation and synaptogenesis to facilitate normal neural circuit activity, and their higher level of expression in seizure may simply reflect the increased level of neural activity (reviewed by Loebrich & Nedivi, 2009). Recent studies have shown that the stabilization of active synapses in mice required the expression of CPG-15 (Fujino et al., 2011); NMDAR-mediated persistent firing patterns in frontal neurons after motor training in mice was regulated by Arc (Ren, et al., 2014); and Homer1a ameliorated brain damage *in vitro* and *in vivo* and improved neurological function after traumatic brain injury (Luo et al., 2014).

Alternatively, the expression of synaptic activity-dependent genes may be part of the neurotoxic mechanism that is induced by the high intracellular calcium levels triggered by seizure. In rats injected with KA directly in the amygdala, the expression of c-JUN correlated with the localisation and extension of the apoptosis caused by KA-seizure induction, suggesting that c-JUN was mediating an apoptotic signalling pathway (Lee et al., 2001).

Some other synaptic activity-dependent genes could be neuroprotective against the cytotoxic effects of this high calcium influx caused by the prolonged membrane depolarisation. Increased transcription of *c-fos* and *BDNF*, or the induction of c-Fos or BDNF protein, have been used extensively in models of epilepsy and other neurological disorders like stroke, as specific markers of neuronal activation in response to the onset of a seizure in epilepsy, or other changes such as inflammation or tissue damage in the CNS. The existing evidence suggests that *c-fos* could fall into this neuroprotective category. *c-fos* null mice treated with KA showed an enhanced seizure severity, increased neuronal excitability and increased apoptosis compared with wild-type mice (Zhang et al., 2002). Moreover, suppression of post-ischaemic *c-fos* expression caused an increase tissue damage following focal cerebral ischemia (Zhang, et al., 1999). These two examples suggest that *c-fos* might be limiting damage caused by different types of insults such as seizures and stroke.

Figure 1.5 sets out a hypothesis that upon seizure onset, some genes that are transcribed might promote the pathological epileptogenesis pathway or alternatively, fulfil homeostatic functions that protect the activated neurons and other circuit components from damage. The same synaptic activity-dependent gene might activate different genes in different types of neurons in order to maintain the homeostasis of the system through different feedback responses. A recent study has shown that Npas4 induced the expression of different but overlapping sets of genes in inhibitory and excitatory neurons, modifying their activity in a specific manner upon the same stimulus (Spiegel et al., 2014).

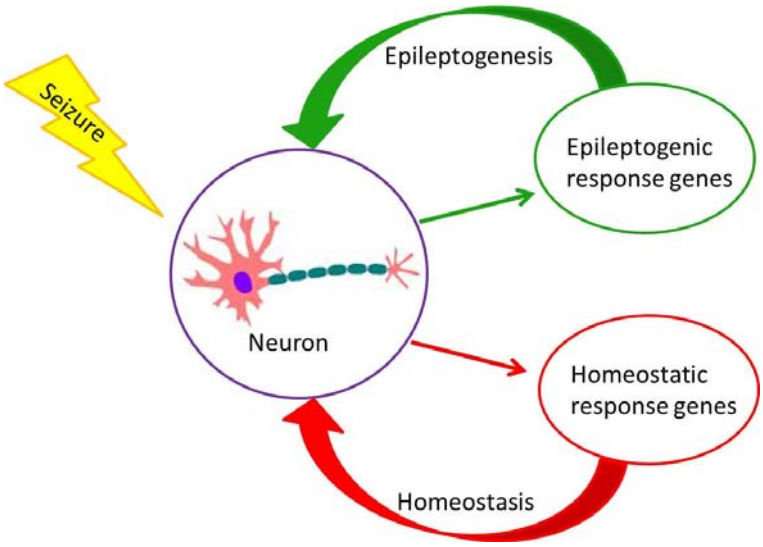


Figure 1.5 Transcriptional response to seizure onset might strengthen the epileptogenesis pathway or promote the homeostasis in the brain to prevent damage.

This hypothesis of altered gene expression contributing to the strengthening of the neuropathological process or attenuating its progression by promoting homeostasis is inspired by studies from the related field of synaptic plasticity.

1.3 Synaptic plasticity

As mentioned before, *CPG-15*, *Arc, Homer1a* and *Bdnf* have been implicated in synapse formation and synaptic remodelling, causing strengthening or weakening of neuronal circuits. Such functional remodelling of neuronal circuits depends on synaptic plasticity, which alters the number, diversity and efficacy of different synapse types (Reviewed by Flavell & Greenberg, 2008). Synaptic plasticity occurs during development, under normal resting physiological conditions (homeostasis) and also during the pathogenesis of neurological diseases. Synaptic plasticity has been particularly well-studied in relation to the processes underlying learning and memory. In 1949, Donald Hebb proposed the first model of synaptic strengthening involved in learning and his principle can be summarised as *'neurons that fire together wire together, whereas neurons that fire out of synchrony lose their connection'* (Hebb, 1949; reviewed by Maya-Vetencourt, 2013).

Sensory experiences can cause changes in the neuronal circuitry of adult brains, therefore giving rise to long-lasting memories or other types of adaptations. Long-lasting synaptic plasticity includes long-term potentiation (LTP) and long-term depression (LTD). The long lasting and stable enhancement of a post-synaptic response resulted from the synchronous stimulation of the neurons is known as LTP (Teyler & DiScenna, 1987); whereas LTD is the weakening of synaptic transmission in response to continuous stimulation (Massey & Bashir, 2007). The establishment of persistent changes such as LTP and LTD depends on the neural activity-induced transcription of activity-dependent genes and their effector proteins to create stable changes in neuronal activity (Cohen & Greenberg, 2008).

Repeated seizures may cause long-term changes in synaptic efficacy and neuronal network organisation, which can alter seizure severity and frequency. Sir William Gowers noticed that seizure severity increasd progressively in some patients suffering from recurrent seizures, therefore he postulated that "seizures beget seizures" (Eadie, 2011; Gowers, 1881). A particularly useful means of investigating the functions of activity-dependent genes in epilepsy, in relation to the elucidating their roles in synaptic plasticity, is by using kindling models. When

an animal is kindled, it is exposed repeatedly to the same stimulus until a permanent change in neuronal excitability is achieved. Even though studies in animal models have confirmed Gower's idea of seizures begetting seizures, there is still some scepticism arguing misinterpretation of data obtained from animal models of epilepsy (Sills, 2006). Graeme Sills believes that preclinical researchers sometimes forget about the importance of clinical relevance, and their interpretation of studies using animal models of epilepsy might occasionally be misguided by the belief that epileptogenesis, pharmacoresistance, and seizures begetting seizures are the same thing (Sills, 2006).

1.3.1 Synaptic plasticity in epilepsy: 'Seizures beget Seizures'

When neurons are depolarised during a seizure, NMDA receptors are activated by the excitatory neurotransmitter glutamate, provoking a conformational change in the receptor that creates a central pore through which calcium can then flow, from the extracellular milieu into the neuronal cytoplasm. This calcium influx leads to the activation of cAMP-dependent protein kinases which phosphorylate the AMPA glutamate receptors. Phosphorylation of the subunits of the AMPA receptors enables the translocation of AMPA receptors to the synapse. Therefore facilitating depolirasation and action potential firing, triggering the early phase of LTP (Boehm et al., 2006). Calcium influx is also responsible for the activation by phosphorylation of the transcription factor CREB, which promotes the transcription of other activity-dependent genes that play roles in eliciting the longer-lasting characteristics of the late phase of LTP, including the development of more new synapses, and the sprouting and re-shaping of dendrites (Malenka & Bear, 2004; Meador, 2007).

The hippocampus is the most commonly analysed area of the brain in epilepsy to study plasticity. The CA3 pyramidal system of the hippocampus is very susceptible to seizures. When seizures are induced in rodents by kainate or PTZ treatment, c-*fos* is activated very strongly and relatively specifically in the hippocampus (Chen, et al., 2009; Esclapez, et al., 1999; Joëls, 2009; Simonato et al., 1991). Extensive dendritic sprouting and a dramatic

increase in the arborisation and formation of synapse by mossy fibres (which are granule cells of the dentate gyrus that project to CA3) have been found in the hippocampus in different models of temporal lobe epilepsy after an status epilepticus is evoked (reviewed by Ben-Ari, 2008; and Scharfman, 2008; Esclapez et al., 1999).

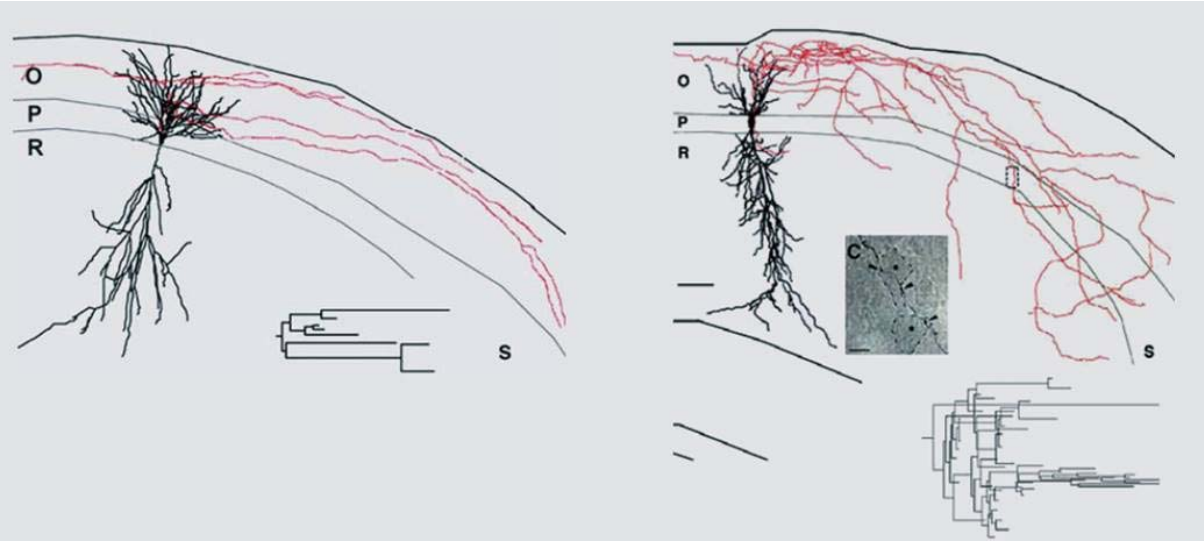


Figure 1.6 Schematic representation of sprouting in the hippocampus of rats after status epilepticus (taken from Ben-Ari, 2008; Esclapez, et al., 1999). The left panel shows a camera lucida tracing of a CA1 pyramidal cell in the hippocampus of a control rat, whereas the right panel shows a camera lucida tracing of a CA1 pyramidal cell in the hippocampus of a pilocarpine-treated rat, after status epilepticus was reached. The two dendrograms beneath the camera lucida tracings iliustrate the extent of branching in each pyramidal neuron.

The expression of the gene *c-fos* is widely use in epilepsy models as a marker to detect neuronal activity and it is one of the genes believed to be involved in synaptic plasticity in epilepsy (Fleischmann et al., 2003; Watanabe et al., 1996). A study comparing *c-fos* null mutant mice with *c-fos* heterozygous mutants and wild-type mice, after being kindled with electrical stimulation, showed that *c-fos* null mutant mice exhibited fewer mossy fibres in the dentate gyrus of the hippocampus than their heterozygous and wild-type siblings; suggesting that *c-fos* is necessary for the formation of new synapses after kindling (Watanabe et al., 1996). Another frequently studied gene in several models of epilepsy is *bdnf*. When brain slices (including the hippocampal region) of rats treated with pilocarpine were exposed to a

bath containing BDNF, the excitatory transmission of the mossy fibre sproutings that develop was enhanced and the granule cells became hyperexcitable only after sprouting had occurred. This results suggest that BDNF might be implicated on the long-lasting adaptations of the neuronal network that occur after status epilepticus (Scharfman, et al., 2008).

The roles of the activity-dependent genes in epilepsy and plasticity remain relatively poorly understood, but by continuing to investigate their functions, deeper insight into the mechanisms underlying epileptogenesis can be obtained in order to develop more efficient treatments. However, it should be taken into account that other factors might be involved in the epileptogenesis process.

1.4 Stress and epilepsy

Stress has been repeatedly identified by patients with epilepsy and physicians as triggers of seizures (Frucht, et al., 2000; Haut, et al., 2007; Nakken et al., 2005; Novakova et al., 2013; Spector, et al., 2000; Sperling, et al., 2008). However, due to differences on perceptions of stress, the results of these types of studies might be somewhat subjective.

The Central Nervous System processes information derived from the periphery that concerns the nature of external stimuli in the external environment, as well as information about internal changes within the body. The brain then uses this information to prioritise and direct physiological responses in order to achieve homeostasis or enhance physiological capabilities. Thus, the CNS matches changes in the environment with internal physiological adaptations which maintain or improve well-being. Kindling is one form of experience that elicits maladaptive changes to CNS function which precipitate seizures. Recently, the clinical and experimental literature has begun to document other aspects of experience-sensitive physiological change which may impact on seizure incidence and severity. Prominent among the experience-sensitive physiological changes thought to modulate seizure severity and frequency are the responses to stressful stimuli, in which activation of the Hypothalamus-

Pituitary-Adrenal (HPA) Axis is a central element. The HPA Axis exerts powerful endocrine impacts that affect synaptic activity within many neural circuits, and co-ordinates a wide variety of physiological responses throughout the body that reduce or eliminate the perceived external threats. (Figure 1.7, adapted from van Campen, et al., 2013).

Novakova and collaborators, wrote a review on how stress can trigger epileptic seizures stating that people with epilepsy present higher levels of psychological distress than healthy people and that according to many studies, stress can provoke the initiation of seizures in patients with epilepsy (Novakova, et al., 2013).

The way the organism responds to stressful events is by activating the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (Figure 1.7, adapted from van Campen, et al., 2013).

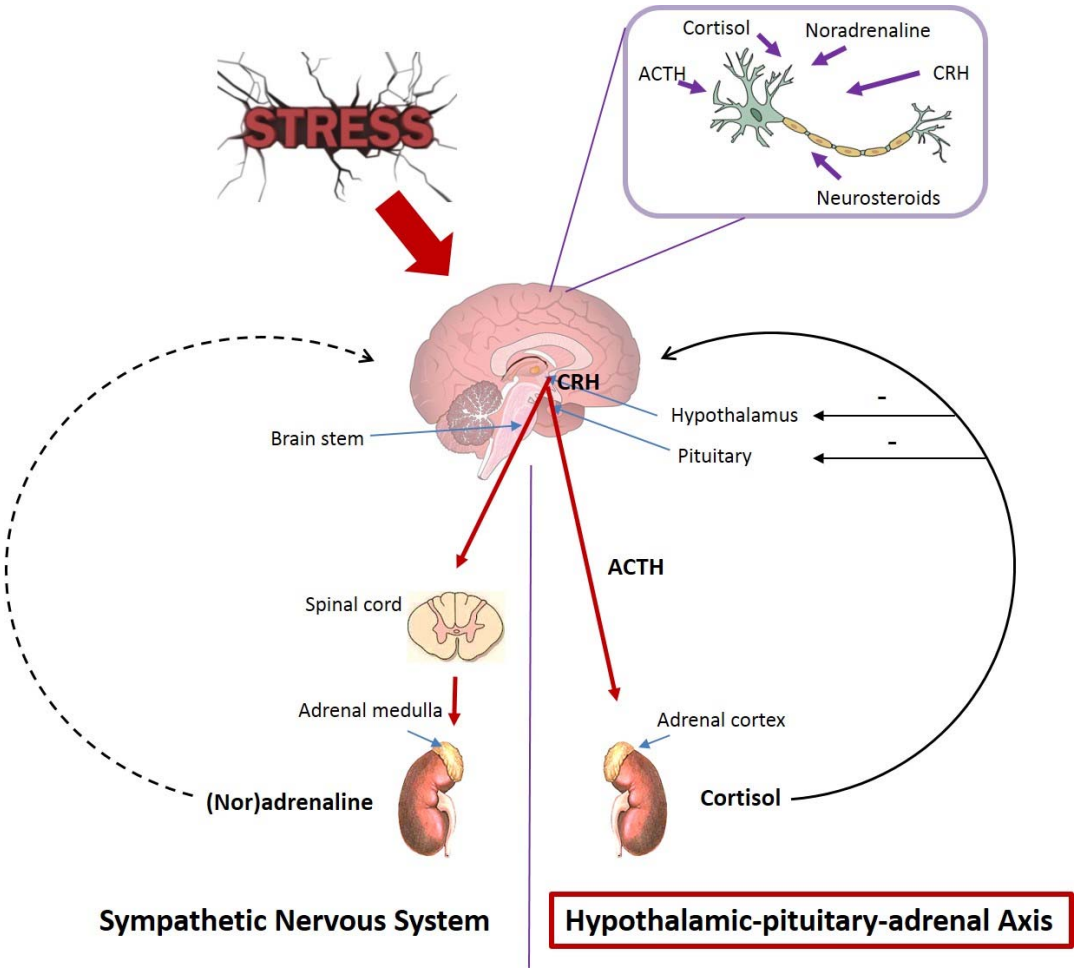


Figure 1.7 Schematic representation of the stress response by the autonomous nervous system (left) and the HPA axis (right) (Adapted from van Campen et al., 2013)

Activation of the HPA axis by stressful experiences leads to the secretion of glucocorticoid hormones such as cortisol (in humans and zebrafish) or corticosterone (in rodents) by the Adrenal cortex, which exerts a remarkably wide variety of physiological changes within the body. Cortisol, along with neuropeptides and other hormones involved in the stress response can affect the function and excitability of neurons. Therefore, stress can influence epilepsy not only by triggering seizures, but it can promote the onset of epilepsy in the first place (van Campen et al., 2013). Several studies using different animal models of epilepsy showed that animals exposed to early-life stress develop epileptic symptoms more rapidly than do non-stressed animals. Chronic stresses in adulthood, such as long-term social isolation, cold

restraint and exposure to the forced swim stress also aggravate seizure responses (De Lima & Rae, 1991; Jones et al., 2013; Matsumoto et al., 2003; van Campen et al., 2013a). Taken together, these findings indicate that stress affects the severity of epileptic seizures. However, more research is required to understand how stress affects both the epileptogenic processes underlying seizure susceptibility, as well as the severity of seizures, at the molecular level. Chapter 5 includes further discussion of this topic.

Aims of this project

The aims of the research described in this thesis are:

- To characterise the transcriptional and behavioural responses to chemically-induced seizures in zebrafish embryos and larvae. (Chapter 3)
- To explore the impacts of repeated seizures and the stress hormone cortisol on seizure-induced gene expression and locomotor convulsive behaviour of zebrafish larvae
- To investigate the functions of a selected subset of synaptic activity-regulated genes whose transcription is a response to seizure induction (Chapters 4 and 5)
- To investigate the function of a new putative seizure susceptibility factor (*sestrin 3*) in our zebrafish epilepsy model, as part of a collaboration with Dr Enrico Petretto and Dr Michael Johnson of Imperial College London (Chapter 6)
- To develop a zebrafish transgenic line exhibiting neuronal-specific expression the Genetically Engineered Calcium Indicator GCaMP3, for *in vivo* imaging of seizure activity. (Chapter 7)

Chapter 2

2 Materials and Methods

2.1 Materials

2.1.1 Zebrafish stocks

- AB wild-type zebrafish were maintained at the aquaria facility of the Bateson Centre of the University of Sheffield.
- Nacre wild-type zebrafish were maintained at the aquaria facility of the Bateson Centre of the University of Sheffield.
- *Npas4a (sa701 allele)* mutant fish were obtained from the Sanger Institute and were maintained at the aquaria facility of the Bateson Centre of the University of Sheffield.
- *NBT:GCaMP3* transgenic fish were maintained at the aquaria facility of the Bateson Centre of the University of Sheffield.
- *ptf1a:GFP* transgenic fish were maintained at the aquaria facility of the Bateson Centre of the University of Sheffield.

2.1.2 Morpholinos

All morpholinos were designed and manufactured by Gene Tools.

- ❖ Gabra1 e2i2 SPL 5'- TAGTGAAACTCATATACTGACCTCC-3'
- ❖ c-fos SPL 5'-GGGAAAATGCAGAATACTTACAGCT-3'
- ❖ Npas4a e2i2 SPL 5'-TGAAATAGCATGTTTACTCACCATT-3'
- ❖ Sestrin3 i3e4 SPL 5'-TGCAGCCTGGAAGACATGGAAAAAA-3'
- ❖ Sestrin3 e4i4 SPL 5'-GACTCCAACTAATGGGTTTACTTGT-3'

2.1.3 In situ probes

Probe name	Gene name	Vector	RE/Polymerase	Reference
bdnf	Brain-derived neurotrophic factor	pCMV-Sport6.1	EcoRI/T7	IMAGE:6797983
cfos	FBJ murine osteosarcoma viral oncogene homolog	pbK-CMV	BamHI/T7	Baxendele, et al., 2012

Crh	Corticotropin-releasing hormone	pME18S-FL3 clonned to pBlueScript KS	EcoRI/T7	IMAGE:7253470
npas4a	Neuronal PAS domain protein 4	pExpress1	BglII/T7	IMAGE :7048053
pomc	Proopiomelanocortin		NotI/Spe6	
pyya	Peptide YYa	pCR4-TOPO	SpeI/T7	IMAGE:9037603
sesn3	Sestrin 3	pCS2+	Eco/T7	BC045518
Th	Tyrosine hydroxylase	pBlueScript KS	XhoI/T3	
tph2	Tryptophan hydroxylase 2	pCR2.1TOPO	BamHI/T7	Teraoka, et al., 2004 (Gift from Claire Russell)
uts1	Urotensin 1	pExpress1	EcoRI/T7	IMAGE:8343300

2.1.4 Primers

All primers were designed using Primer3 web version 4.0.0.

2.1.4.1 RT- PCR

Gene	Forward Primer	Reverse Primer	Product Size
β-actin 1	CTCTTCCAGCCTTCCTTCCT	CACCGATCCAGACGGAGTAT	247bp
bdnf	AGGAGTTGCTTGAGGTGGAA	CCGGCCATTGCGAGTTATAGT	473bp
c-fos	AGCCCATGATCTCCTCTGTG	CTTGCAGATGGGTTTGTGTG	344bp
Il-1β	GACTTCGCAGCACAAAATGA	CAACAGGCCAGGTACAGGTT	399bp
npas4a	AAGGACTTGTTGCCCATCTC	TGTTGAATCTGCAGCGAAAC	400bp
sesn3 e3e4	CTGCGCTGCCACTATTACCT	GCTCAAACTCTTGCACATGG	228/ 133bp
sesn3 e4i4	TGGCTGAGAGGTCTGGAGTT	TGACTTGCCTAATTACTCGCCTA	391bp
sesn3 e4e5	TGGCTGAGAGGTCTGGAGTT	GTGACCCCGTTGACTTCTGT	2084/ 254bp

2.1.4.2 qPCR

Gene	Forward Primer	Reverse Primer	Product Size
Atf3	GAGACCCACCGAACTACCTG	TGCTGCTGCAATTTGTTTC	97bp
β-actin1 (Keeganet al., 2002)	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	102bp
Bdnf	TCGAAGGACGTTGACCTGTATG	TGGCGGCATCCAGGTAGT	112bp
c-fos including intron3-4	TCGACGTGAACTCACCGATA	CTTGCAGATGGGTTTGTGTG	145bp
c-fos	AACTGTCACGGCGATCTCTT	TTGGAGGTCTTTGCTCCAGT	147bp
egr2b	CTGCCAGCCTCTGTGACTAT	GCTTCTCCGTGCTCATATCC	100bp

fosB	CCAGTGCGTCAGTCTCGAAG	CGGCAGCCAGTTTATTTCTC	91bp
gadd45ba	TCTCACAGTCGGCGTTTATG	TCGTCCAGATCCTCCTCATC	99bp
gapdh	GTGGAGTCTACTGGTGTCTT	GTGCAGGAGGCATTGCTTAC	173bp
npas4a	GAGTAACCTGGTGCCTCCAA	TTTGCCTACGCACTGATTTG	86bp
sesn3	CTTCGCAACCTCAACGAGAT	AACAGTACGACCGCGTGAAC	137bp

2.1.5 Solutions and buffers

2.1.5.1 General Solutions

Solution or Buffer	Composition
E3	15mM NaCl, 0.5mM KCl, 1mM $CaCl_2$, 1mM $MgSO_4$,1.5mM KH_2PO_4, 0.05mM Na_2HPO_4,0.7mM $NaHCO_3$ and 3 drops per litre of Methylene blue
Phosphate buffered saline (PBS)	1PBS tablet in 100mls H_2O
Phosphate (PO) buffer	0.02M NaH_2PO_4, 0.08M Na_2HPO_4
Fish fix	0.1M PO buffer, 0.12M $MgCl_2$, 4% Paraformaldehyde powder (w/v), 4% sucrose (w/v)
50X TAE buffer	242g Tris Base, 57.1ml Acetic Acid, 100ml 0.5M EDTA pH8, 1l H_2O

2.1.5.2 Solutions for in situ hybridisation

Solution or Buffer	Composition
PTW	PBS with 0.1% Tween 20 (v/v)
Hybridisation solution	50% Formamide, 5X SSC, 500µg/ml tRNA, 50µg/ml heparin, 0.1% Tween 20 (v/v), 10mM citric acid
20XSSC	3M NaCl and 300mM Sodium Citrate (pH 7.0 with NaOH)
PBT	PTW with 2% sheep serum and 0.2% BSA
Maleic acid blocking buffer	150mM Maleic acid, 100mM NaCl, 2% Blocking reagent (Roche), 0.1% Tween 20 (v/v)
Staining buffer pH 9.5	100mM Tris pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20 (v/v)
NBT/BCIP staining solution	100mM Tris pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20 (v/v), 3.5µg/ml BCIP, 4.5µg/ml NBT
Staining buffer pH 8.2	100mM Tris pH 8.2, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20 (v/v)
Fast red staining solution	100mM Tris pH 8.2, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20 (v/v), 1 Fast red tablet in 4ml buffer.

2.1.5.3 Solutions for in immunofluorescence

Solution or Buffer	Composition
PBS Block	PBS with 0.1% Tween 20 (v/v), 1% DMSO, 10% horse serum.

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Agarose gel electrophoresis

Restriction endonuclease digestion and the probe synthesis were checked by DNA electrophoresis in 1% (w/v) agarose gels containing TAE buffer and 200ng/ml ethidium bromide. For restriction endonuclease digestions the gel was run for 45 minutes at 80 volts, whereas the gels for checking the probe synthesis were run at 160 volts for 10 minutes.

PCR products were analysed in 1.8% (w/v) agarose gels with TAE buffer for 60 minutes at 60 volts.

2.2.1.2 Restriction endonuclease digestion

Restriction endonuclease digestions were carried out using restriction enzymes from New England Biolabs using 1µl of enzyme per every 1µg of DNA. The restriction digest reaction was incubated at 37°C for approximately 2hrs.

2.2.1.3 Phenol extraction and ethanol precipitation of DNA

DNA was diluted to a volume of 400 µl, the same volume of 400 µl of phenol:chloroform:isoamyl-alcohol (24:25:1, pH6.7) was added to the DNA. The mixture was emulsified and then centrifuged at 13000 rpm for 5 minutes at room temperature. The top aqueous layer was collected and 40µl of 3M NaAc (pH 5.2), 1ml of ethanol and 10µg of glycogen were added to it. The solution was mixed and place on dry ice for 20 minutes. The solution was then centrifuged for 30 minutes at 13000 rpm at 4ºC. The pellet of DNA was washed with 200µl 75% ethanol and centrifuged for other 10 minutes at 13000rpm. After the pellet was air dried it was resuspended in an appropriate volume of Milli-Q water.

2.2.1.4 Gel extraction

QIAquick gel extraction kit from QIAGEN was used, following the manufacturer's protocol.

2.2.1.5 DNA ligation and cloning

The ligation reaction was prepared as follows:

10X T4 DNA Ligase Buffer	2µl
Vector DNA (3kb)	50ng (0.025pmol)
Insert DNA (1kb)	50ng (0.076pmol)
Nuclease free water	to 20µl
T4 DNA ligase	1µl

The reaction was mixed gently and incubated at room temperature for 10 minutes for cohesive ends, or 2 hours for blunt ends. The reaction was then chilled on ice and 1 to 5µl were used to transform competent cells.

2.2.1.6 Transformation of competent cells

NEB 10-beta Competent *E. coli* (C3019) bacteria were thawed on ice for 10 minutes. 1 to 5µl of plasmid DNA were added to the tube which was placed again on ice for 30 minutes. The cells were heat shocked at 42°C for 30 seconds and put back on ice for 5 more minutes. Then, 950ul of SOC medium were added and the tubes were placed inside a shaker incubator at 37°C for one hour. Several dilutions of the solution containing the bacteria were spread in LB agar plates containing the appropriate antibiotic according to the plasmid incorporated to the bacteria. The plates were incubated overnight at 37°C.

2.2.1.7 Plasmid DNA purification

To purify DNA plasmids from bacteria, QIAprep Spin Miniprep and HiSpeed Plasmid Midi Kits from QIAGEN were used following the manufacturer's protocol.

2.2.1.8 RNA extraction and purification

Between 15 and 30 larvae were treated with 200µl RNAlater (Ambion) and stored at 4°C, or frozen at -80°C until they were processed for RNA extraction. In some cases, 3 dpf larvae were decapitated and only the heads were processed for RNA extraction. RNA later was discarded and 1ml of TRIzol (Invitrogen) was added to the larvae. The tissue was homogenised using a 5ml syringe with a 21 gauge needle (BD biosciences) or by pipetting continuously up and down. After the homogenisation, the samples were incubated at room temperature 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, 200µl of chloroform (Sigma) were added to the homogenates. Samples were vortexed and incubated at room temperature for 3 minutes. After this short incubation, they were centrifuged at 13000 rpm at 4°C for 15 minutes. The colorless aqueous phase was transferred to a clean tube and the RNA precipitated with 500µl isopropyl alcohol at room temperature for 10 minutes. Samples were centrifuged at 13000 rpm at 4°C for 10 minutes; then the supernatant was discarded and the pellets were washed in 75% 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 20µl DEPC water plus DNase I buffer and 1µl DNase I (Invitrogen). The reactions were incubated for 15-30 minutes at 37°C and the RNA was then precipitated with 130µl of DEPC-treated water, 15µl of 3M NaAc (pH5.2) and 375µl of ethanol as explained for the Digoxigenin-labelled RNA probe synthesis protocol. Alternatively, the RNA was purified using the RNAeasy minikit (QIAGEN).

2.2.1.9 First strand cDNA synthesis

cDNA was synthesised from the total RNA extracted from larvae using SuperScript® II First-Strand Synthesis System for RT-PCR kit (Invitrogen).

2.2.1.10 RT-PCR

0.5µl of a cDNA synthesis reaction was added into a 20µl reaction containing:

5X FIREPol Master Mix	4 µl
Forward primer (10µM)	0.6 µl
Reverse primer (10µM)	0.6 µl
Water	14.3 µl

The reactions were prepared in thin-walled 0.2ml tubes and placed in a thermal cycler PTC-100, MJ Research, Inc. The cycling reaction was carried out as follows: 95°C for 5 minutes, then 30 cycles of 95°C for 30 seconds, 54-57°C for 35 seconds and 72°Cfor 1 minute, followed by 72°C for 10 minutes.

2.2.1.11 qPCR

Each qPCR reaction contained:

Sybr green (Sigma)	5 µl
5µM Fwd primer (300ng final concentration)	0.6 µl
5µM Rev primer (300ng final concentration)	0.6 µl
1:10 cDNA dilution (10ng cDNA final concentration)	1 µl
RNase free water	2.8 µl

The reactions were prepared in Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR Plates (Biorad) and the PCR machine used for the analyses was BioRad CFX96 Touch™ Real-Time PCR Detection System, following this protocol: 95°C for 3 minutes, then 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by an increase of 0.5°C every 5 seconds from 65°C to 95°C.

The efficiency of the primers was assayed at 300ng concentration. All samples were normalised to the levels of the housekeeping gene β-actin1.

2.2.1.12 mRNA synthesis for microinjections

The messenger RNA was synthesised using the mMessage mMachine Kit.

The mRNA synthesis reaction was set as follows:

Linearised DNA (1ug/ul)	1 µl
10X Reaction Buffer	2 µl
2X NTP/CAP	10 µl
Nuclease free water	5 µl
SP6 or T7 enzyme	2 µl

The reaction was incubated at 37°C for 2hrs. 1µl of DNase was added to the reaction and it was incubated for 15 minutes more at 37°C. The mRNA was precipitated by adding 115µl DEPC water, 15µl NH_4Ac and 150µl phenol:chloroform:isoamyl alcohol. After vortexing the solution, it was centrifuged at 13000 rpm for 5 minutes. The top layer was collected, 150µl of isopropanol were added to it and the reaction was incubated on dry ice for 30 minutes. Following the incubation period, the solution was centrifuged at 13000 rpm for 30 minutes. The supernatant was discarded and the pellet was washed with 75% ethanol in DEPC water by centrifuging for 10 more minutes. The pellet was left to air dry after removing the supernatant and was resuspended in 20µl of nuclease free water and 1µl was checked on an agarose gel. The concentration of mRNA was determined using a NanoDrop spectrophotometer and the aliquots were stored at -80°C.

2.2.1.13 Digoxigenin-labelled RNA probe synthesis

An in vitro transcription reaction was prepared with 1µg of linearised DNA, 4µl of 5x transcription buffer, 2µl of 10x DIG-labelling mix, 2µl of RNA polymerase, 1µl of recombinant ribonuclease inhibitor (RNaseOUT) and 10µl of DEPC-treated water. The resulting solution was incubated at 37ºC for 2 hours. 1µl of RNase-free DNase I was added to the solution in order to remove the DNA template and the reaction was incubated for 15 more minutes at

37ºC. After the incubation 130µl of DEPC-treated water, 15µl of 3M NaAc (pH5.2) and 375µl of ethanol were added to the final reaction product and incubated overnight at -20ºC. The next day the solution was centrifuged at 13000rpm for 20 minutes at 4ºC. The pellet was then washed with 200µl of 70% ethanol and centrifuged for 10 more minutes. After the pellet was air dried, it was resuspended in 20 µl of DEPC-treated water and 50µl of deionised formamide were added. The probes were stored at -20ºC.

2.2.1.14 Microinjection

Glass capillaries were used to make needles in a P-97 Flaming/Brown micropipette puller (Sutter Instruments). The needles were placed blunt ends down inside the tubes containing the morpholino or mRNA solutions and filled by capillary action. Once the tip of the needle was filled with solution, it was placed in the microinjector. The tip of the needle was broken using forceps until a volume of 2nl of solution came out of the needle when pressing the injector's pedal. Some needles were also backfilled using long fine bore Gilson tips. The volume to be injected was checked using a microscope graticule under a small petri dish with mineral oil, into which drops of Phenol-red containing morpholino/RNA solution were injected, and the drop diameter estimated using the graticule scale.

Embryos were collected and placed in a 1% agarose injection mould. One cell stage embryos were injected with 2nl of solution inside the yolk. Injected embryos were then transferred into Petri dishes containing E3 media and maintained at 28.5ºC. Penicillin and Streptomycin were added to the medium to prevent infections from becoming established in injected embryos.

2.2.2 Pharmacological induction of seizures

Stocks of pentylenetetrazole (200 mM in water), picrotoxin (100 mM in DMSO), kainic acid (10mM in water) and N-methyl-D-aspartate (170mM in water) were diluted to the required concentration in fresh E3 medium. Embryos and larvae were exposed to the drugs then analysed as required.

2.2.2.1 For in situ hybridisation and immunofluorescence analyses

Following treatment with the compound(s), larvae were transferred immediately into fish fix and incubated at 4°C overnight. The next morning the samples were rinsed in PBS two times, washed once in 50:50 PBS/MeOH for 10 to 15 minutes and washed with 100% MeOH for 5 minutes. They were then stored in MeOH at -20ºC until they were analysed.

2.2.2.2 For total RNA extraction

Following treatment with compound(s), larvae were transferred immediately into RNAlater (Ambion) and kept at 4°C until the RNA extraction was done. 3 dpf larvae were transferred into a plate placed on ice, containing cold E3 to immobilise them. When the larvae were completely unresponsive to touch, their heads (down to the end of the hindbrain) were dissected using syringe needles. The heads were then transferred into RNAlater (Ambion) and kept at 4°C until the RNA extraction was performed.

2.2.2.3 For repeated treatments

Embryos were dechorionated at 24 hpf. Some embryos were exposed to compounds for 1 hour placing the plates in the incubator at 28°C during that time; some were kept untreated as controls. After each hour of treatment, the compound-containing media was removed and replaced with fresh E3, this replacement of media was repeated at least 3 times before putting the plates back into the incubator until the following day.

- All procedures involving experimental animals were performed in compliance with local and national animal welfare laws, guidelines and policies, under the authority of Home Office Personal and Project Licences.

2.2.3 Fixation of embryos and larvae

Embryos (1,2 and 3 dpf) were dechorionated with forceps. After they were subjected to the corresponding procedure, they were transferred into an Eppendorf tube and fixed in fish fix overnight at 4°C. The next morning the fixed specimens were rinsed in PBS two times, washed once in 50:50 PBS/MeOH for 10 to 15 minutes and washed with 100% MeOH for 5 minutes. They were then stored in MeOH at -20ºC until they were analysed.

2.2.4 Whole mount in situ hybridisation

2.2.4.1 Single in situ hybridisation

Day 1. Approximately forty embryos or larvae were placed per Eppendorf tube to carry out the in situ hybridisation analysis. They were washed once in 50:50 PBS/MeOH to rehydrate them, then 4 times in PTW (PBS + 0.1% Tween 20). The samples were incubated in 0.01mg/ml Proteinase K in PTW for several minutes according to their stage, to permeabilise all the membranes. Samples were then fixed in 1ml of fish fix at room temperature for 20 minutes. After the fixation step, five washes in PTW were performed. The samples were then rinsed in 250µl of hybridisation solution without the tRNA, before being transferred into 250µl of pre-hybridisation solution containing tRNA. After 3 hours of incubation at 65-70ºC, the pre-hybridisation solution was replaced by 300µl of a 1:200 dilution of DIG-labelled RNA probe in hybridisation solution and incubated at 70ºC overnight.

Day 2. The probe/hybridisation solution was removed and 1ml of 50:50 hybridisation solution:2X SSC was added to wash the samples for 20 minutes at 65ºC. The samples were washed with 2X SSC for 20 minutes, and then two times with 0.2X SSC for one hour each time. After a final wash with 50:50 PBT:0.2X SSC at room temperature for ten minutes, the samples were incubated in PBT for 10 more minutes. Then, they were incubated for 3 hours in PBT at room temperature. After the 3 hour incubation, the PBT was replaced with PBT containing anti-DIG antibody (Roche, 1:2000) and incubated overnight at 4ºC.

Day 3. The samples were washed 6 times with PBT for 20 minutes each time at room temperature. They were washed with staining buffer pH 9.5 four times for 10 minutes each and then transferred into a 12-well plate and stained in the dark with staining buffer containing 3.5µl/ml of BCIP and 4.5µl/ml of NBT (NBT/BCIP Staining solution). Once desirable levels of staining had been achieved, the embryos/larvae were washed with PBS and then fixed in fish fix overnight. The next day the stained samples were put through glycerol series (25%,50%,75% glycerol/water) and stored in 75% glycerol/water at 4ºC.

2.2.4.2 Double in situ hybridisation

The procedure is exactly the same as for a normal whole mount in situ hybridisation during days 1 and 2.

Day 3. After the desired levels of staining were reached with the NBT/BCIP staining solution, the samples were transferred into Eppendorf tubes. The embryos/larvae were then washed 3 times with PBT for 5 minutes, and then they were incubated in 100mM Glycine for 30 minutes. Subsequently, the samples were washed again 3 times in PBT for 5 minutes and left in maleic acid blocking buffer overnight.

Day 4. The samples were incubated with anti-Fluorescein AB (1:2000) for 5 hours at room temperature. The antibody was removed by washing the samples 7 times with PBT for 30 minutes at room temperature. The samples were left overnight at 4°C in PBT.

Day 5. The samples were washed 4 times in staining buffer pH 8.2 for 5 minutes each time and then transferred into a 12-well plate with fast red staining solution. They were stained in the dark and when the staining was sufficiently strong, they were washed in PBT 8 times for 5 minutes and fixed with fish fix overnight at 4°C. The next day the stained samples were put through glycerol series (25%,50%,75% glycerol/water) and stored in 75% glycerol/water at 4ºC.

2.2.5 Immunofluorescence analysis

Day 1. 4 dpf larvae kept in MeOH at -20°C, were rehydrated through a 10 minute wash in 50:50 MeOH:PBS and then washed 3 times for 5 minutes in PTW. The larvae were incubated in PTW containing 0.01mg/ml proteinase K for 45 minutes at room temperature. Following the proteinase K treatment, the samples were washed 3 more times for 5 minutes with PTW. Samples were fixed in fish fix for 20 minutes at room temperature and then incubated in PBS-Block for 3 hours. The larvae were left overnight in PBS-Block containing anti-cfos antibody (1:200, SantaCruz labs) at 4°C and 1:1000 DAPI.

Day 2. The samples were washed for 30 minutes at room temperature with PBS-Block. Then the PBS-Block was replaced with PBS-Block with cy3 antibody.

Day 3. The samples were washed 5 times at room temperature for 30 minutes with PBS-Block and then 5 minutes with PTW.

2.2.6 Apoptosis assay

TUNEL staining was performed using the Apoptag Plus Peroxidase Kit (Millipore).

Day 1. 4 dpf larvae kept in MeOH at -20°C, were rehydrated through a 10 minute wash in 50:50 MeOH:PBS and then washed 4 times in PTW for 5 minutes each wash. The larvae were incubated in PTW containing 0.01mg/ml proteinase K for 45 minutes at room temperature. Following the proteinase K treatment, the samples were washed 3 more times for 5 minutes with PTW. The PTW was then replaced with 50µl of equilibration buffer, and samples were incubated at room temperature for 1 hour. Then, the equilibration buffer was replaced with TdT enzyme diluted in reaction buffer (16µl enzyme in 38µl buffer, per sample). Samples were incubated at 37°C for 90 minutes. 200µl of stop buffer were added and the samples were left at 37°Cfor 3 more hours. The samples were washed 3 times for 5 minutes in PTW and incubated at room temperature for 2 hours in PTW + 10% heat inactivated horse serum. The solution was then replaced with PTW + 10% heat inactivated horse serum containing 1:2000

AP-anti-DIG antibody conjugate (Roche) and the samples were left incubating at 4°C overnight.

Day 2. The samples were washed 8 times with PTW for 15 minutes each time. The larvae were washed 3 times with staining buffer pH 9.5 for 5 minutes each time and then transferred into a 12-well plate. They were stained in the dark with NBT/BCIP Staining solution. After 20 minutes, the larvae were transferred into fish fix and overnight at 4°C. The next day the stained samples were put through glycerol series (25%,50%,75% glycerol/water) and stored in 75% glycerol/water at 4ºC.

2.2.7 Mounting

The total number of stained embryos was scored, as well as the number of embryos presenting the same intensity of staining. Some embryos with the dominant intensity of staining were deyolked and dissected using syringe needles under a compound microscope. One to four square layers of electrical tape were placed on top of a microscope slide and then a well was cut into the middle of the tape into which the specimen was placed. Imaging of the specimens was performed using a Leica DM2500 microscope with DIC (differential interference contrast) optics.

2.2.8 Imaging

2.2.8.1 Light microscopy

A Leica DM2500 microscope with a Leica DFC 420C digital camera was used to capture light microscopy images. Figures were adjusted and arranged using Adobe Photoshop CS5.

2.2.8.2 Confocal microscopy

Live larvae were imaged on the Perkin Elmer UltraVIEW spinning disk confocal microscope. Images were captured, processed and analysed using the Improvision Volocity suite.

2.2.9 Tracking analysis

The locomotor activity of larvae was recorded using the Zebrabox system (Viewpoint, France). AB larvae at 3 or 4 dpf, were transferred to a 48-well plate, one larva per well in 400 to 500µl E3 medium. PTZ, KA, PTX or NMDA were added into the E3 media to get the desired concentration in a final volume of 500µl. Controls containing E3 alone were also included. Right after adding compounds into the E3 media, the plate was placed in the Zebrabox and the locomotor activity was recorded for 1 to 2 hour period, using a 2 minutes:100% light; 2 minutes:0% light. The data was integrated every 10 minutes during the recording time. The experiments were repeated until the number of larvae analysed per treatment was equal or higher than 12.

2.2.10 Two-colour Microarray-Based Gene Expression Analysis

Total RNA was extracted with TRIzol as described before (2.2.1.8).
The Agilent protocol for Two-color Microarray-Based Gene Expression Analysis, Low Input Quick Amp Labeling was followed for sample preparation, hybridisation, microarray wash and scanning and feature extraction.
(http://www.chem.agilent.com/library/usermanuals/Public/G414090050_GeneExpression_TwoColor_6.6.pdf)

Fluorescence signals were transformed to log base 2 to detect changes in gene expression levels. The values of the signals from the PTZ-treated samples values were subtracted from the values of the control samples to identify genes exhibiting fold-changes greater than 1.00.

Chapter 3

3 Characterisation of a pharmacologically-induced zebrafish model of epileptic seizures

3.1 Introduction

Much of the knowledge about the molecular and cellular mechanisms underlying epilepsy and epileptic seizures have been discovered using rodents as animal models of seizure disorders. However, many other experimental animal models have been developed for this purpose, including *Drosophila melanogaster, C. elegans, Dictyostelium discoideum, Xenopus laevis* and zebrafish (reviewed by Fisher, 1989; White, 2002; Cunliffe et al., submitted).

Studies on zebrafish have revealed that many of the same molecular and cellular mechanisms and principles that govern brain development, physiology and neurological disease in mammals are also operational in this organism (Stewart, et al., 2014). Recently, the zebrafish has emerged as a promising model system in which to elucidate the molecular and cellular mechanisms underlying epilepsy (Baraban, et al., 2005; Baraban et al., 2007;Baraban, et al., 2013; Baxendale, et al., 2012; Hortopan, et al., 2010).

In recent years research in our laboratory has focused on developing a simplified, robust assay for seizure induction and resolution in the zebrafish, which can be used for mechanistic analysis of the responses to seizure induction and in medium-throughput chemical screens to identify compounds with anti-convulsant activity (Baxendale et al., 2012).

This chapter describes the results obtained from experiments to characterise and validate a pharmacologically-induced model of epileptic seizures in zebrafish embryos and larvae. As indicated in Chapter 1, two of the primary responses that can be readily measured in a model of epilepsy are the behavioural and transcriptional responses to seizure induction. In this Chapter, I will describe how these parameters were evaluated to characterise the seizures

and convulsions elicited by a range of different seizure-inducing agents in the zebrafish embryo and larva.

3.1.1 Pharmacologically-induced models of epilepsy

Seizure-inducing chemicals have been used extensively to create a variety of different models of epilepsy. Some of the most commonly used drugs for seizure induction in animals and in *in vitro* studies are Pentylenetetrazole (PTZ), Picrotoxin (PTX) and Kainic Acid (KA). The administration of these compounds can give rise to different types of seizures in animal models, because of the distinct mechanisms of action of these drugs at the molecular level.

Pentylenetetrazole and Picrotoxin cause similar seizures when administered to laboratory animals. Both compounds induce a generalised, tonic-clonic type of epileptic seizures in rodents (reviewed by Fisher, 1989). Generalised tonic-clonic seizures are the most severe seizures in patients with epilepsy. Shortly after the seizure has begun, patients lose consciousness and present with a generalised extensor rigidity (tonic phase), which is then followed by jerking of limbs and face (clonic phase) (Reviewed by Fisher, 1989). Both PTZ and PTX are thought to be non-competitive antagonists of the $GABA_A$ receptor, each reducing the GABA-mediated influx of chloride ions into neurons, and thereby preventing neuronal hyperpolarisation (Huang et al., 2001).

Picrotoxin is a plant-derived toxin which binds to the $GABA_A$ receptor on the cytoplasmic face of the central chloride channel (Olsen, 2006; Yoon, et al., 1993). A second binding site for picrotoxin has also been proposed (Yoon et al., 1993), and a recent study using computer modelling suggests that this binding site facilitates allosteric inhibition of $GABA_A$ receptor function by picrotoxin (Carpenter, et al., 2013). In addition to blocking the $GABA_A$ receptor, picrotoxin also blocks other GABA receptors, as well as glycine receptors and glutamate-gated chloride channels (Pribilla, et al., 1992; Wang, et al., 1995). As mentioned above, picrotoxin has been used to induce generalised tonic-clonic seizures in rodents, but it can also be used

to induce simple partial, acute seizures (reviewed by Fisher, 1989). Simple partial, acute seizures occur in patients with intracranial trauma or haematoma, which give rise to localised seizures affecting a very specific part of the body without causing loss of consciousness (Fisher, 1989; Morimoto, et al., 2004). A more detailed description of the different types of seizures can be found in Chapter 1 of this thesis.

The mechanism of action of pentyleneterazole is not very well understood. However, it is probably the most widely and extensively used drug for the induction of seizures in animal models (reviewed by Fisher, 1989). Despite its popularity as a reliable and consistent research tool, its binding site in the $GABA_A$ receptor has not yet been precisely defined. It was believed that PTZ shared the same site as the benzodiazepines (Squires, et al., 1984), but using radioligand binding studies, Ramamjaneyulu and Ticku (1984) suggested that it shared the picrotoxin domain inside the channel pore of the $GABA_A$ receptor. In 2001, a study by Huang and collaborators proposed that picrotoxin and PTZ bind to distinct but overlapping sites on the $GABA_A$ receptor (Huang et al., 2001).

Kainic acid (KA) is an analogue of glutamate that was first extracted from the red alga *Digenea simplex* by Murakami and collaborators in 1953 (cited by Olney, et al., 1974). The CA3 area of the hippocampus contains a particularly high level of the kainic acid subtype of glutamate receptors. Accordingly, KA is especially toxic to hippocampal tissue even when administered at a site distant from the hippocampus (Velísek, et al., 1995; Vincent & Mulle, 2009). The seizures that develop during the first stages of kainic acid-seizure induction are restricted to limbic areas including the hippocampus and the amygdala. During the initial stages of the response to KA, the induced seizures are comparable to the ones found in human temporal lobe epilepsy (Fisher, 1989; Velísek et al., 1995).

3.1.2 Gene expression in response to exposure to chemical convulsants in animal models.

Several studies have demonstrated that seizure induction by PTZ, PTX and KA in different animal models results in the rapid induction of *c-fos* mRNA and protein synthesis in multiple, distinct structures within the brain (reviewed by Herrera & Robertson, 1996; Shin & Ikemoto, 2010). However, the expression of *c-fos* in the brains of animals treated with PTZ and KA does not occur in the same brain structures. When PTZ is administered to rodents, there is a dramatic increase on the levels of c-Fos in the cingulate and piriform cortices as well as in the granule cells of the dentate gyrus (reviewed by Herrera & Robertson, 1996). By contrast, administration of KA induces *c-fos* expression mainly in limbic structures, and has very little impact in the cortex (reviewed by Herrera & Robertson, 1996) .

3.1.3 Zebrafish model of epileptic seizures

Using zebrafish for research purposes has many advantages compared with rodents and other animal models of disease. They are easy to breed and maintain, their sexual maturity is reached at 2-3 months of age and the embryos have translucent bodies, which readily facilitates the the visualization and analysis of many internal organ systems, including the central nervous system. Another benefit of using zebrafish is that drug administration is easier than with other animal models, because zebrafish absorb the drugs from the aqueous medium which they are immersed. This feature makes zebrafish embryos and larvae particularly useful model organisms in which to perform drug screening (Rubinstein, 2003).

Rodent models of epilepsy are the most used because the complexity of their nervous systems is similar to humans. One possible disadvantage of the use of lower organisms as models for complex disorders as epilepsy, is that their nervous systems are simpler compared to the mammalian brain. However, even though zebrafish does not have a central nervous system as complex as mammals, their ‘simple’ brains possess all the necessary elements to generate abnormal electrical discharges, such as excitatory and inhibitory neurons. Brain development

in zebrafish is guided by the same pathways and genes as in mammals, giving rise to different neuronal subtypes like glutamatergic, glycinergic, GABA-ergic, serotonergic and catecholaminergic along with their receptors (Baraban, 2009; Blader & Strähle, 2000).

Baraban and colleagues were the first research group to use zebrafish as a model of epilepsy (Baraban et al., 2005). 7 dpf zebrafish larvae were treated with the $GABA_A$ receptor antagonist PTZ and induction of seizures was monitored in three distinct ways: by recording swimming behaviour, monitoring transcription of the immediate early gene *c-fos*, and measuring epileptiform-like discharges using electrophysiological tools. Three stages of seizure-induced altered swimming behaviour were described. During the first stage, swim activity increased. Then, at the second stage the larvae swam in circles ('whirpool-like circling swimming behaviour'). The final stage was characterised by clonus-like convulsions until the fish fell to one side and did not move for several seconds. In the elecrophysiological tests both ictal (electrical discharges during seizure) and interictal-like bursts of electrical discharges were observed. In addition, expression of *c-fos* was increased in the brains of fish treated with 15mM of PTZ as compared with the untreated ones. Taken together, these results demonstrated that the zebrafish larva could be used as a model organism for the study of epileptic seizures.

Previous studies in our lab demonstrated that *c-fos* expression was induced in the forebrain and muscle of 2 day zebrafish embryos treated with a concentration of PTZ that was suficient to induce motor convulsions (Baxendale, et al., 2012). In this Chapter, the expression of *c-fos* is analysed in 2, 3 and 4dpf zebrafish larvae to investigate the pattern of *c-fos* expression in response to PTZ treatment more closely. The expression of *c-fos* was also studied in larvae treated with picrotoxin and kainic acid. In addition, the motor behavioural responses of zebrafish larvae exposed to PTZ, PTX, KA and NMDA were investigated.

3.2 Results

As mentioned in the introduction of this chapter, one of the most widely used drugs to induce seizures in animal models of epilepsy is the GABA pathway inhibitor pentylenetetrazole (PTZ). Although PTZ was previously validated as a convulsant agent in zebrafish (Baraban et al., 2005), the larvae used in those earlier studies were older than 5 days post-fertilisation (dpf), when the onset of independent feeding occurs and individual wild-type larvae thus become protected under the Animals (Scientifiic Procedures) Act. In order to optimise application of the principles of the 3Rs in our research, we sought to develop a seizure model that could employ zebrafish embryos and larvae at developmental stages prior to reaching the point of protection at 5 dpf. Since PTZ had already been validated as a useful inducer of seizures in zebrafish, we first sought to determine when and where genes encoding components of the GABA pathway are expressed during early embryonic development. Accordingly, the expression patterns of genes encoding subunits of the $GABA_A$ receptor were determined.

3.2.1 Analysis of the expression of $GABA_A$ receptor subunits α1 and γ2 during early stages of development of zebrafish embryos.

An *in situ* hybridisation analysis of the expression patterns of two subunits of the $GABA_A$ receptor was performed in 24, 48 and 72 hpf (hours post fertilisation) zebrafish embryos. (This work was performed collaboratively with a BMedSci student, James Fox). In order to carry out these experiments, cDNA clones encoding full length sequences of the zebrafish $GABA_A$ receptor subunits α1 (*gabra1*) and γ2 (*gabrg2*) were identified in the EMBL/NCBI DNA sequence database. Mutations in the human orthologues of each of these genes have previosuly been identified in patients with different types of epilepsy (reviewed by Galanopoulou, 2010).

Figure 3.1 The subunits α1 and γ2 of the $GABA_A$ receptor are expressed in the developing brain of zebrafish embryos and larvae. Expression of *gabra1* (left side images) and *gabrg2* (right side images), detected by *in situ* hybridisation analysis, in the developing brain of 24, 48 and 72 hpf zebrafish embryos and larvae.

The *in situ* hybridisation analysis showed that both subunits are expressed in the developing central nervous system of zebrafish embryos from very early stages of development (Figure 3.1). Moreover, the patterns of expression of both *gabra1* and *gabrg2* in the embryonic brain are very similar (Figure 3.2), consistent with their known roles as subunits of the $GABA_A$ receptor, although *gabra1* is expressed more strongly in the telencephalon than is *gabrg2*.

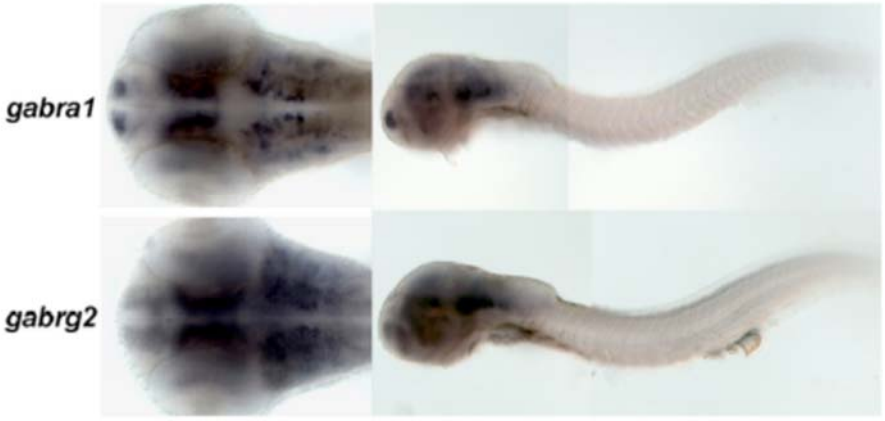


Figure 3.2 *gabra1* and *gabrg2* are expressed in similar domains in the brains of 50 hpf zebrafish embryos (taken from Baxendale, et al., 2012).

These results indicated that components of the likely target for PTZ, the $GABA_A$ receptor, were expressed in the zebrafish embryonic and larval brain at stages prior to the onset of independent feeding, suggesting that PTZ might be might be capable of inducing seizures in embryonic and early larval stages by targeting the $GABA_A$ receptor.

In order to identify a concentration of pentylenetetrazole that could reliably induce measurable, seizure-provoked locomotor convulsions, a dose-response analysis was performed by measuring the distance swam within a 10 minute period by 4 dpf zebrafish larvae, after exposure to a range of different concentrations of PTZ (Baxendale, et al., 2012). This analysis was performed by placing a 48-well plate containing one larva per well, into the Viewpoint Zebrabox system, where the movements of the larvae were recorded (See Materials and Methods for detailed technical description).

3.2.2 Dose-response analysis of PTZ-induced locomotor convulsive behaviour in 3 day-old zebrafish larvae.

In order to combine in situ hybridisation and behavioural analyses in the same early stages of larval development, 3 dpf zebrafish larvae (in which gene expression patterns can be

conveniently imaged) were subjected to locomotor behavioural analysis similar to that previously performed for 4 dpf larvae. With 3 dpf larvae, however, locomotor responses were recorded over a period of treatment lasting one hour, rather than 10 minutes, and only 3 different concentrations of PTZ were tested. Three day old zebrafish larvae treated with 20mM PTZ exhibited greater locomotor convulsive behaviour than did larvae treated with 5mM or 50mM PTZ (Figure 3.3). These data are consistent with previous results obtained with 4 dpf zebrafish larvae (Baxendale, et al., 2012), where 20mM PTZ was also the optimum concentration for inducing a statistically significant convulsive locomotor response.

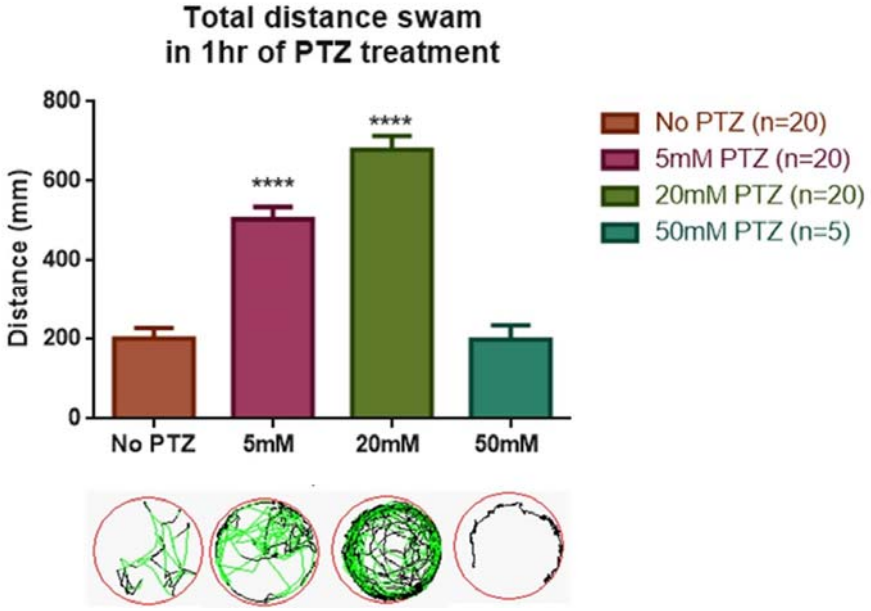


Figure 3.3 Treatment of 3 dpf zebrafish larvae with 20mM pentylenetetrazole induces strong, measurable convulsive movements. 3 dpf zebrafish larvae were treated with 0, 5, 20 or 50 mM PTZ in E3 medium and convulsive locomotor swimming behaviour was recorded from the moment the drug was added to the medium until 60 minutes after the start of treatment. Larvae were analysed using a protocol of 2min-light 100% / 2min-light 0%. Values are given as means, error bars indicate s.e.m. **** indicates a significantly different effect ($P<0.001$) between treated and untreated embryos, using one-way ANOVA with Dunnett's post-test. Representative traces of movements after exposure to each PTZ concentration lie beneath each corresponding value for PTZ concentration in the histogram.

Previous studies indicated that a light protocol of 2min-light 100% / 2min-light 0% in the Viewpoint Zebrabox induced the strongest locomotor response to PTZ treatment as compared to background locomotor activity in 4 dpf larvae (Baxendale et al., 2012). To determine whether this was also the case for 3 dpf larvae treated with PTZ, and also to explore whether background locomotor activity of untreated larvae varied according to the light protocol used, locomotor activity of 3 and 4 dpf larvae was assessed under different light protocols. Accordingly, zebrafish larvae aged 3 and 4 dpf were treated with 20mM PTZ and locomotor behaviour was recorded for 60 minutes under 4 different light protocols: (a) light on (100%) continuously for 60 minutes, (b) light off (0%) continuously for 60 minutes, (c) light 100% for 30 seconds / light 0% 30 seconds over a total of 60 minutes, (d) light 100% for 2 minutes / light 0% 2 minutes over a total of 60 minutes. Interestingly, treatment with 20mM PTZ caused a significant increase in locomotor behaviour with all 4 light protocols. However, robust basal locomotor activity of untreated larvae was most consistently detectable in both 3 and 4 dpf larvae exposed to a light protocol of 2 minutes lights 100% / 2 minutes lights 0% over a total of 60 minutes, indicating that these conditions might be particularly suitable for detecting sedative activities of any anticonvulsant drugs in both 3 dpf and 4dpf larvae (Baxendale et al., 2012). For example, a screened drug can reduce seizure severity, but if the basal activity is also reduced this means that the drug is having a sedative effect and it is not only working as an antiepileptic drug. Since a statistically significant difference in locomotor activity was also detectable between PTZ-treated and untreated larvae under this protocol, in both 3 and 4 dpf larvae, with a P value of P=0.0009, a decision was taken to adopt this protocol as standard for future analysis of convulsive locomotor behaviour.

A video showing the convulsive response to PTZ of 4 dpf zebrafish larvae can be found here:

Movie 1

https://drive.google.com/file/d/0B3wwY1F5s-LKMk9UZ0s5SWpjMm8/edit?usp=sharing

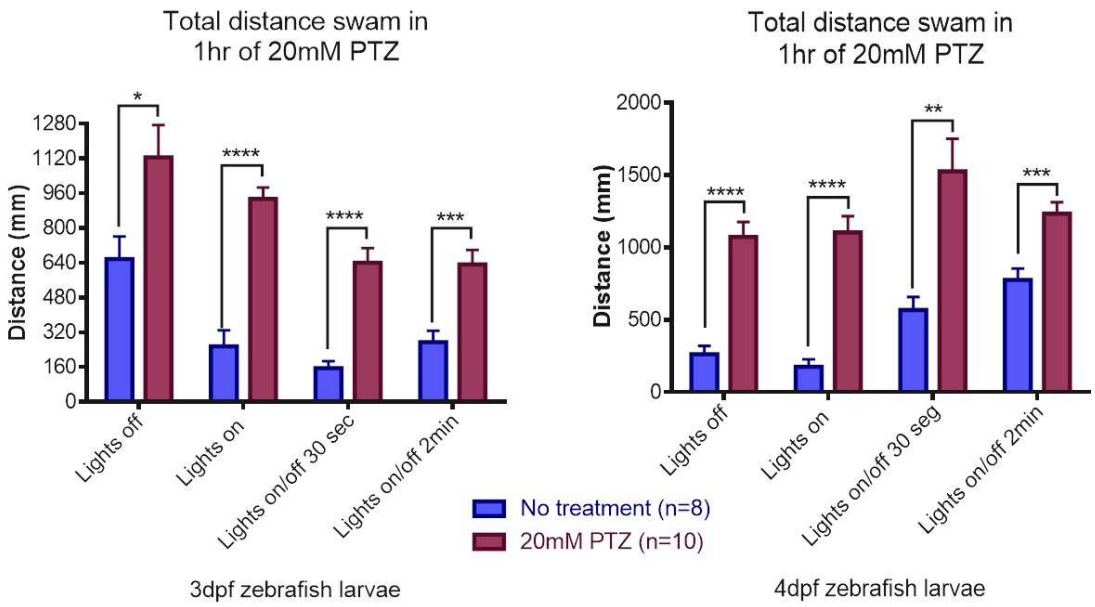


Figure 3.4 Comparison of different light-driving protocols used while recording the locomotor behaviour of 3 and 4 dpf zebrafish larvae during treatment with 20mM PTZ. The graph on the left shows the total distance moved by 3 dpf zebrafish larvae comparing untreated and PTZ treated (20mM) larvae over a monitoring period of 60 minutes, using 4 different light protocols in the Viewpoint zebrabox. The panel on the right shows the results of similar protocols obtained using 4 dpf zebrafish larvae. Values are given as means, error bars indicate s.e.m. **** indicates significantly different (P<0.0001), and *** indicates significantly different (P=0.0009) locomotor behaviour in PTZ-treated and untreated zebrafish larvae subjected to the same conditions of light exposure, using an unpaired t-test.

Although this locomotor assay is robust and reliable when using PTZ, it was decided to test if it could be used to measure convulsions induced by picrotoxin, KA (kainic acid) and NMDA (N-methyl-D-aspartate), which are drugs also known to elicit seizures in other animals.

3.2.3 Multiple convulsants used in rodent models of epilepsy cause convulsive locomotor behaviour in zebrafish larvae.

Seizures can be induced experimentally by either attenuating inhibitory neurotransmission or increasing excitatory neurotransmission. Since both PTZ and picrotoxin are thought to act by

blocking the function of inhibitory neurotransmitter GABA, it was tested whether picrotoxin had a similar effect on zebrafish larvae to that of PTZ. Two analogues of the excitatory neurotransmitter glutamate, Kainic acid and NMDA, were also tested to find out if they could induce seizures in zebrafish larvae.

Three day old zebrafish larvae were treated with PTZ, picrotoxin, KA or NMDA and their locomotor response was recorded using the Viewpoint system over a 2 hour period, following the 2 minutes lights on / 2 minutes lights off protocol described before. The concentrations used for picrotoxin, KA and NMDA were based on concentrations used in other studies on zebrafish larvae (Kim et al., 2010; McDearmid & Drapeau, 2006) , and a dose-response assay was carried out for NMDA, centring on these typical concentrations (Figure 3.5). The NMDA concentraction that induced stronger seizures was 170μM (Figure 3.5).

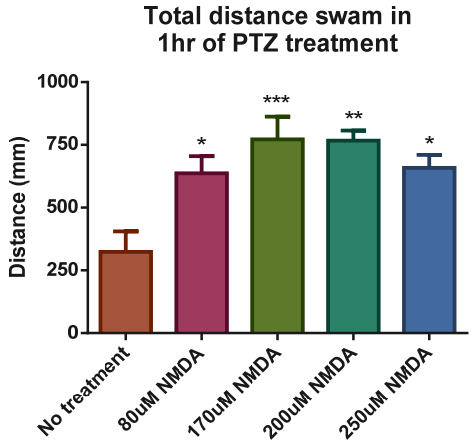


Figure 3.5 Dose response to NMDA treatment of 3 dpf zebrafish larvae. 3 dpf zebrafish larvae were treated with 0, 80, 170, 200 and 250μM NMDA in E3 medium and convulsive locomotor swimming behaviour was recorded from the moment the drug was added to the medium until 60 minutes after the start of treatment. Larvae were analysed using a protocol of 2min-light 100% / 2min-light 0%. Values are given as means, error bars indicate s.e.m. **** indicates a significantly different effect (P<0.001) between treated and untreated embryos, using one-way ANOVA with Dunnett's post-test. (0,80,170μM n=11; 200,250μM n=5)

All of the drugs used in this test elicited a significant difference in the level of convulsive locomotor behaviour (distance swam) exhibited by 3 dpf zebrafish larvae, as compared to untreated larvae after one and two hours of treatment (figure 3.6 A and B). However, the

swimming behaviours of the larvae treated with different convulsants follow distinct patterns. The traces recorded with the Viewpoint system show that PTZ and picrotoxin exhibited a greater tendency to induce whirlpool-like swimming patterns, where larvae swim in circles close to the borders of the wells, whereas KA and NMDA cause an increased swimming activity across the whole area of the wells without whirlpool-like behaviour (figure 3.6 C), potentially suggesting two distinct mechanisms of action of these drugs. Interestingly, picrotoxin induced much stronger convulsive motor response than did PTZ at a 100-fold lower concentration, suggesting it is a much more potent inhibitor of the $GABA_A$ receptor than PTZ.

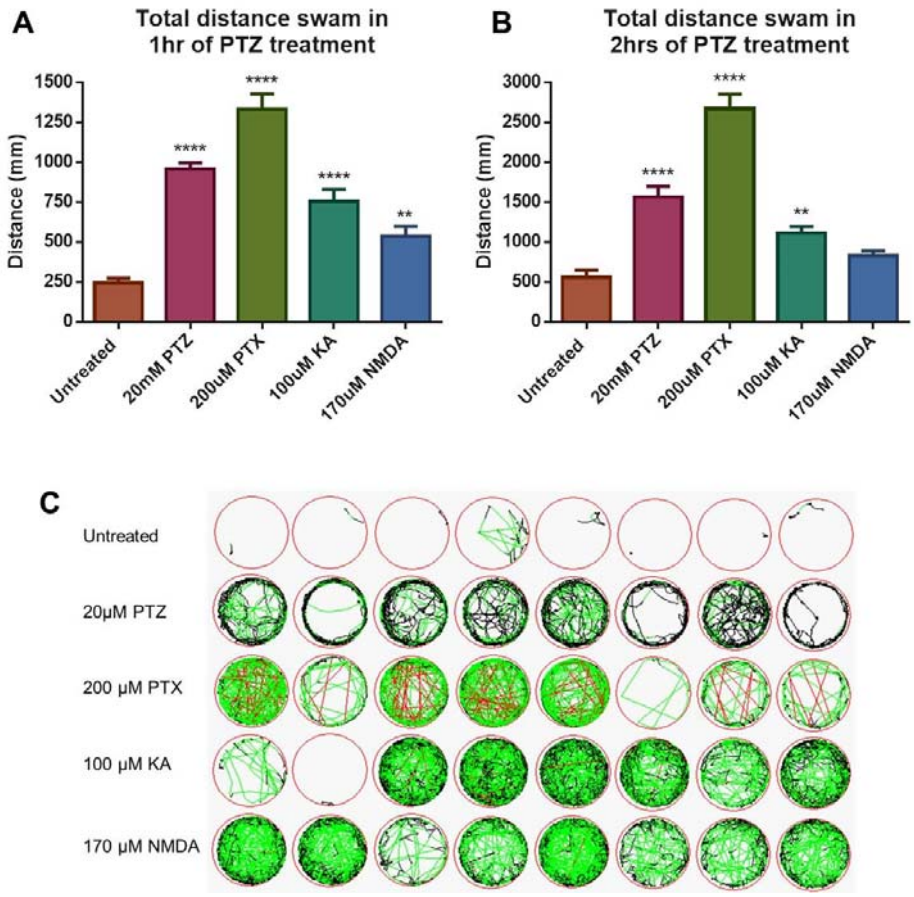


Figure 3.6 Analysis of the convulsive response caused on 3 dpf zebrafish larvae by different convulsant drugs. A) Total distance swam by 3 dpf zebrafish larvae subjected to treatment with PTZ, PTX, KA or NMDA for 1 hour. B) Total distance swam by 3 dpf treated zebrafish larvae after 2 hours of exposure to the drugs. Values are given as means (n=17), error bars indicate s.e.m. **** Significantly different (P<0.001) from untreated embryos, using one-way ANOVA with Dunnett's post-test. C) Example of the traces recorded by the Viewpoint software during 10 minutes of treatment (from minute 10 to 20 of treatment).

The locomotor assay proved to be a robust and reliable technique to detect the convulsive responses caused by PTZ and other convulsant drugs, and indicated that inhibition of GABA pathway function (by PTZ or picrotoxin treatment) could be a more potent mode of convulsion-induction than increased activity of excitatory neurotransmission (by KA or NMDA treatment). However, I also wanted to investigate how PTZ was affecting the function of the nervous system in PTZ-treated zebrafish larvae more directly, using molecular genetic approaches.

The expression of the gene *c-fos* is commonly used as a marker of neuronal activity in epilepsy studies. Hence, the expression of *c-fos* in zebrafish larvae exposed to 20mM PTZ treatment was mapped using *in situ* hybridisation analyses.

3.2.4 Characterisation of PTZ-induced *c-fos* expression in zebrafish larvae.

The expression of *c-fos* was previously analysed in 2 dpf zebrafish embryos treated with 20mM PTZ by colleagues in our laboratory, which showed that *c-fos* is robustly expressed in the developing brain and in trunk muscle after one hour of PTZ treatment (Baxendale, et al., 2012). In the forebrain, *c-fos* is specifically expressed in neurones lying within the ventral telencephalon (subpallium) and ventral diencephalon (preoptic area and hypothalamus). In the hindbrain, *c-fos*-expressing neurones are present in most rhombomeres, but there is a prominent cluster in each half of rhombomere 2, which on the basis of its position may be the locus coeruleus (Figure 3.7).

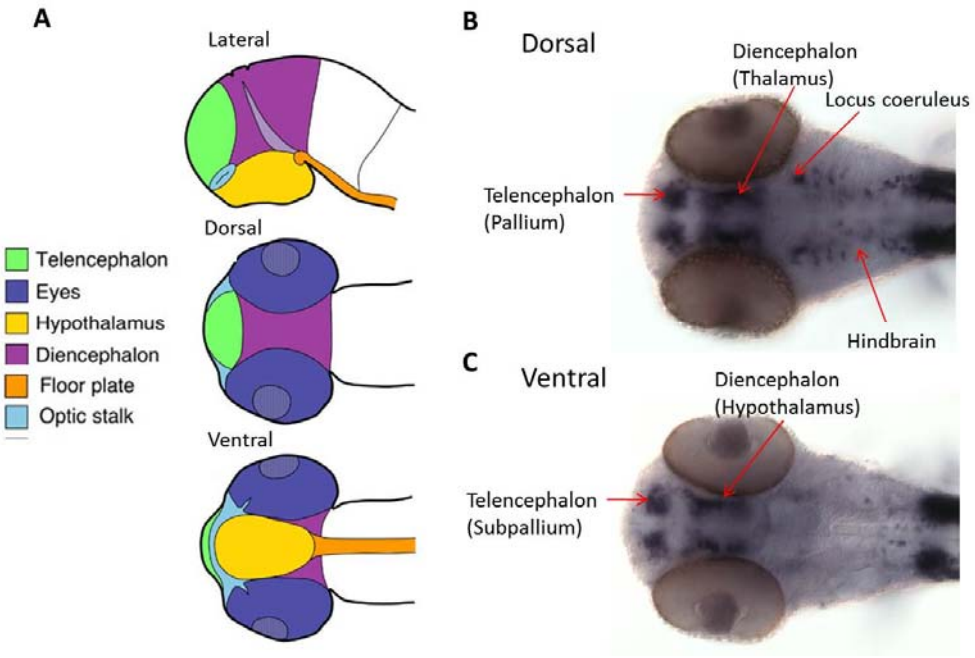


Figure 3.7 Structures of the brain of 2 dpf zebrafish embryos expressing *c-fos* in response to 20mM PTZ treatment. A) Organisation of brain territories in zebrafish (taken from Cavodeassi, et al., 2009). B) Dorsal and C) Ventral views of c-fos expression in the brain after 1 hour of 20mM PTZ treatment of 2 dpf wild-type zebrafish embryos.

A more detailed analysis was done in 3 dpf zebrafish larvae exposed to 20mM PTZ. After 60 minutes of exposure to PTZ, *c-fos* is largely expressed in the brain and in the spinal cord of 3 dpf zebrafish larvae (Figure 3.8A). The expression of *c-fos* increases gradually over the course of time that the larvae are left in PTZ treatment, reaching a peak of expression after 60 minutes of exposure. If the larvae are kept for longer in PTZ, then the expression of *c-fos* starts to decrease in the brain but increases in the muscle (Figure 3.8B).

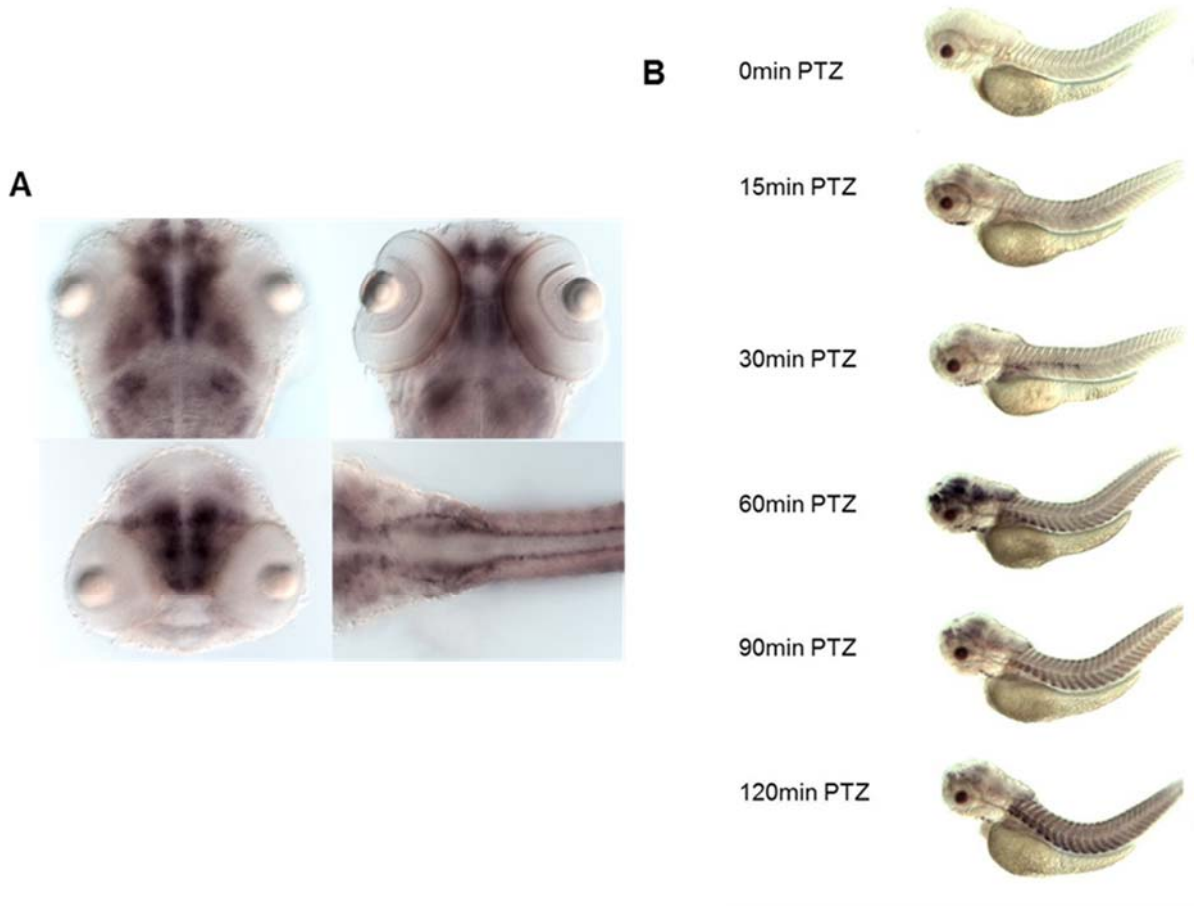


Figure 3.8 In response to PTZ, *c-fos* is expressed in the brain, in the spinal cord and in the trunk muscles of 3 dpf zebrafish larvae. A) After 60min of exposure to 20mM PTZ, *c-fos* is broadly expressed in the brain and the spinal cord of 3 dpf larvae. B) The expression of *c-fos* induced by PTZ, starts in the brain of 3 dpf zebrafish larvae after 30 to 60 minutes of exposure, and it is followed by its expression in the trunk muscles by 2 hours of treatment.

To analyse the expression of c-Fos protein, 4 dpf zebrafish larvae were treated with 20mM PTZ for 90 minutes to give time to the messenger RNA to be translated into protein. Then, the larvae were fixed and processed for immunostaining with an antibody against c-Fos (anti- c-Fos K25, Santa Cruz Biotechnology Inc). This antibody was previously used by Okuyama and collaborators in 2011 in medaka fish. c-Fos immunostaining of 4 dpf zebrafish larvae showed that c-Fos protein is expressed in some neurons of the hindbrain after 90 minutes of 20mM PTZ treatment (Figure 3.9).

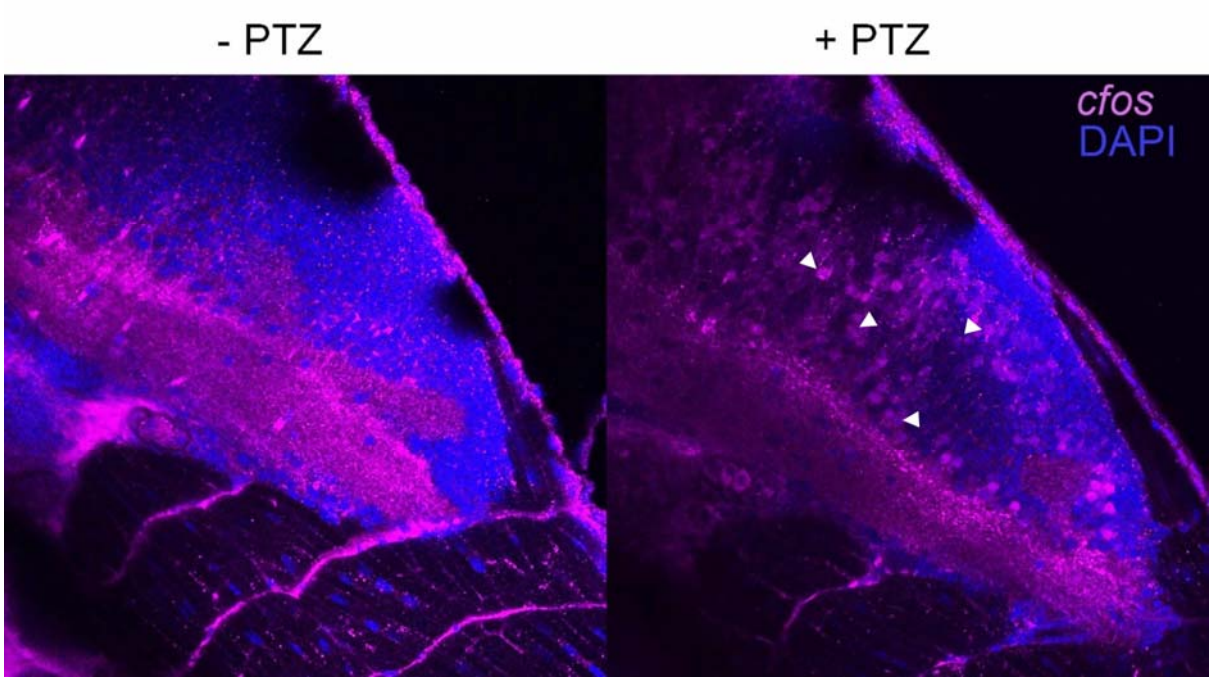


Figure 3.9 c-Fos protein can be detected in the nuclei of hindbrain neurons of 4 dpf zebrafish larvae after 90min of exposure to 20mM PTZ. There is no detectable c-Fos (purple) expression in the nuclei of neurons in the absence of PTZ treatment (left panel). However, after 90min of exposure to 20mM PTZ, c-Fos protein (purple) can be observed in the nuclei of the neurons in the hindbrain of 4 dpf zebrafish larvae (right panel). White arrowheads point to examples where c-Fos is expressed in the nucleus of the neuron.

As observed by *in situ* hybridisation and antibody staining, *c-fos* is expressed broadly in the brain of zebrafish larvae exposed to PTZ. In order to detect what types of neurons express *c-fos* in response to PTZ, double *in situ* hybridisation analyses were performed on 3 dpf zebrafish larvae, treated with 20mM PTZ for one hour. The genes analysed along with *c-fos* were the neuronal markers: tyrosine hydroxylase (*th*), which is a marker of ventral forebrain dopaminergic neurons, tryptophan hydroxylase 2 (*tph2*) which is a marker of serotonergic neurons in the brain, peptide YY (*pyy*) and corticotropin-releasing hormone (*crh*), which are important components of the endocrine response to neural stresses such as those caused by seizure, and which exhibit restricted expression in the ventral diencephalon.

The results of these analyses showed that the PTZ-induced *c-fos* expression domain encompasses a large region within the brain that might include neurons expressing *th* and *crh*

(Figure 3.10). In contrast, *tph2*-expressing neurons did not show a clear colocalisation with *c-fos*-expressing neurons (Figure 3.11B). In the case of *pyya*, its expression colocalises with *c-fos*-expression in some neurons of the hindbrain and spinal cord, but the *pyya*-expressing neurons of the forebrain did not appear to express *c-fos* (Figure 3.11A).

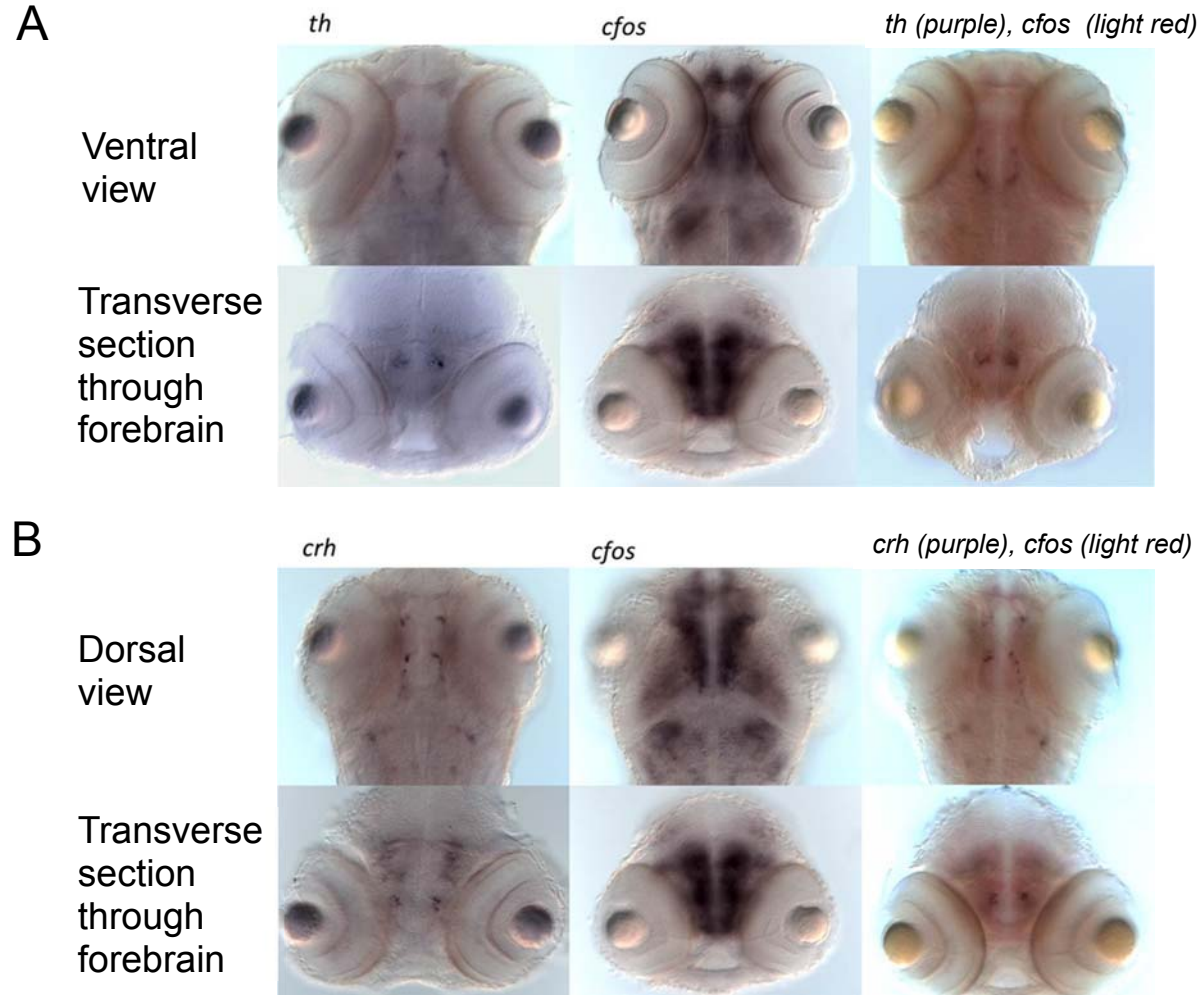


Figure 3.10 Double in situ hybridisation analysis showing colocalisation of the expression of *c-fos* and the neuronal markers *th* and *crh* in 3 dpf zebrafish larvae treated with 20mM PTZ for one hour. A) Ventral view of the head and transverse section through the forebrain showing the expression patterns of *th* and *c-fos* in 3 dpf zebrafish larvae analysed by *in situ* hybridisation. The last column shows that some *c-fos* expressing neurons might also express *th* (*c-fos* in light red, *th* in purple). B) Dorsal view of the head and transverse section through the forebrain showing the expression patterns of *crh* and *c-fos* in 3 dpf zebrafish larvae analysed by *in situ* hybridisation. The last column shows that some *c-fos* expressing neurons might also express *crh*. (*c-fos* in light red, *crh* in purple).

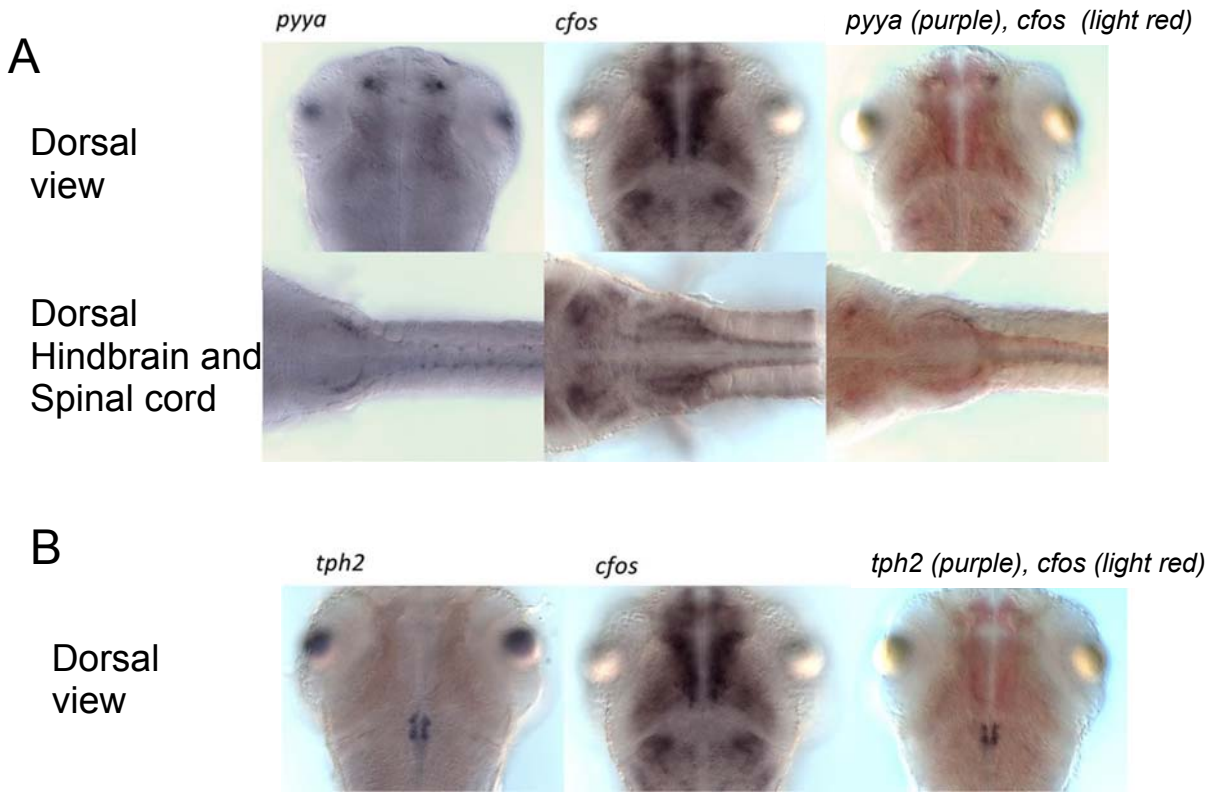


Figure 3.11 Double in situ hybridisation analysis of the expression of *c-fos* along the neuronal markers *pyya* and *tph2* in 3 dpf zebrafish larvae treated with 20mM PTZ for one hour. A) Dorsal view of the head and the hindbrain showing the expression patterns of *pyya* and *c-fos* in 3 dpf zebrafish larvae analysed by in situ hybridisation. The double *in situ* hybridisation staining shows that *pyya* expressing neurons of the midbrain, hindbrain and spinal cord might also express *c-fos*, whereas the neurons of the forebrain that express *pyya* do not show expression of *c-fos* (*c-fos* in light red, *pyya* in purple). B) Dorsal view of the head of 3 dpf zebrafish larvae showing the expression patterns of *tph2* and *c-fos* in analysed by *in situ* hybridisation. The double in situ hybridisation analysis shows that the expression of c-fos and tph2 do not occur in the same neurons (*c-fos* in light red, *tph2* in purple).

Since *crh*, *th* and *pyya* expressing neurons also expressed *c-fos* when exposed to PTZ, the effect of PTZ on the expression of these neuronal markers was analysed by in situ hybridisation. The levels and the pattern of expression of *th* detected by in situ hybridisation did not seem altered by PTZ treatment in 3 dpf zebrafish larvae (Figure 3.12 A). In larvae treated with PTZ, *crh* showed a subtle change in the pattern of expression (Figure 3.12 A, white arrow); whereas *tph2* showed an increased level of expression in response to PTZ (Figure 3.12 A, yellow arrows), even though *tph2* expressing neurons do not express *c-fos*

after PTZ treatment (Figure 3.11 B). The expression of *pyya* was analysed in 50 hpf zebrafish larvae because at this stage there is a more distinctive pattern of expression of *pyya* than at 3 dpf. Figure 3.12 B shows that the expression of *pyya* is increased in response to 20mM PTZ in the hindbrain (B1) and the spinal cord (B2 and B3) of 50 hpf zebrafish larvae.

These results suggest that PTZ is also modifying some neuronal markers in the developing zebrafish brain, especially the ones related to the stress response (*crh* and *pyya*). The importance of these findings is discussed in Chapter 5 where the relationship between stress and seizures is considered in more detail.

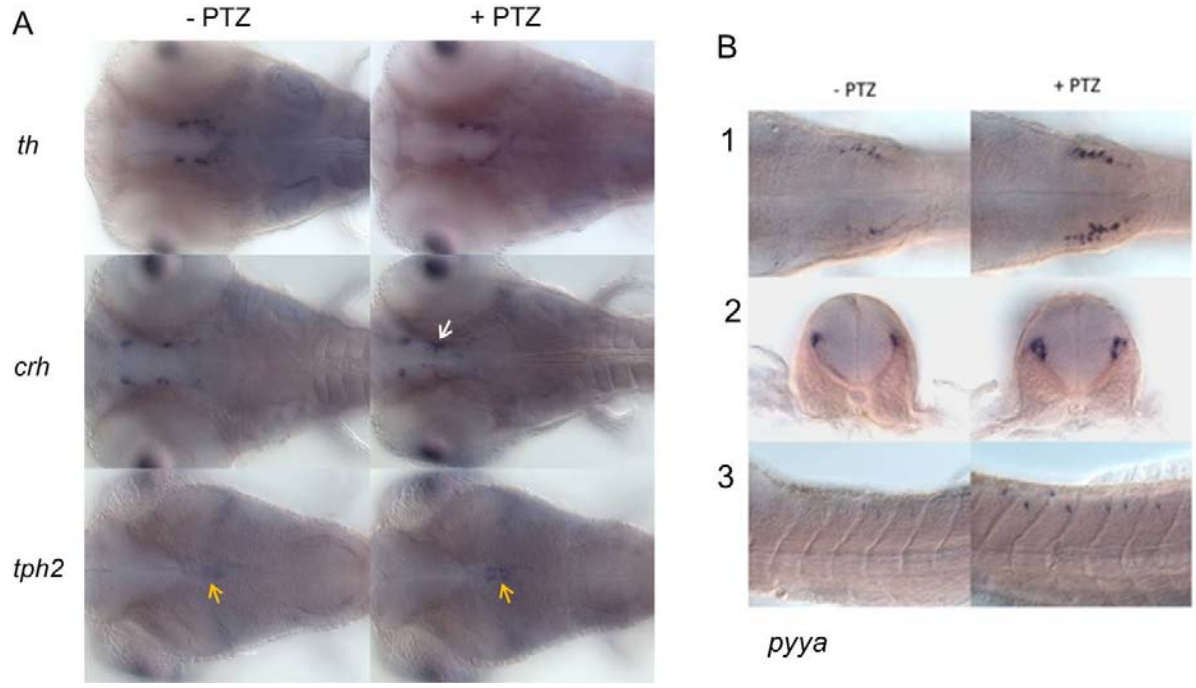


Figure 3.12 Expression of the neuronal markers *th*, *crh*, *tph2* and *pyya* in response to 20mM PTZ treatment. A) Dorsal views of the heads of 3 dpf zebrafish larvae showing the expression of *th*, *crh* and *tph2* in PTZ-treated and untreated larvae analysed by *in situ* hybridisation. White arrow shows a different pattern of expression of *crh* in PTZ treated larvae compared to the untreated ones. Yellow arrows show the expression domain of *tph2,* which is slightly enhanced by PTZ treatment. B) Expression of *pyya* analysed by *in situ* hybridisation in 50 hpf zebrafish larvae treated for 60 minutes with 20mM PTZ. 1 – dorsal view of the hindbrain; 2 (transverse section) and 3 (lateral view) –spinal cord.

3.2.5 Treatment with PTZ induces an ordered sequence of gene expression in 3 dpf and 4 dpf zebrafish larvae.

c-fos is one of a large, growing cohort of synaptic activity-regulated mammalian genes, and it therefore was of interest to explore whether zebrafish orthologues of other synaptic activity-regulated genes were simliarly induced by PTZ treatment. Two previously well-characterised genes are *Bdnf* and *Npas4*. The gene *Bdnf* (brain-derived neurotrophic factor) has previously been investigated in other models of epilepsy, which revealed that it is induced by seizures (reviewed by Koyama & Ikegaya, 2005) and plays important roles in neuronal growth, survival and synaptogenesis (reviewed by Flavell & Greenberg, 2008). A previous study of *Npas4* (*neuronal PAS domain protein 4*) demonstrated that *Npas4* is induced in the prefrontal cortex of wild type mice after 2 hours of PTZ (60mg/kg) treatment (Lin et al., 2008). Moreover, *Npas4* regulates the expression of genes involved in the formation and maintenance of inhibitory synapses, and its expression was previously shown to be induced in cultured neurons by membrane depolarization (Lin et al., 2008).

The expression patterns of *bdnf* and *npas4a* were analysed by *in situ* hybridisation of 3 dpf zebrafish larvae treated with 20mM PTZ for a period of 1 hour and control, untreated sibling larvae. Figure 3.11 shows that there is a very strong induction of *npas4a* expression after 60 minutes of PTZ treatment. The expression of *bdnf* is also increased in PTZ treated larvae after 60 minutes of exposure to PTZ, although the level of induction is not as great as that of *npas4a* (Figure 3.13).

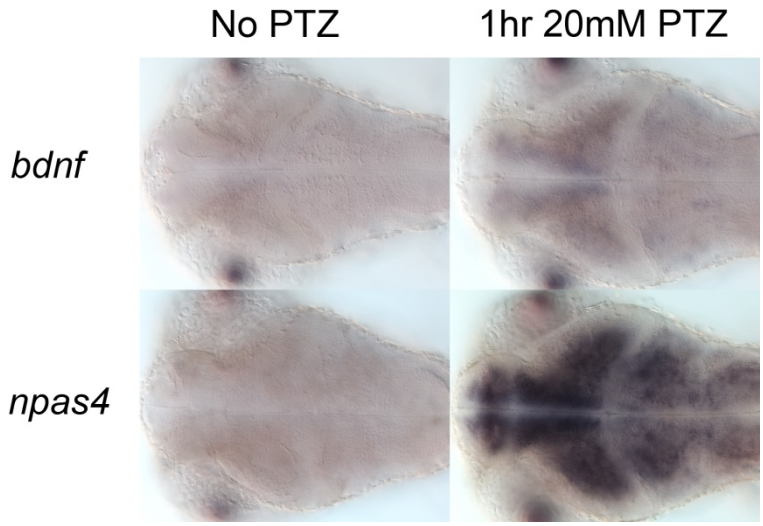


Figure 3.13 The transcription of *bdnf* and *npas4a* is induced after 1 hour of exposure to 20mM PTZ. Increased levels of expression of *bdnf* and *npas4a* can be detected by *in situ* hybridisation in 3 dpf zebrafish larvae after 1 hour of 20mM PTZ treatment.

To further understand the dynamics of *c-fos*, *npas4a* and *bdnf* transcription in response to seizure onset by PTZ, 3 dpf zebrafish larvae were treated for differents periods of time with 20mM PTZ, after which they were fixed and analysed by in situ hybridisation. After only 15min of exposure to PTZ, 3dpf larvae start to express *npas4* in the brain. In contrast, expression of *c-fos* was only detectable after 1hr, and robust expression of *bdnf* required 90 minutes of exposure to PTZ (although a weak signal was detectable after 60 minutes, in agreement with the results in Figure 3.13). The temporal differences in expression of these three genes, and the fact that the most rapidly activated gene *npas4a* encodes a transcription factor, suggested the possibility that Npas4a might be regulating transcription of a set of downstream genes in response to PTZ-mediated inhibition of GABA signalling, that could include the two other genes (Figure 3.14).

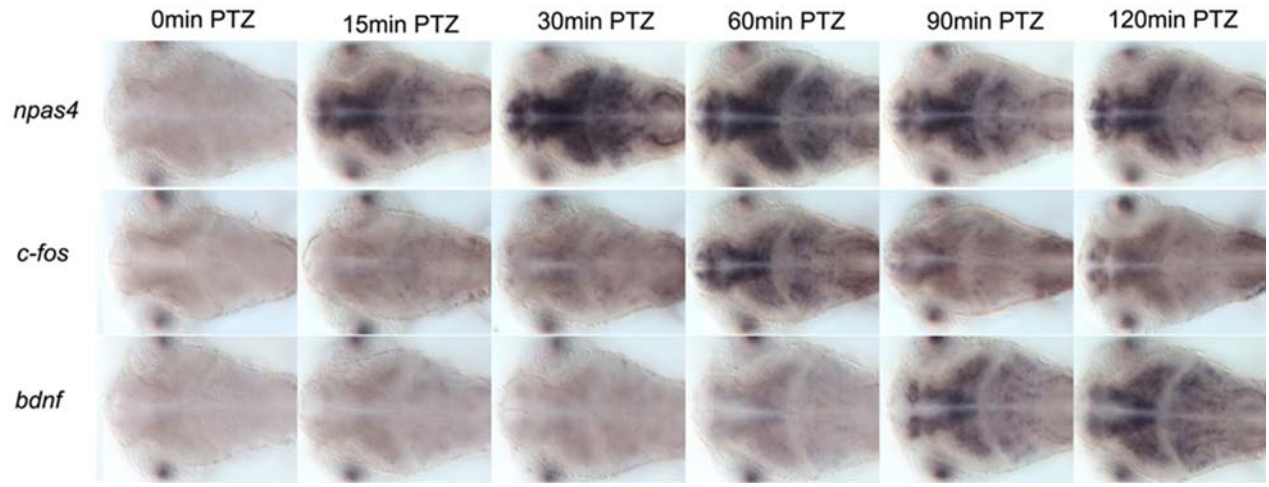


Figure 3.14 Distinct temporal regulation of *npas4a, c-fos* and *bdnf* transcription by the PTZ in 3 dpf zebrafish larvae. *Npas4a* transcripts are synthesised rapidly throughout the brain of 3 day old zebrafish larvae within 15 minutes of exposure to PTZ, whereas *c-fos* and *bdnf* transcripts accumulate to a comparable level within 60 minutes and 90 minutes of exposure to 20mM PTZ, respectively.

Interestingly, if larvae are first treated with PTZ for one hour, then transferred to fresh medium without convulsant, expression of *npas4a* persisted for 30 minutes whereas expression of *c-fos* did not. This suggests that *cfos* may be activated in an Npas4a-independent manner, or alternatively that a high level of *npas4a* expression is required to activate *c-fos*, whose level of expression dropped within 30 minutes after PTZ removal. Furthermore, expression of *bdnf* was induced after PTZ was withdrawn, implying that induction of *bdnf* is a secondary effect of PTZ treatment, and that the initial consequences of PTZ exposure, such as the rapid transcriptional activation of *npas4a*, may be sufficient to induce transcription of genes that are expressed later, such as *bdnf* (Figure 3.15).

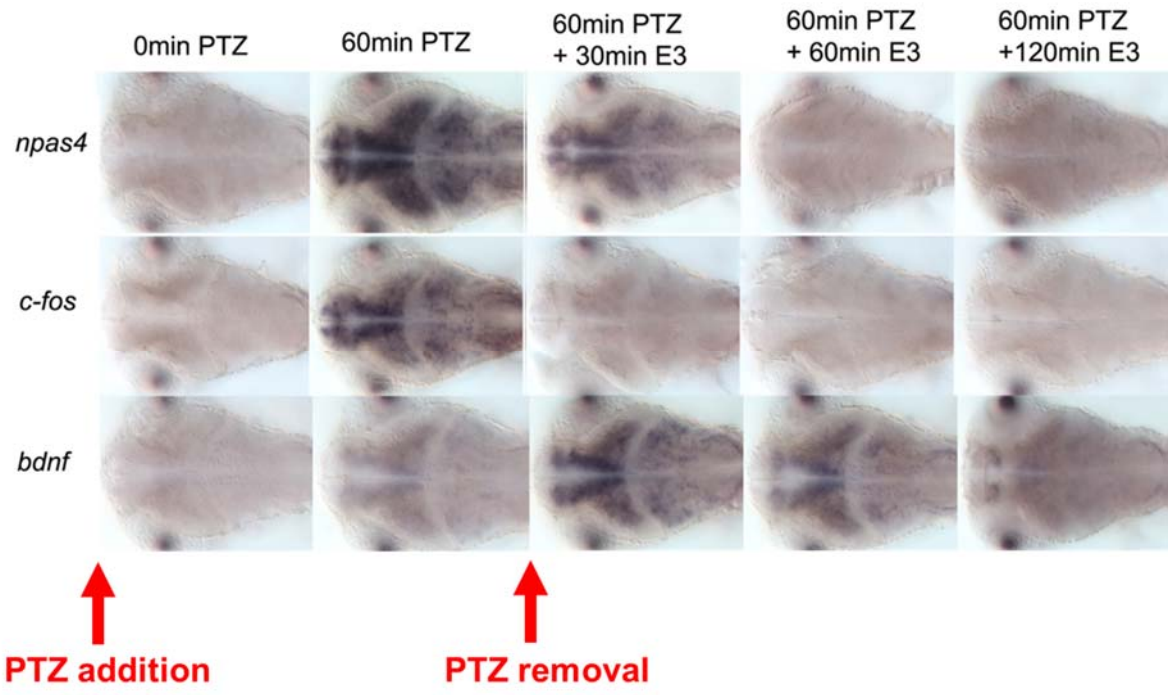


Figure 3.15 Effect of PTZ withdrawal after 1 hour of treatment on the expression of *c-fos*, *npas4a* and *bdnf*. *npas4a* and *c-fos* require the presence of PTZ to be induced, whereas *bdnf* expression can be detected after PTZ treatment is stopped.

3.2.6 Analysis of the expression of *c-fos* and *npas4a* in 3 dpf zebrafish larvae exposed to picrotoxin, kainic acid and NMDA.

A convulsive motor behaviour was induced when 3 dpf zebrafish larvae were exposed to picrotoxin, kainic acid and NMDA. Thus, it was of interest to determine whether these compounds also were able to induce the expression of *c-fos* and *npas4a* as observed for PTZ. Accordingly, the expression patterns of *c-fos* and *npas4a* were analysed by *in situ* hybridisation (Figure 3.16) in larvae exposed to picrotoxin, NMDA or kainic acid.

The *in situ* hybridisation results show that picrotoxin induced strong expression of both *c-fos* and *npas4a* in the brains of 3 dpf zebrafish larvae in similar patterns to those induced by PTZ. However, whereas the PTZ-induced transcription of *npas4a* preceded *c-fos*, the picrotoxin-induced transcription of *c-fos* preceded that of *npas4a* (Figure 3.16 A). These results suggest

that there are differences in the modes of action of PTZ and picrotoxin, which is particularly surprising since both PTZ and picrotoxin are thought to act via inhibition of the $GABA_A$ receptor.

When larvae were treated with the glutamate receptor agonist NMDA, weak induction of both *npas4a* and *c-fos* transcription was observed within 60 minutes of exposure, which did not appreciably increase after a further hour of exposure and was much lower than the level of *cfos* and *npas4a* transcripts produced by exposure to either PTZ or picrotoxin. The induced transcription of *npas4a* and *c-fos* in 3 dpf zebrafish larvae treated with NMDA seemed to be stronger in the forebrain (white arrows, Figure 3.16 B). However, when larvae were treated with kainic acid, no transcripts were detectable for either *npas4a* or *c-fos.*

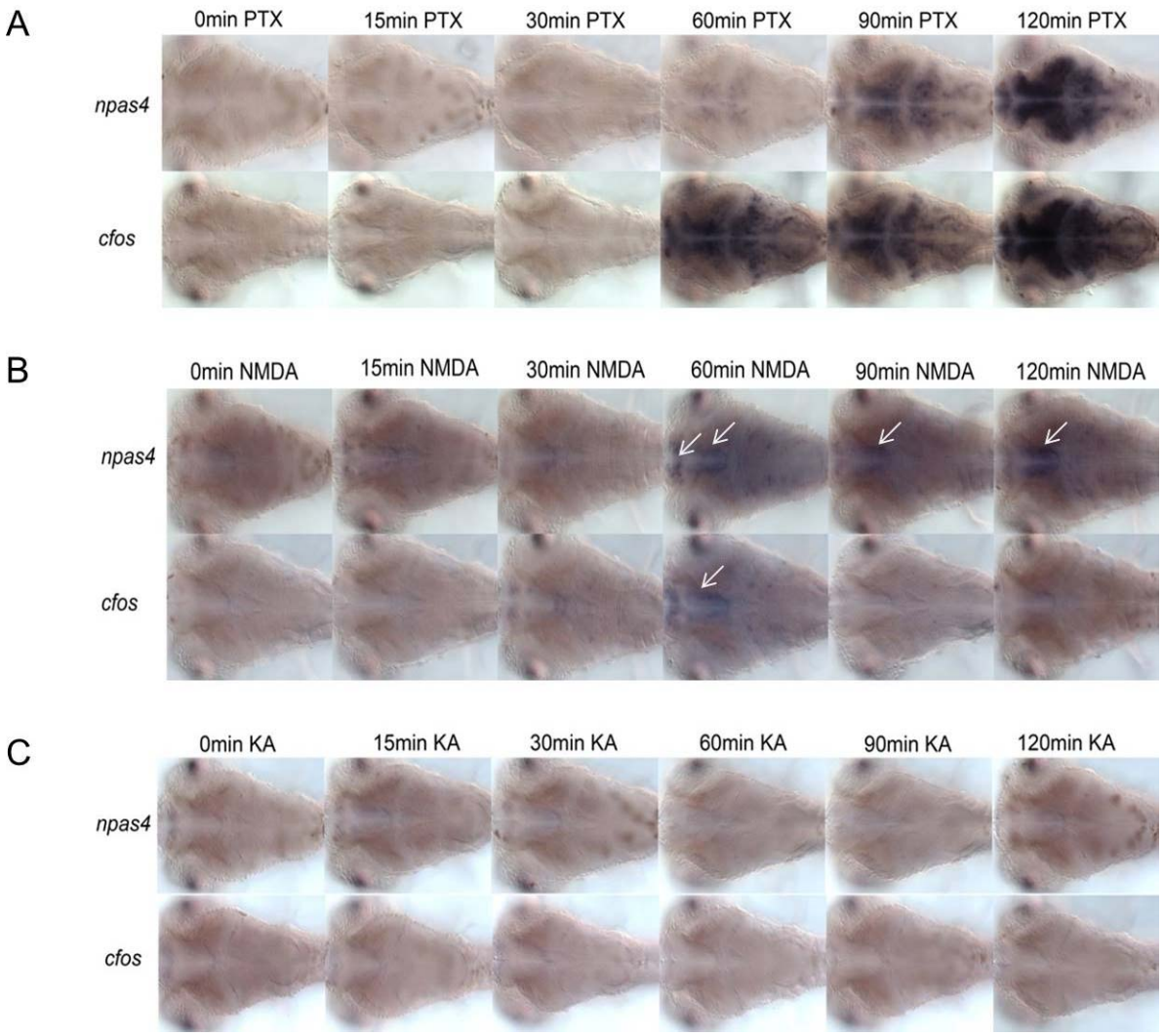


Figure 3.16 *npas4a* and *c-fos* expression during different times of treatment with picrotoxin, NMDA and kainic acid. A) Exposure to 200μM PTX causes a strong expression of *c-fos* and *npas4a* after one hour of exposure. B) 170μM NMDA treatment provoked a weak expression of *npas4a* and *c-fos* in the midbrain and forebrain of 3 dpf zebrafish larvae (white arrows). C) Treatment with 100μM KA does not induce either *npas4a* or *c-fos* expression, detectable by in situ hybridisation.

3.3 Discussion

In order to investigate the possibility of using zebrafish embryos and pre-5 dpf larvae as in vivo models of epilepsy, I sought to characterise their responses to chemical convulsants. Previous studies demonstrated the presence of glutamatergic, glycinergic and GABA-ergic neurones in zebrafish embryos from 20 hpf onwards (Higashijima, et al., 2004; Mueller, et al., 2006). I found that the α1 and γ2 subunits of the $GABA_A$ receptor were found to be expressed in 1, 2 and 3 dpf zebrafish by *in situ* hybridisation (figures 3.1 and 3.2), confirming that the target pathway for PTZ exists in young zebrafish. PTZ is able to cause behavioural changes in 4 dpf zebrafish embryos (Baxendale, et al., 2012), 7 dpf zebrafish larvae (Baraban et al., 2005) and adult zebrafish (Mussulini et al., 2013). The concentrations of PTZ tested in zebrafish range from 2.5 to 80mM (Baraban et al., 2005; Baxendale, et al., 2012; Mussulini et al., 2013; Orellana-Paucar et al., 2012). I found that the optimum concentration of PTZ eliciting a significant convulsive motor response in 3 dpf zebrafish was 20mM, which agrees with the results of previous analyses in the lab for 4 dpf zebrafish larvae (Baxendale, et al., 2012). Exposure of 3 dpf larvae to a 20mM PTZ made 3 dpf swam almost 500mm more distance than untreated larvae. This is approximately 3.5 times more distance than normal basal swimming for 3 dpf larvae (Figure 3.3). By using a light protocol of 2 minutes lights on – 2 minutes light off, as in previous experiments (Baxendale, et al., 2012), appreciable levels of basal activity were detected for both 3 dpf and 4 dpf zebrafish larvae (Figure 3.4). The results of these preliminary experiments thus allowed the parameters of PTZ concentration and light exposure to be optimised for applications to both mechanistic studies of seizures and for high-throughput anti-epileptic drug screening. The fact the basal activity of untreated larvae was relatively high with the selected light protocol, provides a sensitive background in which to detect sedative effects of compounds that could have been identified initially as putative anti-epileptic hits.

Epilepsy is a syndrome with subtypes that are so diverse that a single pharmacological model of seizure-induction is unlikely to be adequate for identifying treatments for all epilepsies. I found that 3 dpf zebrafish larvae exhibited epilepsy-like convulsions in response to picrotoxin,

kainic acid and NMDA, as well as to PTZ. Of all of the convulsants tested, picrotoxin was by far the most potent, whereas KA and NMDA elicited weaker responses than PTZ (Figures 3.6 A and B). These findings suggest that possibility that blockade inhibitory neurotransmission is a more potent means of inducing seizures than is increasing the level of excitatory neurotransmission, implying that inhibitory neurotransmission may provide an endogenous neuroprotective mechanism for over-riding increases in excitatory neurotransmission that could develop into seizures. The effects of picrotoxin and NMDA have not been reported in zebrafish before, but there are studies on the effect of kainic acid administration to zebrafish larvae and adults (Alfaro, et al., 2011; Kim et al., 2010; Menezes, et al., 2014). However, whilst my results showed that 100μM KA caused 3 dpf zebrafish larvae to exhibit convulsive motor movements, Menezes et al., reported that exposure of 7 dpf zebrafish larvae to 100, 200 and 300μM kainic acid actually reduced locomotor activity, as compared to untreated larvae. Nevertheless, the results of Kim et al. are broadly consistent with my observations, since, 50μM KA was reported to be sufficient for the induction of epileptiform discharges in 5 dpf zebrafish larvae, confirming that young larvae exhibit epileptic seizures when treated with kainic acid (Kim et al., 2010b). Taken together with these findings, my results indicate that 3 dpf larvae exhibit KA-induced convulsions that are the result of epileptic seizures, thus providing another pharmacological epilepsy model that may be suitable for anti-epileptic drug screening.

Whilst the convulsive responses to pharmacological agents such as PTZ, picrotoxin, kainic acid and NMDA provide sensitive, quantifiable, non-invasive measures of seizure severity, it is important to have additional independent means of characterising seizures in an experimental model of epilepsy which directly report changes in neuronal activity within the CNS. Transcription of *c-fos* is widely employed as an indicator of synaptic activity and neuronal activation in many experimental models of epilepsy, and its expression in the hippocampus in response to seizures has been particularly well-documented (reviewed by Herrera & Robertson, 1996). In zebrafish, the equivalent of the mammalian hippocampus is most likely

to be located within the medial pallium of the telencephalon (Friedrich, et al., 2010; Santana, et al., 2012). Consistent with this idea, the *c-fos* expression was particularly strongly induced within the pallium of 2 and 3 dpf zebrafish embryos and larvae (Figures 3.7 and 3.8), as well as in 4 dpf zebrafish larvae (as will be seen in Chapter 5). *c-fos* expression was also induced by PTZ in regions of the midbrain (diencephalon), the hindbrain (including the locus coeruleus) and the spinal cord (Figures 3.7 and 3.8). Trunk muscle also expressed *c-fos* in response to PTZ, transcript abundance being particularly high throughout the trunk at 2 dpf, then declining in 3 dpf larvae.

To further characterise the brain structures and neurons involved in responding to PTZ exposure, a double *in situ* hybridisation analysis was performed assessing the expression of *c-fos* simultaneously with specific markers of distinct neuronal subtypes. The results suggest that in PTZ-treated 3 dpf larvae, *c-fos* is expressed in some dopaminergic neurons expressing tyrosine hydroxylase (*th*), neurons expressing corticotropin-releasing hormone (*crh*) and a subset of *pyya*-positive neurons (Figures 3.10 A, 3.10 B, 3.11 A). Over-expression of *c-fos* in neuroendocrine tumour cultured cells can induce the expression of *th* (Sun & Tank, 2002). However, 3 dpf zebrafish larvae treated with PTZ did not show an increased expression of *th* that was detectable by in situ hybridisation (Figure 3.12). In the case of *crh*, when rats were stressed with aversive stimuli, some neurons in the paraventricular nucleus of the hypothalamus co-expressed *c-fos* and *crh* (Pezzone, et al., 1992). My experiments show that 3 dpf zebrafish larvae exposed to PTZ also expressed *c-fos* in *crh*-positive neurons (Figure 3.10 B) suggesting the possibility of an association between seizure induction and the endocrine stress response, which will be further explored in Chapter 5.

Peripheral injection of the neuropeptide PYY in rodents induces c-Fos immunoreactivity in regions of the amygdala and thalamus (Bonaz, et al., 1993), and when PYY is injected locally in the hippocampus of rats subjected to PTZ stimulation, it reduces the latency to the first seizure, but it does not alter the duration of seizures (Reibel et al., 2001). Whilst these observations are consistent with an epileptogenic role for PYY, it remains unclear as to

whether PYY enhances seizure severity in all circumstances or whether in some instances it can be neuroprotective (Baraban, 2004; Reibel et al., 2001; Vezzani, et al., 1999). Our results show that expression of *pyya* was increased in the hindbrain and spinal cord of 50 hpf zebrafish embryos treated with PTZ, the significance of which will be worth exploring in the future.

Another gene that is overexpressed in a variety of different models of epilepsy is the brain-derived neurotrophic factor (*Bdnf*) (Morimoto et al., 2004; Zhang et al., 2002). In epileptic animal models, expression of BDNF follows the seizure-induced expression of c-Fos protein, which suggests that *c-fos* could regulate the expression of BDNF (Dong et al., 2006; J. Zhang et al., 2002). One previous study relevant to this issue analysed expression of BDNF after both 4 hours and 48 hours of kainic acid treatment in wild-type and *c-fos* mutant mice (Zhang et al., 2002). After 4 hours of kainic acid treatment, *c-fos* mutant mice exhibited no increase in BDNF expression, whereas wild-type mice exhibited a two-fold increase in the expression of BDNF (Zhang et al., 2002). However after 48 hours post-seizure induction, the levels of BDNF were similar in wild-type and *c-fos* mutant mice (wild-type levels declined, and mutant levels increased). The results indicated that the initial induction of BDNF is *c-fos* dependent, but over the longer term other mechanisms promote BDNF transcription (Zhang, et al., 2002). We observed that zebrafish larvae also express *bdnf* in response to seizure induction (Figures 3.13 and 3.14), which begins after the peak of seizure-induced *c-fos* expression, suggesting that *bdnf* transcription may depend on the prior induction of other genes that could include *c-fos* (Figure 3.14).

The gene *Npas4* encodes a transcription factor of the bHLH-PAS domain family that regulates the expression of genes involved in the formation and maintenance of inhibitory synapses (Y. Lin et al., 2008). Previous studies demonstrated that *npas4* transcription is induced by KCl-induced depolarisation of cultured mammalian neurons (Y. Lin et al., 2008). The transcription of this gene is also induced in the murine hippocampus by PTZ treatment (Flood, et al., 2004). However, the function of Npas4 in epileptic seizures has not yet been fully elucidated. I found

that *npas4a* is strongly expressed in the brains of zebrafish larvae treated with 20mM PTZ (figures 3.13 and 3.14). The transcription of *npas4a* was induced rapidly and before *c-fos,* starting within 15 minutes after starting PTZ treatment, reaching a peak after 30 minutes of PTZ exposure, after which a high level of PTZ-dependent transcription was maintained for at least 2 hours (Figure 3.14). In contrast, strong induction of *c-fos* required 1 hour of PTZ treatment, and robust expression of *bdnf* required 2 hours of exposure to PTZ. The temporal differences in expression of these three genes suggested the possibility that Npas4a might be regulating the transcription of the two other genes in response to PTZ. However, when PTZ was withdrawn after one hour of treating 3 dpf zebrafish larvae, the expression of *npas4a* persisted for longer than *c-fos* expression, suggesting the possibility that PTZ-induced *c-fos* transcription may be *npas4a*-independent or attenuated by the build-up of an inhibitory factor that is rapidly PTZ-induced. My experiments also demonstrated that *bdnf* expression was induced after PTZ was removed from the medium (Figure 3.15), suggesting that *bdnf* transcription may be induced by a factor whose synthesis is elicited by PTZ treatment, rather than directly by the signalling pathway transducing the effect of PTZ treatment. Since *Npas4* has previously been shown to regulate *Bdnf* levels in cultured neurons, this relationship may also exist in PTZ-treated zebrafish larvae (Lin, et al., 2008). Experiments to investigate the functions of *c-fos* and *npas4a* in the PTZ-induced zebrafish model of epilepsy will be described in detail in later Chapters of this thesis.

The accumulation of *c-fos* and *npas4a* transcripts is a good indicator of seizure induction by PTZ treatment. However, the responses of *c-fos* nor *npas4a* were different in zebrafish larvae treated with picrotoxin, kainic acid and NMDA (figure 3.16). Interestingly, kainic acid did not induce transcription of either *c-fos* or *npas4a* in 3 dpf zebrafish larvae, as tested by in situ hybridisation (Figure 3.16 C), and NMDA only weakly induced these genes. The expression of *c-fos* in response to kainic acid has been reported to be age dependent in rats; FOS immunoreactivity was not detected in P7 rats after 1 and 2 hours of KA administration (Sonnenberg, et al., 1989) Moreover, picrotoxin treatment induced *c-fos* transcription more

rapidly than that of *npas4a*, further suggesting that independent mechanisms may regulate PTZ-induced transcription of *c-fos* and *npas4a*.

Chapter 4

4 Analysis of the function of *c-fos* and *npas4* in response to PTZ treatment.

4.1 Introduction

c-fos is a transcription factor that is expressed in neurons in response to the calcium influx that occurs when they are depolarised (Morgan & Curran, 1986). In addition to neurotransmitters, neurotrophic factors can also induce *c-fos* expression (reviewed by Kovács, 1998). In view of the fact that *c-fos* transcription can be induced by a range of distinct stimuli, it is possible that its function could be either context-dependentent or context-invariant (reviewed by Herrera & Robertson, 1996). In models of epilepsy, even though *c-fos* is the most heavily investigated synaptic activity-regulated gene, its function downstream of synaptic activity is still very poorly understood, although it has been previously shown to mitigate the excitotoxic effect of kainic acid (Zhang et al., 2002).

By contrast, there has been greater progress in elucidating the function of *Npas4,* which was first identified in a human foetal brain cDNA library ten years ago (Ooe, et al., 2004). Npas4 regulates the formation and function of inhibitory synapses in cultured cells (Y. Lin et al., 2008), and *in vivo* Npas4 regulates the balance between excitatory and inhibitory neuronal activity within specific neural circuits (Spiegel et al., 2014). Nevertheless, despite this progress (Bloodgood, et al., 2013; Ramamoorthi et al., 2011; Yun et al., 2013, Spiegel et al., 20104), the role of *npas4* in the response to seizure induction has not yet been elucidated.

4.1.1 Roles for *c-fos* in epilepsy and neural plasticity

As mentioned in Chapter 3, the expression pattern of *c-fos* has been used as an indicator of neuronal activity in tissues due to its low basal levels in resting conditions and its inducibility in response to a wide range of types of neuronal stimulation (Kóvacs, 1998). However, *c-fos*

is not only a marker of synaptic activity, and has been implicated in the regulation of many different types of biological process, including cell proliferation, cell differentiation and apoptosis (Herrera & Robertson, 1996).

When convulsants such as the glutamate receptor agonist kainic acid (KA), or the $GABA_A$ receptor antagonists Pentylenetetrazole (PTZ), Bicuculline or Picrotoxin (PTX) are administered to adult mice, *c-fos* is specifically transcribed to a high level in neurones of the dentate gyrus in the hippocampus, a region of the brain that has been implicated in learning and memory, as well as in seizures (reviewed by Herrera, & Robertson, 1996). *c-fos* has also been found to be a target of *p53* tumour suppressor gene, although whether it promoted apoptosis or facilitated the recovery of the cells from DNA damage was not very clearly determined (Elkeles et al., 1999). Knockout mice lacking *c-fos* function in the hippocampus exhibit stronger seizures and significantly more neuronal cell death as a consequence of seizure induction, in comparison to wild-type littermates (Zhang et al., 2002), implying a role for c-Fos in limiting excitatory activity in neural circuitry. Moreover, *c-fos* knockout mice exhibit defects in learning and memory, as well as reduced levels of *bdnf* expression in the brain (Fleischmann et al., 2003; Zhang et al., 2002). Learning and memory impairment in *c-fos* knockout mice could be the consequence of reduced sprouting from the mossy fibres (Watanabe et al., 1996). Taken together, these results indicate that synaptic activity-dependent induction of *c-fos* transcription in the dentate gyrus may be neuroprotective in seizures, and they also suggest that lower levels of *c-fos* transcription in the dentate gyrus of normal animals promotes learning and memory formation, perhaps via regulation of *bdnf* expression.

4.1.2 Roles for *Npas4* role in epilepsy and neural plasticity

Unlike *c-fos*, whose expression is induced in response to a variety of developmental cues and physiological stimuli, the expression of the bHLH transcription factor *npas4* is restricted to neurons and induced exclusively in response to membrane depolarisation and calcium influx

(Ramamoorthi et al., 2011a). Interestingly, studies in Npas4 knockout mice have shown that short and long-term memory is impaired in *Npas4* lacking mice (Ramamoorthi et al., 2011a), indicating a role for this gene in facilitating the establishment and refinement of circuits involved in creating and/or storing memories.

Npas4 is induced in response to experimentally-induced seizures in rodents (Lin et al., 2008; Yun et al., 2013). This is particularly intriguing in light of the observations indicating that Npas4 promotes the establishment of functional GABA-ergic inhibitory synapses as a direct response to induction of synaptic activity by convulsants (Lin, et al., 2008). The clearest implication of these results is that by promoting inhibitory transmission as a response to the high levels of seizure-induced neuronal excitation, Npas4 may be driving a neuroprotective response which attenuates neural circuit firing which could be neurotoxic if left unchecked. Such a negative feedback loop would thus be a homeostatic mechanism that could help to terminate a seizure and restore brain activity to normal neurophysiological levels.

Like *c-fos, npas4* also stimulates BDNF expression in neurones by binding to neural activity-responsive DNA sequences within the BDNF promoter (Lin, et al., 2008; Loebrich & Nedivi, 2009), which suggests that together these two genes might co-ordinately promote *bdnf* expression.

In this Chapter, I sought to explore the functions of *c-fos* and *npas4a* in the zebrafish model of PTZ-induced epileptic seizures, based on the hypothesis explained in Chapter 1 (Figure 1.5). This hypothesis postulates that in response to a seizure-inducing stimulus, genes that are transcribed in activated neurons may provide either epileptogenic (pathogenic) or homeostatic (neuroprotective) feedback.

4.2 Results

4.2.1 *c-fos* knockdown with a splice blocking morpholino reduced the level of PTZ-induced *bdnf* expression at 50 hpf, but caused a phenotype characterised by small heads and small eyes.

As explained in the previous chapter, the gene *c-fos* is transcribed rapidly in response to PTZ treatment in the developing central nervous system of zebrafish embryos and larvae. In order to study the function of *c-fos,* a splice-blocking morpholino was designed which binds to the junction between exon 3 and intron 3-4 of this gene (Figure 4.1). This splice-blocking morpholino was designed to disrupt the splicing of the primary transcript such that any *c-fos* translation products from the mis-spliced RNA would lack a functional DNA-binding domain. In order to determine whether the morpholino disrupts splicing as predicted, an RT-PCR analysis of *c-fos* RNA transcripts was performed using primers specific for sequences within exons 2 and 4 of the zebrafish *c-fos* gene (Baraban, et al., 2005). These primers are predicted to generate RT-PCR fragments of 1030bp corresponding to the mature mRNA and of 1234bp for the un-spliced primary RNA produced by transcription of genomic DNA (Figures 4.1 and 4.2).

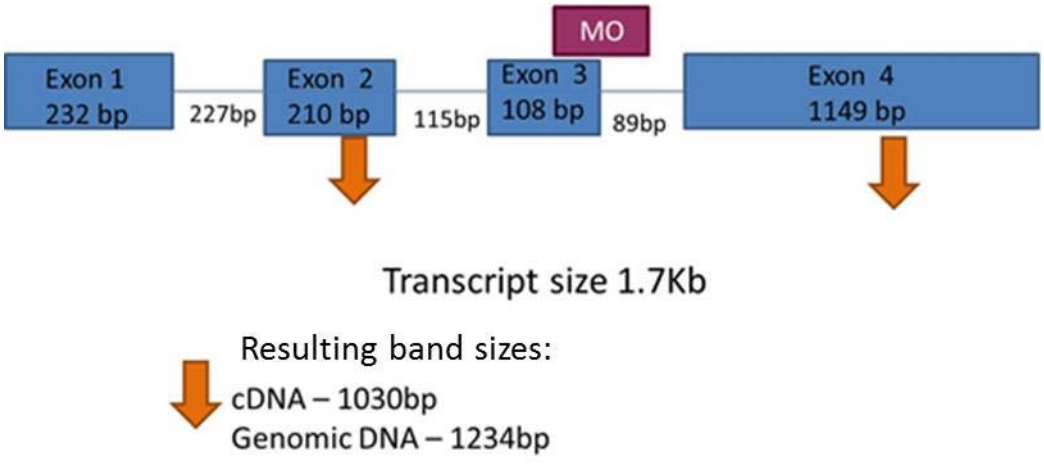


Figure 4.1 Schematic representation of *c-fos* gene, *c-fos* morpholino and primer binding sites. The orange arrows represent the location of the primer sequences (Baraban et al., 2005), the purple rectangle represents the location of the splice blocking morpholino.

To analyse the effect of the *c-fos*-targeted splice-blocking morpholino, one cell-stage zebrafish embryos were injected with 2nl of 0.2mM morpholino and at 50 hpf they were treated with 20mM PTZ for 0,15,30 and 60 minutes. The resulting gel shows that the increase in the levels of the transcript over the time of PTZ treatment does not occur in the morphants as with wild-type larvae. Moreover, more bands are evident in morphant larvae, of which the upper ones corresponds to the morpholino-induced, partially spliced, variant transcripts (include ~100nt intron 3-4) and the lower one corresponds to the properly spliced mRNA (Figure 4.2).

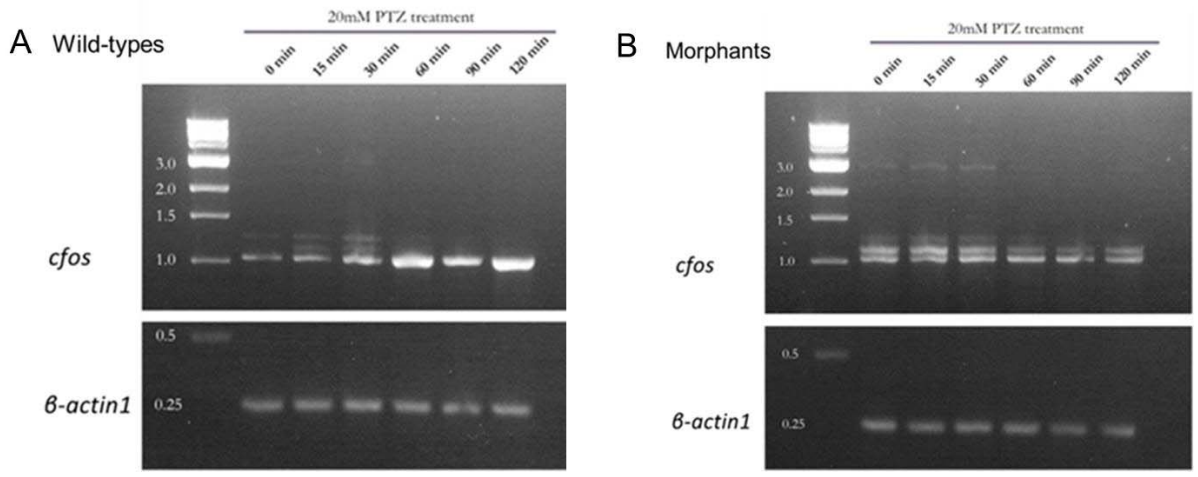


Figure 4.2 *c-fos* morpholino blocks accumulation of PTZ-induced, correctly processed *c-fos* mRNA. 1.6% agarose gel showing the DNA fragments obtained after RT-PCR analysis of 50 hpf untreated wildtype zebrafish larvae (A) and 50 hpf PTZ-treated *c-fos* morphants(B) using primers to amplify *c-fos* cDNA.

The expression of *c-fos* in *c-fos* morphants was also analysed by *in situ* hybridisation. One-cell stage embryos were injected with 2nl of 0.3mM *c-fos* morpholino and treated with PTZ at 50 hpf. The results shown on Figure 4.3 A indicate that the transcript distribution of *c-fos* is not appreciably altered in *c-fos* morphant larvae, in spite of the fact that the RT-PCR analysis showed reduction of mature *c-fos* mRNA after 60 minutes of treatment with PTZ in the morphants compared to the wild-type embryos.

The expression of *bdnf* in *c-fos* morphants was also analysed by *in situ* hybridisation. The majority of the *c-fos* morphant larvae analysed exhibited lower expression of *bdnf* than the wild-type larvae when exposed one hour to 20mM PTZ (Figure 4.3 B), indicating a role for *c-fos* in the regulation of *bdnf* expression.

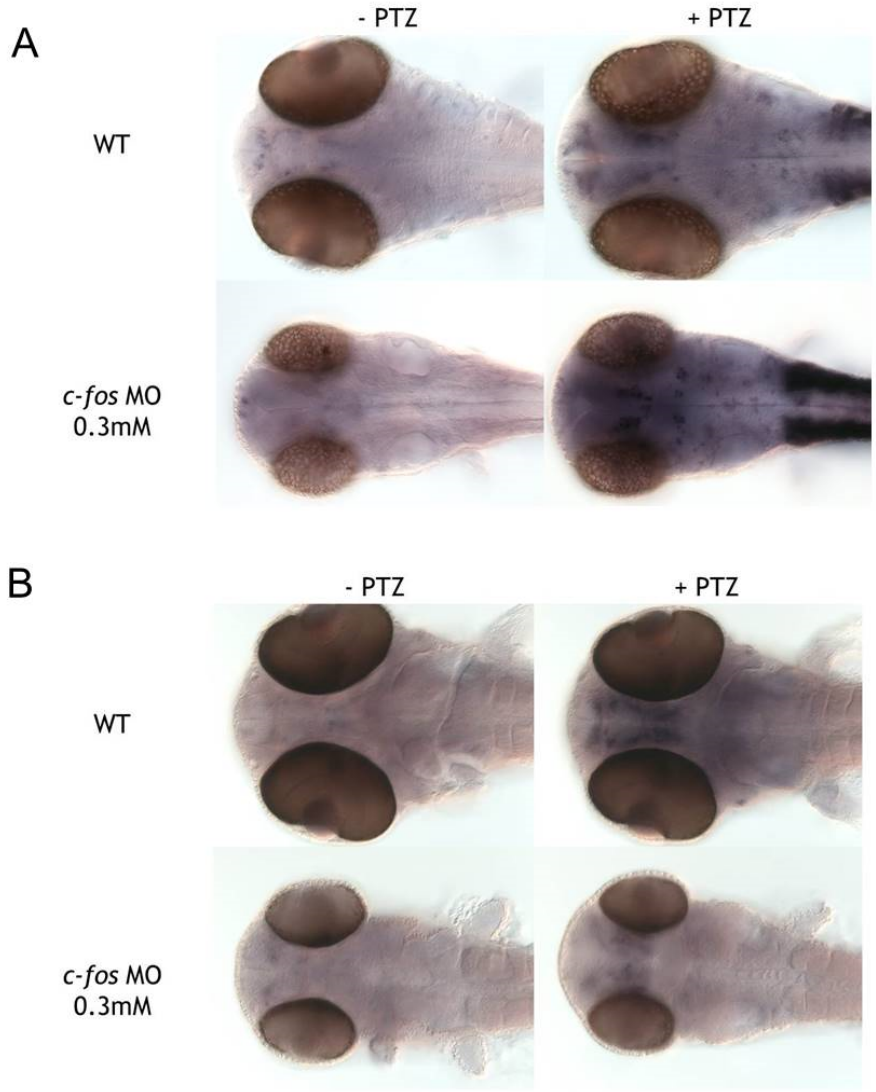


Figure 4.3 *c-fos* morpholino reduces PTZ-induced *bdnf* expression but not *c-fos* expression at 50hpf, and causes a phenotype characterised by small heads and small eyes. *In situ* hybridisation analysis of: A) Dorsal views of 50 hpf zebrafish embryos showing that the pattern of *c-fos* expression is unaltered in the brains of PTZ-treated *c-fos* morphants, as compared to that of uninjected, PTZ-treated, wildtype embryos. B) Ventral views of 50 hpf zebrafish embryos showing a reduction in the expression of *bdnf* in PTZ-treated *c-fos* morphants compared to uninjected PTZ-treated wildtype embryos.

The embryonic phenotype of small heads and small eyes is sometimes caused by the over-activation of the p53 apoptotic pathway, which is a common off-target effect of morpholinos (Bill, et al., 2009; Robu et al., 2007). Therefore, to analyse if this was the case for the *c-fos* splice-blocking morpholino, the *c-fos* morpholino was co-injected along with a *p53*-specific morpholino. The embryos were treated with PTZ at 50 hpf for one hour, then fixed, and an *in situ* hybridisation analysis for *bdnf* expression was carried out. However, when *p53* morpholino was co-injected with *c-fos* morpholino, the small head phenotype was not rescued (Figure 4.4). By contrast, the injection of a standard control morpholino did not cause the small head phenotype. Moreover, the larvae that were co-injected with both *p53* and *c-fos* morpholinos, then treated with PTZ, did not recover the levels of *bdnf* expression, unlike uninjected and standard control morpholino-injected larvae, which exhibited normal levels of PTZ-induced *bdnf* expression (Figure 4.4).

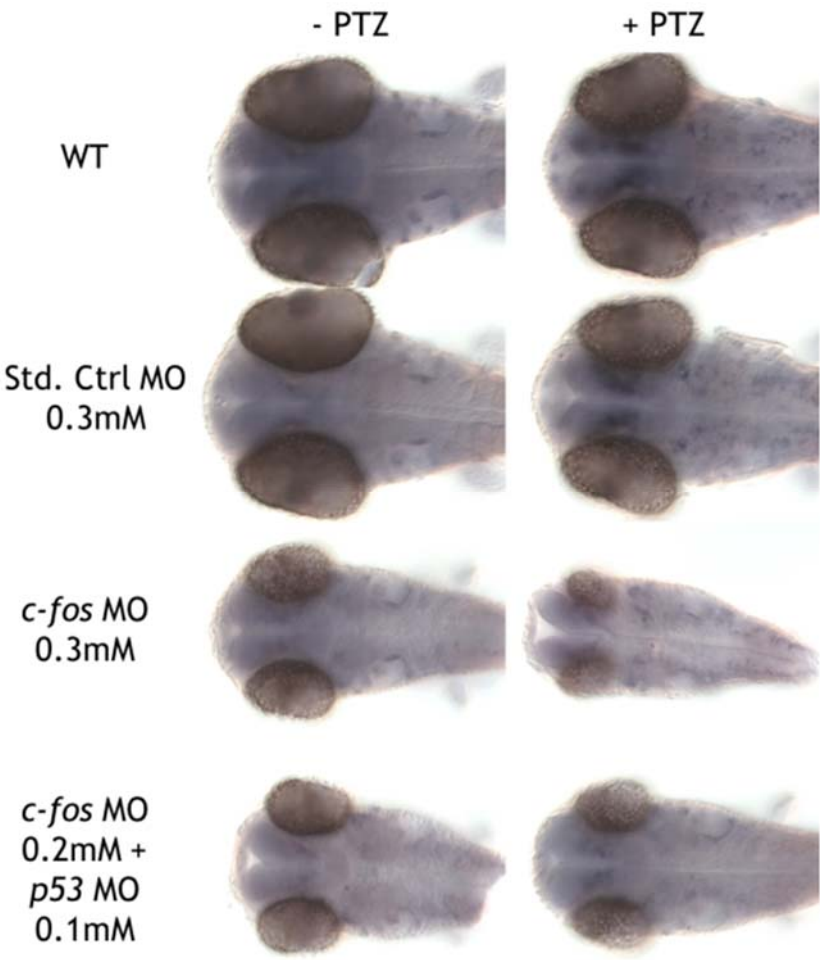


Figure 4.4 Co-injection a *p53* morpholino does not rescue the small head phenotype or the reduced expression of *bdnf* caused by injection of a *c-fos* splice-blocking morpholino. Dorsal views of 50 hpf zebrafish larvae comparing the expression of *bdnf* detected by in situ hybridisation, between PTZ treated and untreated embryos. The levels of *bdnf* expression are similar in PTZ-treated uninjected embryos and standard control morpholino-injected embryos. However, embryos injected with the splice-blocking *c-fos* morpholino have smaller heads and exhibit reduced expression of *bdnf*, neither of which are rescued by the co-injection of a *p53*-specific morpholino with the *c-fos* morpholino.

Definitive demonstration that the morphant phenotype was a specific consequence of inhibiting endogenous *c-fos* expression required an mRNA rescue experiment, where synthetic mRNA representing the properly spliced *c-fos* mRNA was co-injected with the splice-blocking morpholino.

In order to begin these experiments, a full-length *c-fos* cDNA was cloned into the pCS2+ plasmid vector. The plasmid was then linearised and *c-fos* mRNA was synthesized using the mMessage mMachine SP6 kit. Before injecting *c-fos* mRNA into *c-fos* morphants, the mRNA was tested in wild-type embryos to determine whether microinjection of *c-fos* mRNA itself could cause any adverse effect in the normal development of zebrafish embryos.

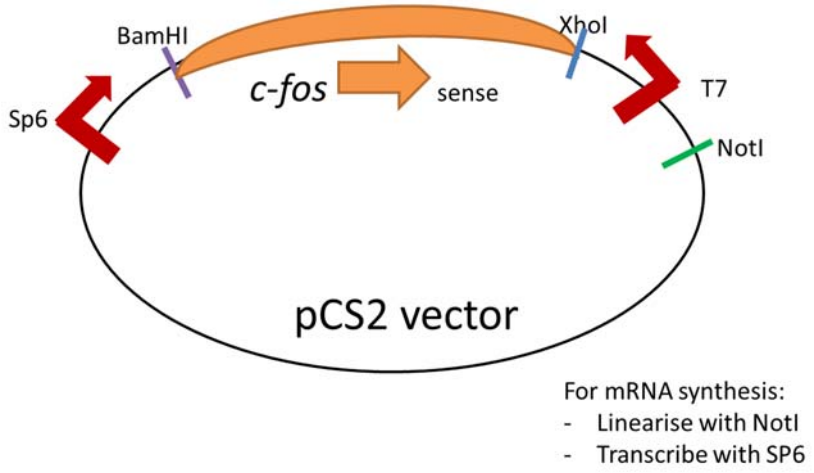


Figure 4.5 pCS2.*c-fos* construct for full-length *c-fos* mRNA synthesis. Map of the plasmid containing the full-length *c-fos* cDNA. The restriction enzyme used to cut the plasmid to synthesize *c-fos* messenger RNA was NotI, and the in vitro transcription reaction was carried out using Sp6 polymerase.

To test whether the phenotype caused by microinjection of *c-fos* morpholino could be rescued by synthetic *c-fos* mRNA, wild-type zebrafish embryos were co-injected at the one-cell stage with 0.2mM of *c-fos* morpholino and 270pg of *c-fos* mRNA. The development of these embryos was then compared with wild-type embryos and embryos that were injected with either 0.2mM of *c-fos* morpholino only or 270pg of *c-fos* mRNA only. At 50hpf, some of the embryos were treated with PTZ for one hour, then fixed and analysed by *in situ* hybridisation for expression of *bdnf*.

Embryos that were co-injected with both *c-fos* morpholino and *c-fos* mRNA did not exhibit a normal morphology and instead resembled embryos injected with *c-fos* morpholino alone,

which had small heads. (Figure 4.6). These preliminary results suggest that the injected dose of *c-fos* mRNA may not completely rescue all of the defects caused by c-*fos* morpholino injection. In addition to the morphological defects, although the *in situ* hybridisation analysis of *bdnf* expression showed unusual high levels of *bdnf* in untreated wild-type embryos, a slight increase of *bdnf* expression was detected in PTZ- treated embryos compared with untreated embryos.

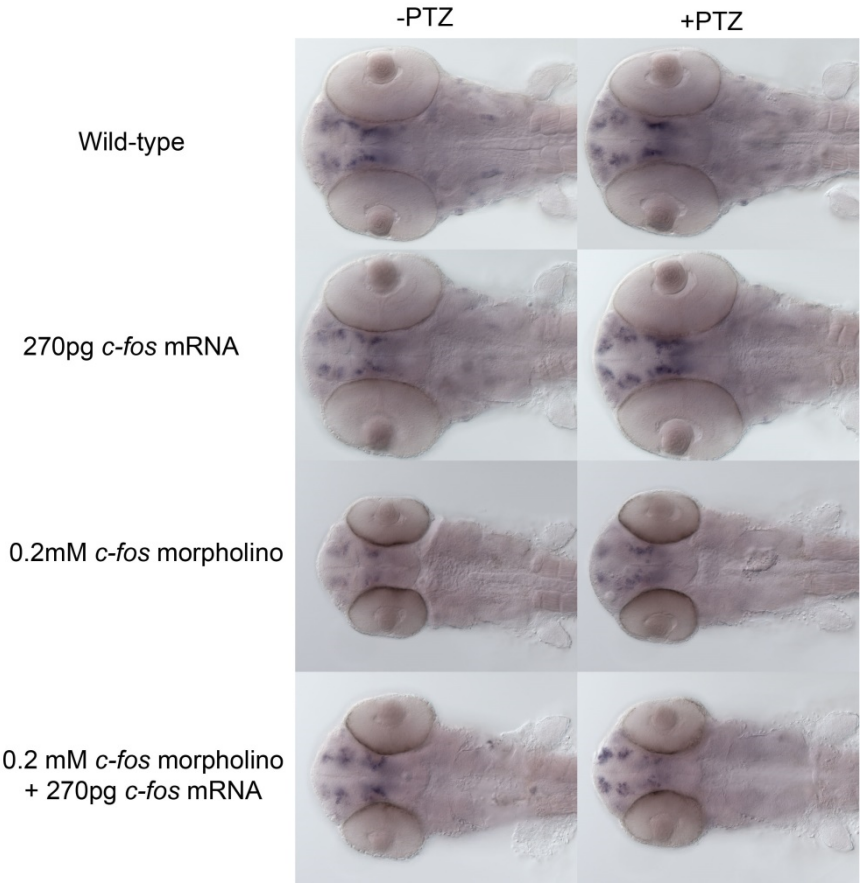


Figure 4.6 Synthetic *c-fos* mRNA was not sufficient to rescue the small head phenotype at 50 hpf, caused by the morpholino injection. The microinjection of *c-fos* mRNA along with *c-fos* morpholino was not enough to rescue the small headed phenotype caused by *c-fos* morpholino. *In situ* hybridisation analysis show an abnormal increase of *bdnf* expression in PTZ-untreated wild-type embryos, but the levels of *bdnf* are slightly higher in PTZ-treated embryos compared with PTZ-untreated embryos.

To test whether the *c-fos* splice-blocking morpholino rendered embryonic neurones more sensitive to the excitotoxic effects of seizures, the level of apoptosis in morphant and control embryos was compared using the TUNEL assay. Wild-type zebrafish embryos were injected at one cell stage either with 0.2mM *c-fos* morpholino alone or with a mixture of 0.1mM *c-fos* morpholino and 0.1mM *p53* morpholino. Some of the injected embryos were treated with PTZ and compared with uninjected embryos, some of which were also treated with PTZ. *c-fos* morphant embryos exhibited more apoptotic cells than wild-type embryos independently of any exposure to PTZ, but this phenotype was rescued by the co-injection with *p53* morpholino (Figure 4.7).

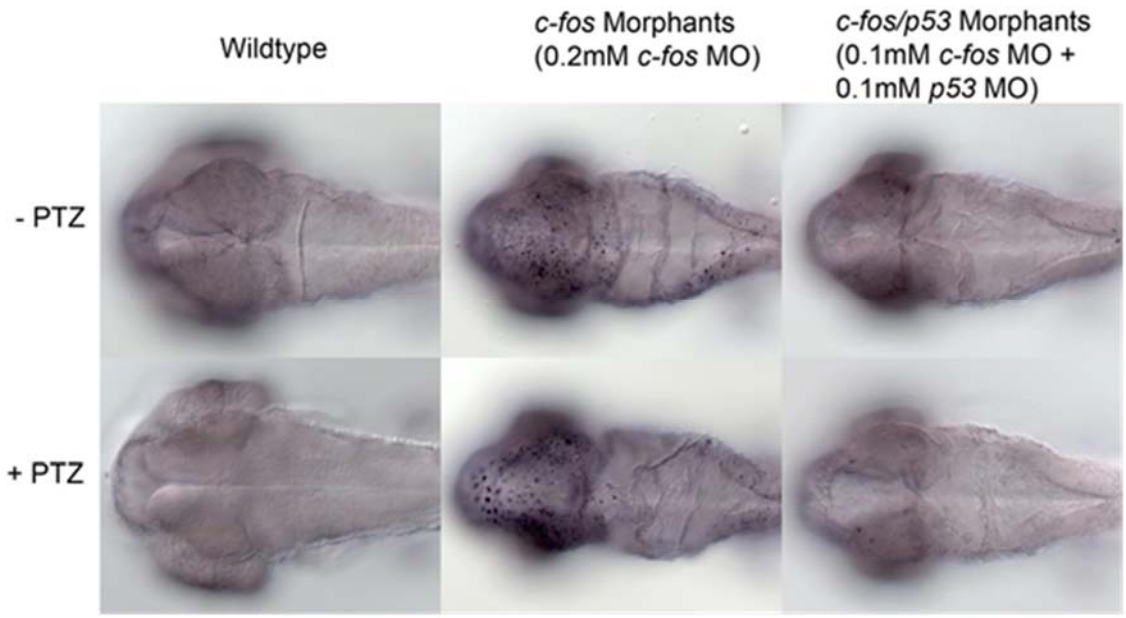


Figure 4.7 *c-fos* morpholino injection causes an increase in apoptotic cell death at 50 hpf, which can be rescued by the co-injection of p53 mopholino along with *c-fos* morpholino. Dorsal views of 50 hpf zebrafish embryos showing that co-injection of *c-fos* and *p53* morpholinos is able to rescue the increased apoptosis seen in *c-fos* morphants.

These results indicate that the *c-fos* morpholino induces limited apoptosis in the CNS which is suppressed by co-injection of the p53-specific morpholino. However this does not account for the small head phenotype, which appears to be due to a p53-independent effect of the *c-fos* morpholino that is not rescued by injection of synthetic *c-fos* mRNA. Whether this small

head phenotype is due to loss of endogenous *c-fos* gene expression remains unclear and will require further investigation in the future, ideally using a mutant that is deficient in *c-fos* function.

As was described in the previous Chapter, transcription of *npas4a* is strongly induced in zebrafish larvae in response to PTZ treatment. Moreover, transcription rapidly reaches a high level well in advance of that of *c-fos* (Figure 3.13), suggesting the possibility that Npas4a plays an important role in the initiation of responses to synaptic activity and seizure onset. In order to begin to investigate the function of Npas4a, a morpholino-based approach was adopted initially, which was then augmented by the analysis of an *npas4a* mutant line (*sa701*) that became available through the Zebrafish Mutation Project co-ordinated by the Sanger Institute.

4.2.2 *Inhibition of Npas4a* expression and function using a splice-blocking morpholino and the *sa701 npas4a* mutant line.

When zebrafish larvae are exposed to 20mM PTZ, one of the first genes to be expressed is *npas4a,* as shown in Figure 3.13 of this thesis, raising the possibility that this gene plays an important role in the initiation of the response to PTZ treatment. To begin to investigate the function of *npas4a*, a splice-blocking morpholino was designed to target the primary transcript sequence lying at the junction between exon 2 and intron 2-3, which would inhibit the production of transcripts encoding a protein that included the the PAS domain of Npas4a. RT-PCR analysis was performed to validate the function of the *npas4a* splice-blocking morpholino, using primers complementary to sequences in exons 1 and 4. The DNA fragment size when using this pair of primers in RT-PCR on uninjected embryos was 400 bp. However, if the morpholino caused an inclusion of intron 2-3, then a larger DNA fragment of 491 bp should be expected in the PCR analysis (Figure 4.8).

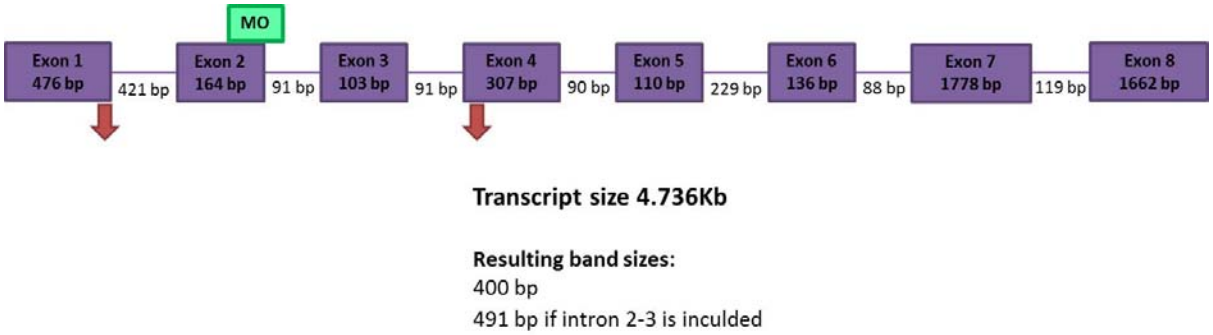


Figure 4.8 Schematic representation of the *npas4a* gene, the locations of the sequence targeted by the *npas4a* morpholino and the sequences complementary to the RT-PCR primers used to analyse the effects of the morpholino on transcript structure. The red arrows represent the location of the primer sequences; the green rectangle represents the location of the splice blocking morpholino.

One-cell stage zebrafish embryos were injected with 2nl of 0.5mM *npas4a* morpholino and treated with 20mM PTZ for 2 hours at 72 hpf, in order to allow sufficient time for potential downstream transcriptional changes caused by alterations in Npas4a function to be monitored. Total RNA was then extracted, cDNA was synthesised and RT-PCR was carried out using the primers designed to validate the function of the morpholino. The RT-PCR results showed that the increased expression of *npas4a* produced by the exposure to PTZ was suppressed by the morpholino (Figure 4.9). Moreover, a band of approximately 491 bp was observed only in the *npas4a* morphants, due to the retention of intron 2-3 in the transcript as a consequence of the action of the morpholino (Figure 4.9).

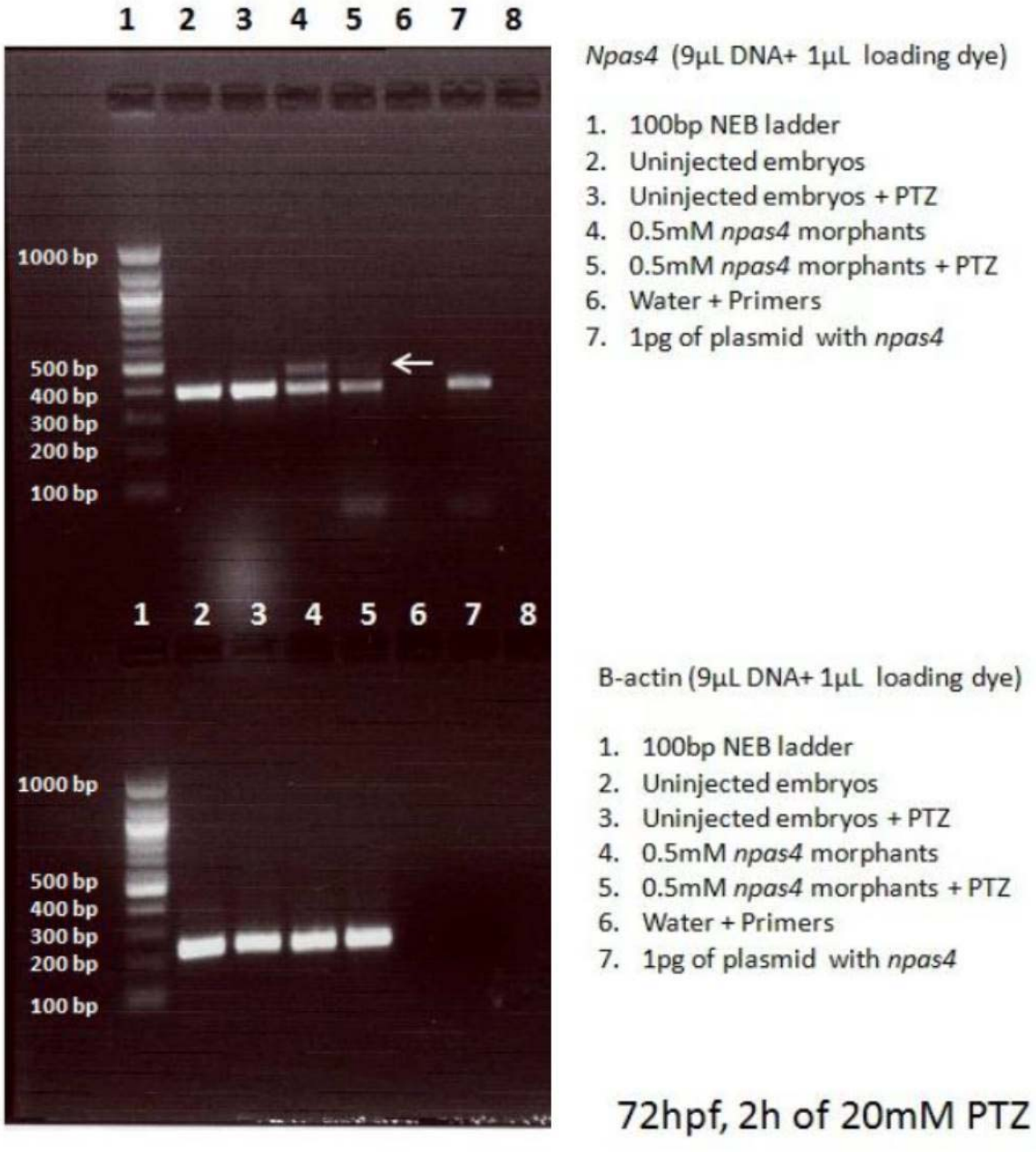


Figure 4.9 *Npas4a* splicing is disrupted by the *npas4* morpholino. PCR amplification of cDNA products derived from *npas4* transcripts. Upper panel: primers used were *npas4* forward and *npas4* reverse. Amplification product of normal primary transcript expected size: 400 bp (lower fragment). Inclusion of a 91 bp intron is marked by the white arrow. Lower panel: primers used were *beta-actin* forward and *beta-actin* reverse, to detect transcripts of *beta-actin* loading control.

Once the *npas4a* morpholino was validated, the morpholino was titrated and it was found that the optimal concentration to use was 0.3mM, since at 3 dpf embryos injected with 0.3mM

npas4a morpholino were morphologically normal, but above this concentration physical defects began to appear.

Taking together the observations that PTZ-induced *npas4a* expression occurs before that of *c-fos* and *bdnf* (Chapter 3, Figure 3.13) and also that *bdnf* expression persists when PTZ is removed from the E3 medium in which embryos are maintained (Chapter3, figure 3.14), I hypothesised that *npas4a* may regulate the expression of *c-fos* and/or *bdnf* in response to PTZ treatment. To investigate this possibility, expression of *c-fos* and *bdnf* was assessed in *npas4a* morphant and control uninjected embryos by *in situ* hybridisation, after they were incubated with or without PTZ for 1 hour (for *c-fos*) or 2 hours (for *bdnf*) with 20mM PTZ. This work was performed collaboratively with MSc student Pablo Silva Rodriguez. The results of the *in situ* hybridisation analysis revealed that the expression of *c-fos* and *bdnf* was reduced when *npas4a* was knocked-down with the splice-blocking morpholino (Figure 4.10).

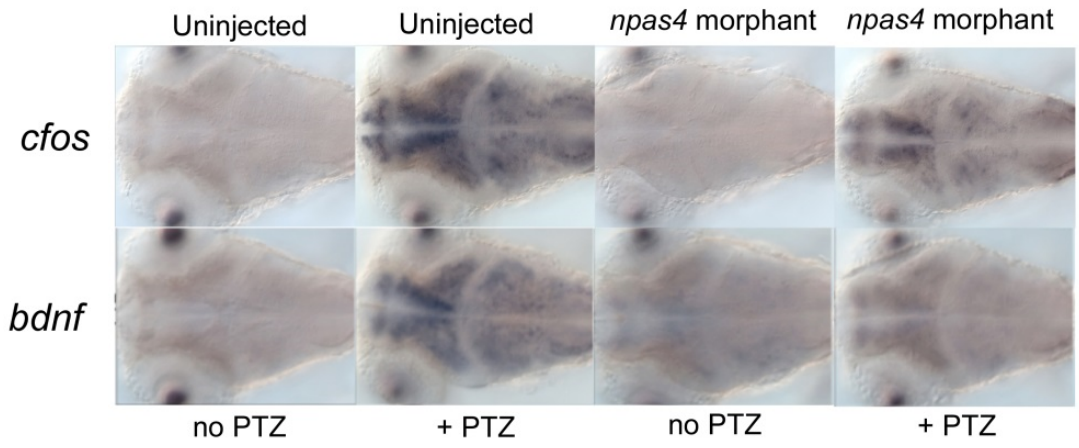


Figure 4.10 PTZ-induced expression of *c-fos* and *bdnf* is reduced in 3 dpf *npas4a* morphants. Dorsal views of 3 dpf zebrafish larvae comparing the expression of *c-fos* (upper row) and *bdnf* (lower row) between uninjected larvae and *npas4a* morphants treated with and without PTZ. The expression of both genes (*c-fos* and *bdnf*) is reduced in *npas4a* morphants treated with PTZ compared to uninjected larvae treated with PTZ.

The motor behavioural response to PTZ exposure was also analysed in *npas4a* morphants. I hypothesised that *npas4a* morphants may be more susceptible to PTZ-induced convulsions

because it has been reported that *Npas4* has a role in the formation of inhibitory synapses (Y. Lin et al., 2008). Thus, if there is less inhibitory input, a higher level of neuronal excitation might arise in the presence of PTZ. At 72 hpf, zebrafish larvae that were injected with 0.3mM *npas4a* morpholino at the one-cell stage were exposed to 5mM, 20mM or 50mM PTZ and compared with uninjected zebrafish larvae aged 72 hpf. The motor behavioural response to PTZ exposure was then recorded over a period of 1 hour. Four larvae were analysed per condition and no significant difference was found between the responses of *npas4a* morphants and wild-types to treatment with each of the three different concentrations of PTZ, although a statistically non-significant tendency of *npas4a* morphants to respond more strongly to PTZ was observed (Figure 4.11).

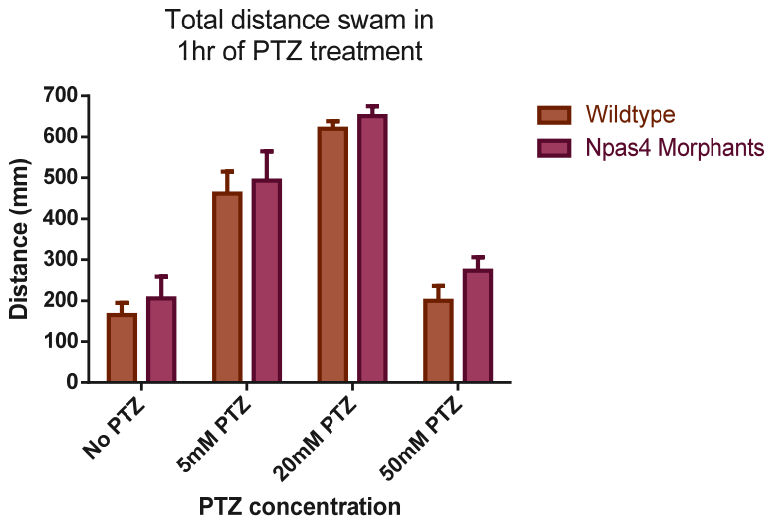


Figure 4.11 The convulsive response caused by PTZ does not change in 3dpf *npas4a* morphants. *npas4a* morphants and wild-type larvae responded similarly to treatment with 5mM, 20mM or 50mM PTZ.

In addition to the analysis of *npas4a* morphants, the phenotype of the *npas4a* mutant zebrafish line *sa701* was also analysed (Zebrafish Mutation Project, Sanger Institute). The *sa701* zebrafish line contains a nonsense mutation in codon 6 of the first exon of the gene *npas4a* and was generated in the Tupfel longfin background. 3 dpf *sa701* zebrafish larvae progeny of

a heterozygous incross were treated with either 5mM or 20mM PTZ for one or two hours while their swimming behaviour was recorded using the Viewpoint system. Since there were no morphological phenotypic differences between mutant and wild-type siblings, larvae were genotyped after the locomotor analysis (these experiments were performed collaboratively with MSc student Bilal Mughal), to identify homozygous mutants, heterozygotes and homozygous wild-type individuals.

After one hour of PTZ treatment, a tendency of the mutant larvae to respond less vigorously to PTZ was observed, which was more appreciable after 2 hours of treatment (Figure 4.12), although the results did not show statistically significant differences between genotypes (unpaired t-test).

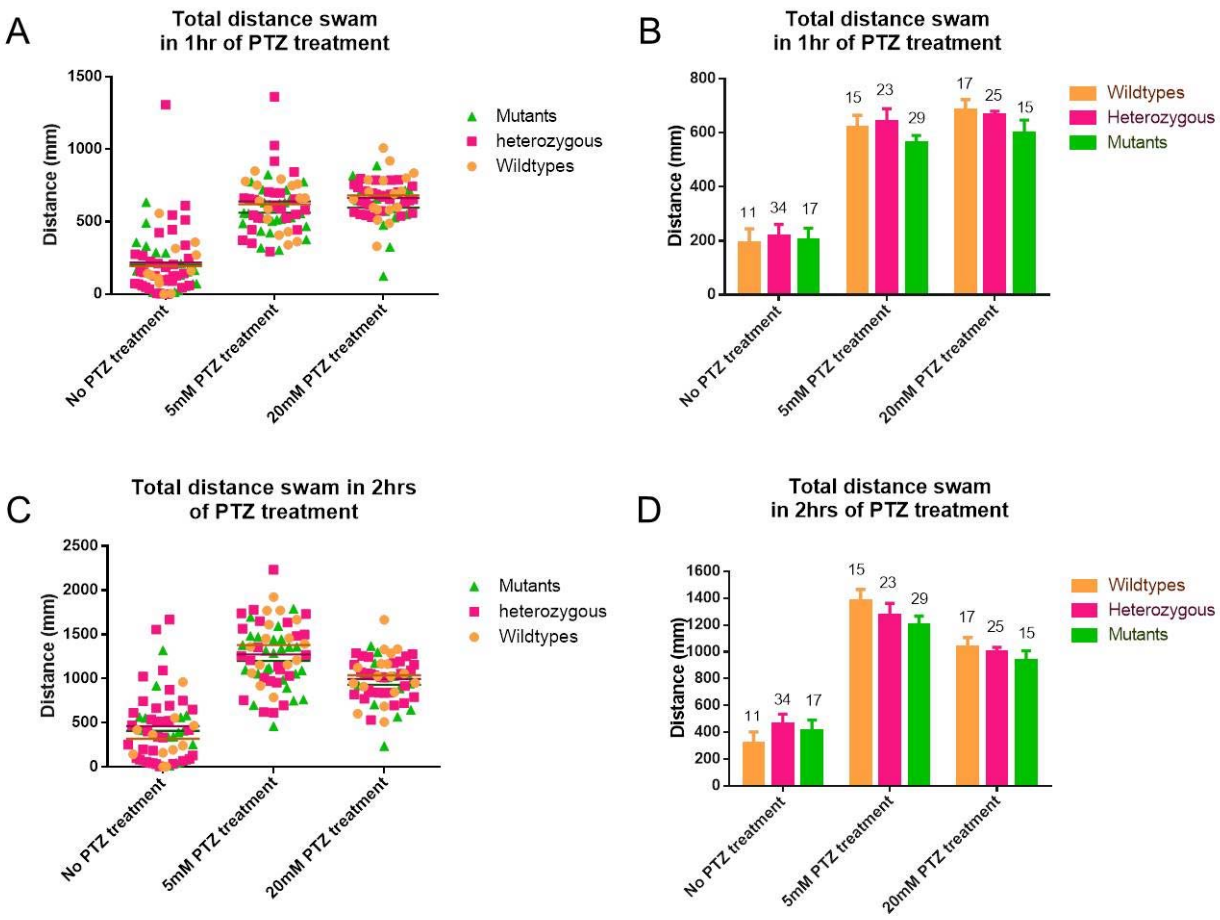


Figure 4.12 3dpf homozygous *npas4a* mutant and wild-type sibling larvae exhibit similar motor behavioural responses to PTZ treatment. Figures A and C show the distribution of homozygous, heterozygous and wild-type zebrafish larvae in response to the different treatments. Figures B and D show the total distance swam by the larvae after 1 hour (B) and 2 hours (D) of treatment (the numbers above the bars show the number of larvae analysed per condition). n=numbers above bars.

At 3 dpf, genotypes *sa701* homozygous mutant larvae developed normally and there were no apparent morphological or behavioural differences between them and genotyped wild-type siblings. There was however a possibility that the homozygotes might exhibit reduced viability at later stages, so the survival rates of *sa701* mutant and wild-type progeny were analysed.

sa701 zebrafish larvae were raised in 12-well plates (one larva per well), and maintained in the aquarium from day 5 until day 18 post fertilisation. The water was changed daily and the locomotor behaviours of the larvae were analysed on days 5, 11, 14 and 18, using the

Viewpoint Zebrabox. Larvae were checked each day and larvae that died were removed and genotyped. At 18 dpf all surviving larvae were then euthanised with tricaine and then genotyped. The survival plot shows that *sa701* homozygous and heterozygous mutant larvae exhibited slightly lower survival rates than their wild-type siblings (Figure 4.13).

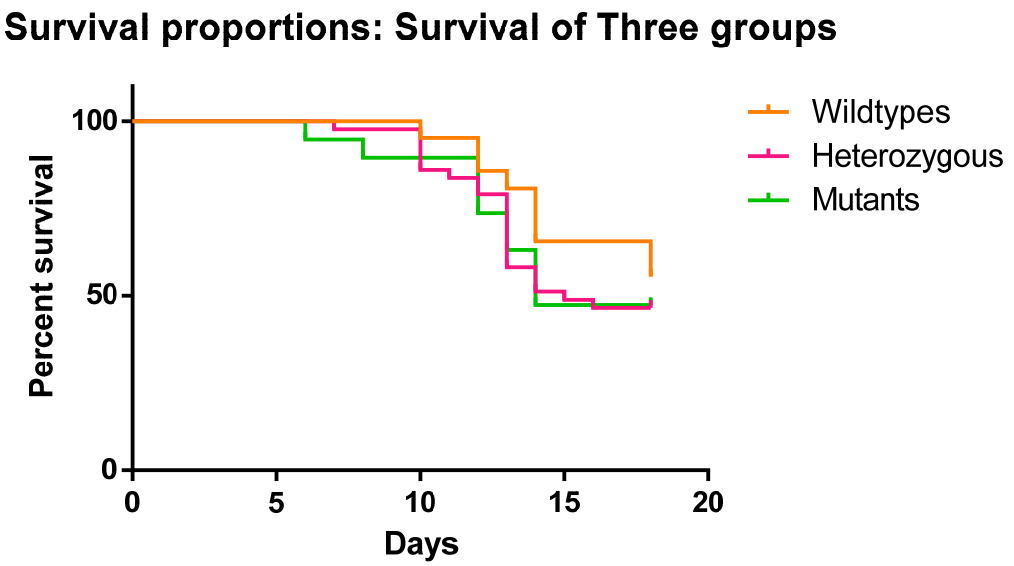


Figure 4.13 Survival rates of *sa701* zebrafish larvae. The survival data show a slightly lower survival rate for both homozygous and heterozygous *sa701* mutant larvae compared to their wild-type siblings, but the results are not statistically significant according to Log-rank (Mantel-Cox) test.

To monitor their locomotor behaviour, larvae were transferred into a 48-well plate (one larva per well) with 500µl of aquarium system water per well and their movements were recorded in the Zebrabox for one hour under a light protocol of 2 minutes lights on / 2 minutes light off (as explained in Chapter 3). After the recording was complete, larvae were returned to 12-well plates and maintained in the aquarium until day 18 post fertilisation. After statistical analysis of the data (t-test) no significant differences were detected in the swimming behaviour of *sa701* mutants compared to *sa701* heterozygous and wild-type siblings at 5, 11, 14 and 18 dpf (Figure 4.14).

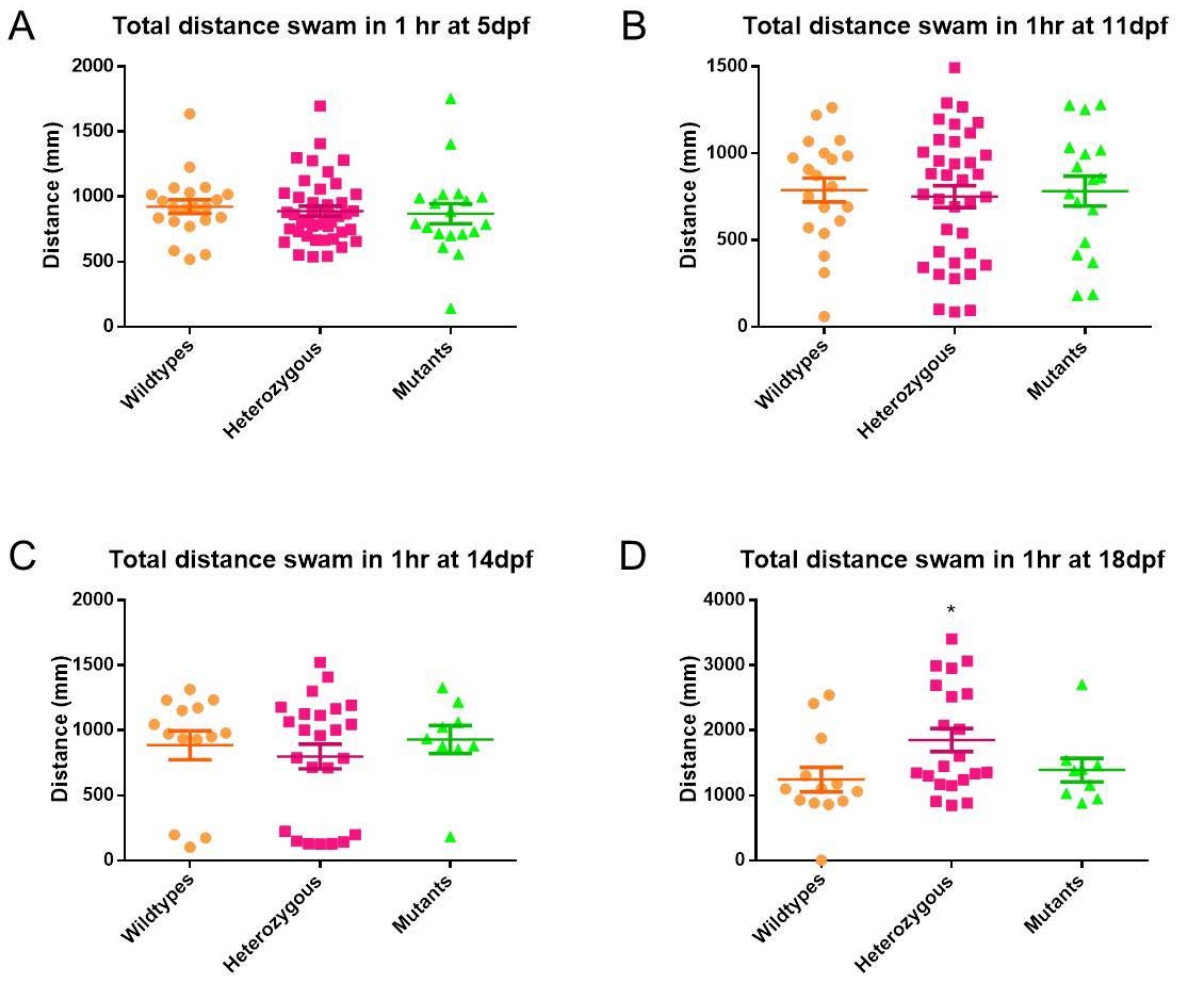


Figure 4.14 The swimming behaviour of *npas4a* mutant larvae is comparable to that of their wild-type siblings at 5, 11, 14 and 18dpf. Figures A, B, C and D show the distance swam by *sa701* larvae at 5 (A), 11 (B), 14 (C) and 18 dpf (D). Each dot, square or triangle represent one individual larva.

PTZ treatment resulted in a tendency of 3 dpf *sa701* mutants to respond less vigorously to PTZ than heterozygous and wild-type siblings (Figure 4.12). Older larvae were exposed to PTZ to determine whether larvae with different genotypes exhibited different locomotor responses to PTZ at later time points. Two tanks of approximately 50 larval progeny from an *sa701* heterozygous in-cross were raised to 10 dpf as a group in an aquarium tank (approx. 50 larvae per tank). At day 10 post fertilisation, larvae were transferred into a 48-well plate. Half of the larvae were treated with 20mM PTZ and the locomotor behaviours of all larvae were recorded for one hour using the Viewpoint system. After the recordings were completed, larvae were euthanised with tricaine and genotyped.

Unfortunately it was not possible to obtain a definitive conclusion from the data generated in this experiment because *sa701* homozygous mutant larvae exhibited an apparent loss of viability, unlike their heterozygous and wild-type siblings, which complicated the analysis in terms of numbers of mutant larvae available to perform a statistically valid test (Figure 4.15).

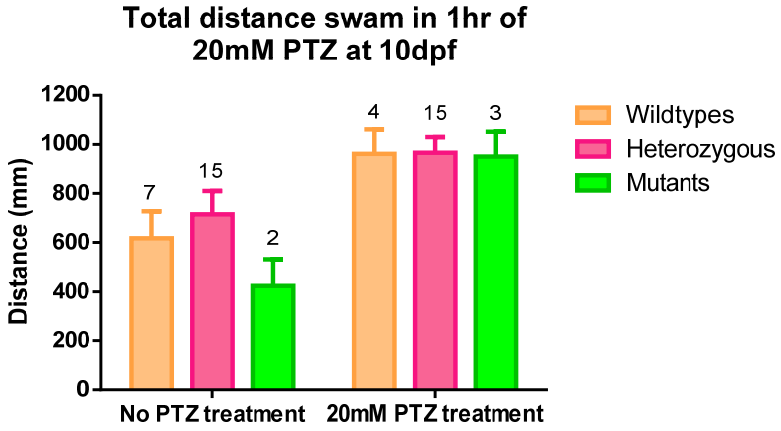


Figure 4.15 10 dpf *npas4* mutant larvae responded to PTZ exposure as strong as their wild-type siblings. Graph showing the total distance swam by 10 dpf larvae for one hour, without and with 20 mM PTZ treatment. The response to PTZ did not change between *sa701* wild-type, heterozygous and homozygous larvae. n=numbers above bars

4.3 Discussion

The synaptic activity-regulated genes *c-fos* and *Npas4* are rapidly induced in mammalian models of epilepsy, and I found that the zebrafish orthologues exhibited similar characteristics. The rapid and robust inductibility of these genes implied roles for them in the response to seizure-inducing stimuli, so I performed experiments to identify these roles using morpholinos to inhibit their expression and with a loss of function mutant in the zebrafish *npas4a* gene.

The approach used to study the function of *c-fos* in our zebrafish model of epilepsy was by using a splice-blocking morpholino. This morpholino bound to the intersection of exon 3 with intron 3-4 (Figure 4.1). RT-PCR analysis demonstrated that whilst wild-type uninjected embyos exhibited a robust increase in the level of correctly processed *c-fos* mRNA in response to PTZ (Figure 4.2 A), *c-fos* morphants did not exhibit a PTZ-induced increase in the correctly processed form of *c-fos* mRNA, and instead exhibited two fragments, one corresponding to the correctly processed transcript and one that is likely to be an incorrectly processed transcript that includes intron 3-4 (Figure 4.2 B), neither of which was increased in abundance by PTZ treatment.

The injection of the *c-fos* splicing blocking morpholino caused morphological defects in the zebrafish embryos, which included reductions in the size of the head and the eyes. This morphological phenotype can be caused by the activation of p53 as an off-target effect of morpholinos (Eisen & Smith, 2008; Robu et al., 2007) However, these morphological defects could not be rescued by coinjecting *c-fos* morpholino with *p53* morpholino, implying that these morphological abnormalities are p53-independent and unlikely to be the consequence of the p53-mediated off-target effect triggered by morpholinos. Synthetic *c-fos* mRNA was injected to zebrafish embryos along with the *c-fos* splice-blocking morpholino in order to determine whether the morphant phenotype was directly caused by the inhibition of endogenous *c-fos* expression. However, 50hpf zebrafish embryos injected with both *c-fos* morpholino and *c-fos* synthetic mRNA exhibited small heads and small eyes similar to those of the embryos injected

with *c-fos* morpholino alone (Figure 4.6). These results suggest that either the injected *c-fos* mRNA did not give rise to sufficient c-Fos protein to rescue the effect of the splice-blocking morpholino, or that a p53-independent off-target effect was responsible for the morphological abnormalities. Previous studies using the mouse line *f/fc-fos-cre*, which lacks the DNA binding domain and the leucine zipper domain of *c-fos*, showed that the expression of *bdnf* is positively regulated by *c-fos* in response to the convulsant kainic acid (Dong, et al., 2006; Zhang et al., 2002). In these published experiments, expression of *bdnf* was analysed after 2 and 4 hrs of kainic acid injection, and it was found that *c-fos* mutant mice exhibited reduced expression of *bdnf* in response to kainic acid compared to wild-type mice. In my experiments, *c-fos* morphants exhibited reduced levels of *bdnf* expression in response to PTZ treatment (Figure 4.4). However, injection of *c-fos* mRNA did not rescue the small head phenotype caused by the *c-fos* morpholino. Thus, the small head phenotype of *c-fos* appears to be a significant off-target effect that complicates the phenotypic analysis of *c-fos* morphants. The previously published studies of *c-fos* function in mouse suggested a role for this gene in protecting neurones from excitotoxic cell death during seizures (Zhang et al., 2002). When zebrafish *c-fos* morphants were analysed by TUNEL assay, I found that the level of apoptosis was significantly reduced in zebrafish embryos injected with a combination of *c-fos* and *p53* morpholinos, compared to the level of apoptosis shown by embryos injected with the *c-fos* morpholino alone, even though the small head phenotype was not rescued by p53 co-injection (Figure 4.7). These results indicate that *c-fos* splice-blocking morpholino might be inducing a p53-dependent apoptotic off-target effect or that the functions of *c-fos* and *p53* might be somehow related, as has previously been suggested (Kley et al., 1992; Elkeles et al., 1999.)

Unfortunately, *c-fos* morpholino injections caused severe developmental defects which included retarded growth within the CNS, making further analysis of the responses to PTZ in 3 dpf larvae, including locomotor behavioural studies, very difficult. To take the functional analysis of *c-fos* forward, ideally a targeted mutation in the *c-fos* gene should be created using

TALEN or CRISPR technology, which could then be exploited to investigate the role of *c-fos* in the responses to seizures at both the behavioural and molecular levels.

Previous studies in mice and rats have demonstrated that *Npas4* is necessary for *c-fos* and *Bdnf* expression in neurons (Bloodgood, et al., 2013; Pruunsild, et al., 2011; Ramamoorthi et al., 2011). Moreover, in a rodent model of learning and memory, *Npas4* expression reached a peak after 5 minutes of training, whereas the *c-fos* transcripts were most abundant after 30 minutes of training (Ramamoorthi et al., 2011). In addition, conditional deletion of *Npas4* in the CA3 region of the hippocampus resulted in reduced *c-fos* expression after kainic acid administration. The Npas4 transcription factor also binds to multiple DNA sequences within the alternative promoters I, IV and IX of *bdnf*, implying that Npas4 regulates *bdnf* expression directly. To investigate whether *npas4a* also regulates the expression of *c-fos* and *bdnf* in the brains of zebrafish larvae in response to PTZ exposure, zebrafish embryos were injected at 1 cell-stage with a splice-blocking morpholino designed to bind to the primary RNA transcript at the junction between exon 2 and intron 2-3 (Figures 4.8 and 4.9).

My results show that *npas4a* morphants treated with PTZ at 3 dpf exhibited reduced expression of *c-fos* and *bdnf*, compared with 3 dpf wild-type zebrafish larvae that were also treated with PTZ, which is in agreement with the previously published studies in rodents (Pruunsild et al., 2011; Ramamoorthi et al., 2011).

Interestingly, however, whilst PTZ-induced *c-fos* and *bdnf* expression was reduced in *npas4a* morphants, their convulsive response to PTZ was not altered (Figure 4.11); however, this might be due to the low numbers of larvae analysed. To further study the role of *npas4a* in seizures, the mutant zebrafish line *sa701* was analysed, which has a nonsense mutation at codon number 6 in the *npas4a* gene. Previous studies have shown that *Npas4* knockout mice (*Npas4* -/-) are prone to seizures, and exhibit behaviours that are characteristic of states of anxiety and hyperactivity (Y. Lin et al., 2008). However, I observed no abnormalities in the locomotor behaviour of *sa701*mutant zebrafish larvae, when they were compared with *sa701* wild-type siblings at 5, 11, 14 and 18 dpf (Figure 4.15). When *sa701* larvae were exposed to

PTZ, homozygous mutant larvae exhibited a small, statistically non-significiant reduction in the magnitude of the locomotor response to PTZ (Figure 4.12). Moreover, although homozygous mutants were morphologically indistinguishable from heterozygotes and wild-type siblings, clutches of larvae produced by in-crossing heterozygotes displayed variable levels of *c-fos* and *bdnf* expression by in situ hybridisation (Figure 4.13), suggesting the possibility that different genotypes within these clutches could express different levels of *c-fos* and *bdnf,* although these distinct phenotypes were not present in Mendielian ratios. Intriguingly, I observed that homozygous and heterozygous *sa701* mutant larvae exhibited a slightly lower survival rate that of their wild-type siblings. Similar observations have been reported for *Npas4* mutant mice (Coutellier, et al., 2012). Future studies should explore this reduced viability closely, along with more detailed analysis of the reduced level of PTZ-induced locomotor activity exhibited by homozygous *sa701* mutant larvae.

Chapter 5

5 Neural activity-dependent and stress hormone-mediated regulation of seizure severity in zebrafish larvae

5.1 Introduction

The occurrence of an epileptic seizure is a major traumatic event within the nervous system, activating many neural circuits within the brain, which can have severe adverse physiological consequences throughout the body. Sir William Gowers, the 19th century English physician who first described the clinical features of epilepsy, postulated that "seizures beget seizures" in some patients suffering from recurrent seizures, such that seizure severity increases progressively, potentially due to alterations to brain function caused directly by the seizures (Gowers, 1881). Other observational clinical studies have challenged the idea that epilepsy is a progressive disease, but a wide range of studies in experimental rodent seizure models have demonstrated that exposure of animals to low, subconvulsive doses of seizure-inducing convulsants can modify CNS function and increase their later susceptibility to spontaneous seizures (Reviewed by Morimoto, et al., 2004). Moreover, other forms of experience-dependent CNS activity, such as the neuroendocrine changes that are elicited by psychological and behavioural stressors, are also thought to lower the threshold for seizure induction in people with epilepsy (De Lima & Rae, 1991; Gilboa, 2012; Lai & Trimble, 1997; Yun et al., 2010a), suggesting that neural stress itself may erode resilience to the onset of seizures. The main aims of the experiments described in this chapter were (a) to investigate the effects on seizure severity of repeatedly exposing zebrafish larvae to seizure-inducing convulsants and (b) to determine the effect of administering glucocorticoid stress hormone on the severity of larval seizures induced by the convulsant PTZ. In addition, I sought to identify novel markers of the transcriptional response to seizure onset that might be useful molecular

markers of seizure severity, and whose potential roles as regulators of seizure severity could be explored.

5.1.1 Kindling

In adult rodent models of epilepsy, administration of a series of subconvulsive doses of convulsants or electrical discharges engenders the eventual occurrence of spontaneous seizures (White, 2002), which is a process known as kindling. The kindling model of epilepsy was developed by Graham Goddard in the late 1960s, although the first scientists to observe the kindling effect in cats were Delgado and Sevillano in 1961 (Delgado & Sevillano, 1961; Goddard, 1983; Morimoto, et al., 2004). Since then, the kindling model has been used as the main model to study temporal lobe epilepsy (TLE) in animals (McNamara, et al., 1980; Morimoto et al., 2004). The reason why kindling is a good model of TLE is because the amygdala, which is located in the temporal lobe, is the most susceptible structure of the brain in which seizure activity can be kindled (Kairiss, et al., 1984).

Kindling can be induced by direct electrical stimulation of the brain with implanted electrodes. Alternatively, rodents may be kindled by either localised injection of chemical convulsant into specific brain regions, or by systemic intravenous administration of convulsant via the blood stream (Reviewed by McNamara et al., 1980). Kindling by either electrical or chemical convulsant exposure may enhance excitatory neurotransmission or suppress inhibitory neurotransmission. Whilst many areas of the brain can be electrically stimulated to induce kindling, different areas can be distinguished by their distinct sensitivities to kindling agents. Goddard characterised these differential sensitivities and found that the amygdala was most easily modified by administration of subconvulsive doses of electrical stimulation in order to precipitate spontaneous seizures. The amygdala is situated in the medial temporal lobe, and the seizures of patients with Temporal Lobe Epilepsy (TLE), are often initiated in either the amygdala or the hippocampus (Falconer, et al., 1964).

Different kindling models of experimental epilepsy offer distinct practical advantages for investigating seizure mechanisms. Although it is a labour-intensive, technically demanding and surgically invasive technique, electrical kindling offers the advantage of targeting a specific area of the brain to induce focal activation of neural circuits (reviewed by Morimoto et al., 2004). Also, once the animals are fully kindled, the resulting spontaneous seizures can be studied without the need for further interventions such as administration of convulsants or other chemicals. Seizures can also be induced by repeated injection of subconvulsive doses of pilocarpine, which establishes spontaneous seizures reminiscent of TLE (Curia, et al., 2008), or by repeated injection of subconvulsive doses of Pentylenetetrazole, which sensitises rodent brains to further acute exposures (Mason & Cooper., 1972; Murphy, et al., 1989). The PTZ-induced kindling state can persist for at least 30 days after PTZ withdrawal in mice, suggesting that relatively long-term, persistent changes are introduced into CNS neural networks (Murphy et al., 1989).

5.1.2 Stress in epilepsy

An association between stress and epilepsy has long been identified by both physicians and patients with epilepsy. Whilst some clinical studies have focused on elucidating the relationship between stress and epilepsy, most of these are retrospective, qualitative analyses of patients' and clinicians' own experiences (reviewed by Lai & Trimble, 1997; van Campen, et al., 2013). The results of these types of studies might thus be somewhat subjective and difficult to interpret, due to individual variation in perception of and resilience to stressful situations, and difficulties in making objective measurements. Nevertheless, a variety of different studies in the topic have helped strengthen the idea of a link between stress and epilepsy (Reviewed by Novakova, et al., 2013). Patients are clearly able to recognise this link, and in one survey that asked patients with epilepsy to describe types of events, experiences and psychological states that triggered their seizures, 60% of all respondents identified psychological stress as a trigger for their seizures (Fisher et al., 2000).

At a physiological level, stressful experiences lead to the activation of the hypothalamic-pituitary-adrenal (HPA) axis. As a result of this activation, corticosteroid hormones are released into the bloodstream by the adrenal cortex and can bind to their cognate nuclear receptors in the brain, leading to altered transcription of Glucocorticoid Receptor-responsive genes. Interestingly, both acute and chronic administration of the rodent glucocorticoid, corticosterone, in rodent models of epilepsy, enhanced sensitivity to convulsions, elevated seizure frequency and increased discharge activity (Joëls, 2009). Moreover, chronic stress induced by repeated exposure of adult rats to restraint stress lowered the threshold for amygdala-kindled seizures and prolonged their duration (Jones et al., 2013). In addition, early-life stress induced in newborn rats by maternal separation increased seizure susceptibility to kindling at 7 weeks of age (Ali et al., 2013). Consistent with these findings, a recent study demonstrated that isolation stress reduced the time for adult zebrafish to develop a severe seizure phenotype in response to PTZ treatment (Pagnussat et al., 2013). Taken together, these studies suggest that stressful experiences cause neuroendocrine effects that can lower the threshold for seizure onset and potentially increase the severity of seizures elicited by seizure precipitants.

5.2 Results

5.2.1 Repeated exposure to PTZ modulates both the severity of the locomotor convulsive response and seizure-induced gene expression in zebrafish larvae.

In rodent models of epilepsy, seizures with convulsions can be triggered spontaneously after repeated exposures to electroshocks or a convulsant drug such as PTZ, kainic acid or pilocarpine. In order to explore the effects of experimentally inducing a series of multiple seizures on seizure severity, a repeated-exposure-to-PTZ paradigm was developed in which AB zebrafish larvae were treated with PTZ for a period of 1 hour on three consecutive days (1 dpf, 2 dpf, 3 dpf), and then either re-exposed to PTZ or analysed with E3 medium only on day 4, before locomotor behaviours and PTZ-induced gene expression were analysed (Figure 5.1 A). The convulsive locomotor behaviour of these larvae aged 4 dpf was monitored by tracking larval swimming movements during one hour of PTZ (or E3 control medium) treatment, using the Viewpoint Zebrabox under the Light Protocol of 2 minutes lights on / 2 minutes lights off as described in Chapter 3. The total distance swam was used as the parameter to quantify the magnitude of the resulting locomotor convulsive behaviour.

The results show that convulsive locomotor behaviour was greater for larvae that were treated with PTZ on each of four consecutive days of development, than that of larvae exposed to PTZ on day 4 only. Moreover, the basal locomotor activity of 4 dpf larvae never exposed to PTZ was greater than that of 4 dpf larvae that were treated with PTZ only on each of the first 3 days of development, and not on day 4 (Figure 5.1 B and C). Taken together, these results demonstrated that PTZ pre-treatment on days 1, 2 and 3 reduced basal locomotor activity, but increased the response to PTZ treatment on day 4. These results were very robust and the experiment was replicated 10 times, with similar results each time.

A

Groups	1 dpf	2 dpf	3 dpf	4 dpf
No treatment	-----	-----	-----	Analysis
Treatment on days 1, 2 and 3	PTZ Pre-treatment	PTZ Pre-treatment	PTZ Pre-treatment	Analysis
Treatment for 4 days	PTZ Pre-treatment	PTZ Pre-treatment	PTZ Pre-treatment	PTZ treatment + Analysis
Treatment on day 4 only	-----	-----	-----	PTZ treatment + Analysis

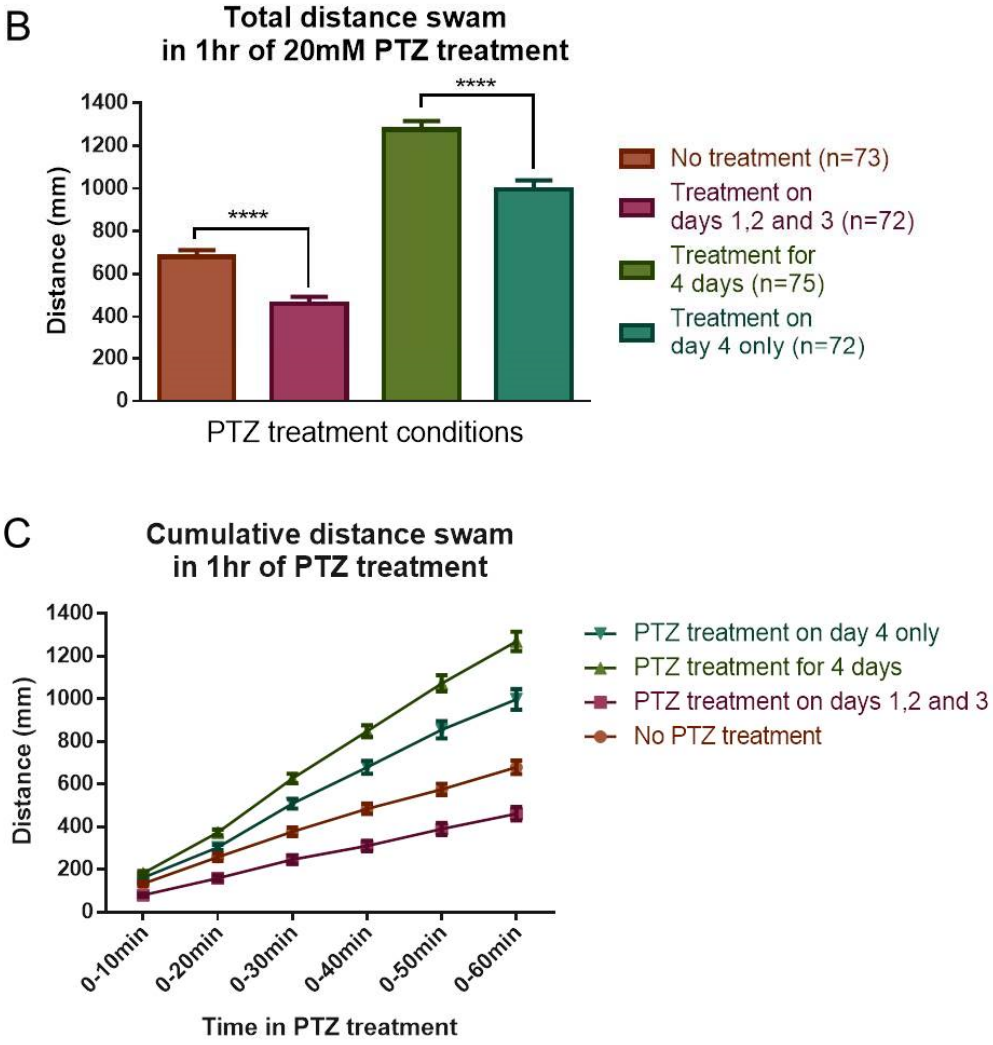


Figure 5.1 Behavioural and transcriptional responses of 4 dpf zebrafish larvae to repeated or single exposures to the seizure-inducing compound PTZ. A) Table describing the different treatments in the repeated exposure experiments. B, C) Locomotor convulsive behaviour of larvae subjected to different treatments. Locomotor activity of larvae that were exposed to PTZ for one hour on each of four days was greater than that of larvae that were exposed to PTZ on day 4 only. Values are given as means, error bars indicate s.e.m. **** indicates significantly different ($P<0.0001$) using an unpaired t-test, n represents he total number of larvae analysed over 10 different experiments (6-8 larvae per condition per experiment)

These results show that zebrafish larvae can be sensitised to PTZ from a very young age. However, it was not clear if three consecutive exposures to PTZ were necessary to enhance the response to the fourth treatment with PTZ. To determine how many times the zebrafish had to be pre-treated with PTZ in order to observe an enhanced locomotor convulsive response at 4 dpf, the developing embryos/larvae were treated with 20mM PTZ for one hour a day during day 1 only, day 2 only, day 3 only, days 1 and 2, and days 2 and 3.

First, 1, 2, and 3 dpf zebrafish were subjected to a single exposure to PTZ before day 4 to see if a single PTZ pre-treatment on any of these three days was sufficient to sensitize the response to the later exposure to PTZ on day 4. Zebrafish embryos/larvae were treated either at 1 dpf, 2 dpf or 3 dpf for one hour and then analysed at 4 dpf while they were in medium containing PTZ or control medium. Locomotor behaviours of 4 dpf larvae were recorded during the hour of PTZ treatment as described before using the Viewpoint Zebrabox. The only statistically significant difference between an experimental and control group was found for the zebrafish larvae that were treated with PTZ at 2 dpf but not at day 4, showing that PTZ treatment at 2 dpf reduced basal locomotor activity in comparison to that of the untreated controls (Figure 5.2 B). The rest of the results showed that pre-treatment of zebrafish embryos and larvae once only on day 1, day 2 or day 3 did not alter the severity of the response to PTZ treatment at 4 dpf (Figure 5.2).

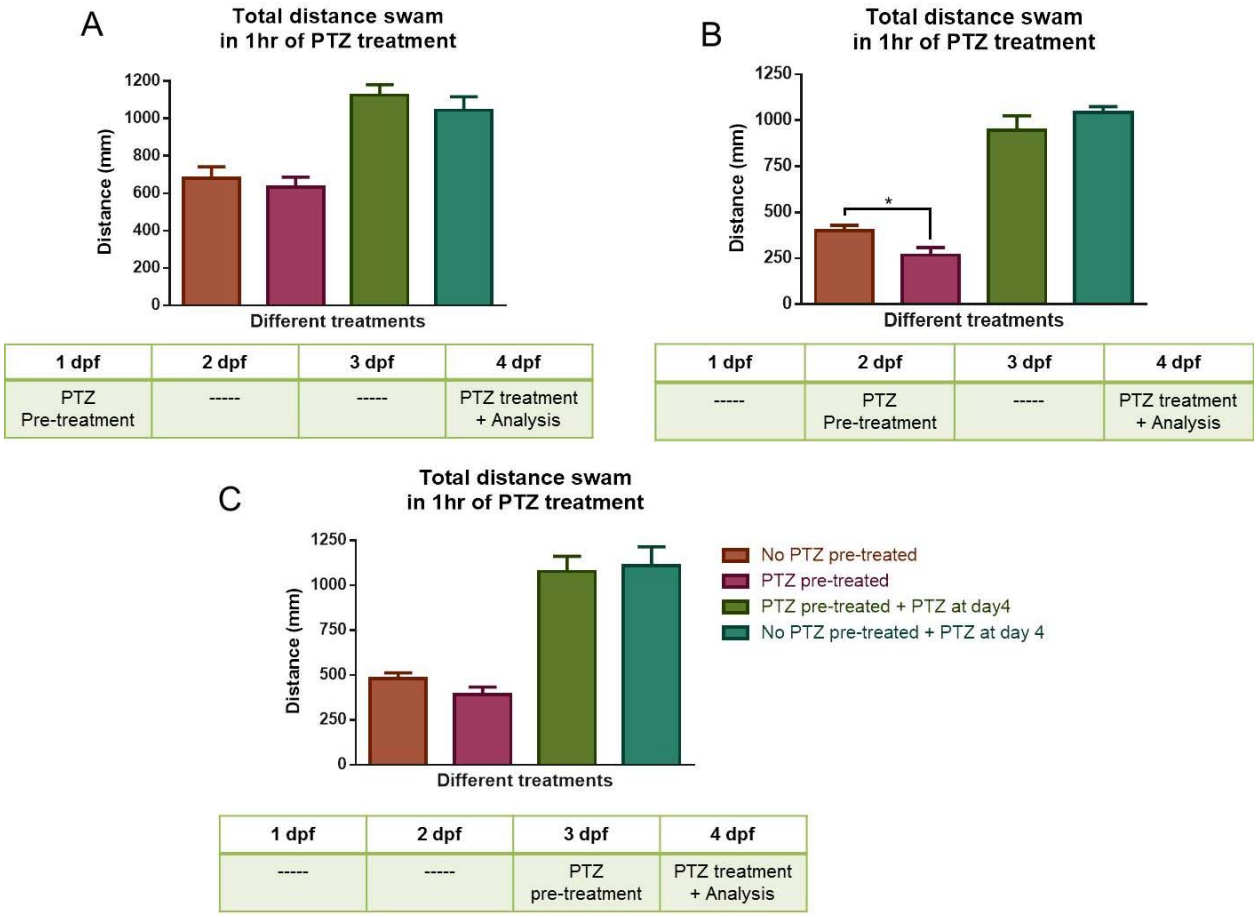
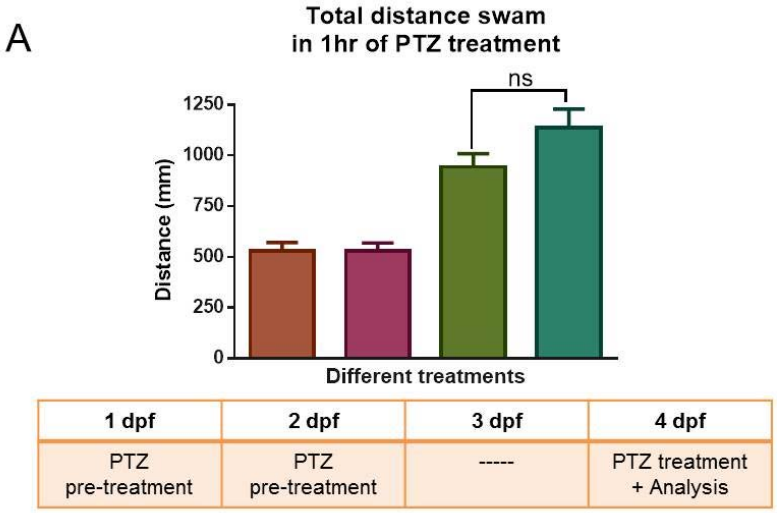


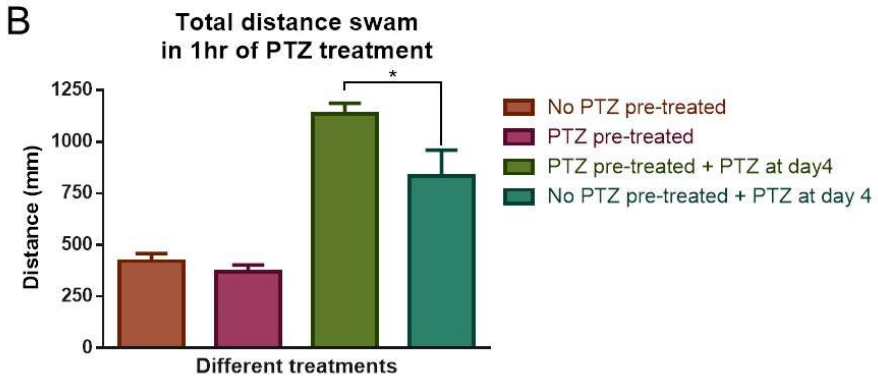
Figure 5.2 One-day pre-treatment of zebrafish embryos/larvae with PTZ did not increase locomotor behavioural sensitivity to a later exposure to PTZ on day 4. Histograms show the total distance swam by 4 dpf zebrafish larvae exposed to PTZ for one hour at 1 dpf (A), 2 dpf (B) or 3 dpf (C). Values are given as means, error bars indicate s.e.m. * indicates significantly different ($P<0.05$) using an unpaired t-test, n=17 to19 per group.

Next, I investigated whether pre-treatment of zebrafish embryos/larvae with PTZ for 1 hour on each of 2 consecutive days of early development was sufficient to change the magnitude of the locomotor response to PTZ at 4 dpf. Accordingly, embryos were treated on either days 1 and 2, or days 2 and 3. Whilst pre-treatment on days 1 and 2 did not affect locomotor responses to PTZ treatment on day 4, pre-treatment on days 2 and 3 significantly increased locomotor responses to PTZ treatment on day 4, in comparison to larvae that received no pre-treatment (Figure 5.3 B). However, the average of distance swam by larvae exposed to PTZ only on day 4 was lower compared to the other experiments shown in figures 5.2 A,B,C and

5.3 A. Therefore, pre-treatment with 20mM PTZ for one hour during days 1, 2 and 3 post fertilisation was the best protocol for inducing an enhanced locomotor response to PTZ during a fourth treatment.



1 dpf	2 dpf	3 dpf	4 dpf
PTZ pre-treatment	PTZ pre-treatment	-----	PTZ treatment + Analysis



1 dpf	2 dpf	3 dpf	4 dpf
-----	PTZ pre-treatment	PTZ pre-treatment	PTZ treatment + Analysis

Figure 5.3 Two-day pre-treatment with PTZ during days 2 and 3 post fertilisation increased locomotor behavioural sensitivity to a later exposure to PTZ on day 4. Graphs showing the total distance swam by 4 dpf zebrafish larvae exposed to PTZ for one hour at 1 and 2 dpf (A) or 2 and 3 dpf (B). Values are given as means, error bars indicate s.e.m. * indicates significantly different ($P<0.05$) using an unpaired t-test, n=12 per group.

To determine whether this treatment protocol affected the expression of synaptic activity-regulated genes, transcription of *c-fos* and *npas4a* was analysed after the last PTZ treatment.

Accordingly, after one hour of treatment on day 4, larvae were fixed in paraformaldehyde for *in situ* hybridisation analysis or transferred to RNAlater (Ambion) for RNA extraction and qPCR analysis (figure 5.4).

In situ hybridisation analysis demonstrated that there was a considerable reduction in the level of *c-fos* transcripts in the CNS of larvae treated with PTZ for 4 consecutive days, in comparison to the non-pre-treated larvae exposed once to PTZ at 4 dpf. A reduction of *npas4a* expression was not as clear-cut by in situ hybridisation as for *c-fos* (figure 5.4 A). However, qPCR analysis of 3 biological replicates showed a 30% reduction of expression of both *npas4a* and *c-fos* in PTZ pre-treated larvae treated on day 4 with PTZ compared with larvae treated on day 4 only (figure 5.4 B).

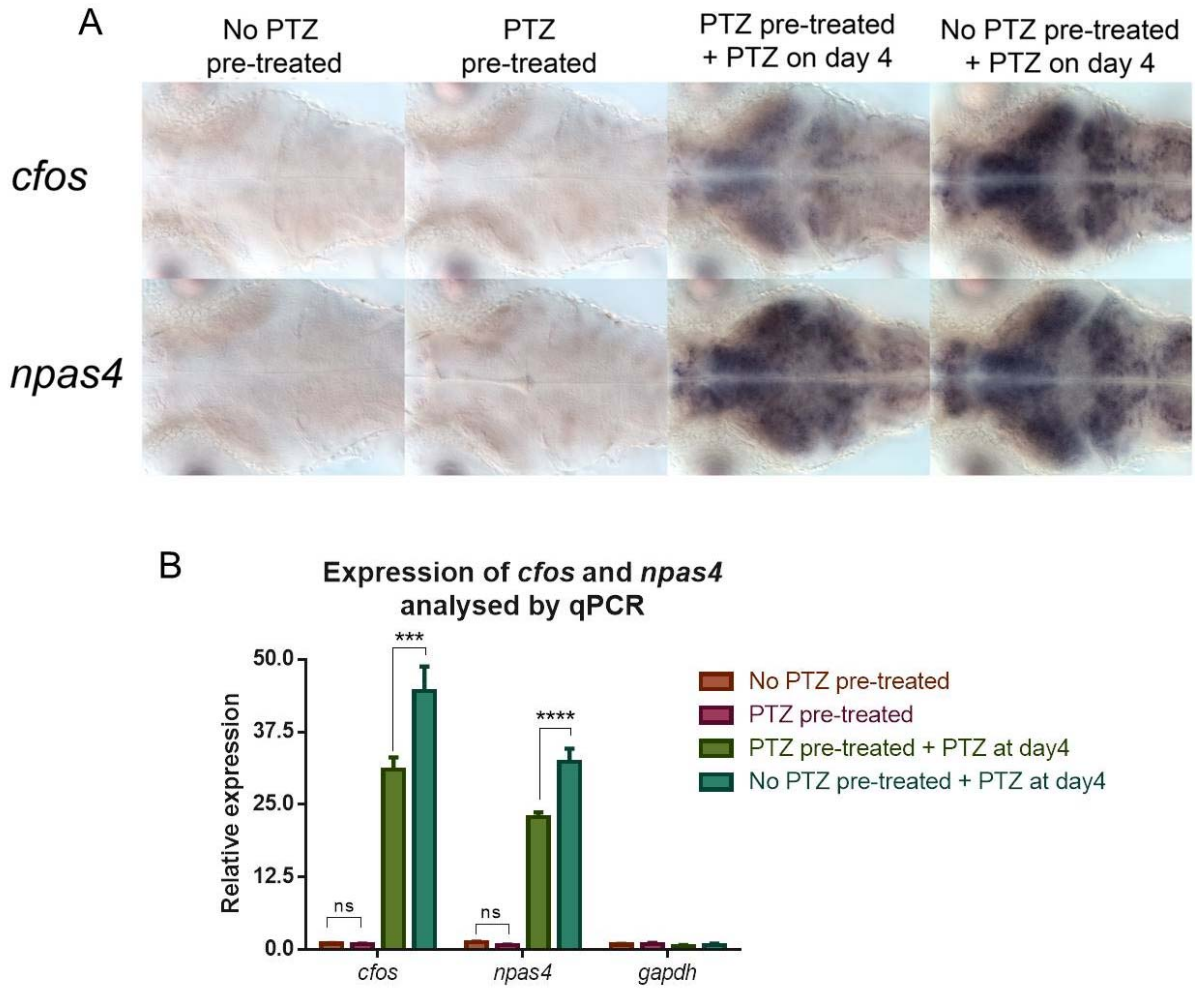


Figure 5.4 Expression of *c-fos* and *npas4a* transcripts in 4 dpf in control and PTZ-treated zebrafish larvae. A) *In situ* hybridisation analysis of *c-fos* and *npas4a* transcripts, showing a clear reduction of *c-fos* expression in zebrafish larvae pre-treated with PTZ on days 1, 2 and 3 then treated once again on day 4, in comparison to the level of *c-fos* transcription in zebrafish larvae treated with PTZ only on day 4. By contrast, a change in *npas4a* transcript levels was not so clear-cut in zebrafish larvae pre-treated with PTZ on days 1, 2 and 3 then treated once again on day 4. B) qPCR analysis *c-fos* and *npas4a* transcripts showing a consistent reduction of *c-fos* and *npas4*a expression in PTZ pre-treated larvae after PTZ treatment on day 4. Values are given as means, error bars indicate s.e.m. **** indicates significantly different ($P<0.0001$) using a one-way ANOVA with a Tukey's multiple comparison test. Every other comparison between groups analysing *c-fos* and *npas* expression, was significantly different according to the one-way ANOVA with Tukey's multiple comparison test with a p-value <0.00001.

5.2.2 Repeated exposure to the glutamate receptor agonist kainic acid does not modulate either the severity of the locomotor convulsive response or the expression of *c-fos* or *npas4a* in zebrafish larvae.

Interestingly, the increase in locomotor convulsive behaviour caused by repeated exposure of developing zebrafish embryos and larvae to PTZ was accompanied by a reduction in the level of *c-fos* and *npas4a* transcripts in the treated larvae, suggesting a possible functional link between the elevated locomotor activity and the reduced PTZ-induced expression of these genes. Alternatively, increased locomotor activity could also be due to altered neural activity that is independent of the transcription of these genes. To explore these possibilities further, locomotor behaviour and expression of *c-fos* and *npas4a* were compared in larvae treated with the convulsant agent kainic acid (Figure 5.5 A), which I previously observed did not induce transcription of either *c-fos* or *npas4a* (Chapter 3 – figure 3.15 C).

Larvae that were pre-treated with kainic acid for one hour, once only, during days 1, 2 or 3 of development did not exhibit an increased convulsive response when they were exposed again to kainic acid on day 4 (Figure 5.5 B and C). However, a statistically significant reduction of basal locomotor activity was observed in larvae that were pre-treated with kainic acid repeatedly, for one hour each day, over 3 consecutive days, compared to control larvae that were not exposed to kainic acid (figure 5.5 B – star).

A

Groups	1 dpf	2 dpf	3 dpf	4 dpf
No treatment	-----	-----	-----	Analysis
Treatment on days 1, 2 and 3	KA Pre-treatment	KA Pre-treatment	KA Pre-treatment	Analysis
Treatment for 4 days	KA Pre-treatment	KA Pre-treatment	KA Pre-treatment	KA treatment + Analysis
Treatment on day 4 only	-----	-----	-----	KA treatment + Analysis

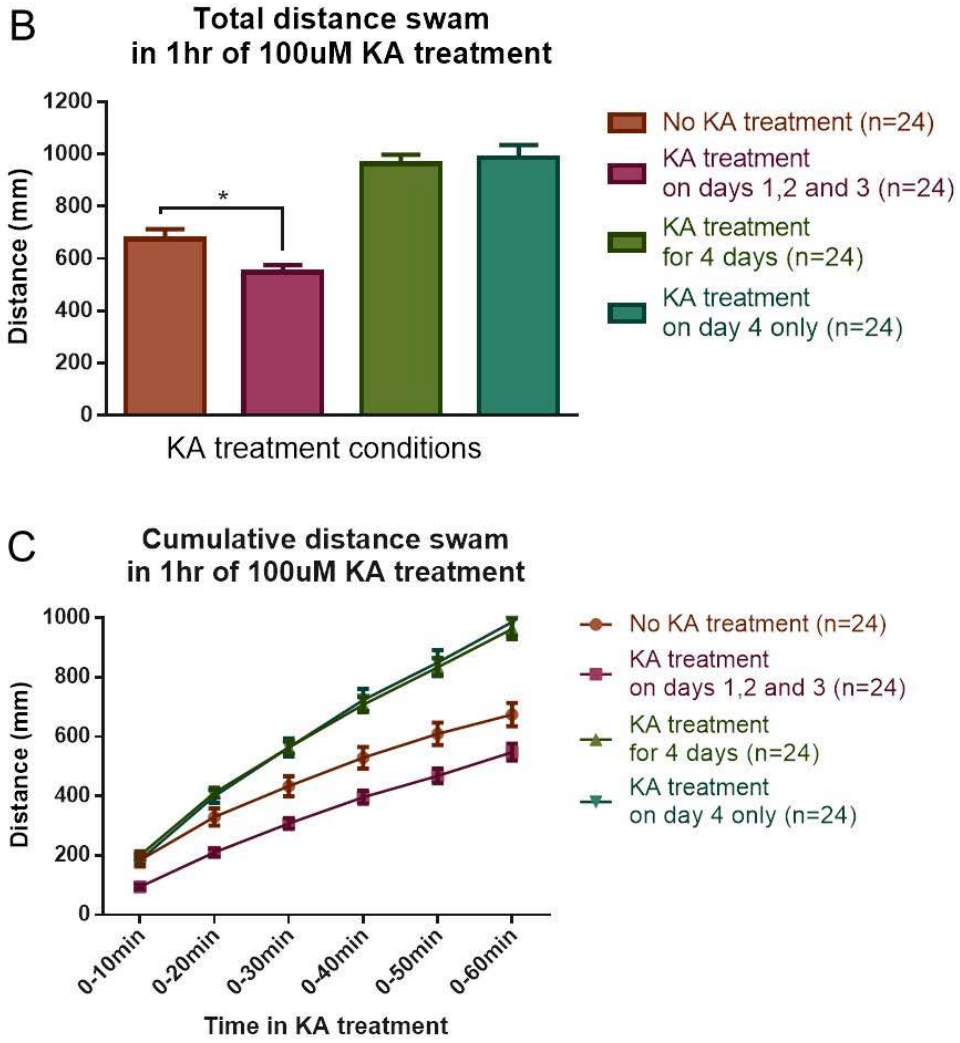


Figure 5.5 Locomotor behavioural responses to single or repeated treatment of zebrafish embryos/larvae with the seizure-inducing compound kainic acid. A) Table describes the different treatments in the repeated exposure experiments. B, C) Locomotor convulsive behaviour of larvae subjected to different treatments. Values are given as means, error bars indicate s.e.m. * indicates significantly different ($P<0.05$) using an unpaired t-test, n indicates the total number of larvae analysed in two separate experiments (12 larvae per condition per experiment).

The expression of *c-fos* and *npas4a* in 4 dpf larvae of the KA pre-treatment experiment was analysed by qPCR. The qPCR analysis showed that KA induced only a very modest, less than 2-fold increase of *c-fos* expression in 4 dpf larvae that were either previously untreated or repeatedly pre-treated with KA on days 1, 2 and 3 of development (Figure 5.6), consistent with the absence of detectable *c-fos* transcripts in KA-treated larvae analysed by situ hybridisation (Chapter 3 - Figure 3.15). Similarly, KA also induced a very modest ~2-fold increase in *npas4a* expression that was statistically significant in the non-pre-treated larvae compared to the untreated larvae when analysed by one-way ANOVA with Dunnett's test (Figure 5.6). Kainic acid pre-treatment did not appreciably alter the expression of either *c-fos* or *npas4a* compared to the larvae exposed to KA only once at 4 dpf.

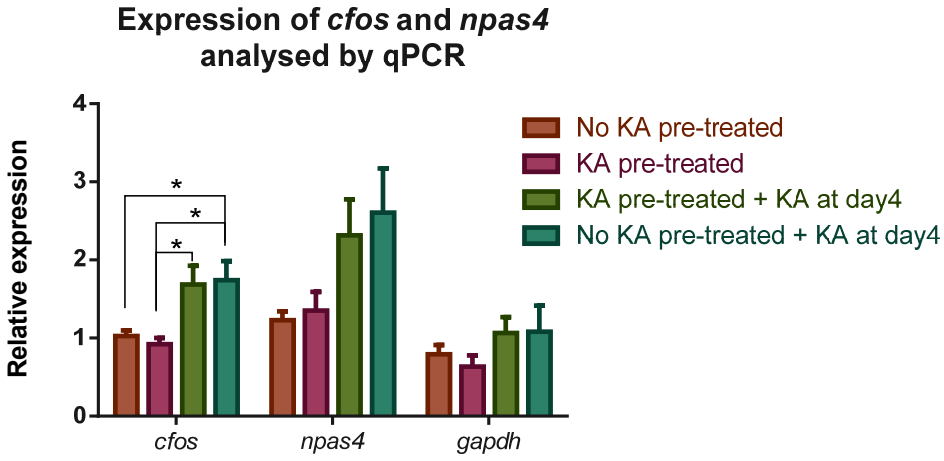


Figure 5.6. Expression of *c-fos* and *npas4a* in 4 dpf zebrafish larvae after single or repeated treatment with the seizure-inducing compound kainic acid (KA). qPCR analysis showing the reduction of *c-fos* and *npas4a* expression in PTZ pre-treated larvae also treated at day 4. Values are given as means, error bars indicate s.e.m. * indicates values significantly different from values in control untreated larvae ($P<0.01$) using a one-way ANOVA with a Tukey's multiple comparison test. Three biological replicate pools of ~20 embryos per condition were used for the analysis.

5.2.3 Analysis of apoptotic cell death and synaptic remodelling in PTZ pre-treated larvae.

My observation that repeated treatment of developing zebrafish embryos and larvae with PTZ enhanced the locomotor convulsive behavioural response to PTZ and reduced expression of synaptic activity-induced genes, suggested that the structure and function of the CNS was being stably altered by repeated exposure to PTZ. Potential mechanisms that might underlie these phenotypic changes include altered patterns of differentiated neurones due to excitotoxic cell death, or synaptic remodelling of neuronal circuits.

To test the possibility that PTZ induced neuronal apoptosis, a TUNEL assay was performed in PTZ-treated 4 dpf zebrafish larvae. As positive controls for apoptosis, larvae injured in the brain by insertion of a hypodermic needle and 4 dpf *gle1* mutant larvae were analysed (Figure 5.7 A). Previous studies of *gle1* mutant and morphant zebrafish embryos and larvae demonstrated that homozygous mutants exhibited an increased number of apoptotic cells (Jao, et al., 2012). *Gle1* mutant larvae were kindly provided by Dr John Wood (SiTraN, University of Sheffield).

PTZ/KA-treated, PTZ/KA-pre-treated and untreated control 4 dpf larvae were fixed for TUNEL assay after one hour of PTZ, KA, or E3 control medium treatment at 4 dpf. Whilst the needle-damaged positive control larvae exhibited extensive neuronal cell death in the injured brain (Figure 5.7 A – yellow arrow), no apoptotic cell death was detected by TUNEL assay in any of the 4 dpf zebrafish larvae that had been exposed to either PTZ or KA (Figure 5.7 B). Interestingly, only very limited numbers of apoptotic cells were visible in *gle1* mutant larvae.

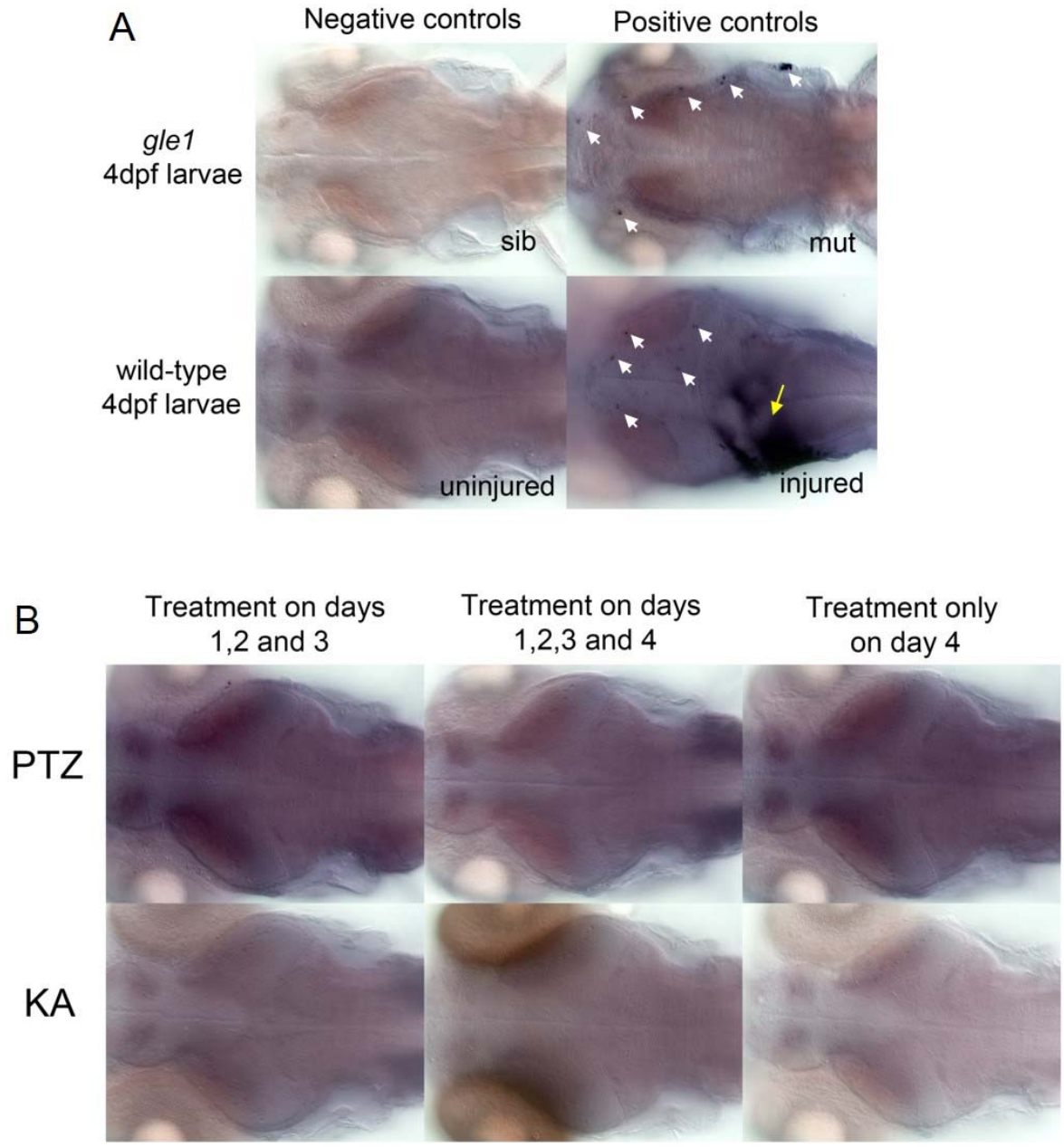


Figure 5.7 TUNEL assay of 4 dpf zebrafish larvae treated with PTZ or kainic acid. A) *gle1* wild-type siblings and uninjured larvae exhibited an absence of TUNEL-positive apoptotic cells, whereas *gle1* mutant larvae and larvae injured by a syringe needle displayed variable numbers of TUNEL-positive apoptotic cells (yellow/white arrows). B) Heads of 4 dpf zebrafish larvae subjected by different PTZ and KA treatments, showing no specific TUNEL staining of apoptotic cells.

To investigate the possibility that altered locomotor behaviour due to repeated exposure of zebrafish embryos/larvae to PTZ might involve changes in dendritic arborization, synapse formation or patterning, a transgenic line, *ptf1a:GFP,* in which GFP expression is restricted to

the cytoplasm (including axons) of *ptf1a*-expressing neurones, was used. *Ptf1a* (pancreas transcription factor 1 complex) is expressed in the cytoplasm of both GABA-ergic and glutamatergic neurons in the cerebellum and spinal cord of zebrafish larvae, and thus provides a good marker of neuronal morphology in these parts of the CNS (Aldinger & Elsen, 2008; Jusuf & Harris, 2009; Yamada et al., 2007). Accordingly, I sought to compare the expression pattern of the *ptf1a.GFP* transgene in PTZ pre-treated and un-pre-treated 4dpf larvae, after further exposure to PTZ or control E3 medium.

Ptf1a:GFP embryos were exposed to 20mM PTZ or control E3 medium only for one hour at 1, 2 and 3 dpf. At 4 dpf, larvae were then exposed to 20mM PTZ or control medium for a further one hour, then fixed in paraformaldehyde and imaged under the Confocal Microscope (Nikon). I found that the most informative region of the CNS in which to image *ptf1a:GFP* expression was the lateral hindbrain (Figure 5.8). The imaging results indicated that there were more complex dendritic networks in PTZ-pre-treated larvae than in control larvae, suggesting that repeated exposure to PTZ re-modelled dendritic arborisation and/or synapse formation. However, the data in the images were difficult to quantify, providing more of a qualitative impression than a definitive quantitative analysis. Further studies will therefore be required in order to draw firm conclusions.

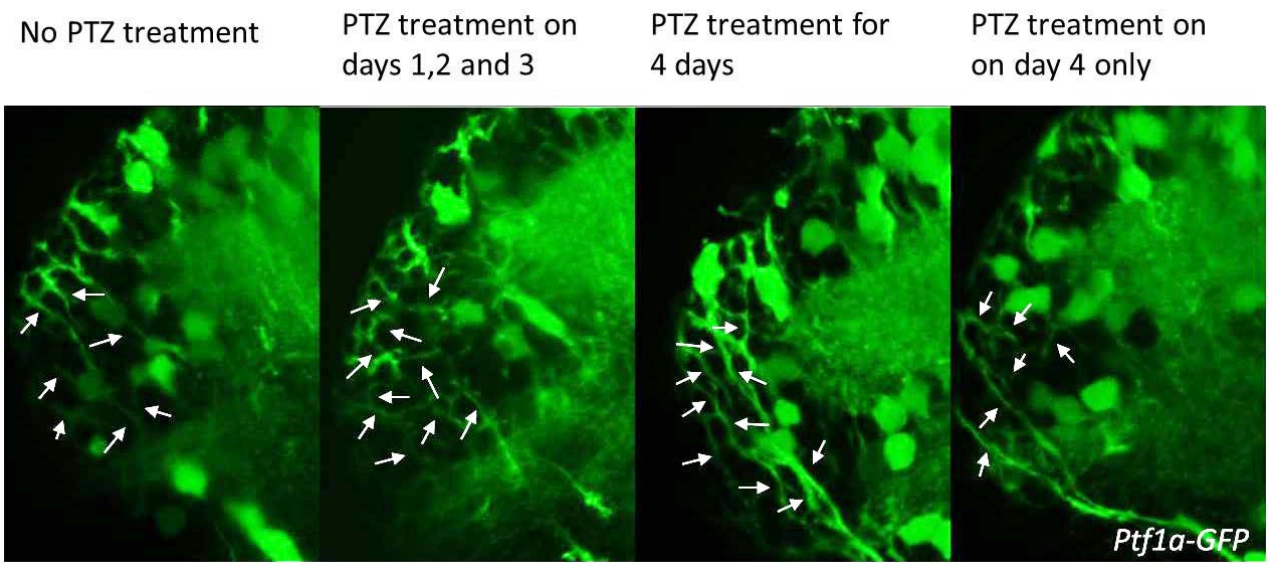


Figure 5.8 Confocal images of part of the lateral hindbrain of 4 dpf *ptf1a:GFP* larvae subjected to the treatments with PTZ or control medium as indicated, showing the expression pattern of ptf1a.GFP. White arrows show visible axon processes.

5.2.4 The enhanced severity of locomotor convulsive behaviour caused by repeated treatment of zebrafish embryos/larvae with PTZ is suppressed by the Histone Deacetylase inhibitor Trichostatin A.

The substantially increased level of locomotor convulsive behaviour in zebrafish larvae repeatedly exposed to PTZ was accompanied by a reduction in the level of PTZ-induced transcription of *c-fos* and *npas4a*, which suggested possible roles for the transcriptional silencing mechanisms which attenuated the transcription of these and potentially other genes, in promoting convulsive locomotor behaviour. Transcriptional silencing mechanisms involve chromatin regulatory proteins such as Histone Deacetylases (HDACs) and DNA methyltransferases, which regulate the distribution of acetylated histones and methylated DNA in chromatin, respectively. Therefore, to test for a potential role for transcription silencing in regulating the severity of locomotor convulsions, larvae were exposed to the HDAC inhibitor Trichostatin A either separately or in combination with PTZ. Thus, zebrafish embryos aged 1, 2 or 3 dpf were treated with either TSA alone for 3 hours or TSA for 2 hours followed by PTZ

for one hour, as required. Some of the larvae were treated again with PTZ only at day four and the swimming behaviours of all larvae (aged 4 dpf) were then analysed using the viewpoint Zebrabox during the one hour of PTZ treatment (Figure 5.9). The results show that treatment with TSA did not appreciably alter basal locomotor activity, nor did TSA treatment prevent the PTZ-induced increase in locomotor convulsive behaviour of 4 dpf larvae that were not pre-treated with PTZ (Figure 5.9). However, TSA pre-treatment did suppress the elevated increase in locomotor convulsive behaviour elicited by PTZ exposure on day 4 of larvae that had been pre-treated with PTZ (and TSA) on days 1, 2 and 3 of development. Thus, inhibition of HDAC activity suppressed the increase in locomotor convulsive behaviour caused by repeated exposure to PTZ, which is consistent with a role for HDAC-mediated transcription silencing in engendering the heightened severity of seizures caused by repeated exposure to PTZ. These results suggested a possible role for epigenetic mechanisms in conferring the heightened sensitivity to PTZ that results from repeated exposure to PTZ.

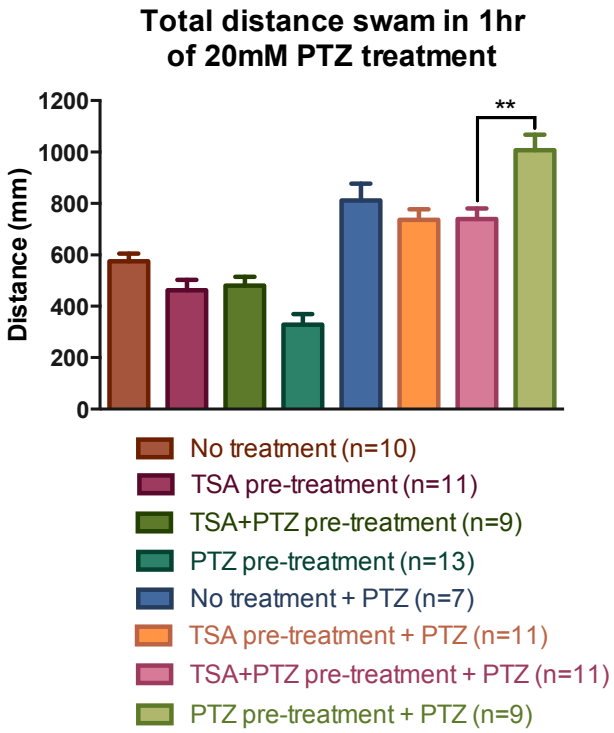


Figure 5.9 Locomotor behavioural responses of 4 dpf zebrafish larvae treated with TSA and/or PTZ. PTZ pre-treated larvae that were also pre-treated with TSA at 1, 2 and 3 dpf showed a statistically significant reduction to the severity of the response to the 4th treatment with PTZ. Values are given as means, error bars indicate s.e.m. ** indicates significantly different (P<0.001) using an unpaired t-test.

To explore the effects of TSA further, expression of *c-fos* was analysed by in situ hybridisation in larvae that were treated with PTZ and/or TSA (Figure 5.10). Interestingly, TSA severely suppressed PTZ-induced *c-fos* transcription, suggesting that the TSA-induced suppression of the elevated, PTZ-induced locomotor convulsive behaviour that was exhibited by PTZ-pre-treated larvae, was unlikely to be caused by TSA-mediated transcriptional de-repression of *c-fos*, and that other transcriptional targets may be involved.

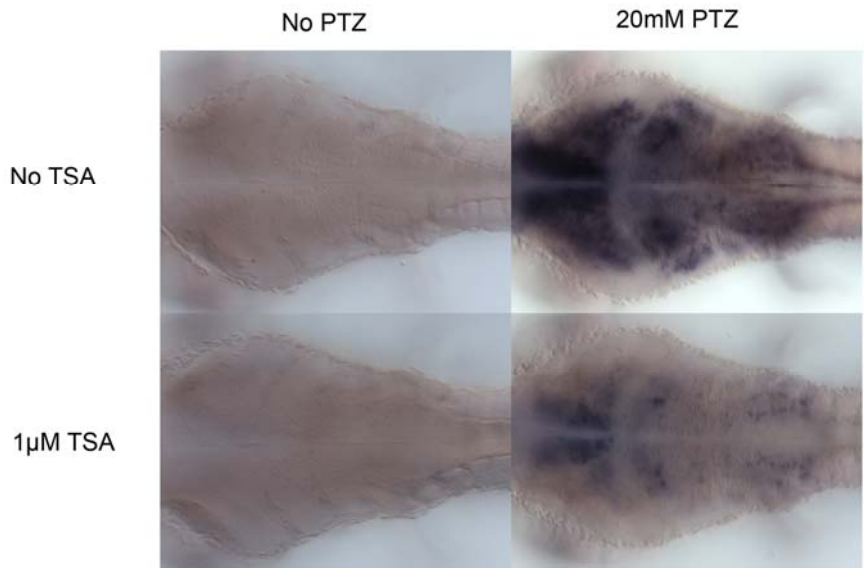


Figure 5.10 Treatment with 1μM TSA suppressed PTZ-induced *c-fos* expression in 3 dpf zebrafish larvae. TSA treatment reduced the expression of *c-fos* in response to PTZ treatment.

5.2.5 Glucocorticoid treatment increases the severity of PTZ-induced locomotor convulsive behaviour in zebrafish larvae

Epilepsy patients often report that stressful experiences are common triggers for their seizures (Frucht, et al., 2000; Haut, et al., 2007; Nakken et al., 2005; Novakova et al., 2013; Spector, et al., 2000; Sperling, et al., 2008), raising the possibility that HPA axis activation, leading to the synthesis and release of stress-responsive glucocorticoids such as cortisol, could lower the threshold for seizure onset. To test the possibility that systemic increases in glucocorticoid might enhance the severity or incidence of PTZ-induced seizures, zebrafish larvae were exposed to a synthetic analogue of cortisol, betamethasone, which had previously been demonstrated to be effective in zebrafish larvae (Griffiths et al., 2012).

3 dpf zebrafish larvae were treated with 25μM or 100μM betamethasone for one hour before they were exposed to 20mM PTZ for a further hour. Their swimming behaviour was monitored using the Viewpoint Zebrabox during the PTZ treatment. The results show that zebrafish larvae exposed to 100μM betamethasone prior to addition of 20mM PTZ exhibited a 55% increase in locomotor convulsive behaviour compared to that of larvae treated with 20mM PTZ

only (Figure 5.11). In contrast, exposure to 25μM betamethasone did not elicit a comparable increase in PTZ-induced locomotor activity. Interestingly, the increase in locomotor activity elicited by 100 μM betamethasone in the presence of 20mM PTZ was apparent from the very beginning of the behavioural recording period, and was maintained throughout the period of monitoring (Figure 5.11B).

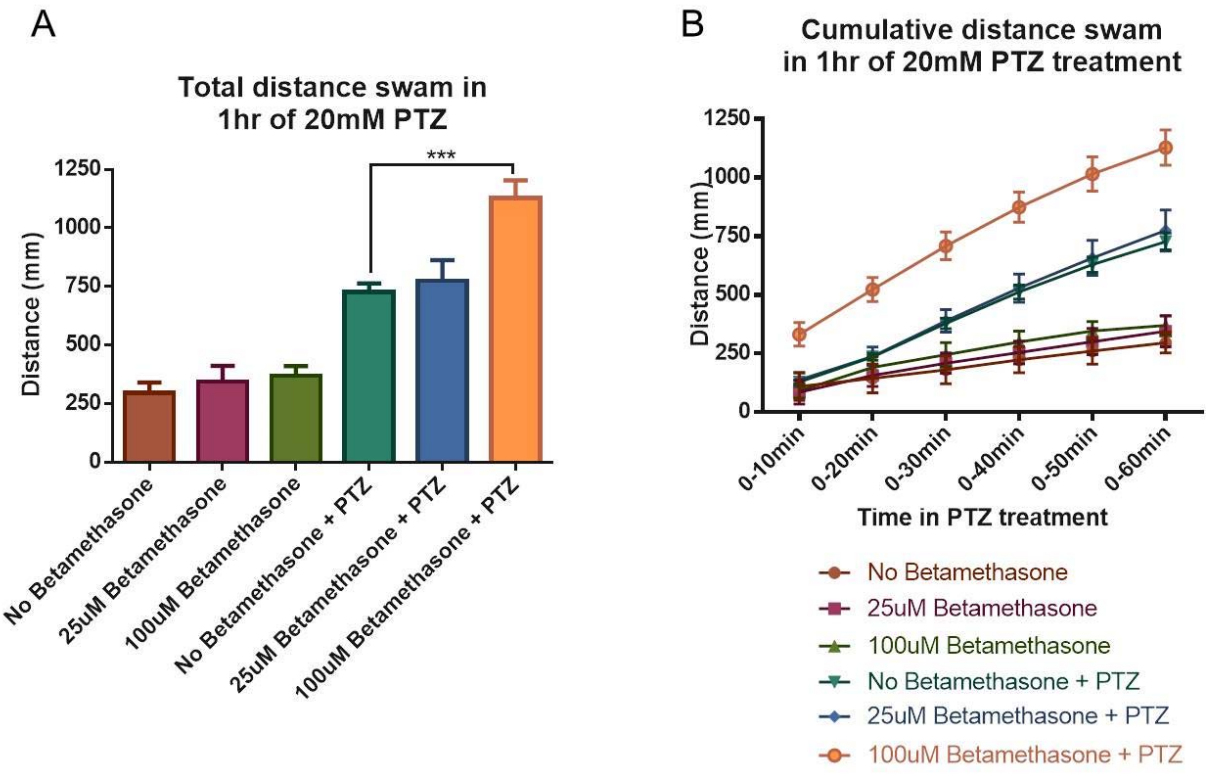


Figure 5.11 Locomotor behavioural responses of 3 dpf zebrafish larvae treated with betamethasone and PTZ. A) Total distance swam during one hour of 20mM PTZ treatment. B) Cumulative distance swam during one hour of 20mM PTZ treatment. Values are given as means, error bars indicate s.e.m. *** indicates significantly different (P=0.0005) using an unpaired t-test.

To determine whether betamethasone elicited effect on the expression of PTZ-inducible genes, an *in situ* hybridisation analysis for *c-fos*, *npas4a* and *bdnf* was performed. The larvae used for this assay were first treated with betamethasone for 2 hours, then fixed after an optimal period of exposure to PTZ, which varied according to the gene to be analysed. Thus, to detect *npas4a* transcripts, 3 dpf larvae were exposed to PTZ for 30 minutes, whereas to detect *c-fos* transcripts, larvae were exposed to PTZ for one hour, and to detect *bdnf*

transcripts, larvae were exposed to PTZ for 90 minutes (some aspects of this work were performed with the assistance of an undergraduate project student, Ellen Buckley). The results of these experiments demonstrated that PTZ-induced transcripts corresponding to *c-fos*, *npas4a* and *bdnf* were markedly reduced in larvae that received treatment with betamethasone before PTZ exposure, whereas all genes were robustly induced by PTZ in the absence of betamethasone treatment (Figure 5.12).

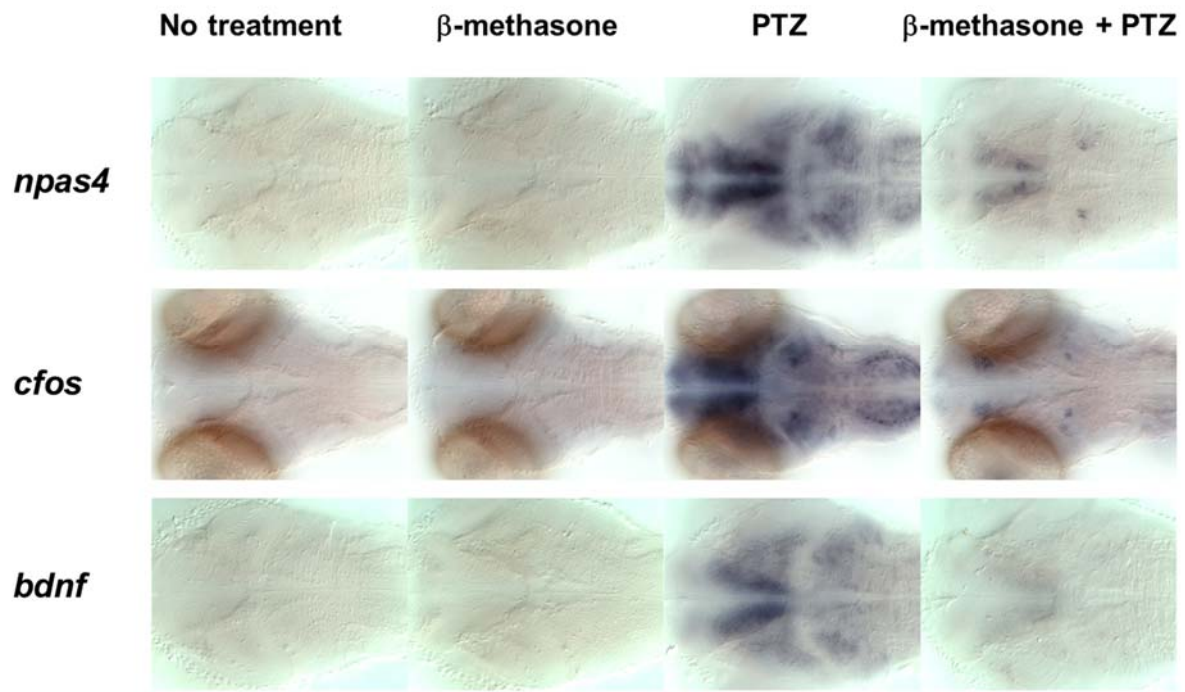


Figure 5.12 Betamethasone exposure extinguishes PTZ-induced expression of *npas4a*, *c-fos* and *bdnf* transcripts in 3 dpf zebrafish larvae. Dorsal views of the heads of control untreated 3 dpf zebrafish larvae and sibling larvae treated with betamethasone and/or PTZ, analysed by in situ hybridisation for expression of *npas4a*, *c-fos* and *bdnf*.

The fact that betamethasone treatment strongly enhanced the magnitude of the PTZ-induced locomotor convulsive behaviour and simultaneously extinguished expression of *npas4a*, *c-fos* and *bdnf* suggested the possibility of a functional link between seizure severity and the expression of these genes. One possibility consistent with these observations is that the seizure-induced expression of *npas4a*, *c-fos* and *bdnf* may be neuroprotective, limiting the severity of the seizure-induced locomotor convulsive behaviour. Moreover, a further possibility

is that betamethasone could reduce the threshold concentration of PTZ required to induce strong locomotor convulsions. Thus, I hypothesised that in addition to increasing the magnitude of the locomotor convulsive response to seizure-inducing concentration of PTZ, betamethasone exposure might also lower the concentration of PTZ that is effective in inducing a clear-cut increase in locomotor convulsive behaviour. To investigate this possibility, 3 dpf larvae were first exposed to betamethasone for one hour after which PTZ was added to the medium at a concentration of either 0, 5 or 20 mM, and larval locomotor convulsive behaviour was monitored using the Viewpoint Zebrabox over an additional period of one hour.

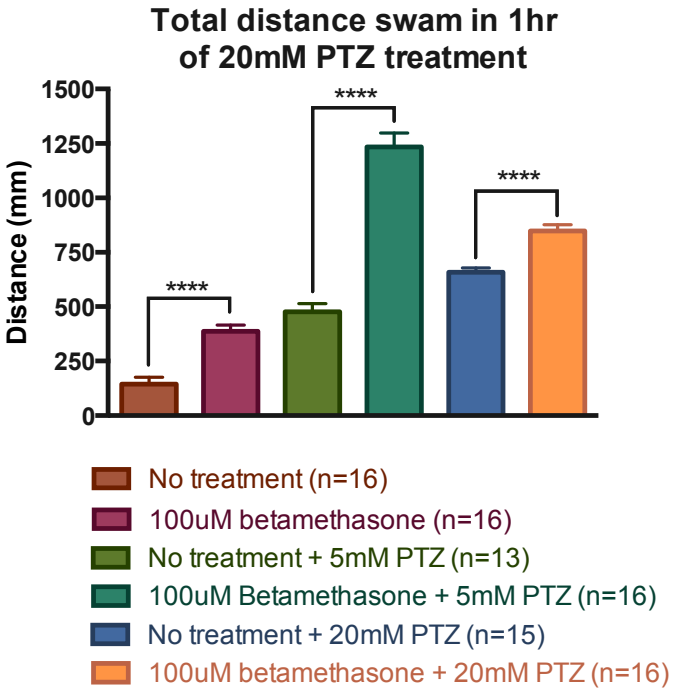


Figure 5.13 Locomotor behavioural responses of 3 dpf zebrafish larvae treated with betamethasone and/or 5mM or 20mM PTZ. Total distance swam during one hour of PTZ treatment. Values are given as means, error bars indicate s.e.m. *** indicates significantly different (P=0.0005) using an unpaired t-test.

The results of this experiment clearly demonstrate that betamethasone robustly enhanced, by more than two-fold, the locomotor convulsive behaviour of larvae triggered by 5mM PTZ (Figure 5.13). Interestingly, the locomotor responses of larvae treated with betamethasone and 20mM PTZ were lower than those of larvae treated with betamethasone and 5mM PTZ,

although 20mM PTZ alone elicited a stronger behavioral response than did exposure to 5 mM PTZ alone.

5.2.6 Microarray analysis of PTZ-induced gene transcription in 3 dpf zebrafish larvae

My results indicated that repeated treatment of zebrafish larvae with PTZ, exposure to the HDAC inhibitor TSA, and exposure to the glucocorticoid betamethasone each modified the magnitude of the locomotor convulsive responses of larvae to subsequent PTZ treatment. Moreover, betamethasone also enhanced larval motor responsiveness to low, minimally convulsive concentrations of PTZ, consistent with the hypothesis that glucocortocoid stress hormones lower the threshold of PTZ required to elicit a strong, convulsion-inducing seizure. Many of the above treatments also exerted effects on the level of PTZ-induced gene transcription that accompanied altered locomotor behavioural responses, suggesting the possibility that gene expression changes might play roles in modulating the locomotor convulsions elicited by subsequent PTZ treatment. In order to adopt an unbiased approach to identifying such gene expression changes, I undertook an initial genome-wide analysis of the transcriptional response to PTZ treatment, employing a two-colour microarray-based gene expression platform (Harrison,et al., 2011). In order to capture both rapid and more delayed (potentially secondary) changes in gene expression, I analysed the transcriptomes of 3 dpf larvae exposed to PTZ for 30 minutes and 90 minutes, along with those of control untreated larvae. Accordingly, 3 dpf larvae were collected after being exposed to PTZ for 30 or 90 minutes. Control untreated larvae were also collected at each time point. Total RNA was extracted, and cDNA synthesis for the microarray analysis was done following the protocol provided by Agilent for two-colour microarray-based gene expression analysis. To identify gene expression changes elicited by 30 minutes of PTZ treatment, cDNA samples from larvae treated with PTZ for 30 minutes and control larvae were labelled with Cy3 and Cy5, respectively, and co-hybridised to duplicate arrays. A second pair of cDNA samples from PTZ-treated and control larvae were labelled with Cy5 and Cy3, respectively (dye-swap samples),

and co-hybridised to a second pair of arrays. To identify gene expression changes elicited by 90 minutes of PTZ treatment, the same protocol was employed: cDNA samples from larvae treated with PTZ for 90 minutes and control larvae were labelled with Cy3 and Cy5, respectively, and co-hybridised to duplicate arrays. A second pair of cDNA samples from PTZ-treated and control larvae were labelled with Cy5 and Cy3, respectively (dye-swap samples), and co-hybridised to a second pair of arrays. The hybridised arrays were washed and scanned, and fluorescence signals were transformed to log base 2 to detect changes in gene expression levels. The values of the signals from the PTZ-treated samples values were subtracted from the values of the control samples to identify genes exhibiting fold-changes greater than 1.00. Only fold-changes that were confirmed for each gene on either 3 or 4 of the 4 arrays analysed were reported (Tables 5.1, 5.2).

The results show that although 30 minutes of PTZ treatment was sufficient to induce a transcriptional response, it was after 90 minutes of exposure to PTZ that a more substantial change in gene expression was observed. As expected, *npas4a* was one of the genes that was strongly up-regulated after only 30 minutes of PTZ treatment. Of the three genes initially used to characterise the PTZ model of seizure induction in 3 dpf zebrafish larvae (*c-fos*, *npas4a* and *bdnf*), *npas4a* was most strongly expressed after 30 minutes of PTZ exposure (Chapter 3 - Figure 3.13). Moreover, *npas4a* was the top hit, showing the greatest increase in PTZ-induced expression (8.5-fold increase) of all genes on the array after 30 minutes of PTZ treatment (Table 5.1). Many of the genes that were found up-regulated after 30 minutes of exposure to PTZ encode transcription factors, such as *c-fos*, *fosb* and *junbb* (Table 5.3).

After 90 minutes of PTZ treatment, more than 50 genes were significantly up-regulated; 16 of these genes encode proteins that are involved in gene transcription, and others are involved in processes such as apoptosis, ion homeostasis, neuronal remodelling and muscle contraction (Table 5.4). Consistent with my observations of gene expression changes after 30 minutes of exposure to PTZ, after 90 minutes of exposure to PTZ, the genes *c-fos* and *npas4a*

were among the genes that were up-regulated the most, showing 10-fold and 8-fold increases, respectively (Table 5.2).

Interestingly, of the 18 genes that were strongly up-regulated after 30 minutes of PTZ treatment and the 50 genes that were up-regulated after 90 minutes of exposure to PTZ, 5 of these genes encode proteins of unknown function, suggesting the potential involvement of novel molecular mechanisms in the pathogenesis of epilepsy and/or the response to seizure onset. The results of the microarray experiments will be useful to understand which genes respond to seizure induction and how they modulate the severity of the seizure response under different types of experiences like seizure recurrence or stress.

Table 5.1 Principal gene expression changes in 3 dpf zebrafish larvae after 30 minutes of exposure to the convulsant agent PTZ

Up-regulated genes

Gene Name	Gene Symbol	Accession Number	Log2 Fold Change	Standard Error	Fold Change
Danio rerio neuronal PAS domain protein 4a	*npas4a*	NM_001045321	3.08936	0.39214	8.511
Danio rerio v-fos FBJ murine osteosarcoma viral oncogene homolog (fos)	*cfos*	NM_205569	2.63798	0.23494	6.225
Danio rerio zgc:175128	*zgc:175128*	NM_001114453	2.34433	0.49062	5.078
Danio rerio cDNA clone FDR107-P00062-BR_H09 5-	*wu:fj62d01*	EH506070	2.02805	0.36345	4.079
Danio rerio suppressor of cytokine signaling 3a	*socs3a*	NM_199950	2.01569	0.27366	4.044
Danio rerio si:ch211-235e18.3	*si:ch211-235e18.3*	NM_001126383	1.93908	0.11580	3.835
Danio rerio cDNA clone FDR306-P00016-BR_L07 5-	*wu:fc62b05*	EH584150	1.79685	0.13846	3.475
Danio rerio FBJ murine osteosarcoma viral oncogene homolog B	*fosb*	NM_001007312	1.73992	0.28173	3.340
Danio rerio immediate early response 2	*ier2*	NM_001142583	1.69453	0.17533	3.237
Danio rerio B-cell translocation gene 2	*btg2*	NM_130922	1.64827	0.18098	3.135
Danio rerio jun B proto-oncogene b	*junbb*	NM_212750	1.61690	0.19397	3.067
Danio rerio zgc:113232, transcript variant 1	*zgc:113232, transcript variant 1*	NM_001014359	1.56589	0.11383	2.961
Danio rerio dual specificity phosphatase 1	*dusp1*	NM_213067	1.44623	0.12226	2.725
Danio rerio zgc:113232, transcript variant 2	*zgc:113232, transcript variant 2*	NM_001127513	1.43290	0.17823	2.700
Danio rerio calymmin	*cmn*	NM_131014	1.28655	0.19166	2.439
Danio rerio collagen, type IX, alpha 1b	*col9a1*	NM_213264	1.20701	0.03710	2.309
Uncharacterised protein	*Uncharacterised protein*	ENSDART00000099252	1.18900	0.06754	2.280
Danio rerio growth arrest and DNA-damage-inducible, beta a	*gadd45ba*	NM_213031	1.18238	0.15880	2.270

Down-regulated genes

Gene Name	Gene Symbol	Acession Number	Log2 Fold Change	Standard Error	Fold Change
Danio rerio desumoylating isopeptidase 1a	*desi1a*	NM_200700	1.64649	0.22911	3.1307

*Fold change = $2^{\text{Log2 fold change}}$

Table 5.2 Principal gene expression changes in 3 dpf zebrafish larvae after 90 minutes of exposure to the convulsant agent PTZ.

Up-regulated genes

Gene Name	Gene Symbol	Accession Number	Log2 Fold Change	Standard Error	Fold Change
Danio rerio v-fos FBJ murine osteosarcoma viral oncogene homolog (fos)	*cfos*	NM_205569	3.32106	0.25313	9.994
Danio rerio zgc:175128	*zgc:175128*	NM_001114453	3.02998	0.50002	8.168
Danio rerio neuronal PAS domain protein 4a	*npas4a*	NM_001045321	2.98672	0.74857	7.927
Danio rerio jun B proto-oncogene b	*junbb*	NM_212750	2.75994	0.29496	6.774
Danio rerio insulin-like growth factor binding protein 1a	*igfbp1a*	NM_173283	2.73886	0.19574	6.675
Danio rerio cDNA clone FDR107-P00062-BR_H09 5-	*wu:fj62d01*	EH506070	2.71951	0.34829	6.586
Danio rerio semaphorin 3d (sema3d)	*sema3d*	NM_131048	2.61936	0.42924	6.145
Danio rerio cytochrome P450, family 24, subfamily A, polypeptide 1	*cyp24a1*	NM_001089458	2.45346	0.37472	5.477
titin-cap (telethonin)	*tcap*	ENSDART00000007293	2.42865	0.39496	5.384
Danio rerio Jun dimerization protein 2	*jdp2*	NM_001002493	2.29578	0.39586	4.910
Danio rerio immediate early response 2	*ier2*	NM_001142583	2.29071	0.22897	4.893
Danio rerio si:ch211-235e18.3	*si:ch211-235e18.3*	NM_001126383	2.22780	0.31824	4.684
Danio rerio B-cell translocation gene 2	*btg2*	NM_130922	2.21798	0.21395	4.652
Danio rerio cytochrome P450, family 1, subfamily A	*cyp1a*	NM_131879	2.18407	0.20244	4.544
Danio rerio FBJ murine osteosarcoma viral oncogene homolog B	*fosb*	NM_001007312	2.16305	0.31199	4.479
Danio rerio matrix metalloproteinase 9	*mmp9*	NM_213123	2.16183	0.31506	4.475
Danio rerio nuclear receptor subfamily 4, group A, member 1	*nr4a1*	NM_001002173	2.15477	0.20494	4.453
cAMP responsive element modulator a	*crema*	ENSDART00000025254	2.10919	0.17135	4.314
Danio rerio suppressor of cytokine signaling 3a	*socs3a*	NM_199950	2.06829	0.33672	4.194
Danio rerio ankyrin repeat domain 9	*ankrd9*	NM_001007294	2.06759	0.36550	4.192
Danio rerio activating transcription factor 3 (atf3)	*atf3*	NM_200964	2.06300	0.34085	4.179
Danio rerio zgc:153932	*zgc:153932*	NM_001083007	2.05732	0.30127	4.162
Danio rerio methylsterol monooxygenase 1	*msmo1*	NM_213353	1.94807	0.33819	3.859

Table 5.2 Principal gene expression changes in 3 dpf zebrafish larvae after 90 minutes of exposure to the convulsant agent PTZ (Cont.)

Gene Name	Gene Symbol	Accession Number	Log2 Fold Change	Standard Error	Fold Change
Danio rerio growth arrest and DNA-damage-inducible, beta a	*gadd45ba*	NM_213031	1.90768	0.19119	3.752
Danio rerio insulin induced gene 1	*insig1*	NM_199869	1.89628	0.14488	3.723
Danio rerio midnolin	*midn*	NM_207052	1.86027	0.18418	3.631
Danio rerio growth arrest and DNA-damage-inducible, alpha, b	*gadd45ab*	NM_001002216	1.80052	0.09473	3.483
Danio rerio pyruvate dehydrogenase kinase, isoenzyme 2	*pdk2*	NM_200996	1.79484	0.21967	3.470
Danio rerio heat shock protein, alpha-crystallin-related, b11	*hspb11*	NM_001099427	1.79127	0.29106	3.461
Danio rerio Ras-related associated with diabetes	*rrad*	NM_199798	1.78530	0.32071	3.447
Danio rerio si:ch73-141c7.1	*si:ch73-141c7.1*	NM_200789	1.77546	0.41293	3.423
Danio rerio fos-like antigen 2	*fosl2*	NM_001082998	1.77068	0.15151	3.412
Danio rerio diablo, IAP-binding mitochondrial protein a	*diabloa*	NM_200346	1.76076	0.15977	3.389
Danio rerio Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4a	*cited4a*	NM_001044982	1.74424	0.18020	3.350
Danio rerio CCAAT/enhancer binding protein (C/EBP), beta	*cebpb*	NM_131884	1.70963	0.16814	3.271
Danio rerio dual specificity phosphatase 5	*dusp5*	NM_212565	1.68929	0.35180	3.225
Danio rerio Rh family, C glycoprotein a	*rhcga*	NM_001089577	1.68819	0.16642	3.223
Danio rerio cDNA clone FDR306-P00016-BR_L07 5-	*wu:fc62b05*	EH584150	1.68679	0.27142	3.219
Danio rerio prostaglandin-endoperoxide synthase 2b	*ptgs2b*	NM_001025504	1.67248	0.19793	3.188
Danio rerio insulin-like growth factor binding protein 1b	*igfbp1b*	NM_001098257	1.67109	0.06490	3.185
Danio rerio zgc:110340	*zgc:110340*	NM_001013472	1.64963	0.27976	3.138
Danio rerio regulator of G-protein signaling 13	*rgs13*	NM_001089512	1.64864	0.19857	3.135
Danio rerio period circadian clock 2	*per2*	NM_182857	1.58656	0.24557	3.003
Danio rerio purine nucleoside phosphorylase 5b	*pnp5b*	NM_001004628	1.57669	0.19752	2.983
Danio rerio cytochrome P450, family 3, subfamily A, polypeptide 65	*cyp3a65*	NM_001037438	1.56189	0.20227	2.952
Danio rerio growth arrest and DNA-damage-inducible, alpha, a	*gadd45aa*	NM_200576	1.53376	0.15848	2.895
Danio rerio brain-derived neurotrophic factor	*bdnf*	NM_131595	1.53139	0.07485	2.891

Table 5.2 Principal gene expression changes in 3 dpf zebrafish larvae after 90 minutes of exposure to the convulsant agent PTZ (Cont.)

Gene Name	Gene Symbol	Accession Number	Log2 Fold Change	Standard Error	Fold Change
Danio rerio LON peptidase N-terminal domain and ring finger 1, like	*lonrf1l*	NM_001277234	1.52804	0.23292	2.884
Danio rerio lipin 1	*lpin1*	NM_001044353	1.52681	0.17204	2.881
Danio rerio nuclear receptor subfamily 4, group A, member 2b	*nr4a2b*	NM_001002406	1.45673	0.13930	2.745
Danio rerio RERG/RAS-like a	*rergla*	NM_001002494	1.45143	0.15911	2.735
Danio rerio titin a	*ttna*	BC091692	1.07403	0.02398	2.105

Down-regulated genes

Gene Name	Gene Symbol	Acession Number	Log2 Fold Change	Standard Error	Fold Change
Danio rerio hatching enzyme 1	*he1*	NM_213635	2.20055	0.17863	4.597
Danio rerio wu:fa26c03	*wu:fa26c03*	NM_001105115	2.13650	0.23250	4.397
Danio rerio si:dkey-26g8.5	*si:dkey-26g8.5*	NM_001103115	2.01236	0.20813	4.034
Danio rerio Cathepsin L1-like	*MGC174155*	NM_001103118	1.96521	0.15803	3.905
Danio rerio zgc:174855	*zgc:174855*	NM_001103122	1.92786	0.30993	3.805
Danio rerio cathepsin L, 1 b	*ctsl1b*	NM_131198	1.92645	0.15464	3.801
Danio rerio lecithin retinol acyltransferase a	*lrata*	NM_001204131	1.42921	0.27269	2.693

*Fold change = $2^{Log2\ Fold\ Change}$

Table 5.3 Function of genes whose expression is altered in 3 dpf zebrafish larvae after 30 minutes of exposure to the convulsant agent PTZ.			
Up-regulated genes			
Gene Symbol	**Type of protein**	**Function**	**Pathways**
npas4a	bHLH: Basic helix-loop-helix proteins	Transcription factor	
cfos	bZIP: basic leucine zipper proteins	Transcription factor	MAPK, Ras, Toll, Nod, KEGG
zgc:175128	zinc finger C2H2	Transcription factor	
wu:fj62d01	*Uncharacterised protein*		
socs3a	STAT-induced STAT inhibitor	Protein kinase inhibitor activity	JAK-STAT, STAT3, IL-9,4,10
si:ch211-235e18.3	serine/threonine-protein kinase SIK1	Protein kinase activity	
wu:fc62b05	*Uncharacterised protein*		
fosb	leucine zipper proteins	Transcription factor	NOD, IL-17, IL-2, TCR
ier2		Transcription factor	
btg2	BTG/Tob family	Anti-proliferative protein/transcription coregulator	cell cycle control by BTG, RNA degradation, p53
junbb	bZIP: basic leucine zipper proteins	Transcription factor	NOD, IL-17, Ras
zgc:113232, transcript variant 1	*Uncharacterised protein*		
dusp1	DUSPT: Protein tyrosine phosphatases / Class I Cys-based PTPs : MAP kinase phosphatases	Dephosphorylates MAP kinase MAPK1/ERK2	MAPK, ERK, EGFR, TGF-beta
zgc:113232, transcript variant 2	*Uncharacterised protein*		
cmn	Constitutive protein of the ECM compartment associated to the perinotochordal sheath in the zebrafish embryo	It is specifically expressed by the differentiating notochord cells	
col9a1	COLPG: Proteoglycans / Extracellular Matrix : Collagen proteoglycans	Structural component of hyaline cartilage	Integrin pathway, ERK, MAPK, PTEN
Uncharacterised protein			
gadd45ba		Involved in the regulation of growth and apoptosis	p53, MAPK
Down-regulated genes			
Gene Symbol	**Type of protein**	**Function**	**Pathways**
desi1a	Belongs to the DeSI family	Protease which deconjugates SUMO1, SUMO2 and SUMO3 from some substrate proteins.	

Table 5.4 Function of genes whose expression is altered in 3 dpf zebrafish larvae after 90 minutes of exposure to the convulsant agent PTZ.			
Up-regulated genes			
Gene Symbol	**Type of protein**	**Function**	**Pathways**
cfos	bZIP: basic leucine zipper proteins	Transcription factor	MAPK, Ras, Toll, Nod, KEGG
zgc:175128	zinc finger C2H2	Transcription factor	
npas4a	bHLH: Basic helix-loop-helix proteins	Transcription factor	
junbb	bZIP: basic leucine zipper proteins	Transcription factor	NOD, IL-17, Ras
igfbp1a	Insulin-like growth factor binding protein (IGFBP) family	Prolongs the half-life of the IGFs, promotes cell migration	IGF1, PERK
wu:fj62d01	*Uncharacterised protein*		
sema3d	Semaphorin	Induces the collapse and paralysis of neuronal growth cones	Axon guidance
cyp24a1	Cytochrome P450 superfamily	Has a role in maintaining calcium homeostasis	cytochrome P450, metabolism of steroid hormones and vitamin D
tcap	Sarcomeric Protein	It serves as a scaffold to which myofibrils and other muscle related proteins are attached	Striated Muscle Contraction
jdp2	bZIP: basic leucine zipper proteins	Transcription factor	ATF-2
ier2		Transcription factor	
si:ch211-235e18.3	Serine/threonine-protein kinase SIK1	Protein kinase activity	
btg2	BTG/Tob family	Anti-proliferative protein/transcription co-regulator	cell cycle control by BTG, RNA degradation, p53
cyp1a	Cytochrome P450 superfamily	It oxidizes steroids, fatty acids, and xenobiotics	cytochrome P450, arachidonic acid metabolism
fosb	bZIP: basic leucine zipper proteins	Transcription factor	NOD, IL-17, IL-2, TCR
mmp9	Matrix metalloproteinase	May play an essential role in local proteolysis of the extracellular matrix and in leukocyte migration	Cell adhesion ECM remodelling, integrin pathway
nr4a1	Nuclear hormone receptors	Participates in energy homeostasis by sequestrating the kinase STK11 in the nucleus, thereby attenuating cytoplasmic AMPK activation	MAPK

Table 5.4 Function of genes whose expression is altered in 3 dpf zebrafish larvae after 90 minutes of exposure to the convulsant agent PTZ. (Cont.)

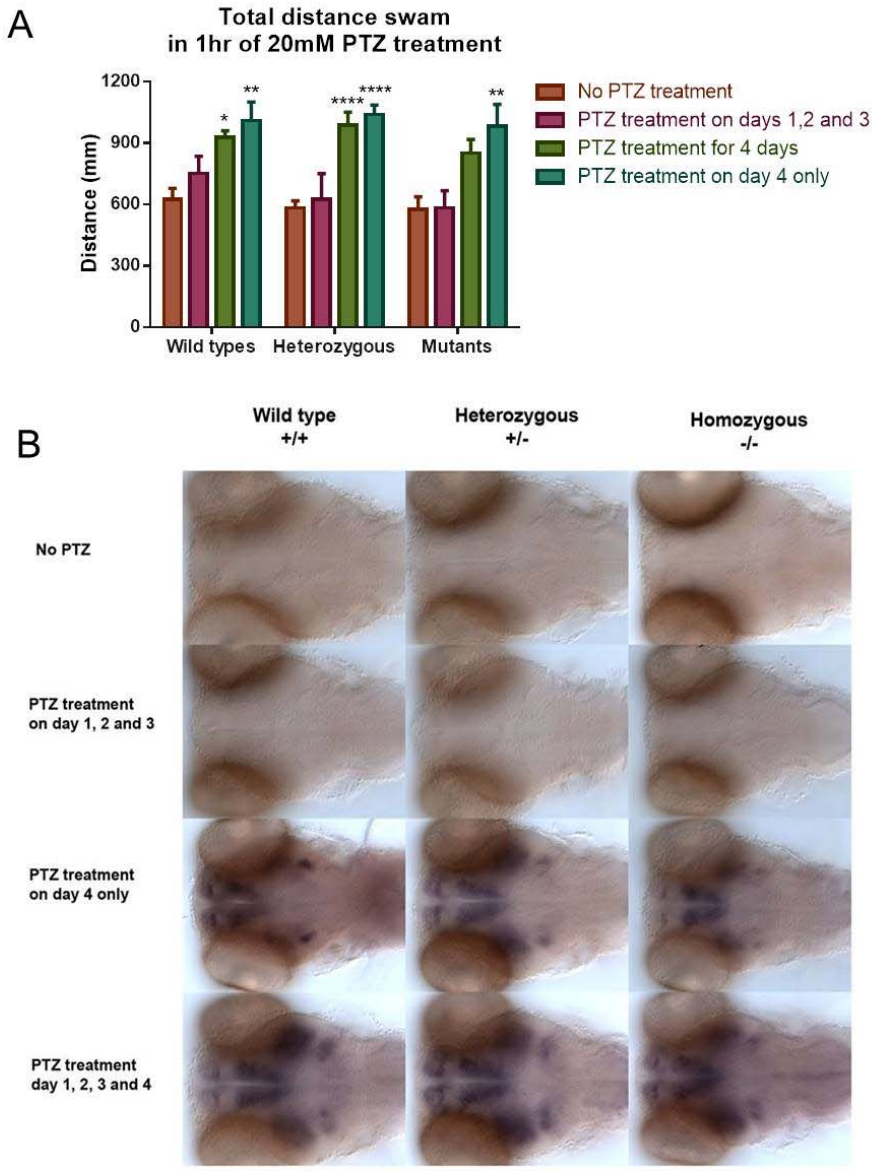
Gene Symbol	Type of protein	Function	Pathways
crema	bZIP: basic leucine zipper proteins	Transcriptional regulator that binds the cAMP response element (CRE)	cAMP, PKA
socs3a	STAT-induced STAT inhibitor	Protein kinase inhibitor activity	JAK-STAT, STAT3, IL-9,4,10
ankrd9	Ankyrin repeat domain containing		
atf3	bZIP: basic leucine zipper proteins	Represses transcription from promoters with ATF sites.	PERK, ERK, MAPK, iNOS, SMAD, TNF
zgc:153932	*Uncharacterised protein*		
msmo1	FATHD: Fatty acid hydroxylase domain containing	C-4 methylsterol oxidase activity and iron ion binding	Cholesterol biosynthesis
dusp1	DUSPT: Protein tyrosine phosphatases / Class I Cys-based PTPs : MAP kinase phosphatases	Dephosphorylates MAP kinase MAPK1/ERK2	MAPK, ERK, EGFR, TGF-beta
gadd45ba		Involved in the regulation of growth and apoptosis	p53, MAPK
insig1	Insulin-induced gene	Mediates feedback control of cholesterol synthesis by controlling SCAP and HMGCR.	SREBP signalling
midn	Contains 1 ubiquitin-like domain	May be involved in regulation of genes related to neurogenesis in the nucleolus	
gadd45ab		In T-cells, functions as a regulator of p38 MAPKs by inhibiting p88 phosphorylation and activity	MAPK, p53
pdk2	Pyruvate dehydrogenase kinase family	Plays a role in p53/TP53-mediated apoptosis	TCA, eNOS
hspb11	HSPB: Heat shock proteins / HSPB	Component of the IFT complex B required for sonic hedgehog/SHH signalling.	
rrad	GTP-binding protein	Regulates voltage-dependent L-type calcium channel subunit alpha-1C trafficking to the cell membrane	Insulin signalling
si:ch73-141c7.1	*Uncharacterised protein*		
fosl2	bZIP: basic leucine zipper proteins	Transcription factor	NOD, IL-17, IL-2, TCR, ERK
diabloa	inhibitor of apoptosis protein (IAP)-binding protein	Promotes apoptosis by activating caspases in the cytochrome c/Apaf-1/caspase-9 pathway.	apoptosis
cited4a	CITED family of transcriptional coactivators	Transcriptional co-activator for TFAP2/AP-2., enhances oestrogen-dependent	

Table 5.4 Function of genes whose expression is altered in 3 dpf zebrafish larvae after 90 minutes of exposure to the convulsant agent PTZ. (Cont.)			
Gene Symbol	**Type of protein**	**Function**	**Pathways**
cebpb	bZIP: basic leucine zipper proteins	Transcription factor	IL-17, MAPK, Ras
dusp5	DUSPT: Protein tyrosine phosphatases	Phosphatase activity toward several substrates, the highest relative activity is toward ERK1	MAPK, p53, ATF-2
rhcga	SLC: Solute carriers	Functions as an electroneutral and bidirectional ammonium transporter	Transmembrane transport of small molecules
wu:fc62b05	*Uncharacterised protein*		
ptgs2b	COX-2 Cyclooxygenase	Mediates the formation of prostaglandins from arachidonate	Arachidonic acid metabolism
igfbp1b	Insulin-like growth factor binding protein (IGFBP) family	Binding of this protein prolongs the half-life of the IGFs and alters their interaction with cell surface receptors	IGF 1, PERK, metabolism of proteins
zgc:110340	*Uncharacterised protein*		
rgs13	Regulator of G protein signalling (RGS) family	Inhibits signal transduction by increasing the GTPase activity of G protein alpha subunits	G-AlphaQ Signaling, cAMP
per2	Member of the Period family	Transcription factor - Component of the circadian clock mechanism which is essential for generating circadian rhythms	Circadian rhythm
pnp5b		Enzyme that reversibly catalyses the phosphorolysis of purine nucleosides.	Purine metabolism
cyp3a65	Cytochrome P450	Metal ion binding, oxidoreductase activity	
gadd45aa		In T-cells, functions as a regulator of p38 MAPKs by inhibiting p88 phosphorylation and activity	MAPK, p53
bdnf	Nerve growth factor family.	Major regulator of synaptic transmission and plasticity at adult synapse	MAPK, ERK
lonrf1l	RNF: RING-type (C3HC4) zinc fingers	ATP-dependent peptidase activity	
lpin1	Lipid phosphate phosphatases	Plays important roles in controlling the metabolism of fatty acids at different levels	Phospholipid metabolism, SREBP signalling

Table 5.4 Function of genes whose expression is altered in 3 dpf zebrafish larvae after 90 minutes of exposure to the convulsant agent PTZ. (Cont.)			
Gene Symbol	**Type of protein**	**Function**	**Pathways**
nr4a2b	Nuclear hormone receptors	Transcriptional regulator important for the differentiation and maintenance of meso-diencephalic dopaminergic (mdDA) neurons	Corticotropin-releasing hormone, Nuclear Receptor transcription pathway
rergla		Binds GDP/GTP and may possess intrinsic GTPase activity	
ttna	Myosin Light Chain Kinase (MLCK) family	Key component in the assembly and functioning of vertebrate striated muscles.	Striated Muscle Contraction, Hemostasis
Down-regulated genes			
Gene Symbol	**Type of protein**	**Function**	**Pathways**
he1		Hatching, metal ion binding, metallopeptidase activity	
wu:fa26c03	*Uncharacterised protein*		
si:dkey-26g8.5	*Uncharacterised protein*		
MGC174155	Cathepsin	Degradation of proteins in lysosomes	Degradation of the extracellular matrix
zgc:174855	Cathepsin	Protein degradation	
ctsl1b		Hydrolase and peptidase activity	
lrata		Retinol metabolism	

5.2.7 Functional analysis of the role of *npas4a* in the response to repeated treatment of zebrafish larvae with PTZ

My studies of the locomotor convulsive behaviour and transcriptional response to repeated treatments with convulsants suggested a correspondence between convulsant-induced, increased severity of locomotor convulsions and changes in the expression of convulsant-induced genes. Thus, increased PTZ-induced locomotor convulsions were accompanied by decreased expression of *c-fos* and *npas4a*. By contrast, repeated exposure to kainic acid neither modified the severity of induced locomotor convulsions nor elicited expression of PTZ-responsive genes *npas4a, c-fos* and *bdnf.* The availability of a homozygous viable loss-of-function mutation in *npas4a*, allele *sa701*, enabled the function of *npas4a* in the enhanced locomotor response to repeated treatment with PTZ to be explored. Accordingly, *sa701* heterozygous adults were in-crossed to generate progeny, which were then exposed to PTZ for one hour on days 1, 2 and 3 post-fertilisation, or maintained without treatment. On day 4, larvae were then either treated with 20mM PTZ for one hour, or maintained without treatment, and over this period their individual locomotor convulsive behaviour was then monitored. After monitoring, larvae were fixed, genotyped and analysed by *in situ* hybridisation to detect expression of *c-fos* transcripts. Analysis of locomotor behaviours and *c-fos* transcripts revealed that wild-type, heterozygous and homozygous mutant larvae all responded similarly to PTZ treatment on day 4. Moreover, pre-treatment of wild-type, heterozygous and homozygous mutant larvae did not enhance the magnitude of the locomotor response to PTZ treatment on day 4, nor did it appreciably alter the level of *c-fos* transcription. These results contrast with the robust increase in PTZ-induced locomotor activity and reduced expression of *c-fos* that was observed for wild-type larvae of the AB strain repeatedly treated with PTZ (Figures 5.1, 5.4). It is possible that genetic background variation in the *sa701* and AB strains could account for the apparent behavioural and gene expression differences between these two lines, or simply that the numbers of larvae analysed were too low to obtain a significant difference. Further experiments will be required to explore these and other possibilities in future.



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	No PTZ treatment	PTZ treatment on days 1,2 and 3	PTZ treatment for 4 days	PTZ treatment on day 4 only
Wild type	n=4	n=4	n=8	n=6
Heterozygous	n=9	n=10	n=13	n=12
Homozygous	n=6	n=4	n=2	n=5

Figure 5.14 Behavioural and *c-fos* expression analyses of *sa701* zebrafish larvae subjected to repeated PTZ-treatment. A) PTZ induces similar levels of locomotor convulsive behaviour in 4 dpf wild-type, heterozygous and homozygous *sa701* mutant larvae, independently of whether they were repeatedly treated with PTZ on day 1, 2 and 3 or not. Statistically significant increases were detected by ANOVA with Dunnett's multiple comparisons test are indicated by asterisks. B) *In situ* hybridisation analysis of *c-fos* expression in 4 dpf *sa701* zebrafish larvae after PTZ treatment(s) with control untreated larvae. C) Table showing the numbers of larvae analysed per condition.

5.3 Discussion

Kindling is a widely used, well established model for the study of epilepsy. Kindling models use repeated subconvulsive stimulation in order to enhance seizure susceptibility. As the kindling process develops, seizures become stronger until spontaneous seizures eventually occur. However, the kindling process is performed in adults and is a lengthy, time-consuming procedure. I therefore adopted a slightly different approach, and investigated the effect of repeated exposures to a known seizure-inducing concentration of PTZ, on the magnitude of the convulsions elicited by the PTZ-induced seizures in 4 dpf zebrafish larvae. I reasoned that this approach would allow me to quickly determine whether multiple, transient exposures to a seizure-inducing concentration of PTZ would sensitise the developing brain to further PTZ exposure and thus increase the magnitude of the convulsions. Accordingly, zebrafish embryos/larvae were treated once a day for one hour with 20mM PTZ on each of the first 4 days of development, and their seizure susceptibility was analysed on the fourth day. This experiment was repeated 10 times using larvae from the strain AB. The results were very consistent throughout the 10 experimental replicates, showing a significant increase in locomotor activity of 4 dpf PTZ-treated larvae that were pre-treated with PTZ during days 1, 2 and 3, in comparison to 4 dpf PTZ-treated larvae that were not previously exposed to PTZ (Figure 5.1). Pre-treating zebrafish embryos/larvae with PTZ on a single occasion was not sufficient to cause a significant increase in the locomotor behavioural response during a subsequent exposure to PTZ at 4 dpf (Figures 5.2, 5.3). Rather, the enhanced severity of the behavioural response to PTZ required repeated treatments with PTZ specifically on days 2 and 3, which is reminiscent of kindling in rodents where the brain becomes more sensitive to seizure induction, and locomotor convulsions become stronger, in older animals (Goddard, 1983; Morimoto et al., 2004).

In addition to the enhanced locomotor behavioural response, reduction of PTZ-induced *c-fos* and *npas4a* expression was also consistently observed in PTZ pre-treated larvae. In rats, PTZ kindling also produces a distinct pattern of *c-fos* expression in different brain structures

compared to the pattern of expression elicited by a single PTZ administration (Szyndler et al., 2009). Interestingly, the levels of expression of *fos* mRNA and protein, along with those of *egr1*, *arc* and *c-jun* were also decreased in rats subjected to repeated, chronic electroconvulsive seizures (Calais et al., 2013; Chen, et al., 2009).

The reciprocal effect of the enhanced seizure severity and the reduction in gene expression suggests that the possibility that *c-fos* and *npas4a* may be part of a neuroprotective transcriptional response to PTZ seizure induction the function of which is to attenuate seizure severity, protecting the brain from the seizure insult. To explore this possibility further, the repeated treatment paradigm was tested in zebrafish embryos/larvae using another commonly used seizure induction drug, kainic acid (Fisher, 1989; Lévesque & Avoli, 2013). Results reported in Chapter 3 showed that 100μM KA can cause a measurable behavioural response (Figure 3.5), but KA-induced expression of *c-fos* and *npas4a* was not detected by *in situ* hybridisation (Figure 3.15). The results described in this chapter show that Kainic acid pre-treatment did not enhance either the severity of the behavioural response to KA exposure at 4 dpf (Figure 5.5), nor did it alter the very modest increase in expression of *c-fos* and *npas4a* in response to KA treatment on day 4 (Figure 5.6), which was more than 30-fold lower than that caused by PTZ treatment (Figure 5.4). These results are consistent with the known differences in the modes of action of KA and PTZ in induction of seizures, and it therefore seems plausible that the effects of pre-treatment, and potentially similar kindling treatments in rodents, are primarily to weaken an inhibitory component of PTZ-activated neurotransmission, which may involve attenuation of genes such as *Npas4* (Bloodgood et al., 2013).

To explore further the consequences of the enhanced seizure severity and the reduced *c-fos* and *npas4a* expression in PTZ pre-treated larvae, apoptosis and synaptic remodelling were investigated. Some seizures are characterised by a high level of excitotoxic cell death that is induced by release of glutamate and the activation of NMDA receptors, followed by massive calcium influx leading to the activation of a signalling cascade that triggers apoptosis (reviewed

by Fujikawa, 2005). When apoptosis was anlaysed in PTZ- and KA-treated larvae with the TUNEL assay (Figure 5.7 B), no significant levels of cell death were observed.

Studies of kindling models have previously reported increased numbers of neurons in the hippocampus, as well as mossy fibre sprouting in kindled rats and mice (Morimoto et al., 2004; Scott, et al., 1998; Watanabe et al., 1996). To explore the possibility that PTZ pre-treatment might remodel synaptic connections between neurones, transgenic larvae expressing *ptf1a.GFP* in GABA-ergic and glutamatergic neurones were pre-treated with PTZ and imaged under the confocal microscope. The lateral views from the most posterior section of 4 dpf zebrafish larvae indicated that PTZ pre-treatment elicited a relatively subtle, difficult to quantify increase in axonal arborisation (Figure 5.8), suggesting the possibility that PTZ pre-treatment may be inducing a moderate level of synaptic remodelling that may be involved in enhancing the severity of the response to seizure induction.

The reduced expression of *c-fos* and *npas4a*, the increased severity of the convulsive response and the apparent synaptic remodelling caused by PTZ pre-treatment might be a consequence of epigenetic modifications that occur in response to the repeated exposure to PTZ. To begin to explore this possibility, zebrafish larvae were treated with the Histone Deacetylase inhibitor Trichostatin A, before each pre-treatment with PTZ. When larvae were exposed to PTZ again on the day of the analysis, 4 dpf larvae pre-treated with TSA and PTZ on days 1, 2 and 3 did not exhibit the increased severity of the locomotor response that was observed for PTZ pre-treated larvae that were not exposed to TSA (Figure 5.9). Moreover, when analysing PTZ-induced *c-fos* expression in 3 dpf TSA+PTZ-treated larvae by in situ hybridisation a noticeable suppression was detected (Figure 5.10). In contrast, TSA treatment before kainic acid stimulation in rats, enhanced the expression of *c-fos* and *c-jun* in different hippocampal subpopulations of neurons (Sng, et al., 2006; Sng, et al., 2005), suggesting that epigenetic mechanisms impact differently on PTZ and KA treatments.

Another trigger of seizures reported by physicians and patients of epilepsy and previously investigated in several rodent models of epilepsy is stress (Castro et al., 2012; Maggio &

Segal, 2012; Novakova et al., 2013; Yun et al., 2010). To study the effects of stress in our zebrafish larval model of PTZ-induced seizures, 3 dpf zebrafish larvae were treated with 25 or 100μM betamethasone for one hour. Betamethasone is a synthetic analogue of cortisol which has been proven to be an effective stress-inducer in zebrafish larvae (Griffiths et al., 2012). After betamethasone treatment, 3 dpf zebrafish larvae were exposed to 20mM PTZ. Larvae treated with 100μM bethametasone exhibited a 55% increase in locomotor activity compared to larvae that were exposed to PTZ alone (Figure 5.11). Furthermore, betamethasone treatment lowered the threshold for seizure induction since the distance swam by zebrafish larvae treated with betamethasone followed by 5mM PTZ was 155% longer than larvae treated with 5mM PTZ only (Figure 5.13). PTZ-induced expression of *npas4a*, *c-fos* and *bdnf* was also heavily suppressed by betamethasone treatment (Figure 5.12). Thus, as was observed for larvae repeatedly treated with PTZ, stress hormone exposure increased locomotor convulsive behaviour and reduced expression of PTZ-responsive genes. These consistent results support the hypothesis that the transcriptional response to PTZ seizure induction elicits feedback signal(s) that limit the severity of seizures, and promote neuronal homeostasis (Figure 5.15).

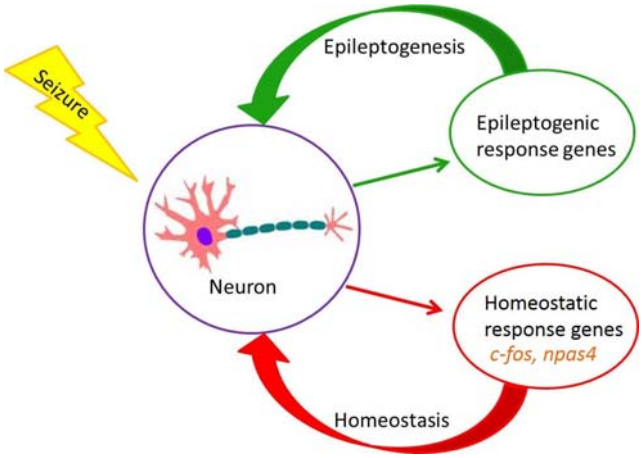

Figure 5.15 Schematic representation of the possible function of *c-fos, npas4* and other genes that are expressed in response to PTZ seizure induction.

To further investigate this possibility, the *npas4a* mutant line *sa701*, was subjected to PTZ pre-treatments to analyse a possible role for *npas4a* in the responses to seizure onset (Figure

5.14). The results indicated that loss of *npas4a* function did not appreciably alter responses to PTZ. One possibility is that *npas4a* exhibits functional redundancy with the related gene *npas4b*. Other possibilities include the existence of modifier loci in the genetic background TL that are not present in the AB line in which all earlier experiments were carried out, or the possibility that too few larvae were analysed in this single experiment to be able to detect statistically significant differences between genotypes.

In order to systematically identify PTZ-induced genes, I performed a gene expression profiling analysis of zebrafish larvae to identify differentially expressed genes after exposure to PTZ for 30 minutes and 90 minutes. The results from the comparison of control and PTZ-treated larvae at the 30 minute time point indicated that *npas4a* exhibited the greatest PTZ-induced fold change of all the genes on the microarray. Moreover, *npas4a* was also among the top 3 up-regulated genes at the 90 minute time point. This suggests that npas4a might have a crucially important role in the initial phase of the response to PTZ seizure induction in zebrafish larvae, a possibility which will be explored in future studies using the *sa701 npas4a* mutant line. The microarray analysis also identified many other seizure-regulated genes. It was remarkable that after only 30 minutes of PTZ exposure, robust transcription of many genes encoding sequence-specific DNA binding transcription factors were identified, including *npas4a*, *c-fos*, *fosb*, *ier2*, *btg2* and *junbb*. 5 out of the 18 genes that were up-regulated after 30 minutes of treatment encode proteins of unknown function. By contrast, only one gene was significantly down-regulated encoding the desumoylating isopeptidase 1a (*desi1a*), which deconjugates SUMO1, 2 and 3 from some substrate proteins (Shin et al., 2012) (Tables 5.1 and 5.3).

Transcriptome analysis of PTZ-treated mice revealed that within one hour of the onset of generalised seizures induced by PTZ administration, *c-fos*, *Nxf* (*npas4)*, *Btg2*, *Nr4a1*, *Egr1 Fbxo33* and *Sgk* were the most strongly up-regulated genes in the hippocampus (Flood, et al., 2004). Four of those genes *(c-fos, npas4a, btg, and nr4a1)* were also transcriptionally up-regulated by PTZ treatement in 3 dpf zebrafish larvae after 90 minutes of exosure to PTZ.

Following 90 minutes of PTZ treatment, more than 50 genes were found to be up-regulated in 3 dpf zebrafish larvae in the microarray analysis. Again, many of these genes encode transcriptional regulatory proteins. Table 5.5 summarises the functions of the genes whose expression was up-regulated after 90 minutes of exposure to PTZ. Among these up-regulated genes was *matrix metalloproteinase 9* (*mmp9*), which exhibited a 4.5-fold increase in response to PTZ. In epileptogenesis, high levels of MMP-9 have been associated with abnormal synaptic plasticity, neuronal death and inflammation (reviewed by Mizoguchi & Yamada, 2013). Furthermore, *mmp-9* has been found to be induced by *c-fos* through the induction of *bdnf* transcription (Kuzniewska et al., 2013). Therefore, this gene would be of particular interest to investigate in the zebrafish model of PTZ-induced seizues.

Taken together, my results indicate that a very complex cascade of gene expression is induced in response to PTZ exposure in zebrafish larvae. This opens new possibilities to identify new genes and identify study the function and interaction in the development of seizure response, modulation of seizure severity and influence of other factors, such as stress.

Table 5.5 Summary of the genes exhibiting increased expression in 3 dpf zebrafish larvae treated for 90 minutes with PTZ.

Function	Genes
Transcription regulation	*c-fos, zgc:175128, npas4, junbb, jdp2, ier2, btg2, fosb, nr4a1, crema, atf3, midn, fosl2, cited4a,cebpb, per2, bdnf, nr4a2b*
Apoptosis regulation	*btg2, dusp1, gadd45ba, gadd45ab, pdk2, diabloa, dusp5, gadd45aa,*
Homeostasis of ions	*cyp24a1,cyp1a, msmo1, rhcga, cyp3a65*
Neuronal formation and remodelling	*npas4, sema3d, midn, bdnf, nr4a2b*
Muscle contraction	*tcap, ttna*
***Sestrin3 Module 1 genes**	*c-fos, fosb, atf3, junbb, gadd45ba*
Unknown	*wu:fj62d01, ankrd9, zgc:153932, si:ch73-141c7.1, wu:fc62b05, zgc:110340*

Chapter 6

6 Sestrin3, a novel genetic regulator of seizure response.

6.1 Introduction

A collaboration was established between our laboratory and Dr Enrico Petretto and Dr Michael Johnson (Imperial College London), who have performed a novel, genome-wide, integrative genomics analysis of human epilepsy patients. Their studies identified a new gene that is highly expressed in surgically-removed hippocampus tissue from patients with pharmacoresistant temporal lobe epilepsy (TLE). This gene is *Sestrin 3,* a member of a family of intracellular proteins involved in regulation of intercellular signalling pathways, including mTOR signalling. In order to obtain information on the function of *sesn3* in epilepsy, a collaboration was established with Drs Petretto and Johnson to investigate the role of zebrafish *sesn3* in the seizures induced by PTZ-treament of zebrafish larvae. Their previous studies had shown that knock-down of Sesn3 in cultured cells using siRNA, reduced expression of *c-fos, egr2, bdnf* and other early response genes. However, an *in vivo* study was needed to confirm the role of *Sesn3* in the pathogenesis of epilepsy. I took advantage of this opportunity and carried out a series of experiments that have revealed a novel role for zebrafish *sesn3* in promoting the epileptic seizures caused by PTZ exposure.

6.1.1 Identification of Sestrin3 in hippocampal tissue of TLE patients.

A large network of 442 co-expressed genes identified in surgically-removed hippocampal tissue of 129 epileptic patients (Petretto et al., manuscript in preparation). Within this network, a smaller sub-network was discovered, designated Module-1, involving 69 genes encoding proconvulsive cytokines and TLR-signalling pathway components. A genome-wide association analysis of the 129 TLE patients using 527,684 Single Nucleotide Polymorphisms

(SNP) allowed the identification of a gene, *SESN3,* whose expression was positively associated with expression of Module-1 genes in epileptic hippocampus tissue both from human patients and in a rodent model of epilepsy (Petretto et al., manuscript in preparation). Furthermore, over-expression of *SESN3* in cultured neurones up-regulated Module-1 genes.

6.1.2 Sestrin3 and the mTOR pathway

SESN3 is a member of a family of stress-inducible proteins called sestrins (Budanov, et al., 2010). Sesn3 expression is up-regulated by serum and growth factors and also by FoxO transcriptional factors (Nogueira et al., 2008). Sestrins have many novel structural features, but they share a conserved N-terminal region that contains a motif also found in the prokaryotic AhpD protein, an alkyl-hydroperoxidereductase that protects bacteria from reactive oxygen species (ROS) (Budanov et al., 2010).

Budanov and collaborators found that the mTOR pathway is a likely target for Sestrins, and two different splice variants of *Sestrin3* inhibited the activity of TORC1 (Budanov & Karin, 2008), a component of the mTOR pathway that is regulated by cytokine and Toll-like receptor (TLRs) signalling (Weichhart & Säemann, 2009). Interestingly, most of the Module-1 genes identified by Dr. Petretto encode cytokines or components of the TLR signalling pathway. Furthermore, the TLR signalling pathway has been previously implicated in epileptogenesis (Vezzani, et al., 2013; Vezzani, et al., 2011). Taken together, these findings suggested that *Sestrin 3* might influence mTOR pathway activity in epilepsy via regulation of Module-1 gene expression. I therefore set out to investigate a possible role for *sesn3* in the PTZ model of epilepstic seizures in zebrafish larvae.

6.2 Results

6.2.1 Analysis of *sestrin 3* expression in zebrafish embryos/larvae.

A full length *sesn3* probe was synthesised to analyse the expression of *sesn3* in zebrafish embryos and larvae by *in situ* hybridisation. The results revealed that expression of *sesn3* mRNA is widespread in the brain of 1, 2, 3 and 4 dpf zebrafish embryos and larvae (Figure 6.1). The widespread expression of *sesn3* was stronger in older larvae than in younger embryos. Transverse sections through the hindbrain revealed extensive expression of *sesn3* in brain tissue, which is increased in 4 dpf larvae compared to 3 dpf larvae (Figure 6.1 B).

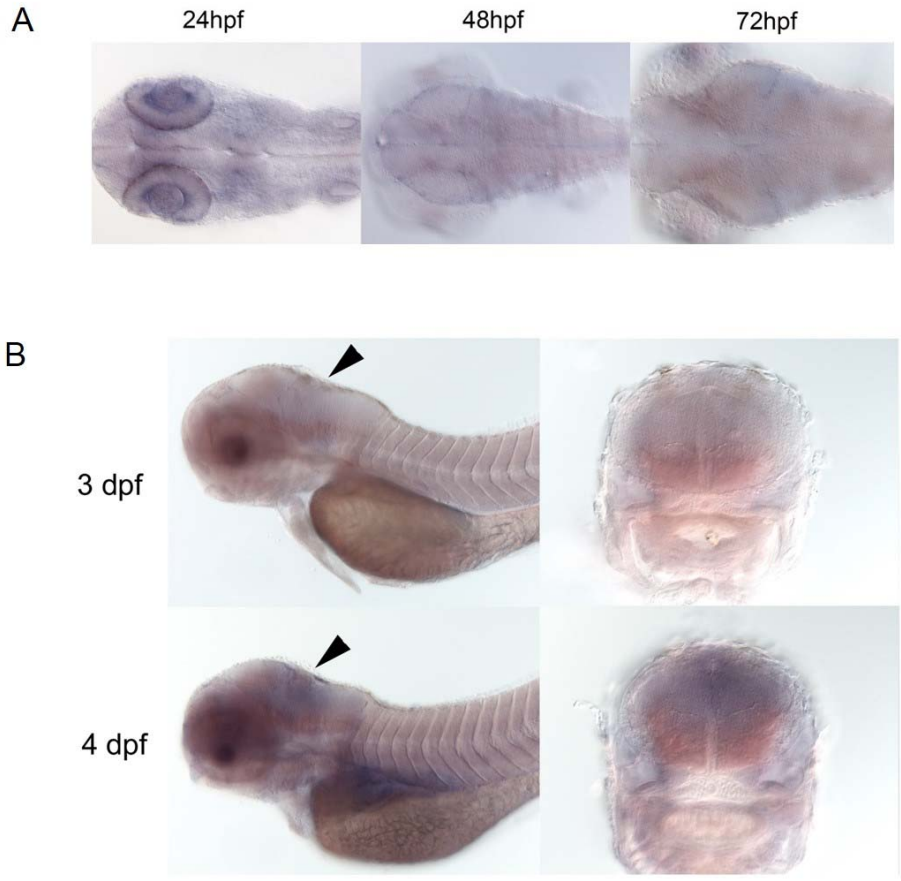

Figure 6.1 *sestrin 3* expression at different stages of development of zebrafish embryos and larvae analysed by in situ hybridisation. A) Dorsal views of the heads of 24, 48 and 72 hpf wild-type zebrafish embryos showing the expression of a full-length probe for *sesn3*. B) Lateral views (left panel) and transverse sections of the hindbrain (right panel) of 3 and 4 dpf wild-type zebrafish larve. Black arrows point to the area with stronger expression of *sesn3* in the hindbrain.

6.2.2 Design and validation of *sestrin 3* morpholinos to investigate the effects of *sesn3* knockdown in a zebrafish larval model of PTZ-induced epileptic seizures.

To study the function of *sestrin3* in response to seizure induction, a morpholino-based approach was adopted to inhibit *sesn3* expression. Two splice-blocking morpholinos were designed to target zebrafish *sesn3* RNA. One morpholino was designed to bind to a sequence in the intersection between intron 3 and exon 4 (*i3e4*), whereas the other was designed to bind to the sequence at the junction between exon 4 and intron 4 (*e4i4)* (Figure 6.2 A).

Three different pairs of primers were used to determine whether injected morpholinos were having the desired effect of reducing the level of *sesn3* RNA transcripts and/or altering the splicing of the primary *sesn3* transcript. The best pair of primers for the morpholino validation was the pair e4e5, which recognised sequences in exon 4 and exon 5, and thus reveal whether RT-PCR products retained intron 4 as a result of interference with splicing by the *e4i4* morpholino binding to the junction between exon 4 and intron 4 (Figure 6).

One-cell stage zebrafish embryos were injected with 2nl of 0.05mM of either *sesn3 i3e4* MO or *sesn3 e4i4* MO, or both morpholinos together. Injection of higher concentrations of either morpholino caused phenotypic malformations and mortality. Injected embryos were collected at 32 hpf for total RNA extraction and cDNA synthesis. RT-PCR analysis showed that the injection of both *sesn3 i3e4* and *e4i4* morpholinos reduced the level of processed *sesn3* mRNA (Figure 6.2 B - arrowhead) to a much greater extent than did injection of either morpholino alone. Moreover, larger PCR products were detected in embryos injected with *sesn3 e4i4* MO than were observed in controls (Figure 6.2 – yellow rectangle), confirming that *sesn3 e4i4* morpholino blocked normal splicing of the *sesn3* RNA transcript, leading to its retention of intron 4.

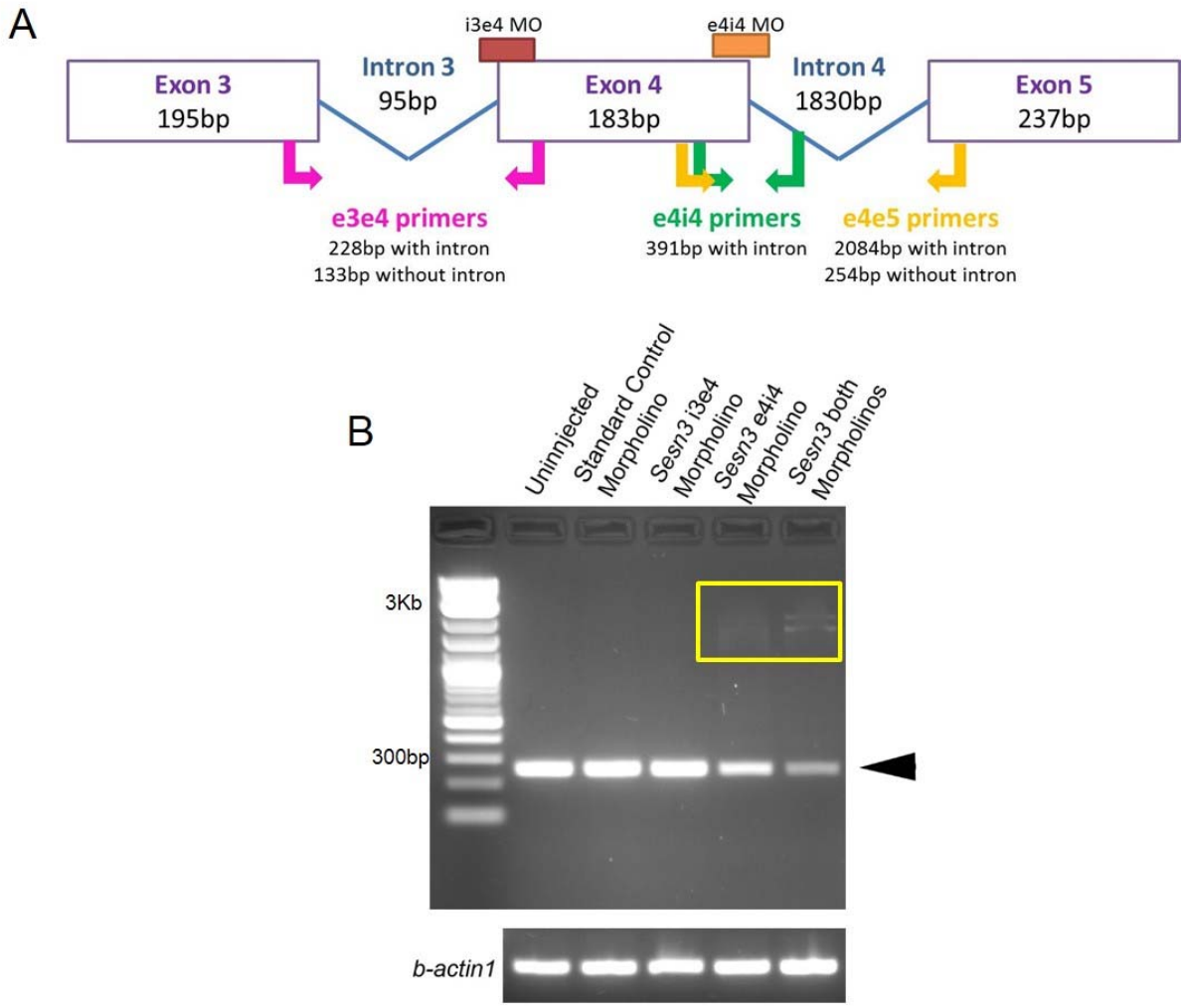

Figure 6.2 *sestrin 3* splice-blocking mopholino strategy design and validation. A) Schematic representation of *sestrin 3* gene, showing the binding sites for both morpholinos, and the pairs of primers used for their validation. B) 1.6% agarose gel showing the bands produced by RT-PCR analysis (using e4e5 primers) of *sesn3* mRNA expression in uninjected embryos and embryos injected with standard control morpholino, *sesn3 i3e4* morpholino, *sesn3 e4i4* morpholino, or *sesn3 i3e4* and *sesn3 e4i4* morpholinos. Black arrowhead shows the 254bp product which corresponds to the normal *sesn3* transcript without intron, yellow rectangle shows unspliced RT-PCR products. NEB *Log2* DNA ladder was used as size marker (left).

The results showed that the injection of both morpholinos (*sesn3 i3e4* and *sesn3 e4i4*) in combination caused a greater reduction of the normal *sesn3* transcript level, suggesting that double morphant larvae would exhibit a stronger phenotype than single morphant larvae.

6.2.3 Analysis of the behavioural and transcriptional response of *sesn3* morphants to treatment with the convulsant agent PTZ.

To determine whether *sesn3* knockdown had an effect on seizure severity, 3 dpf *sesn3* single and double morphants were treated with 20mM PTZ for one hour while their locomotor response was recorded with the Viewpoint Zebrabox.

The results of the behavioural analysis show that combined microinjection of *sesn3 i3e4* and *e4i4* morpholinos inhibited PTZ-induced locomotor activity more strongly than microinjection of either morpholino separately (Figure 6.3). The percentage of reduction of locomotor activity in response to PTZ exposure, as compared with the locomotor activity of PTZ-treated control larvae, was 58%. Notably, microinjection of either morpholino singly either showed no significant effect or very slightly increased locomotor activity as compared to larvae injected with control morpholino, after PTZ treatment. Black bars indicate cumulative locomotor activity in zebrafish exposed to 20mM PTZ.

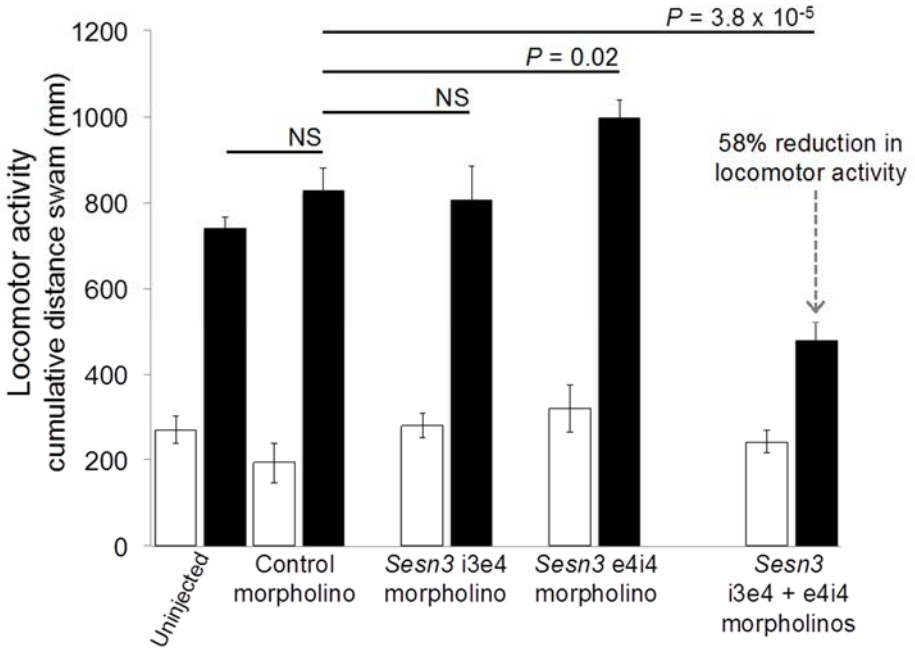

Figure 6.3 Total distance swam by 3 dpf control larvae and *sesn3* morpholino injected larvae after one hour of 20mM PTZ treatment. Data is reported as means, error bars indicate s.e.m. and P-values were calculated by t-test (2-tailed) and after adjusting for unequal variances across different groups.

In addition to assessing the effect of *sesn3* knockdown on the behavioural response, I also sought to investigate whether PTZ-induced *c-fos* expression was also altered in *sestrin 3* single and double morphants. Dr. Petretto and his colleagues had previously observed that the expression of *sesn3* correlated with the expression of a set of genes that they designated as Module-1 genes, and hypothesised that *sesn3* is regulator of these genes, which include *c-fos*, *fosb*, *egr2* and *atf3.*

The expression of *c-fos* was analysed by in situ hybridisation in 3 dpf control and *sesn3* single and double morphants. As with the behavioural analysis, single morphants did not exhibit a clear change in PTZ-induced *c-fos* expression. However, *sesn3* double morphants showed a noticeable reduction in *c-fos* expression in response to PTZ exposure (Figure 6.4).

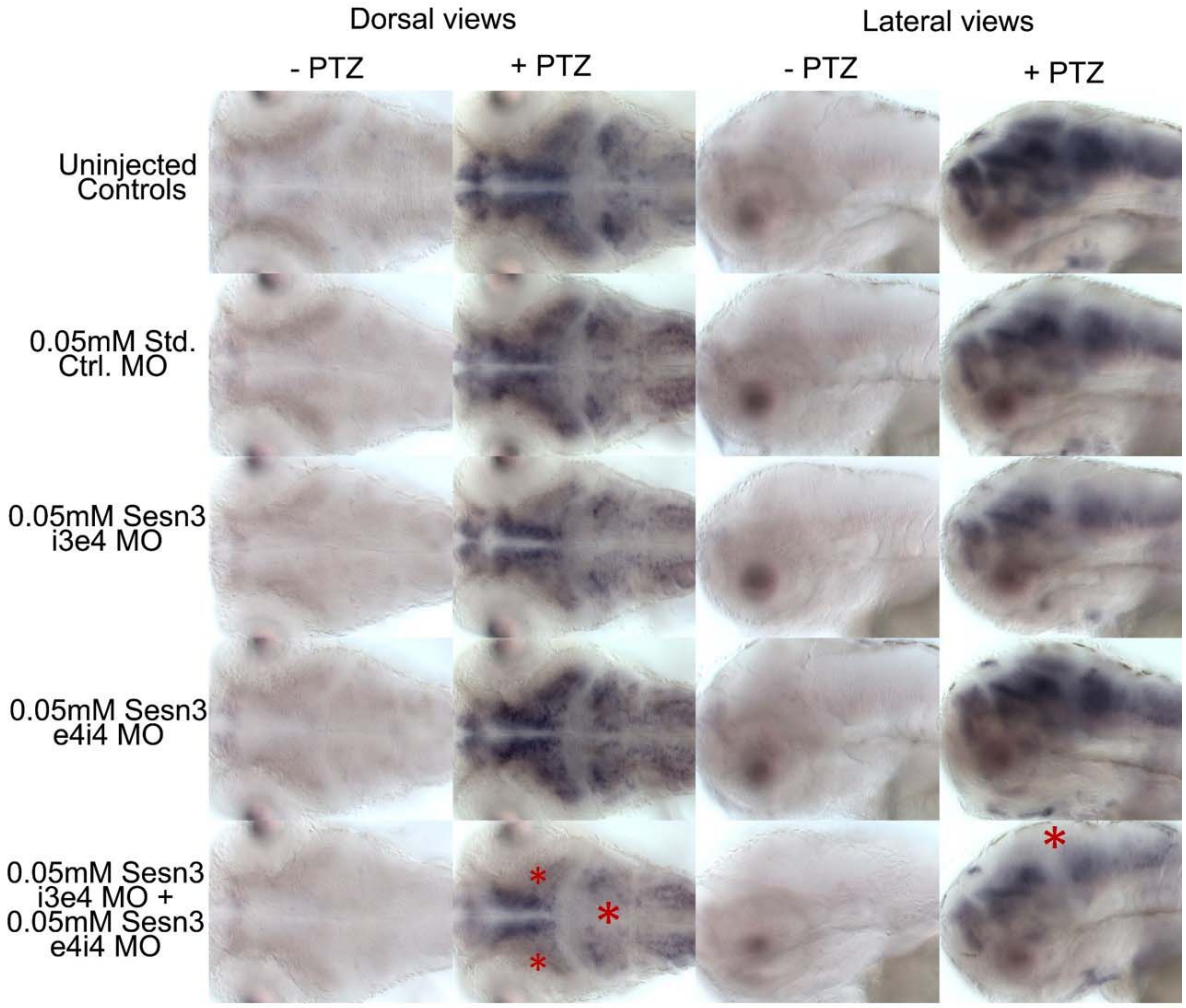

Figure 6.4 *c-fos* expression analysis by *in situ* hybridisation in control and *sesn3* single/double morphants treated with 20mM PTZ. Red asterisks indicate the regions where the expression of *c-fos* is reduced in *sesn3* double morphants.

These results indicated that the double (*sens3 i3e* MO + *sesn3 e4i4* MO) morphants might provide the clearest insights into understanding the role of *sesn3* in regulating the responses to seizure induction. Accordingly, expression of Module-1 genes: *c-fos*, *fosb*, *atf3* and *egr2b* was analysed by qPCR in 3 dpf *sesn3* double morphants treated with 20mM PTZ, which revealed that expression of *c-fos*, *fosb*, *atf3* and *egr2* was reduced in *sesn3* moprhants treated with PTZ (Figure 6.5).

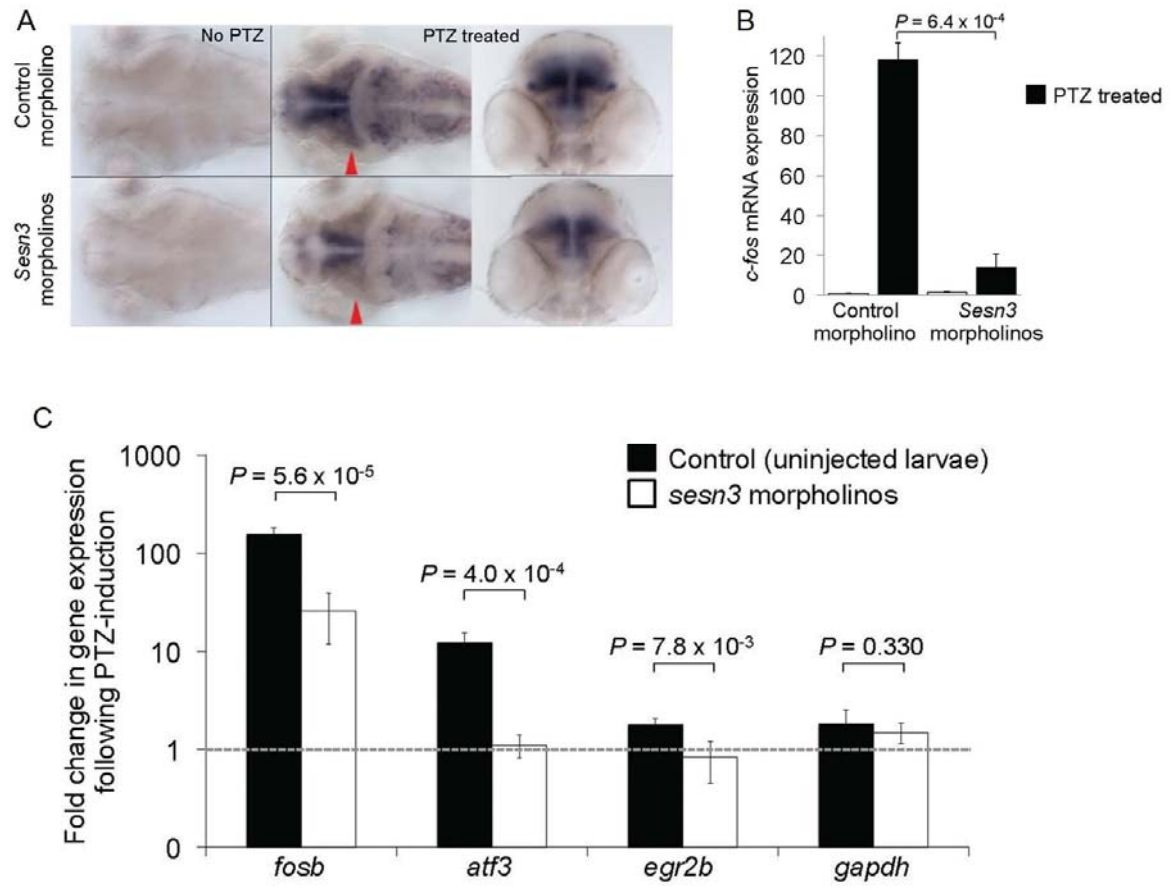

Figure 6.5 Expression of Module-1 genes in 3 dpf *sestrin 3* morphants treated with PTZ. A) Expression of *c-fos* in *sesn3* double morphants analysed by *in situ* hybridisation. Red arrowheads indicate the area of the brain where the reduction of *c-fos* expression was more noticeable. B and C) qPCR analysis of the expression of *c-fos*, *fosb*, *atf3* and *egr2b*. For each sample, total RNA was extracted from 8-20 stage-matched, sibling zebrafish larval heads. Relative expression levels, normalised to beta actin gene expression, were determined by using the $2^{-\Delta\Delta CT}$ method. At least 4 biological replicates were analysed. Data is reported as means, error bars indicate s.e.m. and P-values were calculated by t-test (2-tailed).

These results suggested that Sestrin 3 regulates seizure severity and gene expression in response to PTZ. However, to corroborate that these observations were solely caused by *sesn3* knockdown, a rescue experiment where synthetic *sesn3* mRNA was co-injected along with *sesn3* morpholinos was performed.

6.2.4 Behavioural and transcriptional response to PTZ seizure induction of *sestrin3* morphants injected with synthetic *sesn3* mRNA.

A full-length zebrafish *sestrin 3* cDNA clone (IMAGE:2601412) was subcloned into the pCS2+ expression vector to create *pCS2+.sestrin3*. A linearized template of *pCS2+.sestrin3* was used to synthesize *sesn3* RNA using the mMessage mMachine Kit (Ambion). The synthetic *sesn3* RNA (which cannot be targeted by either of the splice-blocking morpholinos) was then injected into one-cell stage AB wild type zebrafish embryos alone (2nl of 0.3ng/nl *sesn3* mRNA) or in combination with *sesn3* morpholinos (2nl of 0.3ng/nl *sesn3* mRNA + 0.05mM *sesn3 i3e4* MO + 0.05mM *sesn3 e4i4* MO). In addition, some embryos were injected with *sesn3* morpholinos alone (2nl of 0.05mM sesn3 *i3e4* MO + 0.05mM sesn3 *e4i4* MO). The locomotor activities of these larvae, in response to PTZ exposure, was analyzed at 3 dpf using the Viewpoint Zebrabox.

The results show that the co-injection of the synthetic *sesn3* mRNA combined with *sesn3* morpholinos was able to rescue the reduced locomotor response to PTZ of 3 dpf *sesn3* morphant larvae, confirming that this reduction was a specific consequence of the suppression of *sesn3* expression by *sesn3* morpholinos (Figure 6.6).

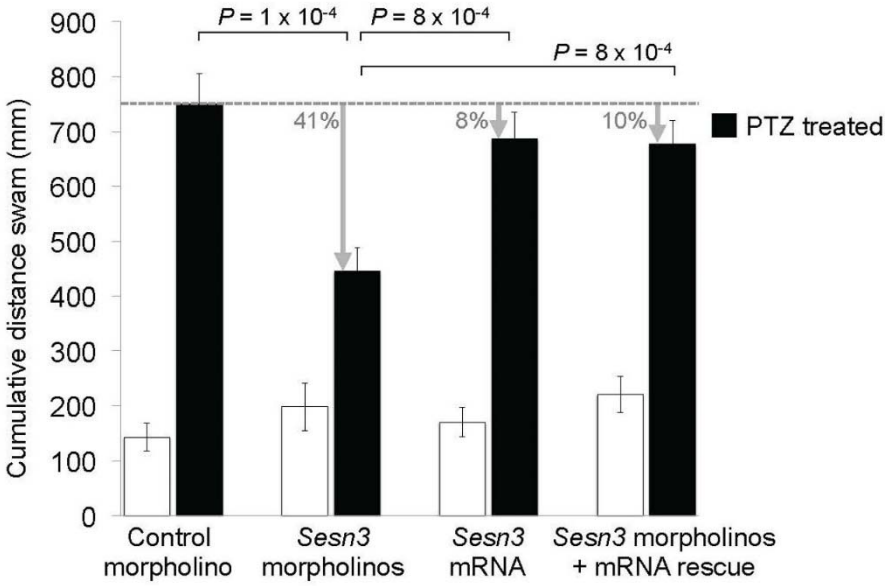

Figure 6.6 Co-injection of *sesn3* morpholinos with synthetic *sesn3* mRNA rescued the morphant phenotype. For each group, 16 to 18 larvae were analysed. Black bars, 1-hr PTZ treatment (20mM). Data is reported as means, error bars indicate s.e.m. and P-values were calculated by t-test (2-tailed).

To analyse if the reduction of PTZ-induced expression of Module-1 genes *c-fos*, *fosb* and *erg2b* in sesn3 double morphants was caused by sesn3 knockdown, the expression of these genes was analysed by qPCR in 3 dpf zebrafish larvae that were injected with *sesn3* mRNA plus *sesn3 i3e4* and *sesn3 e4i4* morpholinos. A partial rescue of gene expression was achieved by the co-injection of *sesn3* mRNA with *sesn3* morpholinos (Figure 6.7), however, the degree of rescue was not as great as was observed for the locomotor assay (Figure 6.6).

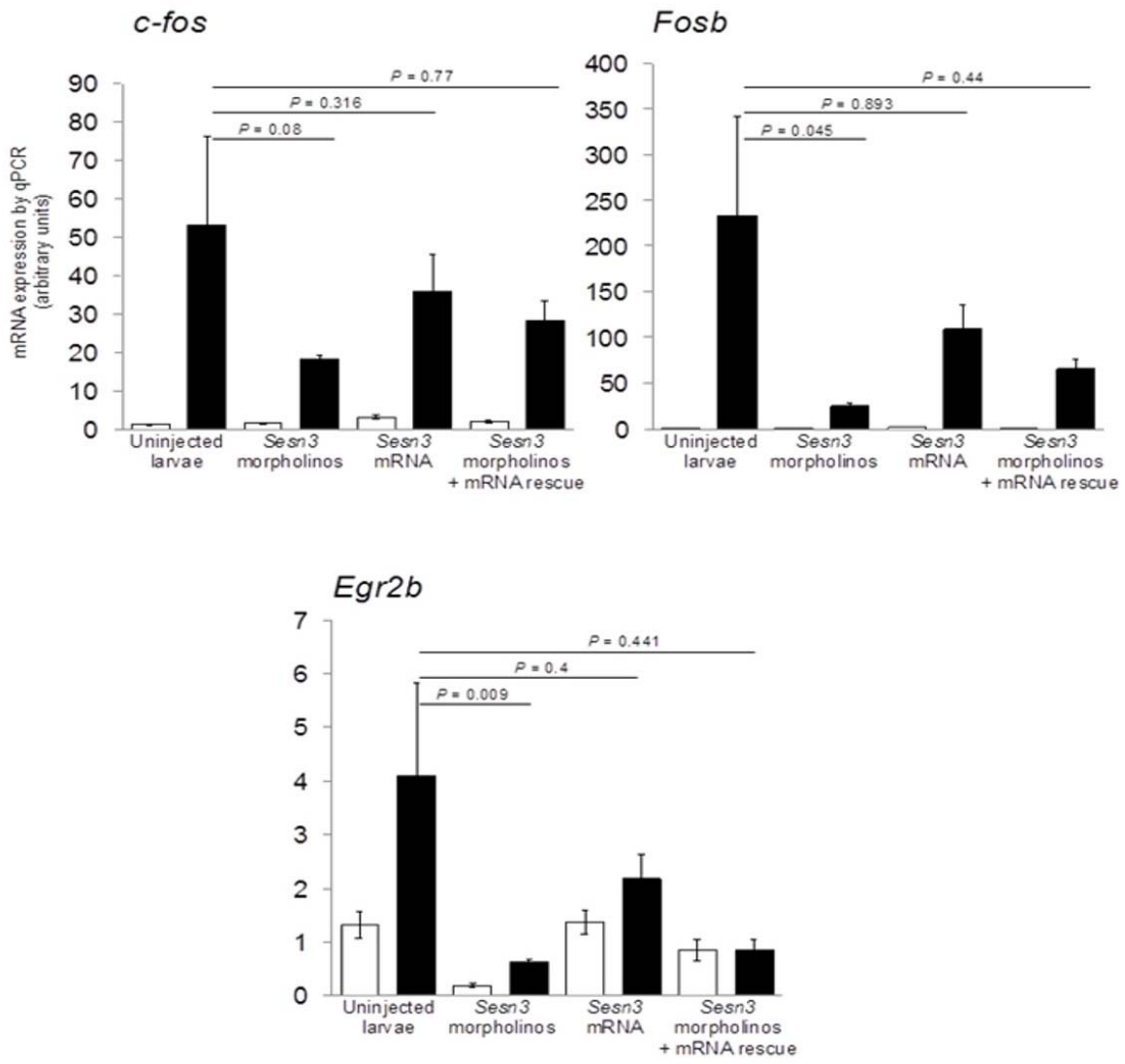

Figure 6.7 The expression of *c-fos*, *fosb* and *egr2b* was partially rescued by co-injecting *sesn3* mRNA with *sesn3* morpholinos. Black bars correspond to PTZ treated larvae. Data is reported as means, error bars indicate s.e.m. and P-values were calculated by t-test (2-tailed) and adjusting for unequal variances across different groups.

To further investigate the regulatory relationship between *sesn3* and Module-1 genes and to overcome the possible problem of there being significant turnover of injected sesn3 mRNA by 3 dpf, 2nl of 0.3ng/nl synthetic *sesn3* mRNA were injected to wild-type zebrafish embryos and expression of Module-1 genes was then analysed at 32 hpf. s*esn3* overexpression caused a statistically significant increase in the expression of *fosb*, *atf3* and *egr2b* in 32 hpf zebrafish embryos (Figure 6.8).

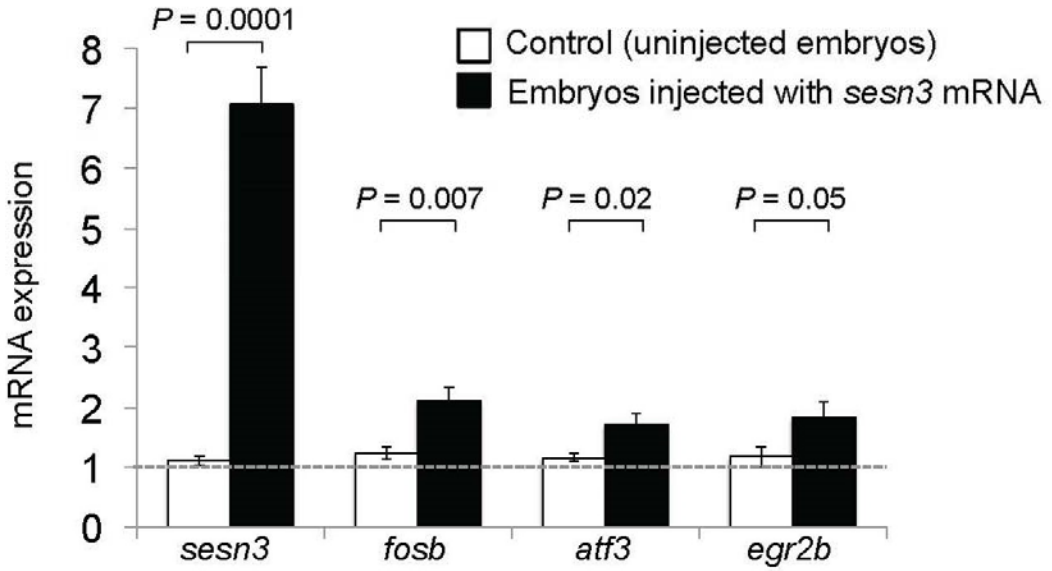

Figure 6.8. *sesn3* overexpression caused a significant increase on the levels of *fosb*, *atf3* and *egr2b* mRNA in 32 hpf zebrafish embryos. Data is reported as means, error bars indicate s.e.m. and P-values were calculated by t-test (2-tailed) and adjusting for unequal variances across different groups.

6.2.5 Analysis of the expression of *sestrin 3* in zebrafish larvae in response to PTZ treatment.

Finally, the inducibility of *sestrin 3* expression by PTZ treatment was analysed in 3 and 4 dpf zebrafish larvae. 3 and 4 dpf wild-tye zebrafish larvae were treated with 20mM PTZ for one hour and fixed for *in situ* hybridisation analysis of *sesn3* expression.

The results show that even though the expression of *sesn3* in the brain of zebrafish larvae was widespread, a slight increase was noticeable both in lateral views (Figure 6.9 A) and transverse sections of the hindbrain (Figure 6.9 B). This result was consistent throughout 3 different replicates.

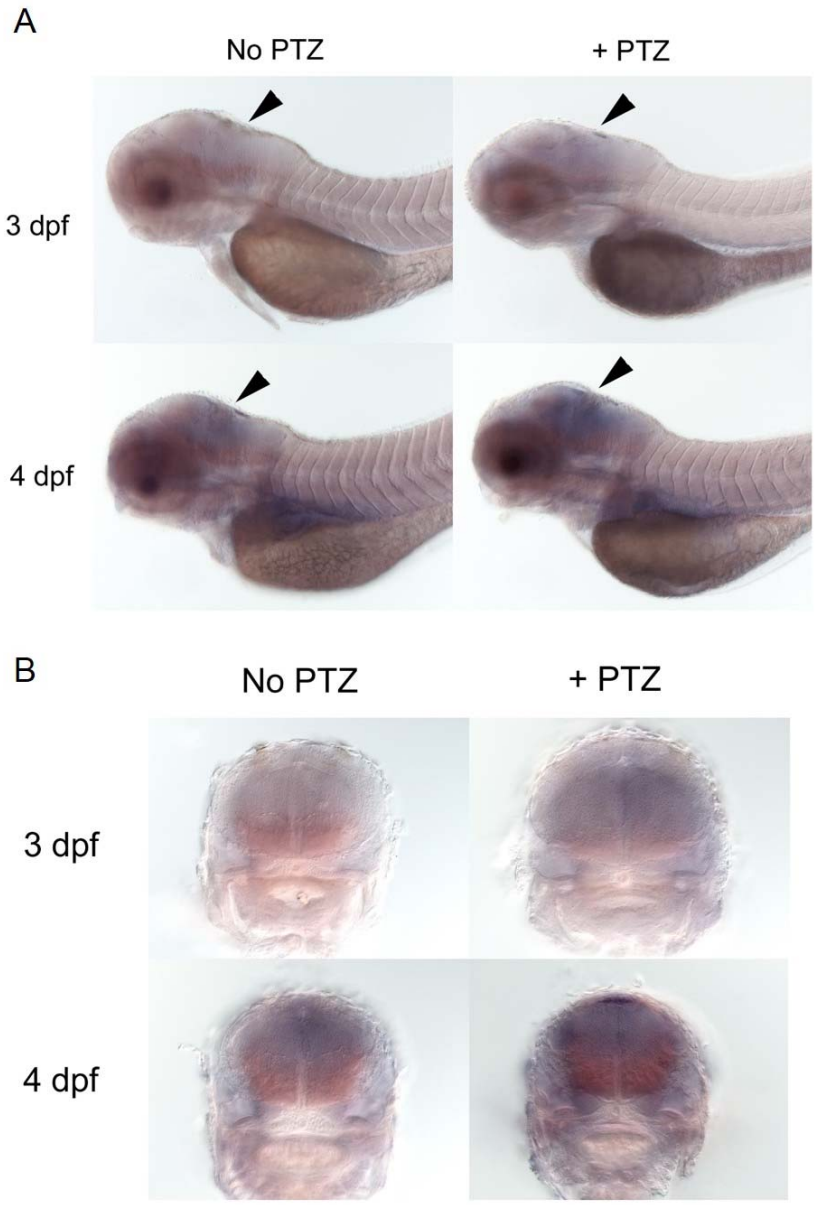

Figure 6.9 *sesn3* expression was slightly increased by PTZ treatment in zebrafish larvae. A) Lateral views of the heads of 3 and 4 dpf zebrafish larvae -/+ PTZ treatment showing the widespread expression of *sesn3* throughout the brain. Black arrowheads show the areas of the brain with strongest *sesn3* expression. B) Transverse sections through the hindbrain of 3 and 4 dpf zebrafish embryos -/+ PTZ treatment.

6.3 Discussion

Sestrin 3 was identified as a positive regulator of a network of genes (Module-1 genes) that were found up-regulated in surgically-removed hippocampal tissue of patients with temporal lobe epilepsy (Petretto et al., manuscript in preparation). To test the function of *sesn3* in our zebrafish model of chemically-induced epileptic seizures, I investigated whether there was a correlation between the magnitude of locomotor behaviour and the transcriptional activation of *c-fos* and a subset of Module-1 genes, in response to PTZ-treatment in *sesn3* morphant and control morphant larvae.

Expression of *sesn3* was first analysed by in situ hybridisation in zebrafish embryos and larvae. *sestrin 3* showed widespread expression in the brain of 3 dpf and 4 dpf zebrafish larvae (Figure 6.1), implying that many neurones within the CNS express this gene.

To study *sesn3* function in zebrafish, a knockdown approach was designed using two different splice-blocking morpholinos for *sesn3* (Figure 6.2 A).The injection of both *sesn3* morpholinos together resulted in a higher suppression of *sesn3* expression than with the injection of either morpholino alone (Figure 6.2 B). 3 dpf *sesn3* single and double morphants were exposed to 20mM PTZ for one hour while their locomotor activity was recorded in the Viewpoint Zebrabox. Neither morpholino when injected alone was sufficiently potent to reduce the severity of the locomotor response to PTZ treatment. However, when both morpholinos were injected together, 3 dpf injected larvae swam 58% less distance than control larvae (Figure 6.3). The fact that the combination of two *sesn3* morpholinos yielded a phenotype that was not apparent when either morpholino was injected singly is an indication of the specificity of the combination of the two morpholinos for the same target, i.e. *sesn3* RNA. The rescue of the double morphant phenotype by injection of synthetic *sesn3* mRNA is a further confirmation of the specificity of the morpholinos for endogenous *sesn3* RNA.

My gene expression studies confirmed that *sesn3* is a positive regulator of Module-1 genes, including *fosb, atf3* and *egr2b*, as well as the seizure-induced gene *c-fos*, which have been implicated in the regulation of neuro-inflammatory processes in the CNS and elsewhere

(Vezzani & Granata, 2005). Sestrins are known to regulate intracellular levels of reactive oxygen species that cause oxidative stress (Nogueira et al., 2008), which is a common feature of neurodegenerative and excitotoxic processes in the brain. It is therefore possible that *sesn3* alters the threshold for activation of an excitotoxic cascade that could underpin transformation of an epileptogenic process to an epileptic seizure. Indeed, the increased expression of Module-1 and *c-fos* gene transcripts caused by *sesn3* mRNA over-expression, albeit relatively modest, suggests that the threshold for neuronal excitability may have been reduced by increasing *sesn3* mRNA levels. Further studies could explore this possibility more closely, for example by investigating whether *sesn3* overexpression increases the locomotor convulsive behaviour of embryos or larvae treated with a low concentration of PTZ which does not by itself induce seizures. The apparent increase in *sesn3* transcript levels caused by PTZ treatment (observed in 3 independent experiments) suggests that if the blockade of inhibitory neurotransmission by PTZ increases neuronal excitability which in turn increases *sesn3* expression, then this increase in *sesn3* expression could further lower the threshold for neuronal excitability and trigger a feed forward loop of rising neuronal activity that might eventualy culminate in a seizure. Further studies of the function of *sesn3* are therefore merited, for which a loss-of-function *sesn3* mutant would be very useful.

Chapter 7

7 An NBT.GCaMP3 Calcium-sensitive transgenic reporter line for the *in vivo* study of seizures.

7.1 Introduction

In recent years, the zebrafish has proved to be a valuable animal model for the study of epilepsy mechanisms and the identification of new potential antiepileptic drugs (Baraban, et al., 2005; Baraban et al., 2007; Baraban, et al., 2013; Baxendale et al., 2012; Hortopan, et al., 2010; Orellana-Paucar et al., 2012a; Orellana-Paucar et al., 2012b).

The most common technique for making direct, real-time recordings of epileptic seizures in various animal models of epilepsy is to use electrographic recordings (Reviewed by McNamara, 1994). In order to obtain an electrographic recording, an electrode has to be introduced into the brain of the analysed animals, making this a very invasive and complicated technique. Among all the advantages that zebrafish can offer, the transparency of young zebafish embryos and larvae is without a doubt a great feature that no other animal model has. Combining this transparency with the recent development of a range of transgene-encoded fluorescent proteins, which exhibit optically measurable responsiveness to ion fluxes, offers the possibililty of monitoring ion flows within cells of the zebrafish embryo. In this respect, one particularly valuable family of proteins are the Genetically Encoded Calcium Indicator (GECI) proteins, which allow direct visualisation of the calcium influx into neurones that occurs in response to excitatory neurotransmission. I therefore sought to construct a transgenic line in which GECI activity was targeted to neurones in the zebrafish larval CNS, which would enable seizures to be visualized directly.

7.1.1 Genetically-encoded calcium indicators (GECIs)

Genetically-encoded calcium indicators (GECIs) consist of a recombinant fluorescent protein fused to a calcium-binding domain. Two types of GECIs have been developed: the ratiometric Fluorescent Protein (FP) FRET-donor-acceptor pairs, and non-ratiometric types such as GCaMP (Hires, et al., 2008).

In GCaMP, circularly permuted GFP is fused directly to a calcium-binding domain from calmodulin, which elicits a strong increase in fluorescence intensity when calcium is bound. Unlike FRET-based sensors, the fluorescence changes exhibited by GCaMP in response to changes in intracellular calcium concentration can be captured directly by conventional fluorescence microscopy (Hires et al., 2008).

The sensitivity of GCaMPs to calcium concentration has improved in recent years. GCaMP6 is the latest version of this type of calcium indicators (Ding, et al., 2014). However, at the time I began this work, GCaMP3 was the best available GECI (Dreosti & Lagnado, 2011; Tian et al., 2009).

Different GCaMP variants have been developed to analyse synaptic activity in zebrafish. They have been used to study the spatiotemporal activation of spinal motor-neurons (Muto et al., 2011), to image the optic-tectum during visual stimulation (Del Bene et al., 2010) and to image retinal responses (Akerboom et al., 2012; Nikolaev, et al, 2013). Compared to its predecessors, GCaMP3 has a stronger baseline fluorescence and was validated as a highly sensitive *in vivo* reporter of neuronal activity-dependent calcium flux in Drosophila, *C. elegans* and mouse (Tian et al., 2009).

The creation of a transgenic zebrafish line expressing GCaMP3 in the entire nervous system represented a potentially extremely powerful tool for the high-resolution, *in vivo* visualisation of neurophysiological activity in intact zebrafish larvae.

7.2 Results

7.2.1 Making of the *NBT:GCaMP3* transgenic zebrafish line.

A transgenic zebrafish line expressing the calcium reporter GCaMP3 under the control of Neuronal beta-tubulin (NBT) promoter was generated by transgenesis using the Tol2 system (Kawakami, 2005, 2007; Suster, et al., 2009). The resulting *NBT:GCaMP3* cassette was then subcloned into a miniTol2 vector and injected into one-cell stage nacre zebrafish embryos along with Tol2 transposase mRNA (Figure 7.1). The injected embryos were checked at 2 dpf and embryos that exhibited mosaic expression of GFP in the CNS were raised in the aquarium until they were mature enough to be crossed to search for founders.

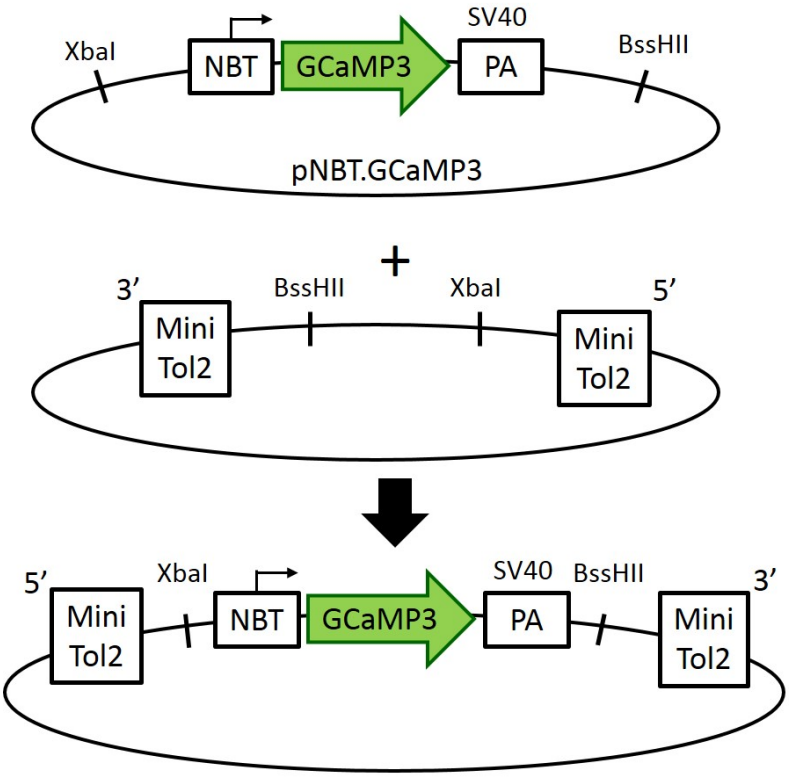

Figure 7.1 Schematic representation of *NBT:GCaMP3* plasmid subcloning into MiniTol2 vector. *pNBT.GCaMP3* was cut using the restriction enzymes XbaI and BssHII and was inserted into the MiniTol2 vector.

When the founder fish were 3 months old, they were crossed with nacre wild-type fish. The fish that had integrated the *NBT:GCaMP3* transgene into their germlines were able to transmit it to their progeny (F1) (Figure 7.2). Some of these non-mosaic *NBT:GCaMP3*expressing embryos were raised to establish the line and others were used for imaging.

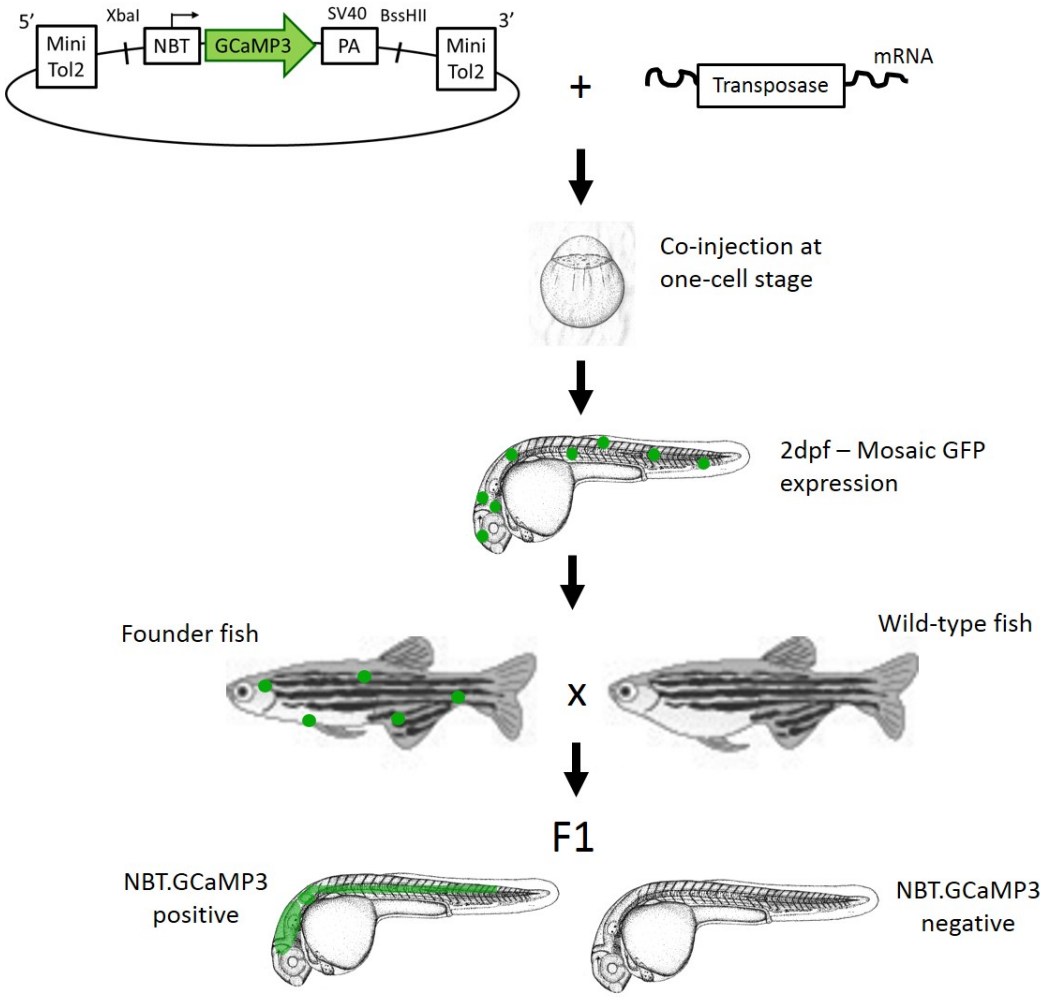

Figure 7.2 Schematic representation of the making of NBT:GCaMP3 zebrafish line. The plasmid containing *NBT:GCaMP3* flanked by Tol2 sites was co-injected along with transposase mRNA into one-cell stage embryos. GFP expressing embryos were selected and raised. When the fish were 3 months old they were crossed with wild-type fish and the NBT:GCaMP3 positive F1 progeny were raised to establish the line.

7.2.2 Analysis *of NBT:GCaMP3* expression in zebrafish larvae in response to PTZ exposure

3 dpf *NBT:GCaMP3* positive larvae exhibited a weak, basal level of GFP expression throughout the CNS, which was strong enough to be detected under the fluorescence microscope (Figure 7.3 – No PTZ). Expression of GFP increased in the CNS when larvae were stimulated by mechanically touching them. However, the expression was strongly enhanced throughout all the brain when the larvae were exposed to 20mM PTZ (Figure 7.3).

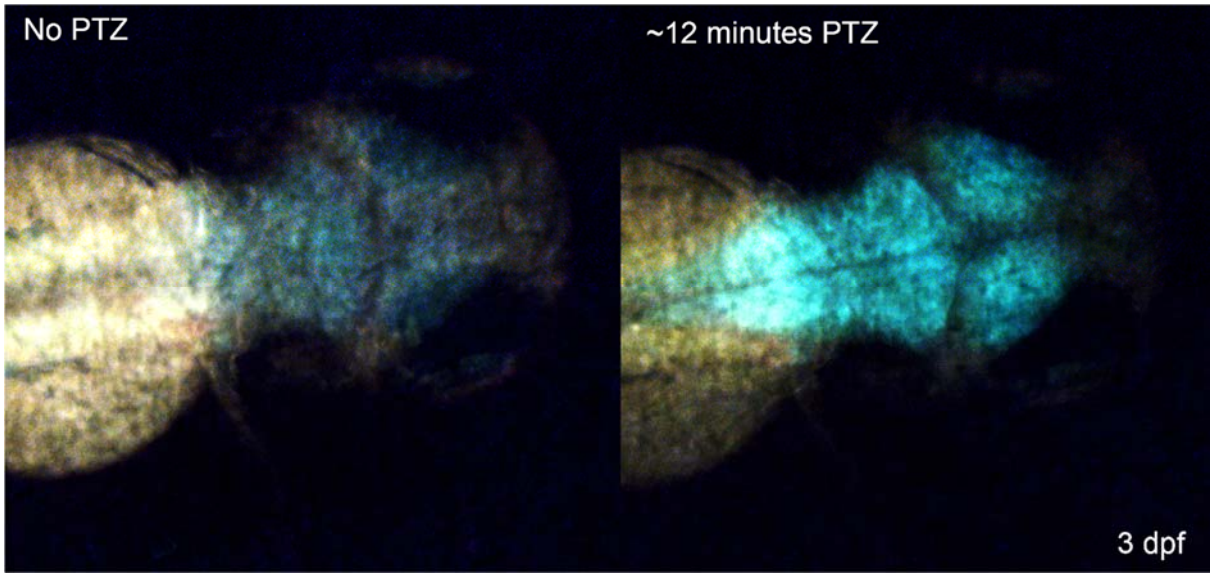

Figure 7.3 *NBT:GCaMP3* larvae could be detected under the fluorescence microscope. Picture of 3 dpf NBT:GCaMP3 larvae taken with a camera connected to the fluorescence microscope. The expression of GFP increased substantially in the presence of PTZ.

To get a better resolution, 3 dpf *NBT:GCaMP3* zebrafish larvae were mounted in agarose and imaged under the confocal spinning disc microscope (Perkin Elmer Ultraview VoX spinning disc confocal system running on an Olympus IX81 motorized microscope). First, the larvae were treated with 50μM Blebbistatin (Sigma) to render them immotile when PTZ was administered. (Kovács, et al., 2004). Larvae were then mounted in 1% low melting point (LMP) agarose in a small glass-bottomed petri dish for imaging on the spinning disk confocal microscope.

Images were recorded using the Improvision Volocity Suite in the spinning disc confocal system. 200µl of 20mM PTZ in E3 medium were added 5 minutes after starting imaging. The time to respond to PTZ was variable from fish to fish, as was the intensity of GCaMP3 fluorescence. Most of 3 dpf zebrafish larvae responded to PTZ exposure between 6 and 12 minutes after PTZ was added. Increased GCaMP3 fluorescence always started in the hindbrain, then spread to the forebrain and quickly extended to the hindbrain and spinal cord.

Figure 7.4 shows a series of still images from time-lapse videos taken under the spinning disc confocal system. After 4 minutes of PTZ exposure, few cells in the hindbrain began to increased GCaMP3 fluorescence; at 6 minutes the increased signal had spread to the forebrain and by 8 minutes after PTZ was added, a wave of increased GCaMP3 fluorescence had flowed through the hindbrain and caudally along the spinal cord (Figure 7.4 A). The increased GCaMP3 fluorescence caused by exposure to PTZ was blocked by pre-treating larvae with the anti-epileptic drug Valproic Acid along with blebbistatin before mounting them in agarose (Figure 7.4 B).

The *NBT.GCaMP3* zebrafish transgenic line shows considerable promise as a novel tool for spatio-temporal mapping of how the zebrafish brain responds to PTZ treatment.

The videos from which the pictures were taken can be found here:

Figure 7.4 A (Movie 2):

https://drive.google.com/file/d/0B3wwY1F5s-LKOURkYm5aOWRfUkU/edit?usp=sharing

Figure 7.4 B (Movie 3):

https://drive.google.com/file/d/0B3wwY1F5s-LKeFBFc2VvVTJXTFU/edit?usp=sharing

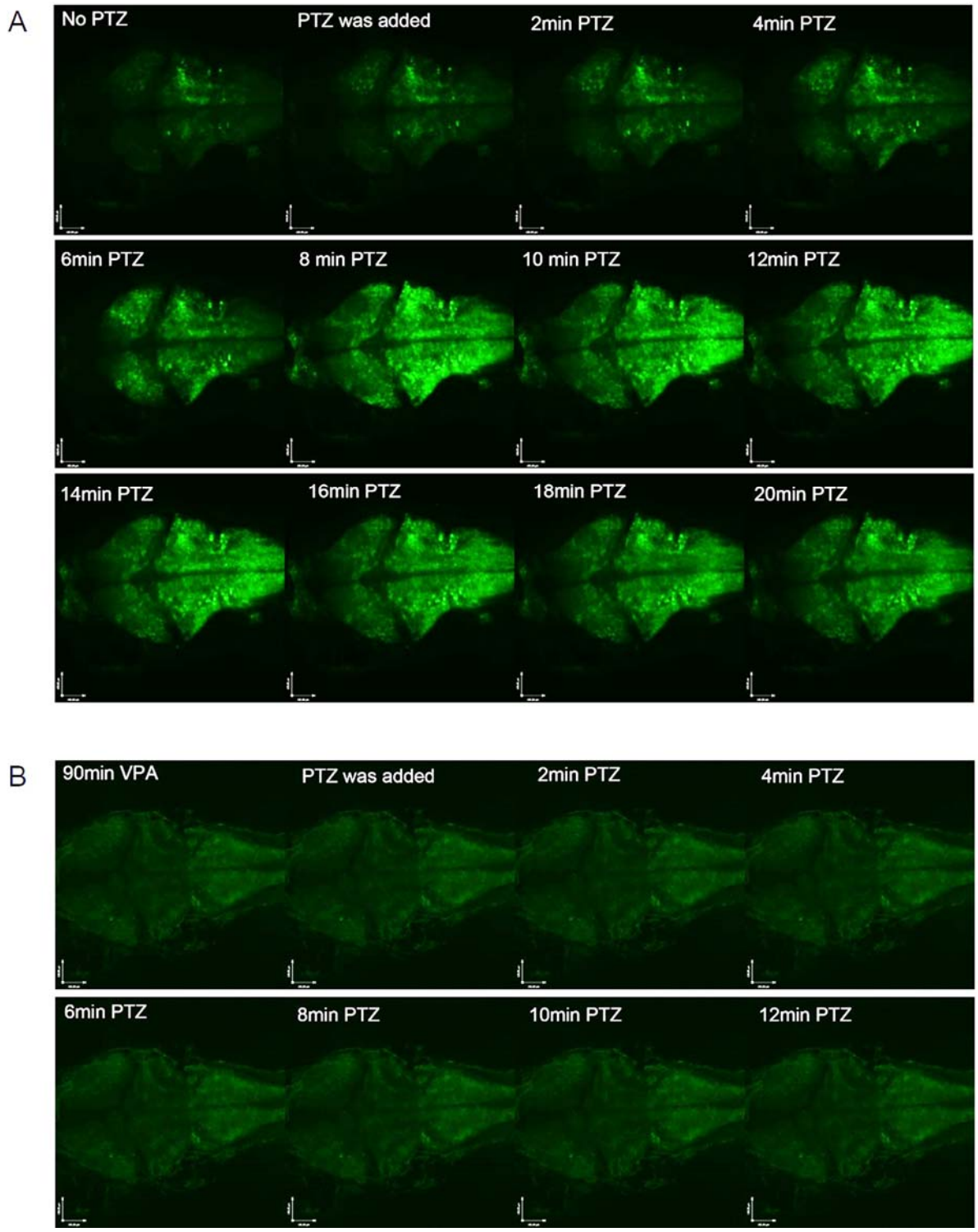

Figure 7.4 Still images taken from time-lapse videos showing GCaMP3 fluorescence in response to PTZ exposure. A) A strong increase in GFP expression was detected when 3 dpf *NBT:GCaMP3* zebrafish larvae were exposed to PTZ. B) When larvae are treated with VPA before PTZ exposure, no GFP increase was detected.

7.3 Discussion

GCaMPs are an extremely powerful tool for the high-resolution*, in vivo* visualisation of calcium changes in a variety of tissues. GCaMPs have been used for the study of calcium flux mainly in neurons of worms, flies, zebrafish and mice (Ding et al., 2014; Muto & Kawakami, 2011; Tian et al., 2009), but they have also been used in other cell types such as astrocytes (Tong, et al., 2013) and muscle cells (Baxendale, Holdsworth, Meza Santoscoy, et al., 2012; Tallini et al., 2014).

I created the *NBT:GCaMP3* transgenic zebrafish line for the *in vivo* imaging of seizures. The most widely used technique for detecting electrical activity in the brain of animal models of epilepsy is by placing electrodes in the brains of surgically operated animals to get electrographic recordings. Although this is a well-stablished technique, it is very laborious, invasive and limiting, because it only captures the activities of individual neurones in specific regions of the brain where the electrodes are placed (McNamara, 1994). Using the *NBT:GCaMP3* line, I was able to visualise for the first time a seizure in real time in a living animal using a GECI. The *NBT:GCaMP3* line offers the opportunity of visualising the spatiotemporal pattern of how seizures are initiated and propagated across the whole brain. In order to characterise the neurotransmitter phenotypes of the responding neurones, *NBT:GCaMP3* line could be crossed to different RFP transgenic lines of glutamatergic (*vglut*), glycrinergic (*glyt2*) and GABAergic (*gad*) neurons (Satou et al., 2013), in order to visualise the excitability of these specific neuronal sub-types.

This transgenic line will be an immensely useful resource not only for the study of epilepsy and for the screening of nee anti-epileptic drugs, but also for the elucidation of the mechanisms underlying the assembly and refinement of neuronal circuits, gene function analysis in neurons, and the *in vivo* mapping of neurotransmitter phenotypes.

Chapter 8

8 Concluding remarks and future prospects

Epilepsy is a common neurological disorder characterised by recurrent epileptic seizures that affects approximately 0.7% of the worldwide population. Even though many patients respond to the available treatments, around a third of them do not respond to existing ant-epileptic drugs (AEDs) (Remy & Beck, 2006). Therefore, there is a need to develop new therapeutic strategies for those epilepsies that are resistant to the current treatments.

In order to develop new anti-epileptic drugs, a better understanding of the pathogenesis of epilepsy is needed, as well as new approaches that enable a cost-efficient way to screen for novel AEDs. To date, research done in rodent models of epilepsy has provided most of the knowledge that we have regarding epileptogenesis. However, in recent years, non-mammalian models of epilepsy have proven to be a reliable, cheaper alternative to the use of high-cost, labour-intensive rodent models of epilepsy (Cunliffe et al., submitted).

Zebrafish (*Danio rerio*) is among those non-mammalian organisms that had emerged as a promising model for the study of epilepsy (Baraban,et al., 2005; Baraban, et al., 2013; Baxendale et al., 2012; Orellana-Paucar et al., 2012). The use of zebrafish as a model organism offers many advantages due to its genetic tractability, easy drug administration and relatively low-cost maintenance (Lieschke & Currie, 2007; Stewart, et al., 2014). Scott Baraban and colleagues first described the behavioural response zebrafish larvae exhibited when exposed to the convulsant drug pentylenetetrazole; three stages of behavioural seizure activity in 7 dpf zebrafish larvae were identified (Baraban et al., 2005; Hortopan, et al., 2010). In order to explore the possibility of using younger zebrafish larvae for the study of epilepsy, I characterised the behavioural response of 3 and 4 dpf zebrafish larvae to PTZ treatment.

Consistent with previous studies in the lab (Baxendale, et al., 2012), the optimum concentration of PTZ to obtain a significant convulsive response in 3 and 4 dpf zebrafish larvae was 20mM. 3 dpf zebrafish larvae treated with 20mM PTZ exhibited the three different stages

of convulsive behaviour described by Baraban and Hortopan. Moreover, 3 dpf PTZ-treated larvae swam 250% more distance than untreated larvae. These results demonstrated that even though the nervous system of zebrafish larvae is very immature at 3 dpf, it is still able to respond to PTZ. Therefore, zebrafish larvae as young as 3 dpf can be used as a model organism for the study of epilepsy.

Since one model of pharmocologicaly-induced seizures would likely be inadequate for identifying AEDs that could be effective for many different types of seizure, I wanted to investigate whether zebrafish larvae responded to other seizure inducing drugs used in different animal models of epilepsy. 3 dpf zebrafish larvae that were exposed to picrotoxin, kainic acid or NMDA responded with an increased convulsive locomotor behaviour, suggesting that zebrafish larvae may be used to find drugs that prevent seizures that have distinct mechanistic origins.

In addition to increased locomotor activity, PTZ treatment also induced the expression of the synaptic activity-dependent genes *c-fos*, *npas4a* and *bdnf* in the central nervous system of 2, 3 and 4 dpf zebrafish embryos/ larvae. The transcription of these three genes showed a distinct temporal regulation in response to PTZ exposure in 3 dpf zebrafish larvae. *npas4a* transcripts were synthesised rapidly throughout the brain within 15 min of exposure to PTZ, whereas *c-fos* and *bdnf* mRNAs accumulate much more slowly. Moreover, transcriptional induction of *npas4a* and *c-fos* required continuous exposure to PTZ, whereas induction and maintenance of *bdnf* transcription occurred after PTZ withdrawal, suggesting that early responses to PTZ include expression of genes that promote *bdnf* transcription. The temporal differences in expression of these three genes suggested the possibility that Npas4a might be regulating the transcription of the two other genes in response to PTZ. Previous studies have shown that *Npas4* expression is required for the expression of *c-fos* and *BDNF* in neurons of rats and mice during memory formation and neuronal depolarisation (Bloodgood, et al., 2013; Pruunsild, et al., 2011; Ramamoorthi et al., 2011). The expression of *BDNF* can also be regulated by c-*fos* expression; studies carried out in *c-fos* null mice showed a reduced KA-

induced *BDNF* expression in *c-fos* null mice compared to wild-type mice (Dong, et al., 2006; Zhang et al., 2002).

The effects of *c-fos* and *npas4a* knockdown by splice-blocking morpholinos were analysed in zebrafish embryos and larvae treated with PTZ. *c-fos* morpholino caused morphological defects probably as a consequence of developmental delays. The small head phenotype caused by *c-fos* morpholino prevented the execution of further studies on the effect of *c-fos* knockdown using this approach. *Npas4a* knockdown in zebrafish larvae by the injection of a splice-blocking morpholino resulted in the reduction of PTZ-induced *c-fos* and *bdnf* expression in 3 dpf zebrafish larvae compared to wild-type controls. However, the behavioural convulsive response was not altered by *npas4a* suppression in 3 dpf PTZ-treated zebrafish larvae. To further study the effects of the loss of *npas4a* function, the *npas4a* null mutant line *sa701* (Zebrafish Mutation Project, Sanger Institute) was also analysed. Differences in the genetic background of Tupfel longfin strain compared to AB strain and low viability made it difficult to obtain any conclusive data. To overcome the possible off-target effects of morpholinos and differences between strains, targeted mutations in *c-fos* and *npas4a* genes could be generated using TALEN or CRISPR technology in an AB background. Moreover, further work needs to be done to investigate whether there is functional redundancy between *npas4a* and its close relative *npas4b* in zebrafish larvae.

Taken together, these results indicate the onset of epileptic seizures triggers the transcription of a tightly regulated programme of gene transcription, that may affect the functional properties of neurons and the neural circuits of which they are components. Specifically, the rapidly-transcribed *npas4a* gene encodes a transcription factor whose expression might be involved in promoting transcription of *c-fos* and *bdnf*. If such a transcriptional cascade is able to stably alter the functional characteristics of neuronal networks, then exposure of zebrafish larvae to PTZ might also alter their subsequent behavioural characteristics.

Severity of seizures is not constant in patients with epilepsy. There are triggers that initiate seizures and others that increase their severity. Sir William Gowers postulated that “seizures

beget seizures" in some patients suffering from recurrent seizures, such that seizure severity increases progressively, potentially due to alterations to brain function caused by the seizures (Gowers, 1881). In rodent models of epilepsy, the kindling model has demonstrated that seizures get worse over time in rats and mice repeatedly stimulated with sub-convulsive doses of electrical stimulation or convulsant drugs, until the animals present with spontaneous seizures (McNamara, et al, 1980).

In order to determine whether multiple, transient exposures to a seizure-inducing concentration of PTZ would sensitise the developing brain of zebrafish embryos/larvae to further PTZ exposure and consequently increase the magnitude of the convulsions, repeated exposures to 20mM PTZ, were carried out for one hour a day during 4 consecutive days. Both the behavioural and the transcriptional responses were analysed at 4 dpf. Throughout 10 experimental replicates, the results showed a consistent increase in the locomotor response to PTZ of 4 dpf larvae pre-treated with PTZ during days 1, 2 and 3 as compared with 4 dpf larvae that were not previously treated with PTZ. In addition, PTZ-induced expression of *c-fos* and *npas4a* was reduced in PTZ pre-treated larvae. This reciprocal effect of enhanced seizure severity and reduced gene expression suggested that stable changes in the structure and function were occurring in the central nervous system of PTZ pre-treated zebrafish larvae.

Given the fact that such a robust programme of new gene transcription accompanied the onset of seizures induced by PTZ, it seemed reasonable to consider the possibility that transcriptional regulatory mechanisms involving proteins such as Histone Deacetylases (HDACs), Histone Acetyltransferases (HATs) or DNA methyltransferases might be involved in the attenuation of the transcription of *c-fos*, *npas4* and other genes and/or promoting an enhanced convulsive behaviour, as was observed in response to repeated treatments with PTZ. Interestingly, the HDAC inhibitor, Trichostatin A (TSA) was administered just before PTZ treatment during days 1, 2 and 3, which attenuated the increased the severity of locomotor convulsive behaviour caused by PTZ treatment at 4dpf. Moreover, 3dpf larvae treated with TSA and PTZ showed a noticeable reduction in *c-fos* expression as compared with 3 dpf

larvae treated with PTZ only. It will now be interesting to explore whether other inhibitors of transcriptional silencing proteins such as the DNA methyltransferase inhibitor 5-azacytidine has a similar suppressive effect on the enhancement of seizure severity caused by repeated exposure to PTZ.

The influence of stress in response to seizure induction was analysed by exposing 3 dpf zebrafish larvae to betamethasone, which is a synthetic analogue of cortisol, before treating them with PTZ. Consistent with the reports that stress is a trigger of seizures in patients with epilepsy (Frucht, et al., 2000; Haut, et al., 2007; Nakken et al., 2005; Novakova et al., 2013; Spector, et al., 2000; Sperling, et al., 2008) and rodent models of epilepsy (Herpfer et al., 2012; Maggio & Segal, 2012; Yun et al., 2010c), treatment with betamethasone caused an enhanced seizure severity in 3 dpf PTZ-treated zebrafish larvae. Furthermore, betamethasone treatment lowered the threshold for seizure induction and strongly suppressed PTZ-induced expression of *npas4*, *c-fos* and *bdnf*. Thus, as was observed for larvae repeatedly treated with PTZ, stress hormone exposure increased locomotor convulsive behaviour and reduced expression of PTZ-responsive genes.

Taken together, these results suggest that there is a complex programme of gene expression involved in the response to seizure induction. To identify PTZ-induced genes, a gene expression profiling analysis of 3 dpf zebrafish after exposure to PTZ for 30 minutes and 90 minutes was performed to identify differentially expressed genes. As expected from the results obtained in the *in situ* analyses, the gene *npas4a* exhibited the greatest fold-change in PTZ-treated larvae after 30 minutes of PTZ exposure, compared with untreated controls. Moreover, *npas4a* was also among the top 3 up-regulated genes at the 90 minute time point. Most of the genes found increased in 3 larvae treated with PTZ for 30 and 90 minutes were genes that encode transcriptional regulatory proteins. The genes *fosb* and *junbb* were found highly up-regulated in response to PTZ treatment. These genes along with *c-fos* form the transcription factor Activator Protein 1 (AP-1) (Morgan & Curran, 1991; Shaulian & Karin, 2002), which has been involved in CREB-mediated long term plasticity in Drosophila (Sanyal, Sandstrom,

Hoeffer, & Ramaswami, 2002). Two genes of the *nr4a* family of transcription factors were also found up-regulated after 90 minutes of PTZ treatment in zebrafish larvae. It would be interesting to study the functions of *nr4a1* and *nr4a2b* in the zebrafish model of epileptic seizures as they have been associated with memory formation and regulation of the hypothalamic-pituitary-adrenal axis in response to stress (Hawk & Abel, 2011; Helbling, et al., 2014; Volakakis et al., 2010). Another gene worth studying from those found in the microarray is *mmp9*; increased levels of MMP-9 have been associated with abnormal synaptic plasticity, neuronal death and inflammation in epilepsy (reviewed by Mizoguchi & Yamada, 2013). The microarray analysis also identified many proteins of unknown function, suggesting that novel molecular mechanisms might be involved in the response to seizure onset or the pathogenesis of epilepsy.

Some of the genes whose expression was up-regulated after 90 minutes of PTZ treatment in 3 dpf zebrafish larvae were also up-regulated in surgically removed hippocampal tissue of patients with temporal lobe epilepsy (TLE) by Dr. Enrico Petretto, Dr. Michael Johnson and their colleagues. These genes included *c-fos, fosb, atf3, gadd45ba* and *junbb*, a group of mostly transcriptional regulatory genes, which is related to a network of inflammatory cytokine and TLR-pathway genes known as Module-1. Previous studies had shown that Module-1 is regulated by *Sestrin 3* (*sesn3*), and so a decision was taken to investigate the function of *sesn3* in zebrafish larvae, using two co-injected splice-blocking morpholinos to disrupt the normal splicing of *sesn3* transcript. 3 dpf *sesn3* morphants showed a reduced locomotor response to PTZ treatment in comparison to uninjected and standard control morpholino injected larvae also treated with PTZ. Furthermore, PTZ-induced expression of Module-1 genes: *c-fos*, *fosb*, *atf3* and *egr2b* was reduced in *sesn3* morphants. When synthetic *sesn3* mRNA was co-injected with *sesn3* morpholinos, the convulsive behaviour was rescued in 3dpf *sesn3* morphants treated with PTZ, and over-expression of *sesn3* by mRNA injection caused an increase in the expression of a subset of Module-1 genes. These the results suggest that *sesn3* might have a role in regulating seizure severity in response to PTZ treatment. A

zebrafish *sesn3* mutant line is currently being raised which will allow the function of this gene in epileptogenesis to be elucidated further. Finally, I have created the *NBT:GCaMP3* transgenic line in order to visualize *in vivo* how a seizure is initiated and propagated, and to map the neuronal circuits involved in the seizure response. This line, together with new zebrafish lines with CRISPR- and TALEN-targeted mutations in orthologues of genes known to be mutated in human epilepsy, may now be used in novel mechanistic studies to investigate the pathogenesis of epilepsy, as well as in screening assays to discover novel anti-epileptic drugs.

Table of Abbreviations

AEDs	Anti-epileptic Drugs
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activator Protein 1
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bHLH	Basic helix-loop-helix
CA1	Cornu Ammonis Area 1
CA3	Cornu Ammonis Area 3
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
CREB	cAMP Response Element-binding Protein
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dpf	Days post-fertilisation
EEG	Electroencephalogram
EMBL	European Molecular Biology Laboratory
FP	Fluorescent Protein
FRET	Förster Resonance Energy Transfer
GABA	Gamma-Aminobutyric Acid

GECIs	Genetically Encoded Calcium Indicators
GFP	Green Fluorescent Protein
HAT	Histone Acetyltransferases
HDAC	Histone Deacetylases
HPA	Hypothalamic-Pituitary-Adrenal
hpf	Hours post-fertilisation
IEGs	Immediate Early Genes
KA	Kainic Acid
LMP	Low Melting Point
LTD	Long-term Depression
LTP	Long-term Potentiation
MeOH	Methanol
MO	Morpholino
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
NBT	Nitro Blue Tetrazolium Chloride; or Neural Beta Tubulin
NCBI	National Center for Biotechnology Information
NMDA	N-methyl-D-aspartate
PBS	Phosphate buffered saline
PO	Phosphate
PTX	Picrotoxin

PTZ	Pentylenetetrazole
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROI	Region of Interest
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
SSC	Saline Sodium Citrate
TAE	Tris-acetate
TLE	Temporal Lobe Epilepsy
TLR	Toll-like Receptors
tRNA	Transfer Ribonucleic Acid
TSA	Trichostatin A
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
VPA	Valproic Acid
WHO	World Health Organization

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