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Chemical Genetics in zebrafish:

Modulation of cAMP and MAPK pathways in behaviour

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ABSTRACT TO THESIS

The prevalence of stress and anxiety disorders in modern society is increasing, but the development of new treatments decreasing due to high research costs and low success rates in clinical trials. The latest type of compounds introduced to treat anxiety and depression was the specific serotonin reuptake inhibitors (SSRI), which was introduced in 1987. Since then, no new class of compounds have been introduced, suggesting that the need to find alternative targets in treating mental disorders is needed.

In this thesis I have used the zebrafish as a model organism to study the modulation of behaviours through intracellular signalling pathways, known to be involved in learning, memory and anxiety.

First, using the pro-convulsant compound, pentylenetetrazole (PTZ), an automated tracking system was established to quantify and analyse swimming behaviour in larvae zebrafish. Pentylenetetrazole induces seizures in zebrafish at high concentrations, however this thesis identifies that the combination of a low level of PTZ and subjecting the fish to alternating cycles of light and dark induced a reversed response to light and dark. A group of compounds with known anti-seizure effects were subsequently screened, which found that a combinational treatment with diazepam and two types of neurosteroids reversed the PTZ-induced light dark response.

Secondly, using the same automated analysis setup, the effect of cAMP modulators was studied on behaviour in zebrafish larvae. Our lab has previously established that Rolipram, a PDE4 inhibitor, causes anxiety thigmotaxis in zebrafish larvae. In this thesis we treated zebrafish larvae with Rolipram and other compounds modulating cAMP, which greatly increased the swimming activity, which was reversed by subsequently treating with PD0325901. To test if the pharmacological modulation of cAMP-levels through the inhibition of other PDEs would lead to increased locomotor activity, a small library of PDE inhibitors was screened, and 4 compounds were identified that caused an increase in locomotion – three of these compounds were PDE4-inhibitors.

Finally, by using two behavioural assays, I found that in adult fish Rolipram cause anxiety-like phenotypes, which is also reversible by MAPK-inhibition.

LAY SUMMARY

The number of people suffering from mental disorders, like depression and stress, are increasing. However, the available treatments for these types of diseases are not very effective, and the development of new drugs is slow. New drugs introduced into the market are often variants of already existing drugs, and often have limited effect or unwanted effects. The development of new drugs for mental illness is a slow and often un-successful task. The lack of good animal models to be used in the initial drug development process is lacking, as the cost and ethical reasons in utilizing rodents in drug discovery screening approach is not feasible. In this thesis I have used the zebrafish as an alternative screening model, to attempt to identify alternative pathways that can be involved in the regulation and development of mental illness, like anxiety or stress. Previously our lab has shown that drugs known to target signalling pathways in the brain can have an anxiety-enhancing effect on zebrafish. Through behavioural studies in zebrafish I have found that drugs, that are primarily used to treat different types of cancer, can in low doses be beneficial in relieving anxiety-like symptoms in zebrafish, that has are caused by the activation of pathways involved in cell signalling. This indicates that the search for alternative pathways in the treatment of mental illness should be extended to include more general signalling pathways, and instead aim at fine-tuning over-active pathways.

I hereby declare that this thesis has been composed by me, this work has not been submitted for any other degree or professional qualification, the work is my own and contribution from others has been clearly indicated.

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1 Introduction

1.1 Introduction

The number of people suffering from mental illness in the 21st century is increasing. In the UK alone it is predicted that 1 in 6 adult experiences some form of mental illness in their lifetime, which makes mental illness a major contribution to the health care costs in the UK per year (Choices 2013). The consequences of mental illness are not only devastating for the patients, but also for the relatives. Due to the increasing number of people affected by mental illness there is a growing need for new treatments. However, the number of drugs being approved and introduced into the clinic is declining, and the cost of developing new drugs is increasing. Especially in the area of mental illness, the development of new drugs is very limited, which reflects poorly on the need for new treatments (Craven 2011). Despite this need for new treatments, there has been an increase in the number of pharmaceutical companies that pull out of research and drug development in the field of psychiatric illness (Craven 2011), due to reasons primarily attributed to long development timelines, which often ends with a very low success rate in the final stage of clinical trials (Agid et al. 2007; Craven 2011; O'Brien et al. 2014). These factors together with the loss of revenue due to expiring patents, together with very high research costs, have made pharmaceutical companies less inclined to take risks when venturing into new, un-explored areas of drug development (Craven 2011). Historically, the use of rodent models in drug development has been a gold standard, but the use of these models in drug development screens is time-consuming, low-throughput, expensive and ethically problematic. Furthermore, there is a growing

concern about the number of rodents used for research and drug development, and a wish to reduce these numbers (Festing and Wilkinson 2007). Thus the need for a complementary model which can bridge the gap between in vitro studies and rodent studies is eminent, that can help to cut down on the number of rodents being used in research and to decrease some of the research costs which are limiting the discovery and development of new drugs. A possible vertebrate model organism that could fit this purpose is the small freshwater teleost, *Danio rerio*, the zebrafish.

1.1.1 The zebrafish embryo model

One of the main advantages of zebrafish as a model organism is the ability to use both the larval and the adult stage of the fish. Zebrafish embryos and larvae have been a favourite model organism in genetics and development for almost three decades, and as a vertebrate model it offers an excellent compromise between higher vertebrate models and popular invertebrate models. The zebrafish is easy to maintain at a low cost, they produce a high number of offspring as one breeding pair can produce approximately 200 embryos a week, and the development of the embryos is rapid. Moreover, the zebrafish embryos develop *ex utero*, at 28°C and in a transparent chorion, which allows for the visualization of developmental progress in a standard light microscope. The potential of the zebrafish as a model organism in genetics and development, was solidified by the publication of a large mutagenesis screen identifying several genes involved in a wide range of processes, among others

the regulation of behaviour, development and visual function (Felsenfeld 1996; Granato et al. 1996; Haffter et al. 1996). The screen was performed by recovering mutants from an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen, depending on a specific phenotype. Granato and colleagues identified mutants with movement defects, representing 30 different genes shown to be important in the development of movement in zebrafish larvae (Granato et al. 1996).

Furthermore, broad use of zebrafish in research has expanded immensely, and in 2000 Peterson and colleagues published a proof-of-principle paper, using small molecules to elucidate gene function on development (Peterson et al. 2000). Using available transgenic and mutant lines available to the zebrafish community (Peal et al. 2011) a wide range of biological processes, amongst them melanocyte development and regulation (Colanesi et al. 2012), cardiac function (Peal et al. 2011), light response (Kokel et al. 2010) and sleep regulation (Rihel et al. 2010) has been studied in zebrafish.

1.1.1.1 The movement repertoire in zebrafish

One of the main objectives when studying behaviour is to understand how the function of the nervous system works. To elucidate on the function of the genes involved in neuronal development and function, the use of genetic models can help to reveal what developmental and genetic pathways underlying these behaviours, and ultimately help to develop new and better types of treatments.

The zebrafish embryo develops into free-swimming larvae at 4-5 days post fertilization (dpf) (Figure 1.1.1). As early as 17 hours post fertilization (hpf) the

embryo develop spontaneous movements, consisting of coil-movements of the tail (Fero, Yokogawa, and Burgess 2011), and at 21 hpf the zebrafish respond to touch. At 30 hpf the fish responds to a flash of bright light (Kokel et al. 2010), and at the time of hatching (3 dpf) they begin to engage in evoked swimming behaviour (Brustein et al. 2003). The swim bladder inflates around 4 – 5 dpf, enabling the larvae to engage in free swimming behaviours, mainly characterized by a combination of burst and glide movements (Fero, Yokogawa, and Burgess 2011). This also allows the larvae to engage in food-seeking behaviours. By utilizing the rapid development of the nervous system combined with the small size, several reports have demonstrated the usability of zebrafish larvae in neuroscience.

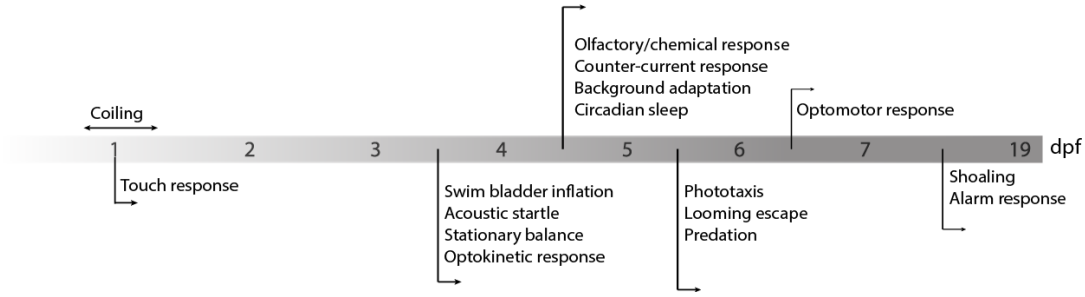

Figure 1.1.1: Developmental timeline of larval behaviour.

The development of the zebrafish larvae is rapid, for that of a vertebrate organism. The earliest movement displayed by the developing zebrafish embryo is the coil movement of the tail, visible around 17 hpf, which can be provoked by a touch to the tail of the embryo. At 21 hpf the embryo respond to high-intensity light flashes, and shortly thereafter (around 48 hpf) the first swimming episodes can be observed, although these are limited due to the un-inflated swim bladder, which is not inflated before 4-5 dpf. The inflation of the swim bladder also promotes the free swimming behaviour of the larvae, and the active feeding behaviour. Reproduced from (Fero, Yokogawa, and Burgess 2011).

1.1.2 Translational validity of zebrafish in anxiety research

The translational validity of zebrafish in behavioural science has been demonstrated during the last decade. Important contributions to this have come from studies using mutants generated from forward genetic screens, compounds known to target specific neurotransmitter systems in humans and rodents and testing them in zebrafish models.

Compounds targeting specific neurotransmitter systems, and with known behavioural effect in rodent models of anxiety, has been tested in zebrafish models of anxiety. Levin et al. use the novel tank test, and show that nicotine causes an anxiolytic effect in adult zebrafish, when they are placed alone into a novel environment. This causes them to explore more of the tank, reduce the number of erratic movements and bottom dwellings (Levin, Bencan, and Cerutti 2007).

The light-dark preference test is a test of boldness and has been demonstrated as a valid assay to asses anxiety-like behaviours in zebrafish (Maximino, da Silva, et al. 2011; R. E. Blaser, Chadwick, and McGinnis 2010; Maximino et al. 2010; Steenbergen, Richardson, and Champagne 2011b). Utilizing the zebrafish natural preference for the dark area of the tank, this assay is comparable to the light-dark test in rodents, a test often employed to test for anxiolytic effects of compounds (Schmidt and Müller 2006). In zebrafish, the pharmacological modulation using known anxiolytic compounds, buspirone and fluoxetine, increases the time the fish spend in the white compartment, and decreases the latency to enter the white compartment,

with less erratic behaviours when the fish are in the white compartment of the test tank (Maximino et al. 2013).

In 2009, Egan and colleagues show that the physiological effect of known anxiogenic and anxiolytic compounds can be measured on both the behavioural and at the neuroendocrine level, in adult zebrafish. By extracting cortisol from whole adult fish, they show that stressing the zebrafish through exposure to alarm pheromone or anxiogenic compounds, causes an increase in whole body cortisol levels (Egan et al. 2009). Cortisol is also the main stress hormone employed by humans, whereas mice use corticosterone. Cortisol is produced in the head kidney of fish, which corresponds to the adrenal gland in mammals. The Hypothalamus – Pituitary – Adrenal/Hypothalamus-Pituitary-Interenal (HPA/HPI) axis is responsible for the control and release of cortisol in response to stress and anxiety in humans and fish (Ramsay et al. 2006).

Taken together, these reports suggest that the translational value of zebrafish behaviour is comparable to that of rodents in behavioural research.

1.1.2.1.1 Behavioural assays in larvae zebrafish

The major advantage of the zebrafish in neurobiological research has been the use of the translucent, rapid developing embryo. Despite the primitive developmental stage of the larvae, it offers an unique opportunity to study the effect of genetic mutations or chemical modulators on both neurodevelopment and behavioural output (Granato et al. 1996; Irons et al. 2010).

The small size of the fish and the large number of offspring generated by a single breeding pair of adult fish, has been utilized in genetic and chemical behavioural screens, and facilitating the use of zebrafish in high-throughput chemical screens increasingly popular (Kokel et al. 2010; Rihel et al. 2010).

Modulation of behaviour has also been developed in more low-throughput settings, including tests for evaluation of anxiogenic- and anxiolytic-like behaviours, for which the light dark-test (Steenbergen, Richardson, and Champagne 2011b) and the open field test have been described in zebrafish larvae (Richendrfer et al. 2012; Schnörr et al. 2012). Other behavioural tests complementing rodent assays have been developed in zebrafish larvae, including locomotor assays (L. Ellis, Seibert, and Soanes 2012), pre-pulse inhibition (Best et al. 2008), anxiety (Richendrfer et al. 2012) and seizure (S. C. Baraban et al. 2005).

The use of larvae zebrafish in these behavioural tests instead of rodents, could serve as an initial screening platform for neuroactive compounds, thereby reducing the number of rodents used in research.

1.1.3 Understanding behaviour

Modelling human behaviour using animals has long been recognized as a valuable tool in biomedical research, and especially in the quest to understand the genetics and biology behind illnesses of the mind, have they proved very useful (Cash-Padgett and Jaaro-Peled 2013, 1; Clapcote et al. 2007; Egan et al. 2009; Schmidt and Müller 2006, 1). Studying behaviour offers an insight into the neurobiology and

neurochemistry of the brain, thus by using animals the understanding of how behaviour correlates to neurochemical or developmental defects have greatly aided in the understanding of the development and management of diseases of the mind. Furthermore the majority of drugs available for treating mental illness has been discovered and developed using animal models of behaviour, further underlining the importance of understanding behaviour.

1.1.3.1 Symptoms of anxiety and depression

1.1.3.1.1 Anxiety

As described previously, anxiety is the most prevalent psychiatric disorder in the western world. Anxiety is a feeling of apprehension or fear, and can often be precipitated by stress. Fear is the emotional response to a real or perceived imminent threat, whereas anxiety is the anticipation of future threat, therefore it is a preparation for future danger and consists of avoidance behaviours (Adhikari 2014). Anxiety is a natural feeling, and healthy individuals often experience moments of anxiety. When the feeling become persistent and disruptive, it progresses into a pathological state.

Symptoms of anxiety disorders are often described as a disturbance in the mood and thought process, which leads to excess worrying and apprehension of fear. These symptoms are often followed by more physical reactions, like dizziness, increased blood pressure and heart rate and insomnia (Adhikari 2014). Anxiety is divided into a range of different symptoms, covering generalized anxiety disorder, obsessive-Compulsive Disorder (OCD), phobias and panic disorders (Cryan og Sweeney 2011).

1.1.3.1.2 Depression

It is not rare for a healthy person to experience feelings associated with depression. Feeling sad or depressed is not uncommon, but in healthy persons these feelings will often pass. This is not the case in people suffering from depression. The feelings of hopelessness, sadness and emptiness are often persisting, which eventually will lead to a chronic state of sadness, low energy and altered temperament. Anhedonia and suicidal thoughts are often associated with depression, along with cognitive deficits.

1.1.4 Modelling anxiety and depression in rodent models

Rodents have been a favoured model in behavioural science for many years. Especially in the drug discovery field the use of mice and rats been extensively utilised. The innate behaviour of rodents is well documented, and therefore the chemical modulation of these behaviours has been used to elucidate on the function of potential drug candidates for the treatment of mental disorders in humans (Fernando and Robbins 2011).

Rodents have an innate drive to explore a new environment, a behaviour which can be manipulated using compounds with known anxiogenic or anxiolytic function. For this the open-field exploration test has proven useful. In this test, the animal is placed into an arena, and the light conditions can be altered, depending on the natural preference of the animal. Anxious animals show a less degree of exploration and stay close to the walls of the arena, a behavioural trait commonly described as thigmotaxis, or wall-hugging (Bouwknecht 2014). Another parameter determined in

this assay is the amounts of time spend in the centre of the arena and the total distance moved. The total distance moved is an important parameter, as this indicates if there is a potential side effect of the compound, causing decreased locomotor behaviours (Prut and Belzung 2003). When mice are treated with anxiolytic compounds, they spend more time at the centre of the arena, and show a greater degree of exploration and movement (Prut and Belzung 2003).

Another test commonly used in anxiety research is the elevated plus maze. In this test the animal is placed on a maze consisting of two open arms and two closed arms. The whole maze is elevated 60-80 cm above the floor, to stress the animal. Animals displaying anxiogenic-like behaviours will spend significantly more time in the closed arms, less time exploring and less time rearing up to examine their environment (Pellow and File 1986). These parameters can be altered when treating animals with anxiolytic compounds e.g. buspirone, a 5-$HT1_A$ agonist, buspirone,

1.1.4.1 Current drug treatments for anxiety

The treatment of anxiety often consists of a combination of cognitive treatment and pharmacological treatment. The goal of the cognitive treatment is to help the patients with managing the negative thoughts and behavioural patterns, which is often the disablitating factors in anxiety disorders.

Drug treatment is often divided into acute and maintenance treatment. The acute treatment is administered during a panic attack and has a fast acting effect, whereas the maintenance treatment is a chronic treatment, often used in an attempt to fine-tune the neurotransmitter systems known to be important in anxiety.

A majority of the treatments available for treating anxiety today have been validated using some of the behavioural assays described above. Below is a brief description of the most common anxiolytic treatment approaches used in the clinic.

1.1.4.1.1 Targeting the gamma-amino butyric acid receptor

Abnormal neuronal excitability is often due to deregulation of excitatory or inhibitory neurotransmission. In the central nervous system (CNS) glutamate is the major excitatory neurotransmitter, whereas gamma amino butyric acid (GABA) is the inhibitory. A deregulation of any one of these two neurotransmitters can lead to adverse neurotransmission, and thereby psychiatric disorders, like depression and anxiety. Therefore, the focus of current treatments for anxiety and depression has been focusing on the regulation and modulation of these two neurotransmitter signalling pathways.

Barbiturates are a very effective class of compounds, with strong sedative effects. This is thought to be archived through its blockage of the AMPA-receptor, inhibiting the excitatory mechanism of glutamate. Furthermore, it also binds to the gamma amino butyric acid (GABA) $_{A}$ receptor, as an allosteric modulator, thereby potentiating the inhibitory neurotransmission of GABA. The broad effect of barbiturates can cause a wide variety of side-effects observed in patients treated with these compounds, including sedation, slurred speech, dependence and ataxia (López-Muñoz, Ucha-Udabe, and Alamo 2005; Cryan and Sweeney 2011).

These adverse effects of barbiturates have made them less favourable in treatment of anxiety and highlight the need for finding alternative treatments with less side-effect.

The development of another class of compounds targeting the $GABA_A$-receptor came with the development of the benzodiazepines. This class of compounds bind to the benzo-site of the $GABA_A$-receptor, as an allosteric modulator, prolonging the opening of the channel, thereby enhancing the GABA-signalling. The compound is not, contrary to the barbiturates, capable of inducing a constant opening of the channel, which makes the risk of over-doses very low. Therefore, the use of benzodiazepines has been favoured over the use of the barbiturates, although there are aversive effects associated with the treatment (Argyropoulos and Nutt 1999). Generally, compounds that modulate GABA signalling are fast acting, making them effective in acute treatment of e.g. panic attacks. This is contrary to the most recent developed anxiolytic compounds, which are in general slower in their mechanism of action.

1.1.4.1.2 Antidepressants used for the treatment of anxiety

Anxiety and depression are often co-morbid, which means that patients diagnosed with one, often show symptoms of the other as well (Cryan and Sweeney 2011). It is therefore not surprising that compounds targeting the same neurotransmitter systems are used to treat both indications. These systems include the serotonin (5.HT) and the noradrenalin (NA) systems. Anxiolytic treatment is often targeting the serotonin system. Serotonin in a neurotransmitter, which acts through binding to the 5-HT-receptors.

The first line of treatment for anxiety is using a selective serotonin re-uptake inhibitor (SSRI) (Hoffman and Mathew 2008). The most widely used SSRI is

fluoxetine (Prozac®). The mechanism of action of SSRI is not well understood, but the prolonged availability of serotonin in the synaptic cleft is one of the proposed mechanisms.

From a clinical trial using a benzodiazepine in rats, it was observed that the rats treated with benzodiazepine had a much lower serotonin-turnover rate compared to that of vehicle treated rats (Wise, Berger, and Stein 1972). These data led to the suggestion, that serotonin play an important role in anxiety. This has been further substantiated from genetic studies, as mice lacking the 5-HT1a-receptor show an increase in anxiety-like behaviours in the elevated plus maze and the open field test (Toth 2003). The realization that serotonin plays an important role in anxiety resulted in the development of compounds specifically targeting this pathway. Partial 5-HT1a agonists, like buspirone, produces anxiolytic-like effects in rodent models, and have been used to treat anxiety in humans (Cryan and Sweeney 2011). These compounds utilized an alternative approach to treating anxiety, which was not through the GABA-receptor modulation. However, due to the slow onset of action of serotonin partial agonists, compared to that of benzodiazepines, the use of Buspirone to treat anxiety is limited (Hoffman and Mathew 2008).

The above mentioned treatment strategies, have all been in clinical use for several years(Wise, Berger, and Stein 1972; Hoffman and Mathew 2008). The lack of new treatments developed for the treatment of psychiatric illness is staggering. Although the constant development of new and better animal models, used to model the behavioural endophenotypes, there has not been a new class of novel anxiolytic

compounds approved and introduced into the clinic since the approval of the first SSRI, fluoxetine, in 1987 (Cryan and Sweeney 2011).

Therefore, there is a need for an alternative approach to treat psychiatric illness. One alternative could be to target the range of intracellular components of the signalling cascades regulated by neurotransmitter activity. One such pathway is the cyclic AMP pathway.

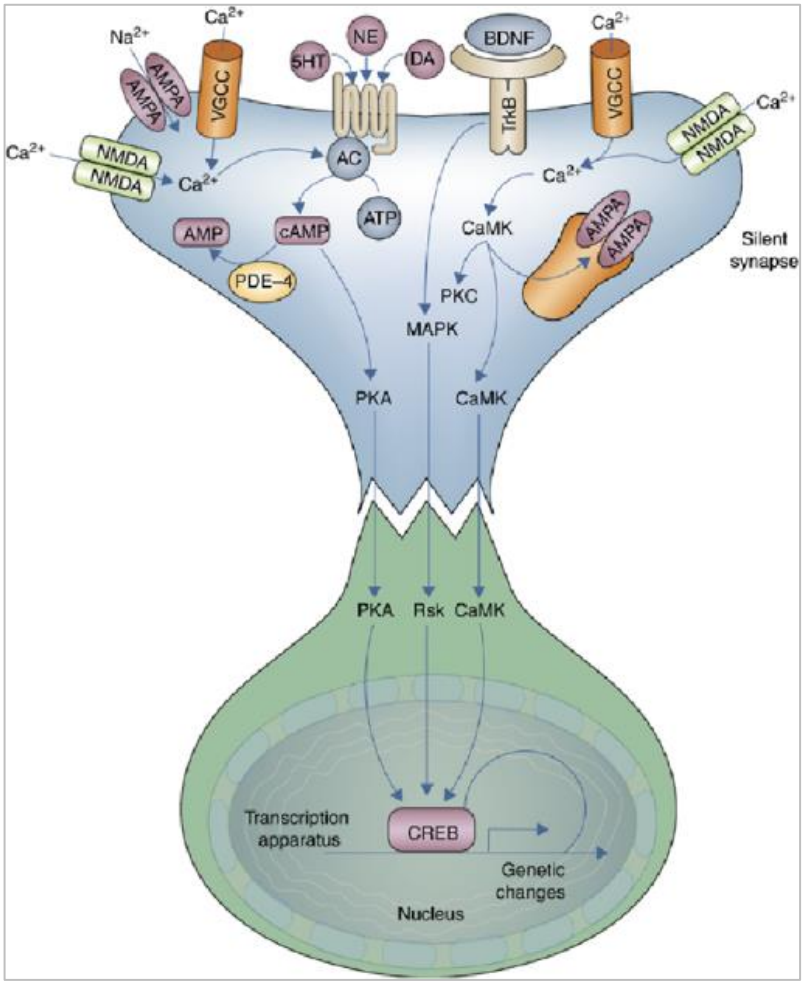

Figure 1.1.2: Schematic drawing of the intracellular signalling pathways involved in regulating neurotransmitter signalling.

Serotonin, dopamine and norepinephrine all signal through G protein coupled receptors, and cAMP. Upon neurotransmitter binding to the receptor, an increase adenylate cyclase is activated, causing an increase in cAMP levels. Activation of PKA by increase in cAMP levels, ultimately results in the translocation of the cyclic AMP Response Element Binding protein (CREB) to translocate to the nucleus, resulting in gene transcription (Drawing from Pittenger and Duman 2007).

1.1.4.2 The cAMP signalling pathway

The cyclic adenosine 3'5' monophosphate (cAMP) is a small secondary messenger. It is the key component in a ubiquitous second messenger signalling system, that relay effects of extracellular neurotransmitters or hormones, when they bind their designated receptors (Beavo, Francis, and Houslay 2006). The formation of cAMP is catalysed by adenylate cyclase (AC), an enzyme that catalyses the synthesis of cAMP from ATP. To date ten adenylate cyclase isoforms have been identified. Of these 10 isoforms, 9 have been identified as being highly expressed in the brain, indicating the importance of cAMP signalling in the nervous system (Chern 2000).

Several neurotransmitters important for the function and modulation of behaviour and memory processes are known to signal through the cAMP cascade, via the G proteins coupled receptors (GPCR; Figure 1.1.3). Serotonin, adrenergic and GABA receptors are among some of the neurotransmitters that signal through G protein couple receptors and the cAMP cascade (Figure 1.1.2) (Lauder 1993).

G proteins are guanine nucleotide binding proteins. They act as a signal transducer, mediating the signal of ligand binding to the receptor, across the cell membrane to the effector systems, like adenylate cyclases. The G protein can either activate or inhibit the AC, and thereby alter the cAMP production in the cell (Chern 2000).

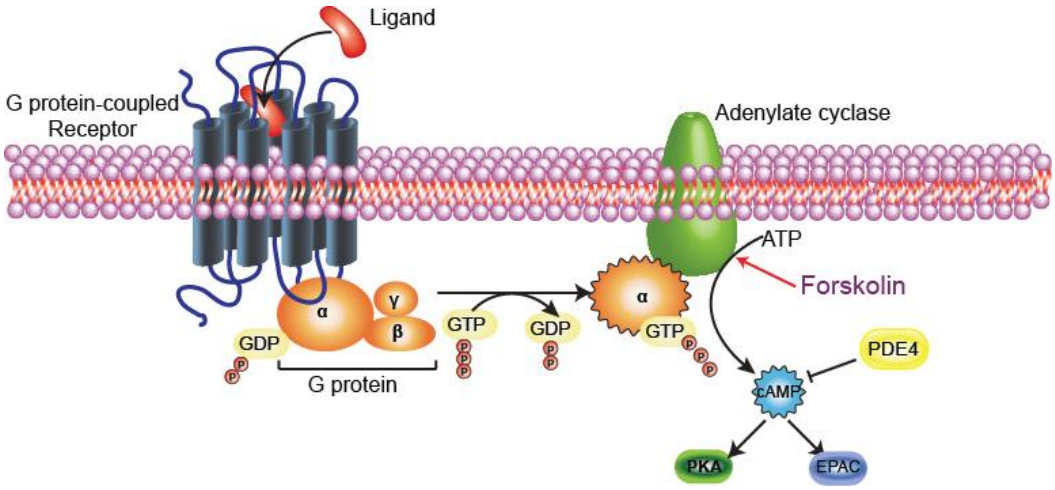

Figure 1.1.3: Schematic of the activation of the adenylate cyclase through a G protein coupled receptor.

A schematic drawing that illustrates the stimulation process of cAMP production, through G protein activation. Upon ligand binding, the G-protein coupled receptor is activated, and the α-subunit is released, and GPT is bound. The GTP-binding subunit directly activates the adenylate cyclases which catalyse the formation of cAMP (*drawing from K. Anastasaki, PhD Thesis, 2011*).

Each G protein is a heterotrimeric protein consisting of three subunits: α, β, γ. There are four families of G proteins, based on the similarity of the α-subunit. They are called Gs, Gi, Gq and G12 (Nicol and Gaspar 2014). The Gs activates all membrane bound AC enzymes, and thereby cause an increase in cAMP levels. The Gs family can be activated by Forskolin treatment (Seamon, Padgett, and Daly 1981). Gi proteins inhibits the cAMP production through AC inhibition, causing a decrease in cAMP levels (Nicol and Gaspar 2014).

Cyclic AMP has been shown to be involved in regulating metabolism, cell growth and differentiation, muscle contractions (Stork and Schmitt 2002), memory formation (Bellen et al. 1987), stress (Cherry, Thompson, and Pho 2001) and insulin

secretion (Yajima et al. 1999). When activated, cAMP interacts with several downstream effectors. The first described target of cAMP was the protein kinase A (PKA). PKA is a serine/threonine kinase, consisting in its inactive state, as a tetramer (Taylor et al. 2005). The tetramer consists of two regulatory units and two catalytic units. When cAMP levels increase, four cAMP molecules bind to the regulatory subunits of the holoenzyme, causing the release of the catalytic units, thus activating PKA, which results in a release of the catalytic (C) subunits from the regulatory (R) subunits (Taylor et al. 2005), leading to a range of cellular responses, ultimately leading to the phosphorylation of the transcription factor cAMP Response Element Binding protein (CREB). CREB binds to the cAMP response element present in the promoter region of the majority of cAMP regulated genes (Carlezon Jr, Duman, and Nestler 2005). Other downstream effectors of cAMP include the Exchange Protein activated by cAMP (EPAC) (Bos 2006), and the mitogen activated protein kinase pathway (Waltereit and Weller 2003).

1.1.4.3 Regulation of cAMP signalling by the phosphodiesterase family

The regulation of cAMP signalling is tightly controlled by the phosphodiesterase (PDE1-11) family of proteins. The PDEs are the sole mechanism of cAMP-hydrolysis in the cell, and thereby cAMP homeostasis, making this group of proteins extremely important. Their functional importance is reflected in the myriad of different isoforms so far identified (Beavo, Francis, and Houslay 2006) This diverse group of proteins consists of 11 different protein families, comprising more than 21

genes so far (Beavo, Francis, and Houslay 2006). Through alternative splicing and alternative promoter usage, these genes encode more than 100 different isoforms of PDEs (Marco Conti, Mika, and Richter 2014).

Of the many different isoforms of PDE that exists, the PDE4 family has been described extensively (M. Conti et al. 2003; Beavo, Francis, and Houslay 2006; Miles D. Houslay, Baillie, and Maurice 2007). PDE4s are highly expressed in the brain and cells of the immune system. The PDE4 family consists of four genes, *PDE4A, 4B, 4C* and *4D* (Beavo and Brunton 2002), and thus far there have been identified more than 20 different isoforms of PDE4 proteins, all of which exclusively catalyse the hydrolysis of cAMP, thus regulating the downstream signalling pathway of cAMP. PDE4 proteins contain a unique amino acid sequence, called the upstream conserved region (UCR) 1 and 2. Depending on the isoform, these two regions are present in the protein, and are important in the regulation of the activity of the proteins (see Figure 1.1.4).

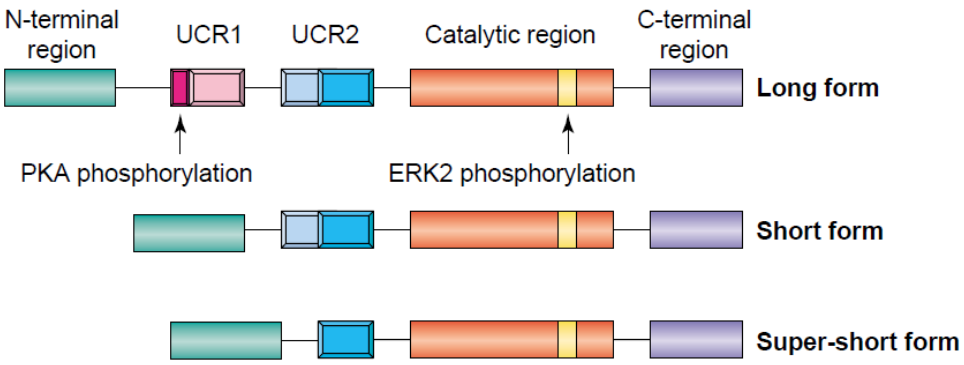

Figure 1.1.4: Structural organisation of the PDE4 family.

The protein structure and organisation of the different isoforms of Pde4 proteins are highly similar. Each family contain different splice forms, resulting in long-form, short form and super short form. The regulation of the different isoforms is determined by the presence of the phosphorylation sites present in the different motifs (*Figure from O'Donnell and Zhang, 2003, Trends in Pharmacology, license no. 3564841347281*).

The UCR1 contains a PKA phosphorylation site, allowing for the phosphorylation of the PDE4 protein by PKA. This cause a conformational change in the structure of the PDE4 protein, allowing UCR1 to interact with UCR2, thereby activating the PDE4 enzyme activity (Beard et al. 2000). Thus the regulation of PDE4 and cAMP are through a feedback loop, where cAMP regulates PDE4 expression and activity, and PDE4 regulates cAMP levels in vivo.

The function of PDE4 has largely been discovered through studies using the highly specific PDE4-inhibitor, Rolipram (S J MacKenzie and Houslay 2000). Rodent studies has demonstrated that Rolipram has antidepressant, anxiolytic and cognitive enhancing properties (Lelkes et al. 1998; Y.-F. Li et al. 2009a). Furthermore, there have been reports of beneficial effect of Rolipram in inflammatory diseases likes

asthma and chronic obstructive pulmonary disease (COPD). This effect is likely due to the decreased production of TNFα and cytokines, following PDE4-inhibition (Zhu, Mix, and Winblad 2001). Roflumilast, the first PDE4-inhibitor approved for clinical use, et, is approved for COPD treatment (Hatzelmann et al. 2010).

Rolipram was withdrawn from clinical development, due to its emetic side effects, (Heaslip and Evans 1995). However, Rolipram is still used extensively used as a tool compound to modulate cAMP levels through PDE4-inhibition. The development of isoform specific PDE inhibitors has proven extremely difficult, due to the high degree of structural similarities (Burgin et al. 2010, 4; Boswell-Smith, Spina, and Page 2006).

To gain insight into the specific function of different PDE4 isoforms, the development of knockout models has greatly increased the current knowledge of the function of specific PDE4-isoforms, and their involvement in regulating behaviour. To date, three knockout models in mice have been reported, for the three types of PDE4-genes primarily expressed in the brain; PDE4A, PDE4B and PDE4D. Interestingly, the behavioural phenotypes of PDE4A and PDE4B both show anxiogenic-like phenotypes. (Zhang et al. 2008; R. T. Hansen, Conti, and Zhang 2014), whereas mice lacking a functional PDE4D display behaviours similar to that observed in rodents subjected to anti-depressant treatment (Zhang et al. 2002b). These findings suggest there are distinct functions of the different isoforms on PDE4 in regulating behaviour in rodents, and that targeting specific PDE4-isoforms could

be potentially powerful in the treatment of psychiatric illness or memory enhancement.

The importance of the PDE4-function in psychiatric illness was further established when Miller and colleagues reported on the disruption of the PDE4B gene function in two patients with psychotic illness, further underlining the importance of PDE4-signalling in mental illness (Millar et al. 2005, 1).

1.1.4.4 Cyclic AMP in Stress and Anxiety

From rodent studies it has been shown that the area of the brain responsible for the regulation of anxiety and stress are the prefrontal cortex and the amygdala (Ganella and Kim 2014). Mounting evidence from both behavioural and pharmacological studies have demonstrated the importance of the cAMP signalling pathway in the brain of rodent models of stress and anxiety (Davidson 2002). Rats subjected to stress show increased activity of cAMP in the response to different stressors, e.g. electrical foot shock, immobilisation and cold: The most extreme form of stress, e.g. foot shock, cause the highest increase in cAMP levels (Kant et al. 1982). Also in zebrafish has the modulation of cAMP been shown to regulate different behaviours. Using two types of phosphodiesterase inhibitors, Rolipram and Roflumilast, Wolman and colleagues show that cAMP is important for regulating learning, but not memory in zebrafish larvae,(Wolman et al. 2014) . Caffeine, another compound known to increase cAMP levels in vivo (Schreiner et al. 1986), has been shown to induce thigmotaxis in both larval and adult zebrafish, a behavioural phenotype often associated with anxiety-like behaviour in animal models (Egan et al. 2009;

Richendrfer et al. 2012) This implies that levels of cAMP can regulate behaviour in zebrafish, suggesting an important function for cAMP-regulation in behaviour, and thus potentially a promising target in treatment of mental illness.

Potentially, behavioural research using zebrafish could be a useful approach to screen compounds for their ability to prevent or induce behavioural phenotypes associated with anxiogenic behaviour.

1.1.4.5 Phenotypic drug screening

Drug development is often driven by a target-based approach. Using cell-based assays many potential drug candidates are screened for specific effects on known targets (Benson et al. 2006). This approach is efficient, when the target is well known and characterized. However, for many types of psychiatric illness the genetics and biology is complex, and often the genetics unknown. Therefore, the target-based approach in drug discovery is not applicable to drug development in psychiatric illness. An alternative and possibly more successful approach would be the phenotype-based approach. Phenotype based drug screens screen for compounds modulating a specific behaviour, instead of compounds targeting a specific cellular target. Using this approach, Rovira and colleagues screened a library of FDA approved drugs on zebrafish larvae. Using a transgenic reporter line, they screened for compounds capable of inducing β-cell differentiation in the pancreas, important for insulin production. From this screen they identified two pathways important for β-cell differentiation in zebrafish, which have not previously been reported to be important in this process (Rovira et al. 2011). Others have used a similar approach,

but used the swimming activity of zebrafish larvae as an output to identify compounds that block to reward-effects of nicotine and alcohol (Cousin et al. 2014). From this screen Cousin and colleagues identified a FDA approved anti-epileptic compound, Topiramate, as being able to attenuate the psychomotor stimulating effects of nicotine, and suggest that the treatment for cessation of smoking (Cousin et al. 2014).

These studies demonstrate the functionality of zebrafish in phenotypic screens. The combination of using the zebrafish and compound libraries consisting of compounds already approved for other indications can help to propel the drug development process for mental illness forward. The zebrafish provides a cost-efficient, high-throughput approach to screen compounds for specific phenotypes, making it a promising model in behavioural drug screens.

1.1.4.5.1 Setting up a phenotypic screen in zebrafish larvae

Using the zebrafish for phenotype based screens has been demonstrated as described above. However, there are a number of papers describing the use of zebrafish in screening for a specific phenotype or reversal of a phenotype. Baxendale and colleagues demonstrated how the use of a zebrafish model of epilepsy could be used to screen 2000 small bioactive compounds, for their effect to reverse the PTZ induced phenotype and suppression of the immediate early gene, *c-fos* expression (Baxendale et al. 2012), a marker for increased neuronal activity (Sheng and Greenberg 1990). The PTZ model offers the opportunity to screen for a phenotypic

effect, and therefore, I used this to evaluate the potential of zebrafish in automated phenotypic screens.

1.2 Aims of this thesis

(i) To setup an automated method to analysis zebrafish larvae behaviour.

(ii) Validate the automated method by testing drugs with known or un-known effects on zebrafish behaviour.

(iii) Evaluate the effect of cAMP modulation on zebrafish larval behaviour.

(iv) Setup behavioural assays in adult zebrafish

(v) Study the effects of cAMP modulators on adult zebrafish

2 Materials and Methods

2.1 Materials

2.1.1 General buffer recipes

PBS (pH7.4)1L

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g
ddH_2O	up to 1L

The buffer is sterile filtered before use.

TBS 10x stock solution (1L)

NaCl	87.66g
Tris Base	12.11g

ddH_2O up to 1L. Adjust pH to 8.0 and sterile filtered before use.

1M Tris Buffer (1L)

Tris Base	121.1 g
ddH_2O	800 mL

Adjust pH accordingly (6.8; 7.0; 8.8) using 10M HCl. Fill ddH_2O up to 1 L and sterile filter.

0.5M EDTA (pH 8.0), 1L

Na_2EDTA	186.1g
ddH_2O	500mL

Adjust pH to 8.0 with 10M NaOH. Fill up with ddH_2O to 1L and filter sterilise.

60 x stock of E3 embryo medium (1L)

NaCl	17.2g
KCl	0.76g
$CaCl_2 \cdot 2H_2O$	2.9g
$Mg_2SO_4 \cdot 7H_2O$	4.9g

ddH2O up to 1L, autoclave to sterilise.

E3 Embryo Medium (1L)

60x E3 stock solution 16.6ml

ddH_2O up to 1L. For storage of embryos 1ml of 0.5% Methylene Blue in aqueous solution is added to the 1x E3.

List of compounds used in the different projects

Compound	*Vehicle*	*Vendor*
Rolipram	DMSO	Tocris
PD0325901	DMSO	Tocris & University of Dundee
IBMX	DMSO	Tocris
Forskolin	DMSO	Tocris
F152611	DMSO	Lundbeck A/S
F152497	DMSO	Lundbeck A/S
F152498	DMSO	Lundbeck A/S
F152497	DMSO	Lundbeck A/S
F152439	DMSO	Lundbeck A/S
Buspirone	DMSO	Sigma
Pentylenetetrazole (PTZ)	E3	Sigma
Diazepam	DMOS	Sigma
Ethosuximide	DMSO	Sigma
Valproate	E3	Sigma
Allopregnanolone	DMSO	Sigma
Alfaxalone	DMSO	Sigma
Pentobarbital	DMSO	Sigma

Acetazolamide	DMSO	Sigma
Pentobarbital	E3	Sigma
Oxacarbamazepine	DMSO	Orgamol S.A.
Lorazapam	DMSO	Sigma
Retigabine	DMSO	NeuroSearch A/S
Zonisamide	DMSO	NeuroSearch A/S
Carbamazapine	DMSO	Sigma
Phenytoin	DMSO	Sigma

Table 2:1: List of compounds used in project

2.2 Methods

2.2.1 Fish husbandry

Zebrafish are a fresh water fish, and in our facility the fish are kept in re-circulating stand-alone aquarium racks from Aquatic Habitats (PentAir, *Florida, USA*). Room temperature was kept at 23°C and the water temperature around 28°C. The room was on a diurnal light schedule, allowing 14 hours of light (7am to 9pm) and 10 hour of darkness (9pm to 7am).

2.2.1.1 Setting up a zebrafish facility

A stand-alone system from Aquatic Habitats were purchased and installed by MBK Installations (*MBK Installations Ltd, Nottingham, UK*). The stand-alone is equipped with an YSI-probe unit, allowing for the continued monitoring and adjustment of the water quality parameters, with an automated dosing system for salt (to control salinity and conductivity) and bicarbonate, to maintain pH values. The system was supplied with RO water and circulation was initiated. The probe head consists of a pH probe, an oxygen probe and a combined conductivity and temperature probe. The probes were cleaned and calibrated every 4 weeks to ensure correct measurements.

2.2.1.2 Water quality parameters

Parameter	*Set value*	*Recommended range*
pH	7.4	7.3-7.8
DO	7.0 mg/ml	5-10 mg/ml
Conductivity	700µS	400-1000µS
Salinity	0.4 ppm	< 2 ppm
Temperature	28°C	27-30°C

Table 2:2: Table of water quality parameters in the Aquatic Habitats stand-alone flow-through system

2.2.1.3 Filtration systems

As the system is a re-circulating system, the water is filtered and sterilised through different steps. First step of water sanitation are the mechanical filters, consisting of a horizontal filter, with a pore size of 100 µm and a cylinder filter, with a pore size of 25 µm. Activated carbon filters remove any traces of chlorine and lastly water is sterilized by passing through a UV-lamp unit.

2.2.1.3.1 Bio filters

The bio filters were initiated by adding 5 dpf old larvae into four 3L tanks, and feeding them 3 times daily with ZM000 (ZM food systems, UK). After 2 weeks, an

additional 4 tanks of fry were added. During this "seed" period, the water parameters were checked once a week, to monitor the establishment of the bio filters. This is done by monitoring the levels of the components of the nitrogenous cycle.

The following parameters are measured once a week, to check that the bio filters are working properly:

Parameter	Acceptable value
Total ammonia nitrogen	<1 mg/L
Nitrite (NO_2^-)	<0.05 mg/L
Nitrate (NO_3^-)	< 20 mg/L

Table 2:3: Acceptable values of components of the nitrogenous cycle in the Aquatic Habitats stand-alone flow-through system.

2.2.1.4 Maintaining zebrafish

Adult fish were fed three times a day with Zeigler Adult Zebrafish diet (MBK systems, UK). In the mornings and afternoons a supplementary feed with live artemia was included (MBK systems, *UK*).

2.2.2 Raising zebrafish larvae

Fertilized zebrafish embryos were collected shortly after onset of light in the fish room. Embryos were cleaned and sorted, and unfertilized or damaged embryos were removed. Embryos were then washed thoroughly in E3, and distributed in petri

dishes. ~50 embryos in a 9cm dish or ~100 in a 15 cm dish, in E3 with 0.005% Methylene blue (Sigma Aldrich, Denmark). The larvae were substituted with fresh E3 every day, and dead or sick embryos were removed to maintain a healthy clutch. At 5 dpf the larvae were introduced into the zebrafish system in 2 cm of system water, and fed with a fry feed, with granule size <100µM (ZM-000, ZM Systems, UK). Every second day 2 cm of system water was introduced into the tank, and when the fish are 21 days the water supply is turned on. At 21 days post fertilization, fish are fed ZM-100, a fry food with granule size of 80-200µM and two daily feeds of live artemia. Around 2 months of age the fish are fed an adult zebrafish diet (Zeigler, Aquatic Habitats, FL, US) three times a day, and two daily feeds of live artemia.

2.2.3 Behavioural assays

For this project, several behavioural assays were established in the lab. First and foremost, setting up of the automated tracking equipment was established, followed by two different assays to measure adult behaviour.

2.2.3.1 Larvae behaviour assays

2.2.3.1.1 Swimming activity

WT embryos were raised in the incubator, with a 14/10 hour light/dark cycle, at 28.5°C. At 5 dpf larvae were distributed into individual wells of a 96-well flat-bottomed plate (*Nunc MicroWell, P7491, Sigma-Aldrich)*, 1 larvae per well, in E3, and allowed to acclimatize in the incubator for 1 hour prior to treatment and testing. After 1 hour of acclimatisation, treatment was added to allocated wells, and the

larvae were put back in the incubator until the next treatment was added or the experiment could be analysed. The multi well plate was moved to the testing setup, and placed in the DanioTrack (Noldus Information Technology, NL) for tracking. The EthoVision-software was programmed depending on the protocol needed. The plate is placed in the DanioVison box, and the lid is closed. The EthoVision software is opened, and the protocol of choice is loaded. The arena settings and the detection settings could be adjusted, so that optimal tracking is archived. When the experiment finished, the larvae were either euthanized or collected in Eppendorf tubes and snap-frozen in liquid nitrogen and kept at -80°C until further analysis were done.

2.2.3.1.2 Data Analysis

2.2.3.1.2.1 Normalizing data

Each individual data set (well) in the experiment was then normalised to this value by dividing each individual well-value by the total average for the whole plate. Comparison of the median in each group, in each of the experiments, indicates whether it is valid to pool all the data into one group, and analyse them together. The normalized data from the dark-experiments were analysed using a Bootstrap method. Using this method we analysed the data from the seven individual experiments pooled together. The Bootstrap method allocates the normalised means into bins, creating defined groups of data. These data were plotted as histograms, and were used to give a graphic representation of the collated data.

2.2.3.1.2.2 Statistical analysis

For statistical analysis GraphPad Prism 5 and R (v3.0.2; www.r-project.org) software were used. Test included One-Way and Two-way ANOVA, followed by Tukey's post-test (One-Way ANOVA) or Bonferroni's posthoc test (Two-Way ANOVA). For data that was found to be non-normal distributed and with unequal variances across groups, 95% confidence intervals of the group means and the difference between group means were estimated by bootstrapping the data (CIs were bias-corrected and accelerated and based on 10.000 resampling's). Means were considered significantly different when the bootstrapped 95% CI (of the difference between means) did not include zero.

2.2.3.1.3 Visual Motor Response

5 dpf zebrafish larvae were distributed into individual wells of a 96-well plate (*Nunc MicroWell, P7491, Sigma-Aldrich*) in 100µL of E3. After the fish were allocated to the individual wells, they were examined using a stereo microscope (Zeiss, Denmark), to make sure all larvae are at the same developmental stage and the any dead or un-healthy larvae are replaced prior to the experiment begins. The larvae were allowed to acclimatize for 1 hour before drugs were added. All larvae received the same number of pipetting events and larvae that did not receive any drug treatment were mock treated with vehicle or E3. After treatment the multi-well plate is placed in the DanioTrack (Noldus Information Technology, NL), and the Visual Motor Response protocol is loaded.

2.2.3.1.3.1 Visual Motor Response Protocol

1: Start of protocol

2: 10 min dark

3: 10 min light

4: 10 min dark

Steps 3&4 three are repeated 3 times in total

End of protocol

2.2.3.1.4 Data analysis

The locomotor analysis was done by analysing the distance travelled in 2 minute time bins, and exported in Excel-format. The date was then transferred to GraphPad Prism, and plotted using an X-Y graph format. Average distance moved during light and dark periods were calculated and compared to that of the DMSO control fish. The total distance moved in all the light and dark periods were calculated as fractions of the DMSO control data in the corresponding periods. Data was analysed using a t-test.

2.2.3.2 Adult behavioural assays

2.2.3.2.1 Shoaling assay

Fish used in the shoaling assay were raised in the facility, at a stocking density not higher than 4 fish per litre of water. On the day of the assay, adult fish were removed from the zebrafish room, and moved to an adjacent room, and allowed to habituate to the room for 2 hours prior to testing. After video capture, the fish were euthanized in ice water, and snap frozen in liquid nitrogen and stored at -80°C, until further analysis was done.

2.2.3.2.1.1 Drug treatment

For drug experiments, the fish were treated for 20 minutes in the test tank (Small Geo tank, Ferplast, Italy), which had the following dimensions; L:18 x D: 11.5 x H: 14 cm, and contained a volume of 1L. The compound or vehicle was added to the water, with a final DMSO concentration of 0.001%. The group of fish were transferred to the tank, and incubated in the compound for 20 minutes prior to recording.

2.2.3.2.1.2 Video recordings

Movies were recorded in AVI for MPEG4 format, and recordings were made from the side and from the top using a digital camera (Panasonic Lumix DMC-TZ8 or Canon IXUS 80 IS). The tank was placed on a white surface and with a white back ground on a RS 2 XA camera stand (Kaiser, Germany). The side recordings were captured by placing the camera on a level surface approximately 50 cm away from

the tank, and the behaviour was captured. When capturing movies from the top, the camera was mounted on the camera stand, with a distance from the tank of approximately 50 cm.

2.2.3.2.1.3 Data analysis

After capture, the data was analysed using either Actual Track (Actual Analytics, Edinburgh, UK) or EthoVision XT9. For the software to calculate the distance travelled and time spend in different zones, the user needs to define the area of interest and the dimensions of this area. This is what the software uses as a reference parameter, when calculating the x-y coordinates used to generate the data. For the side tracking, the entire arena was divided in two equal sized half's, for the further determination of the time spend at the top and bottom of the tank. In the EthoVision XT9, there is a multiple animal tracking module, which can be used to track multiple fish.

2.2.3.2.2 Novel Tank Test

The novel tank test was performed in a trapezoid tank, measuring 27 (L) x 15 (H) x 10 (W) cm (Aquatic Habitats, FL, US). The tank was placed on a level, white surface, against a white background. The fish used for the experiment were AB/TL fish (6-12 months of age) or Pde4d+/+ or pde4d-/-. The fish were stocked at a stocking density of maximum 4 fish per litre, to avoid stress through crowding. On the day of the test, the fish were moved into the testing room in their home tank, and allowed to habituate for 2 hours prior to testing.

2.2.3.2.2.1 Drug treatments

For experiments involving drug treatment, the fish were treated in a 1 L beaker (Pyrex, VWR, Denmark) in 200 mL of system water containing the compound or 0.001% DMSO. The pre-treatment time was 20 minutes, and after treatment the fish was gently poured into the test tank, containing 1.8 L of system water.

After each trial the tank was cleaned and re-filled with fresh system water.

2.2.3.2.2.2 Video Recordings

Movies were recorded in AVI for MPEG4 format, and recordings were made from the side and from the top using a digital camera (Panasonic Lumix DMC-TZ8 or Canon IXUS 80 IS). The tank was placed on a white, level surface and against a white background. Movies were recorded from the side, for 6 minutes. The camera was placed on a level surface approximately 80 cm away from the tank, and the behaviour was captured.

2.2.3.2.2.3 Data analysis

The data was analysed using EthoVision XT9 (Noldus Information Technology, NL). The arena was defined in the software, and the top half of the tank was indicated as the top half of the area filled with water. The parameters measured were time spent in top of zone, latency to enter the top zone and total distance moved. Moreover, the frequency of erratic movements and freezing incidents, and total time of erratic and freezing episodes, was also determined. Data was plotted using GraphPad Prism 5, and analysis was done using a t-test.

2.2.4 Drug treatments

2.2.4.1 Rolipram

50 mg of Rolipram (Tocris Biosciences, Bristol, UK) was dissolved in 3.6mL of DMSO to make a stock concentration of 50mM. The stock was frozen at -20ºC in 20µL aliquots, and stored for 4-8 months. Larvae behavioural assays were done using a final concentration of 15µM of Rolipram, and adults were treated with a final concentration of 30µM Rolipram.

2.2.4.2 PD0325901

10 mg of the MEK inhibitor, PD0325901 (MW = 482.19 g/mole), was purchased from Dundee University (Hilary McLaughlan, University of Dundee, UK), or Tocris (Tocris bioscience, Bristol, UK) and diluted in 2.07 mL of DMSO to make up a 10 mM stock. This stock was stored at -20°C in 10 µL aliquots for 1 year. After thawing the aliquot was not frozen again.

2.2.4.3 IBMX

10 mg of IBMX (MW=222.24 g/mole) was purchased from Tocris (Tocris Biosciences, Bristol, UK) and diluted in 7.5 mL of DMSO to make a stock concentration of 30mM. 20µL aliquots of the stock were stored at -20°C for up to 1 year. 5 dpf embryos were treated with a maximum of 30µM IBMX for 1 hour. The stocks were not re-frozen.

2.2.4.4 Forskolin

10 mg of Forskolin (MW: 410.51 g/mole) was purchased from Tocris (Tocris Biosciences, Bristol, UK) and diluted in 3.25 ml of DMSO, to make up a stock solution of 7.5mM. 20µl aliquots were made up, and stored at -20°C. Once defrosted, the stock was not re-frozen.

2.2.5 Drug screens

2.2.5.1 PDE inhibitor screen

5 dpf WT zebrafish larvae were added to 96 well plates (Nunc MicroWell, P7491, Sigma-Aldrich), 1 larvae per well. The larvae were allowed to acclimatise in the incubator for 1 hour before the compounds were added, in a concentration curve format. Each compound was tested in concentrations 10nM, 30nM, 100nM, 300nM, 1µM, 3µM and 10µM, with a final amount of DMSO of 0.1% in E3. 100µL of each compound was added to each well, totalling of 12 larvae per concentration. The effect of the compound was tested during constant light for 1 hour. Data was analysed as total distance moved, and statistical analysis was done, using a one-way ANOVA, followed by a Tukey's post-test.

2.2.5.2 PTZ screen

Five day old larvae were sorted and added into individual wells of a 96-well plate (Nunc MicroWell, P7491, Sigma-Aldrich, Denmark) in 100µL of E3. The larvae were allowed to acclimatise for 1 hour in the incubator. After one hour, 50µL of compound was added in a dose-curve format. Concentrations were done in log-scale

format. Larvae were incubated for 1 hour in the incubator after the drugs were added. After one hour, 50µL of 20mM of PTZ was added, resulting in a final concentration of 5mM PTZ. Immediately after PTZ addition the 96-well plate was placed in the DanioVison chamber, and the VMR protocol was loaded. The data was exported as an Excel sheet, and the graph plotted using GraphPad Prism 5.

2.2.6 Protein analysis

2.2.6.1 Protein extraction

After compound treatment embryos were snap frozen liquid nitrogen, and 100µL of lysis buffer was added, and the embryos were transferred to a screw-cap tube containing ceramic beads (Precellys, VWR Denmark). The fish were homogenised using a PreCellys 24 tissue homogenizer for 3x30 seconds, at 5000 rpm. Samples were transferred to ice, and spun at 10000 rpm for 15 min. Supernatant was removed and stored at -80°C until protein quantification.

2.2.6.1.1 10 ml lysis buffer for protein extraction

Tris-HCl	20 mM, pH7.5
NaCl	150 mM
EDTA	1 mM
EGTA	1 mM
Triton-X-100 1%	1 %
Sodium Pyrophosphate	2.5 mM
β-glycerophosphate	1 mM
NaF	30 mM

Before use a final concentration of 1mM Na_3VO_4 was added, and 1 tablet of protease inhibitors (ComPlete Mini, Roche, Denmark) and 1 tablet of phosphatase inhibitors (PhosStop, Roche, Denmark) was dissolved in the buffer.

2.2.6.2 Protein determination

To determine the concentration of extracted proteins, a DC protein assay was performed, using the Bio-Rad DC assay (Bio-Rad, Denmark). The assay was done in a 96-well plate format. The A' reagent was made up, by adding 20µL of reagent A to each ml of reagent S that is needed for the assay. A standard curve using bovine serum albumin (BSA) was prepared for each assay. The following standards were prepared from a stock of 1.5 mg/ml of BSA: 0, 0.25mg/ml, 0.50 mg/ml, 1.0mg/ml and 1.5mg/ml. The standard curve was prepared in the same buffer used for the protein extraction. 5µl of standards and samples were added in triplicate to the 96-well plate. The 25µl of Reagent A' was added to each well, followed by 200µL of reagent B. The reaction was incubated at room temperature for a minimum of 15 minutes, before absorbance was measure at 690 nm using a plate reader. The standard curve was plotted in Excel, and protein concentration of unknown samples was determined using the equation for the standard curve.

2.2.6.3 Western Blotting

2.2.6.3.1 Buffers for Western Blotting

2.2.6.3.1.1 Running buffer

To make a 1 L of 10x stock

SDS	10g
Tris-Base	36.3g
Glycine	144 g.

100 ml stock is diluted in 900 mL of dH_2O and used for running the gel.

2.2.6.3.1.2 Transfer buffer (Towbin)

10 x stock solution:

Tris-base (0.25M)	30.3g
Glycine (1.92 M)	144g
dH_2O add	1 L

To make 1 x Towbin buffer:

10x stock solution	100ml
Methanol	200ml
dH_20	700ml

2.2.6.3.2 Sample preparation

The samples from the protein extraction were prepared by adding 3 x Blue Loading buffer (Cell Signalling Technology, AH Diagnostics, DK) with a final concentration

of 3 x DTT (Cell Signalling Technology, AH Diagnostics, DK). The samples were prepared, so that the samples contain a final loading buffer and DTT of 1x. Prior to loading on the gel, the samples were denatured in a heat block at 95°C for 5 min, and immediately placed on ice, until the gel was ready to load.

2.2.6.3.3 SDS page

A precast polyacrylamide gradient gel (4-20% MINI protean TGX gel, Bio-Rad, Denmark) was prepared and mounted in the electrophoresis chamber (Bio-Rad, Denmark) and filled with 1 x running buffer. A total of 15mg of protein from each sample was loaded in the gel, and 5 µL of Spectra multicolour broad-range protein ladder (Thermo Scientific, VWR, Denmark) was loaded as a marker. The gel was run at a constant voltage of 120V for 50 minutes, or until the loading buffer had run off the bottom of the gel.

2.2.6.3.4 Protein Transfer

The transfer buffer was made up (see 2.2.6.3.1.2) and 200 ml was poured into a tray containing 4 pieces of Whatman paper and 2 sponges. The FL-Immobilon PVDF membrane (Millipore, Denmark) was prepared by quickly soaking it in 100% methanol, and rinsing it in transfer buffer. The gel cassette was opened, and the gel was washed in transfer buffer prior to assembling the transfer cassette. The cassette was assembled on the clear side of the transfer cassette, with 1 sponge, 2 pieces of Whatman paper, PVDF membrane, gel, 2 pieces of Whatman paper and 1 sponge. Air bubbles were removed with a roller after the membrane and gel had been placed in the cassette. The cassette was carefully closed and placed into the electrode

module, with the black side facing the black face in the electrode module. The module was put in the buffer tank, and an ice block was placed next to it. Transfer buffer was poured in, until the cassette was covered, and a magnet was placed into the tank. The transfer unit was placed on a magnetic stirrer and the transfer was performed at 100V for 30 minutes.

2.2.6.3.5 Protein detection

After transfer the transfer sandwich was disassembled and the membrane was rinsed in TBS. To block for unspecific binding of the antibody, the membrane was incubated in Odyssey blocking buffer (Li-Cor Biosciences, UK) for 1 hour with constant agitation at room temperature. After blocking, 5 ml of primary antibody solution was poured on and the membrane was incubated over night at 4 °C with constant agitation. Next day the primary antibody was removed, and the membrane was washed 3 x 10 minutes in TBS + 0.1% Tween (TBS-T). After washing 5 ml of secondary antibody solution was added, and the membrane incubated for 1 hour at room temperature, under constant agitation and protected from light, to avoid photo bleaching of the secondary antibodies. Both primary and secondary antibodies were diluted in the blocking buffer. After incubation with the secondary antibody, the membrane was washed for 3 x 10 min in TBS-T under constant agitation, and before imaging the membrane was washed for 5 min in PBS. To detect protein expression, the membrane was imaged using the Odyssey CLX system.

2.2.7 Cortisol determination

To determine whole-body levels of cortisol in adult fish, cortisol extraction was performed, followed by an enzyme immunoassay.

2.2.7.1 Cortisol extraction

Adult fish were euthanized in ice water and snap frozen in liquid nitrogen. For cortisol extraction the fish were allowed to slowly defrost on ice. The fish were weighed and cut into smaller pieces, on ice. Using a hand held homogenizer (Tissue master homogenizer, Omni International, UK) the fish were homogenized in a 50ml falcon tube, containing 1 mL of ice cold, sterile filtered PBS. Following homogenization, 2 mL of diethyl ether (Sigma Aldrich, DK) was added to the tube and the tube was vortexed for 2 min. Samples were then spun down at 3,000 rpm for 15 min. After spinning the tubes were snap-frozen in liquid nitrogen and the solvent layer containing the cortisol was transferred to a glass tube. The ether was evaporated overnight in the fume hood. Next day the samples were transferred to an Eppendorf tube, and re-constituted in ice cold PBS. The samples were kept in the fridge overnight, before being assayed the next day.

2.2.7.2 Cortisol assay

For the cortisol determination the High Sensitivity salivary Cortisol enzyme immunoassay kit from Salimetrics were used (Salimetrics Europe Ltd, UK). The standard curve was fitted using a 4-parameter sigmoid function, plotted in GraphPad

prism, and the concentration of the unknown samples was extrapolated from the curve.

2.2.8 Cyclic AMP assay

To determine the total levels of cAMP an Elisa assay was performed. This assay is a direct Elisa assay, where a multi-well plate is coated with anti-cAMP. The cAMP in the sample compete with the cAMP is the conjugate, thus the higher levels of cAMP in the sampled, the lower colorimetric signal.

2.2.8.1 Cyclic AMP extraction

5dpf old zebrafish larvae (n=15 per treatment per experiment) were treated with 0.1%DMSO, 15µM Rolipram, 7.5µM Forskolin, 1.5µM PD0325901 or the combination of Rolipram + PD0325901, Forskolin + PD0325901 or DMSO + PD0325901 for 1+1 hour, and then euthanized by snap freezing in liquid nitrogen. Cyclic AMP were extracted using 200µL 0.1M HCL and samples were homogenized using a hand-held homogenizer with pestle (Pellet-Pestle, VWR, DK). Following homogenization samples were sonicated for 15 seconds (MFI Sonicator, MSE, Svend Schröder, Denmark). After sonication the samples were incubated on ice for 15 min, followed by centrifugation at 13,000 rpm for 15 min at 4°C. After centrifugation supernatant was removed and transferred to Eppendorf tube, and stored at -80°C until analysis.

2.2.8.2 ELISA assay

The concentration of cAMP in each sample was determined using a Direct Elisa cAMP kit (Enzo Life sciences, AH diagnostics, Denmark). The samples with extracted cAMP were diluted 1:1 in 0.1M HCL and assayed in duplicates. To increase the sensitivity of the assay, all the samples and standards were acetylated, using the kit that was provided by the manufacture. The manufactures instructions were followed and the cAMP levels were determined. All samples and standards were assayed in duplicates, and extra care was taken when wells were washed. All excess washing buffer was blotted off on tissue paper between each wash, and a total of four washes were performed. The absorbance was read using a plate reader (MultiSkan Ascent, Thermo Labsystems), at a wavelength of 450 nm.

2.2.8.3 Data analysis

The data were analysed using GraphPad prism 5. The absorbance was plotted against the concentration of the standards, and unknown samples were extrapolated from the standard curve, that was fitted using a linear regression function in GraphPad prism 5. The cAMP levels were adjusted according to total protein concentration, measured using the Bio Rad DC protein assay (see 2.2.6.2)

2.2.9 Quantitative PCR

To determine the gene expression levels of c-fos genes in larvae treated with PTZ and neurosteroids, quantitative qPCR was performed.

2.2.9.1 RNA Extraction and quantification

After respective compound treatments the 5 dpf old larvae (12 per group) were transferred to RNAse/DNAse free tubes, and euthanatized in liquid nitrogen. Total RNA was extracted using the RNEasy Micro Kit (Qiagen, DK). RLT buffer containing 1% β-mercaptoethanol was added to the larvae and incubated at room temperature for 15 minutes. Following incubation, the larvae were homogenised using a hand-held homogeniser (Pellet-Pestle, VWR, DK), before nuclease-free water containing 1.7% of proteinase K was added, and samples were incubated at 55 °C for a minimum of 15 minutes, to ensure that the larvae had completely dissolved. After incubation, the samples were centrifuged at 13,000 rpm for 3 minutes. The supernatant was collected and mixed gently with 100% ethanol, corresponding to half of volume of the supernatant, and loaded onto the column. The column was spun at 10,000 rpm for 15 seconds (s) and the flow through discarded. After the first wash with RW1-buffer, and on-column DNAse 1-digestion was performed, to remove all traces of genomic DNA. The column was washed with 2 additional wash steps and one ethanol wash, before the RNA is eluted with nuclease-free water.

After elution, the RNA concentration was determined using the NanoDrop 2000 (Fisher Thermo Scientific, Mytogen, DK).

2.2.9.2 Reverse Transcription

The RNA was reverse transcribed to cDNA using the Precision nanoScript ™ 2 Reverse Transcription kit (PrimerDesign Ltd, UK). Using Oligo-dT primers included in the kit, 600 ng of RNA was reverse transcribed to cDNA, and the final

concentration of cDNA was determined using the NanoDrop 2000 (2000 (Fisher Thermo Scientific, Mytogen, DK).

2.2.9.3 GeNorm reference kit

Errors do occur throughout the PCR process, meaning that the precise amount of cDNA loaded into each well of the qPCR reaction is uncertain. Therefore, the need to normalise the data when doing quantitative PCR is important. One way to do this is to analyse the expression of certain housekeeper genes as reference genes, and uses the expression levels of these genes to normalise the expression level of your gene of interest against. Some important features when selecting which housekeeper gene to use are listed in the MIQE guidelines (Bustin et al. 2009), which also specify the importance of normalising to more than one reference gene. To determine which reference genes to use, I used the geNorm reference gene selection kit for zebrafish (PrimerDesign Ltd, UK), which was specifically designed upon request by our laboratory. The geNorm kit consists of assays for 12 reference genes from the zebrafish genome, and it measures the gene stability as the average pairwise variation between a particular reference gene and all other reference genes in a given samples. Using the inter-gene stability, the geNorm ranks the reference genes according to their expression stability in the cDNA from different tissue samples (Vandesompele et al. 2002).

The geNorm analysis of expression of the 12 reference genes was measured in a representative set of zebrafish cDNA samples. The PCR was run as described in 2.2.9.4.

The zebrafish geNorm reference gene selection kit targeted the following genes: 16S ribosomal RNA (*16S*), cytochrome P450 K (*cyp2k*), topoisomerase II (*top2*), beta actin 1 (*bact1*), tyrosine 3-monooxygenase (*ywhaz*), succinate dehydrogenase complex, subunit a (*sdha*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), ATP synthase (*atp5d*), Ribosomal protein L13A (*rpl13a*) and NADH dehydrogenase (*nadh*). The data from the geNorm qPCR was analysed using qBase PLUS software (Biogazelle, NL), which ranks the relative stability of each reference gene in the samples and determine the number of reference genes needed to normalise the data from the given samples (Vandesompele et al. 2002).

All the primers for the zebrafish geNorm reference gene kit were designed by PrimerDesign Ltd. However, the company does not give out the sequences for reference genes, as this is considered commercially sensitive information.

Gene	*Accession number*	*Anchor nucleotide*	*Context length*
16S	AF036006	813	107
actb1	NM_131031	144	116
ATPsynth	BC083308	709	128
CYP2K17	DQ097890	852	112
EIF1B	BC067620	468	102
GAPDH	AY818346	1128	89
NADH	BC059660	840	111

RPL13	BC075977	345	104
SDHA	AY391458	1403	124
TOP2	BC086970	3810	83
USP5	BC097033	159	81
YWHAZ	AB194124	2062	128

Table 2:4: List of genes contained in the geNorm Reference Gene kit from PrimerDesign.

2.2.9.4 Quantitative PCR

Quantitative analysis of specific genes of interest within the cDNA samples was determined using Precision-iC SYBR green master mix (PrimerDesign Ltd, UK) with the CFX96 Real-Time PCR Detection System (Bio-Rad, Denmark).

Duplicate reactions were performed in 20 µl volumes containing 10 µl Precision-iC SYBR green master mix (PrimerDesign Ltd.), 300 nM primer (PrimerDesign Ltd, UK), 15 ng cDNA and made up to 20 µl with nuclease-free water. The following cycling conditions were used: initial activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, and 60 °C for 1 min.

Data was collected during each cycling phase. Melt curve analysis, to ensure each primer set amplified a single, specific product, completed the protocol.

RT- samples and no-template controls (NTCs) were run alongside all reactions to assess contamination. Quantification cycle (Cq) values were determined using Bio-Rad CFX96 Manager 3.0 software.

2.2.9.4.1 Analysis of qPCR data

The relative gene expression of the gene of interest (GOI) was normalised against the expression levels of the reference genes (RG). The relative level of gene expression is calculated using the below listed method.

The average reference gene expression was calculated using the following formula:

$$Cq(CqRG) = \frac{Cq^{NADH} + Cq^{RPL13A}}{2}$$

The normalized expression of the GOI:

$$\Delta Cq = Cq^{GOI} - Cq^{RG}$$

The normalised expression of the GOI

$$GOI = 2^{-\Delta Cq}$$

To determine the fold-change in gene expression between the different samples:

Control sample ΔCq *– Treatment sample* $\Delta Cq = \Delta\Delta Cq$

$$Fold - change\ in\ gene\ expression\ between\ samples = 2^{-\Delta\Delta Cq}$$

3 An alternative PTZ model in zebrafish larvae

3.1 Introduction

Small molecules can be screened in zebrafish embryos either manually or automatically. The manual approach allows for the concurrent screening for different effects in one screen, e.g. development of pigmentation and patterning, general development, morphology and movement defects. Compounds identified using this approach can then be divided into phenoclusters based upon their particular effect, allowing the compound to be studied in more detail (Colanesi et al. 2012). Neuroactive compounds are identifiable through a simple touch assay, where a gentle prod with an insect pin can evoke a swimming response in the larvae. To monitor and report changes in basal behaviour an automated tracking system is employed which allows for the tight and standardised control of external stimuli, e.g. light and sounds, and is unbiased in the analysis of the behaviour. Light and sound are two types of external factors that are known to affect zebrafish behaviour and swimming activity (Best et al. 2008; MacPhail et al. 2009). Five-day-old zebrafish larvae can recognise changes in light and register different types of sound stimuli, therefore light and sound are often useful when modulating the swimming activity and behaviour of zebrafish. This is often desirable when studying the behaviour of young larvae, below 5 dpf, as their basal swimming activity is generally low (Budick and O'Malley 2000).

3.1.1 Automated analysis of zebrafish behaviour

A significant progress in automated analysis of zebrafish behaviour was the development of automated tracking software by academic groups (H. A. Burgess and

Granato 2007) and commercial companies (Noldus, Spink, and Tegelenbosch 2001). This facilitated the quantifiable analysis of specific behaviours, such as general locomotor activity (Irons et al. 2010), non- associative learning (Best et al. 2008), light adaptive behaviours (Burgess and Granato, 2007b) and seizure activity (Afrikanova et al. 2013). Moreover, the small size of the larvae allows for the screening in 96-well format, thus greatly increasing the number of compounds that can be screened in a day (Bruni, Lakhani, and Kokel 2014). Further developments to increase throughput have been the development of systems for automated embryo sorting and distribution and automated compound addition.

3.1.1.1 Large scale small molecule screens in zebrafish larvae

Kokel and colleagues (2010) screened approximately 14000 small molecules, a selection of different of compound libraries with known and un-known functions. The behavioural output was a light-induced behaviour, termed the photomotor response (PMR), and observed in 30 hours post fertilization (hpf) old embryos. The screen identified 1627 hits, which were phenotypically clustered. Analysis of these compounds showed that they targeted similar pathways (Kokel et al. 2010). Another study described the identification of molecular pathways involved in the regulation of rest/wake behaviour in zebrafish larvae (Rihel et al. 2010). Using automated analysis of behaviour, Rihel and colleagues screened 5648 compounds on 4 dpf larvae, and continuously monitored the activity for a total of 3 days. Behavioural parameters included rest latency, waking activity, number and duration of rest bouts, and wake latencies. Using this approach more than 60000 behavioural profiles were

recorded, and the data was used to phenotypically cluster the compounds. Further analysis found that the behavioural profile and the therapeutic activity of the compounds often correlated. For example, a class of compounds all causing highly increased waking activity were all agonists of sodium channels. Using this approach, they identified 547 compounds specifically altering the rest/wake cycle of zebrafish larvae (Rihel et al., 2010).

3.1.2 Available commercial tracking software solutions

The first commercially available zebrafish tracking equipment was the ZebraBox/ZebraLAB (ViewPoint, France). The system consists of a box with an infrared light source and a camera, capable of recording movies with 30 frames per second (Figure 1.1.1). The ZebraLab software controls the box and analyse the swimming activity of the larvae. The infrared light source allows for behavioural monitoring in the dark, as the fish do not see infrared light (Emran et al. 2010). Initially our laboratory used the ZebraBox to analyse larval behaviour, measuring swimming distance, velocity, and placement. However, the available version of ZebraLab had very limited flexibility with respect to off line data analysis and did not permit analysis of externally recorded movies of zebrafish movement. Current versions of the ZebraBox and the software have been greatly updated, and now have a fully flexible software platform, capable of analysis recorded data retrospectively (www.viewpoint.fr). In 2010, Noldus introduced the DanioVison system (Noldus Information Technology, NL), which consists of the DanioVison chamber for recording the behaviour of larval zebrafish, and the software platform, EthoVision XT9.0, for analysing the data. EthoVision has a flexible data analysis module, and

separately acquired movies can be loaded into the software enabling automated analysis of adult zebrafish behaviour.

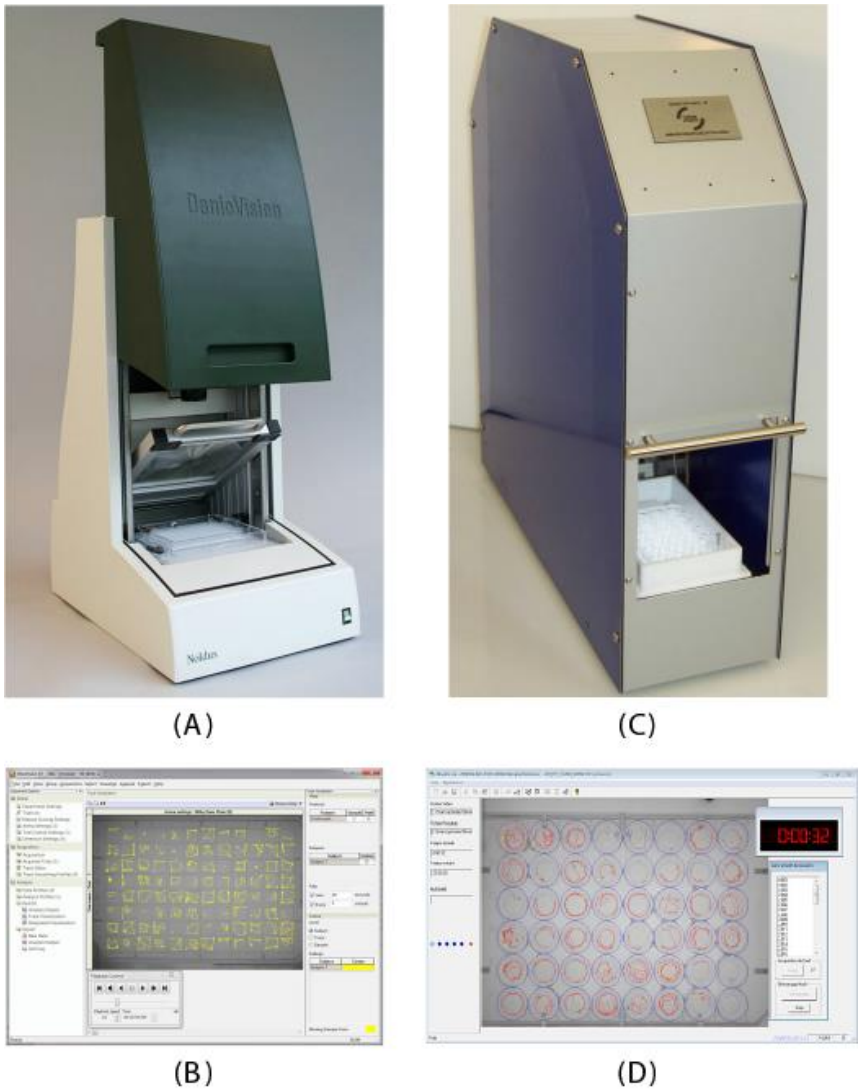

Figure 3.1.1: The two commercially available tracking systems.

A: The DanioVison from Noldus Information Technology, NL.B: Screenshot from the acquisition module in EthoVision XT9, the software used by DanioVison (Noldus, NL) C: The ZebraBox from ViewPoint, Fr. Both systems consists of a tracking box with LED lights, an infrared light source, and an infrared camera, capable of recording movies at 30 frames per second. D: Screenshot from the ZebraLab software, from ViewPoint, Fr.

3.1.3 Epilepsy models

Epilepsy is a heterogeneous group of disorders, characterised by a predisposition for spontaneously occurring seizures (Jacobs et al. 2009). The seizures are the result of an imbalance in the excitation and inhibition of neural signalling, ultimately leading to a synchronous discharge of neurons in the brain (Cunliffe et al. 2015). It is a common neurological disorder, affecting around 65 million people worldwide (Thurman et al. 2011). A wide range of anti-epileptic drugs (AED) are available in the clinic, however 30% of patients suffering from epilepsy do not respond to treatment with the available AEDs (Perucca and Tomson 2011). The reason for this is most likely due to the heterogeneity of the syndromes, commonly caused by multiple genetic mutations (Reviewed in Jacobs et al., 2009).

3.1.3.1 Genetic models of epilepsy

Genetic models in rodents have aided in the understanding of epileptogenesis and epilepsy on the molecular and genetic level (Singh et al. 2008; Yu et al. 2006). Genetic models often recapitulate some of the aspects of the human disease, such as spontaneous, infrequent seizures (Martin et al. 2010). Martin and colleagues generated a knock-in transgenic mouse that harboured a specific mutation in SCN1A, a voltage-gated sodium channel. Mutations in this channel are known to be associated with several forms of epilepsy (Reviewed in Escayg and Goldin, 2010). Heterozygous mutants have a normal lifespan but a reduced threshold to seizures. However, homozygous mice show very frequent generalized seizures, and have reduced lifespans, ultimately leading to mortality (Martin et al. 2010). From and

ENU-mutagenesis screen, a zebrafish harbouring a mutated version of the homologous gene in zebrafish, *scn1Lab*, has also been identified. This mutant shows some of the same features observed in the mouse model of SCN1a; for example the homozygous fish die around 10-12 dpf and have spontaneous seizure activity (Scott C. Baraban, Dinday, and Hortopan 2013). Baraban and colleagues screened a library consisting of 320 FDA US approved and toxicology tested compounds, on 5dpf old *scn1Lab* homozygous mutant larvae, screening for compounds capable of suppressing convulsions. Using this approach they identified the antihistamine clemizole,, which was capable of suppressing seizure activity in the mutants (Scott C. Baraban, Dinday, and Hortopan 2013). This study demonstrates the possibilities of using zebrafish genetic models of epilepsy.

3.1.3.2 Pentylenetetrazole-induced model of epilepsy

For over 60 years drug discovery within epilepsy research have been based on chemically induced seizure models in rodents, (Reviewed in Löscher, 2011), and in particular, the pro-convulsive drugs, pentylenetetrazole (PTZ) and pircrotoxin, are still used extensively in epilepsy and seizure research.

PTZ is a gamma-amino butyric (GABA) receptor antagonist used to induce seizures through its binding to the $GABA_A$ receptors (S. L. Hansen, Sperling, and Sánchez 2004; Löscher 2011). PTZ causes tonic-clonic like seizures in rodents, causing the rodent to lose posture and rigidness of the limbs (Fisher 1989).

3.1.3.2.1 GABA signalling

GABA is the major inhibitory neurotransmitter in the central nervous system responsible for the hyperpolarization of neurons at the end of an action potential (Treiman 2001). The GABA($_A$) receptor is a pentameric ligand-gated ion channel, which is widely expressed in the brain and CNS (Olsen and Sieghart 2009). There are multiple subtypes of the receptor due to the molecular diversity of the different subunits comprising the receptor (Olsen and Sieghart 2009). Each subunit possesses specific binding sites, which, depending on subunit-composition, regulate the receptor activity. The function of the channel is to regulate the Cl^- influx into the cell, leading to a hyperpolarization of the membrane, which blocks the immediate firing of another action potential.

Several AEDs have been shown to bind to the GABA receptor and act as a positive allosteric modulator (PAM) like diazepam, which enhances the GABA activity, thus causing relaxation, sedation and anxiolytic effects (S. L. Hansen, Sperling, and Sánchez 2004). The GABA receptor plays an important role in seizure activity, which makes this neurotransmitter and its receptor a favourable target for anti-seizure drug development.

3.1.3.3 PTZ-models in zebrafish larvae

Baraban and colleagues reported in 2005 on the effect of PTZ in larval zebrafish. This group showed that 7 dpf old larvae respond to PTZ treatment in a dose dependent manner, manifested as 3 defined stages of convulsive behaviour, and abnormal electric discharge (S. C. Baraban et al. 2005). They also showed that

known AED's, like diazepam and valproate, could reduce the abnormal electrical discharge induced by PTZ (S. C. Baraban et al. 2005). This has since led to several reports on the use of zebrafish in AED research (Afrikanova et al. 2013; Baxendale et al. 2012; Berghmans et al. 2007; Gupta, Khobragade, and Shingatgeri 2014), all of which describe the use of PTZ at concentrations of 15-20 mM, which induces convulsions in the zebrafish larvae. Compounds with known AED effect were screened, for either a protective effect against the PTZ induced convulsions (Afrikanova et al. 2013; Baxendale et al. 2012; Berghmans et al. 2007), a reversal or an improvement of PTZ induced seizures (S. C. Baraban et al. 2005). The locomotor assay described in these studies were performed under constant darkness, to avoid photo bleaching of compounds (Afrikanova et al. 2013; Berghmans et al. 2007). Tracking larvae during constant darkness can, in some situations, lead to the identification of false positives, which is partly due to the sedative effects caused by a majority of compounds used to treat seizures (Nadkarni and Devinsky 2005), and the properties of the natural swimming response in zebrafish larvae (MacPhail et al. 2009). In constant darkness, zebrafish larvae will initially have a high level of baseline swimming activity, which after 10-20 min will drop and become a low, steady-state level of activity (MacPhail et al. 2009). Under changing light conditions, however, zebrafish larvae show a distinct movement pattern, induced by regular intervals of light and dark periods, a response known as the Visual Motor Response (VMR) (MacPhail et al. 2009; Steenbergen, Richardson, and Champagne 2011a). Therefore, this assay might be more suited for screening for compounds, allowing the elimination of compounds with a sedative effect.

3.1.4 Aim of chapter

Zebrafish embryos can be part of a very useful screening platform for testing and screening small molecules with neuroactive effects. The NeuroSearch (Copenhagen, Denmark) library consisted of a collection of ~200,000 compounds developed mainly for treating central nervous system disorders, e.g. epilepsy, Huntington's disease, depression, anxiety, and neuroinflammation. As part of my collaborative PhD with NeuroSearch, we set up an automated screening method for identifying compounds with neuroactive effects. The aim of this chapter was to implement a zebrafish model of chemically induced seizures in zebrafish larvae that would be able to discriminate between compounds with a sedative effect and true seizure-modulating effect.

3.2 Results

3.2.1 Sub-convulsive levels of PTZ induce a distinct change in the PTZ response

Using the VMR, the effect of increasing concentrations of PTZ was tested on the swimming activity on five dpf larvae (Figure 3.2.1). PTZ perturbed the orderly activity pattern of the VMR in a concentration dependent manner. From previous reports using PTZ in zebrafish, it is known that a high level of PTZ (15 – 20 mM) will induce seizure activity causing an increase in the swimming activity (Afrikanova et al. 2013; S. C. Baraban et al. 2005; Berghmans et al. 2007). Under control conditions, alternating light-dark cycles induced changes in the swimming activity of the larvae. In the light phases, the fish displayed quiescent levels of activity, whereas in the dark phases their activity regularly increased above 200 mm/2min (Figure 1.2.1A). Exposure to 1mM PTZ resulted in a small increase in swimming activity in both light and dark periods. The larvae exposed to the low concentration of PTZ (1mM) also showed a delay in decreasing locomotor activity during the dark periods. An intermediate concentration of PTZ (5mM) resulted in a striking change in the responsiveness to the alternating lighting conditions, a reversed dark-light locomotor pattern. Larvae responded to darkness with no or very low levels of activity, but with the onset of visible light the swimming activity increased instantly. Larvae treated with the highest tested dose of PTZ, 15 mM, did not respond to changes in the light-dark cycles and showed an overall increase in activity, which decreased gradually over time (Figure 3.2.1).

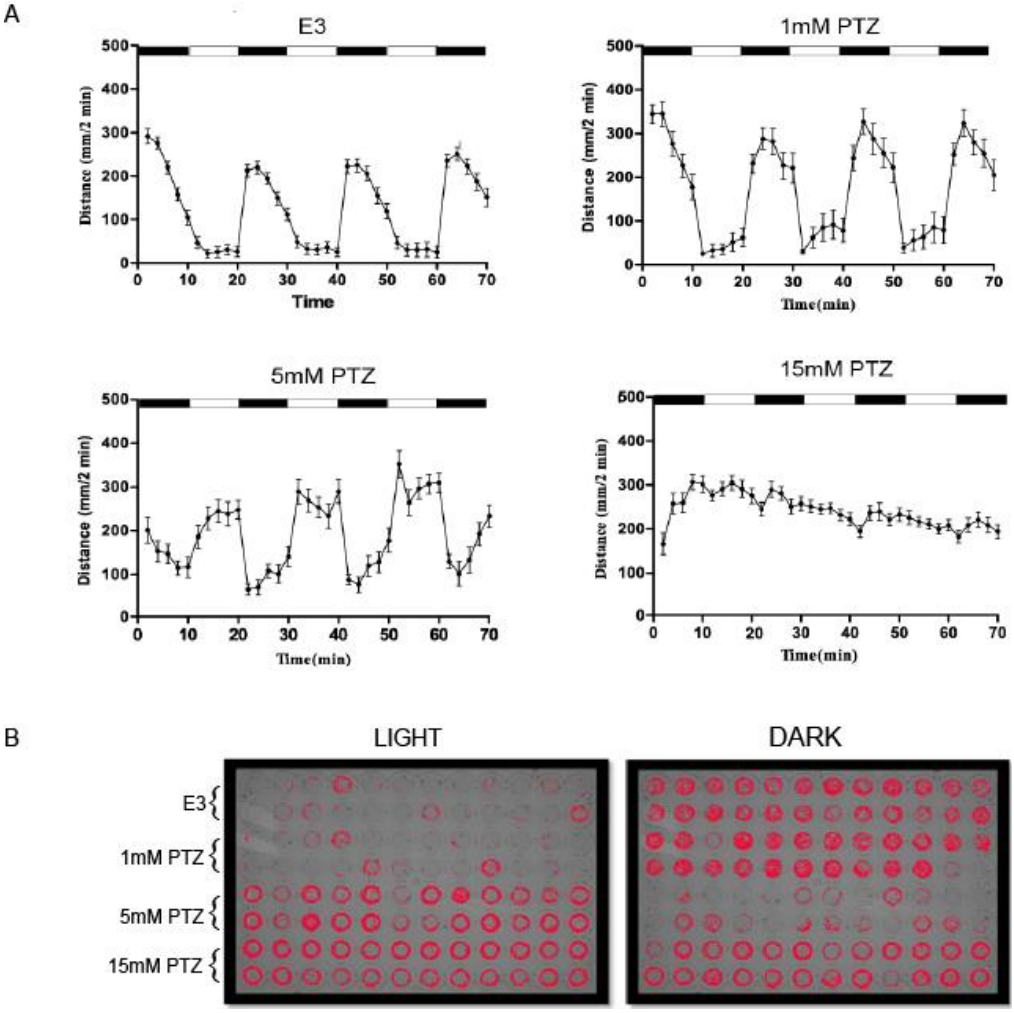

Figure 3.2.1 A: Pentylenetetrazole (PTZ) induces a behavioural phenotype in larval zebrafish.

Plots of 5 dpf old zebrafish treated with increasing concentrations of PTZ. Tracking performed under alternating light conditions, 10 min dark (black bar) and 10 min light (white bar), for a total of 70 min. Data are presented as mean +/- SEM of 12 larvae. Embryo media (E3) are used as vehicle control, and the swimming activity of the PTZ treated larvae are compared to that of E3-treated larvae. B: The distinct concentration-dependent locomotor profile of PTZ in five dpf larvae visualised as the tracking plots from the DanioVison-system. The red tracks are the movement of the individual larvae plotted over a two-minute time period, in either the light or in the dark.

3.2.2 Poor effect of compounds on the PTZ-induced phenotype

The intermediate concentration of PTZ leads to a highly specific and reproducible behavioural phenotype. To investigate if this phenotype it seizure related, I next investigated whether compounds previously been reported to be effective in the PTZ-assay using the higher concentration of PTZ or compounds with known GABA-receptor function, could reverse the inverted VMR behaviour caused by low concentrations of PTZ (5mM). Fourteen compounds were tested in a dose-curve response in combination with 5mM PTZ. The compounds were not tested alone, as the criteria for efficacy in this assay is only a reversal of the phenotype. Of the 14 compounds tested, five compounds showed a complete or partial reversal of the PTZ-induced phenotype (Table 3:1). Of these five compounds, three were classic AEDs, namely diazepam, ethosuximide and valproate, and two were neurosteroids, alfaxalone and allopregnanolone.

3.2.2.1 Effect of valproate and ethosuximide

Treatment with ethosuximide, a Ca^{2+} channel blocker, could not reverse the VMR-response at the majority of doses tested in the assay except at the two highest doses tested (10mM and 30mM). At these concentrations the larvae showed a higher swimming activity when tracked in the dark, compared to the lower doses (100µM – 3mM), however the sedating effect of ethosuximide affected the general locomotor activity of the larvae, making the effect minor (Figure 3.2.2). A similar dose-response was present in larvae treated with increasing doses of valproate, a K^{+}-

channel blocker, and at the highest concentrations, the compound caused a decreased locomotor activity, indicating sedation (Figure 3.2.3).

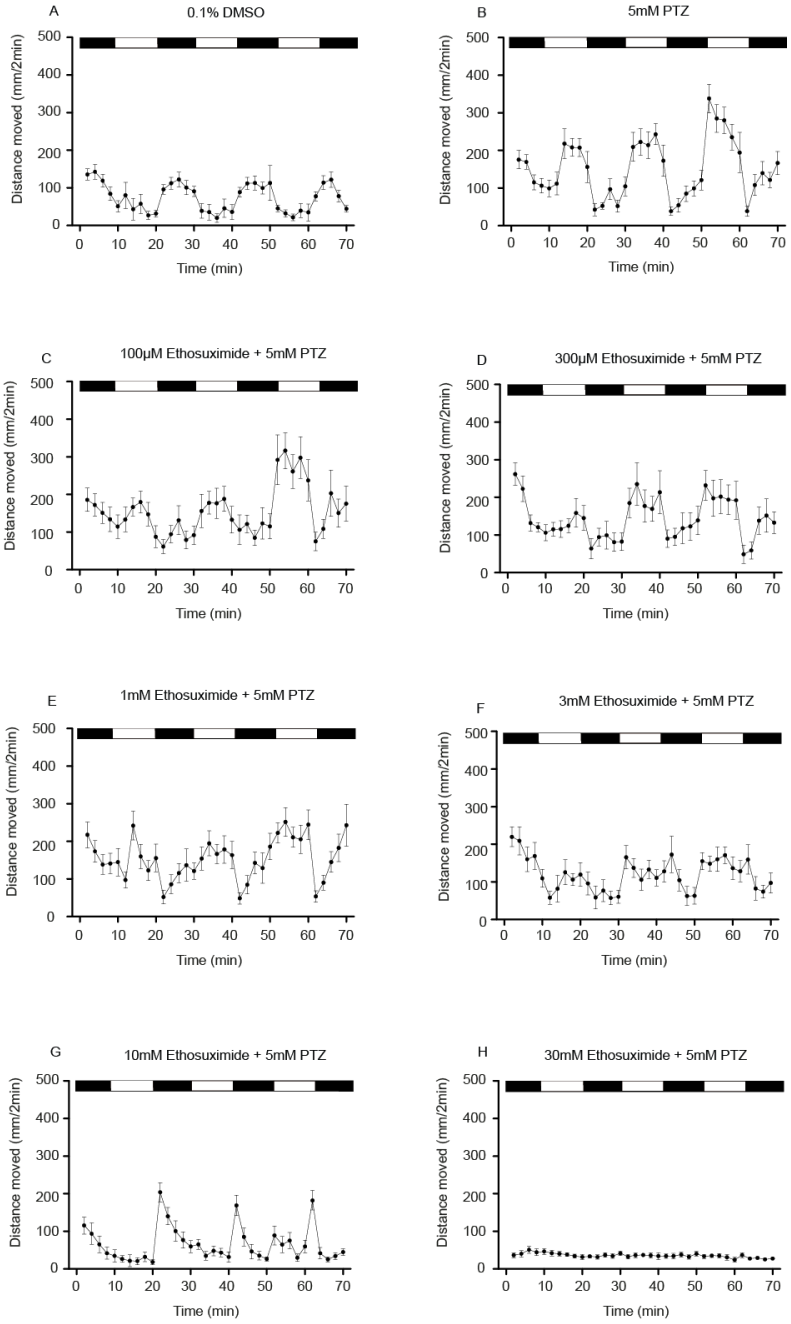

Figure 3.2.2: Ethosuximide cause partial rescue of PTZ phenotype.

Ethosuximide partially reversed the light/dark switching in phenotype, induced by 5mM of PTZ treatment. Tracking curve of 5dpf old zebrafish larvae treated with increasing levels of diazepam. 10mM of ethosuximide show partial reversal of the light/dark response, whereas 30mM ethosuximide dose cause a complete sedation of the larvae. Data plotted as mean+/- SEM of 12 larvae treatment group. The depicted results are representative of a total of three independent experiments.

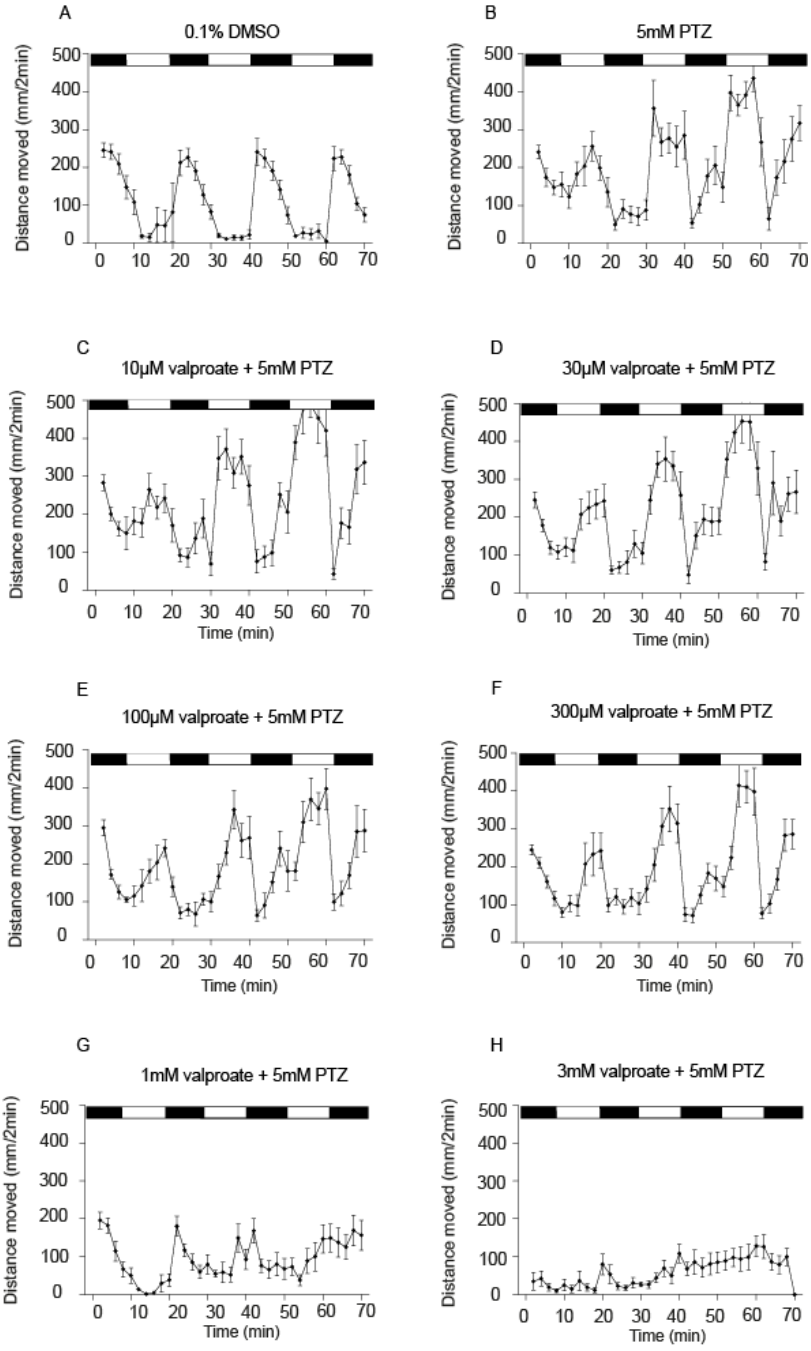

Figure 3.2.3: Tracking plots of valproate dose curve with 5mM PTZ.

Tracking curve of 5dpf old zebrafish larvae treated with increasing levels valproate. At 1mM of valproate, there is a partial reversal of the light/dark behaviour, caused by 5mM PTZ treatment. Data plotted as mean+/-SEM of 12 larvae treatment group. The depicted results are one representative experiment, from a total of three independent experiments.

3.2.3 Diazepam-rescue of PTZ-induced phenotype

At concentrations up to 100 nM, diazepam, a $GABA_A$ receptor agonist, showed no effect on the larvae phenotype (Figure 3.2.4). However, treatment with 300 nM diazepam caused a decreased responsiveness in the light periods, as well as the subsequent dark periods. Subjected to a concentration of 1μM diazepam, larvae displayed a reversal in the PTZ-induced VMR response, similar to that seen with valproate and ethosuximide. At this concentration, there was also a slight sedating effect on the larvae, visible as a reduction in larval swimming activity (Figure 3.2.4). When tested at concentrations of 3 and 10 μM, the larvae showed a marked reduction in swimming activity with no significant response to the alternating light and dark periods (Figure 3.2.4).

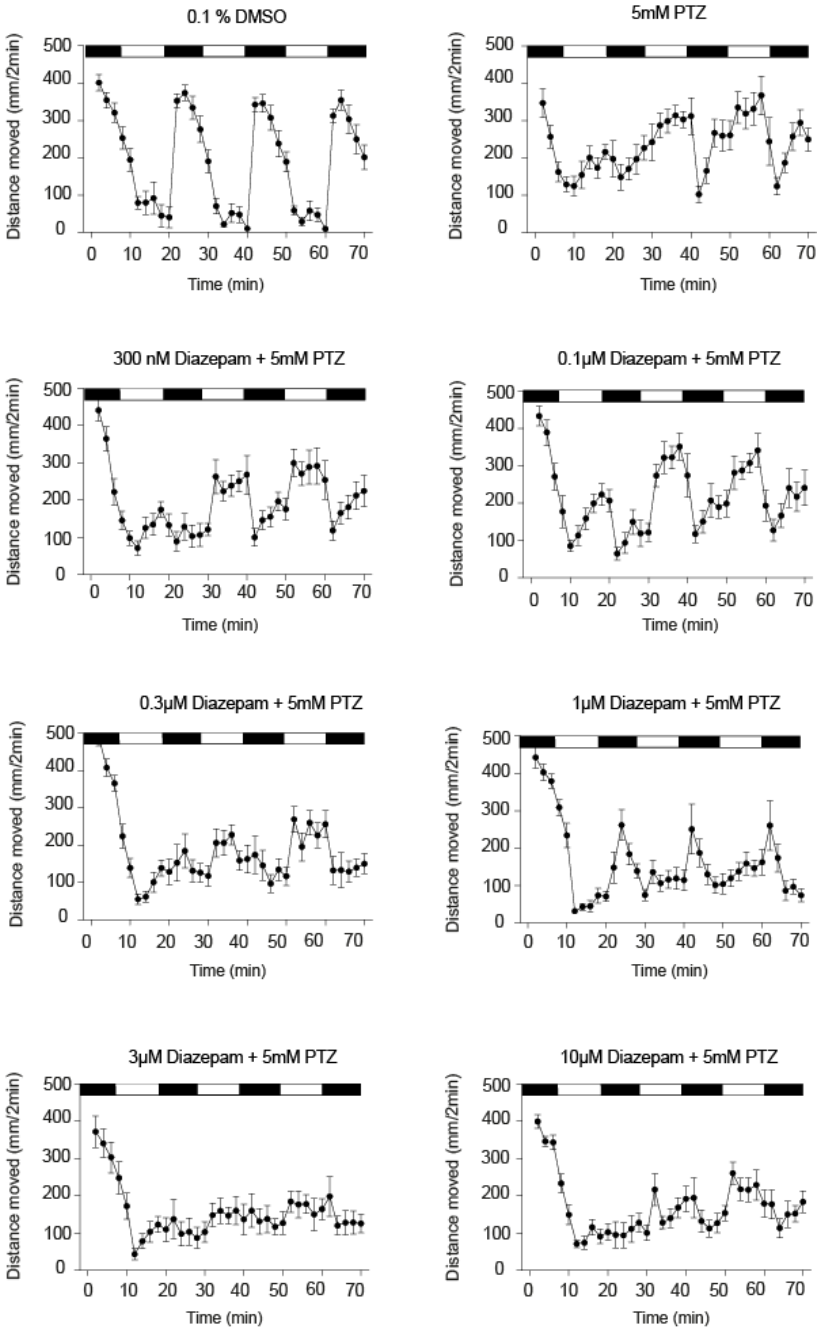

Figure 3.2.4: The classic benzodiazepine, Diazepam, can partially reverse the light/dark switching in phenotype, induced by 5mM of PTZ treatment.

Tracking curve of 5dpf old zebrafish larvae treated with increasing levels of diazepam. 1µM of diazepam show partial reversal of the light/dark response, indicating that the partial rescue observed could be masked by the sedative effect of the compound at the higher doses. Data plotted as mean+/-SEM of 12 larvae per treatment group. Experiment repeated 3 times.

3.2.4 Neuroactive compounds protect against the 5mM PTZ induced locomotor phenotype

Alfaxalone, a synthetic neurosteroid, tested at a concentration range from 100 nM to 30 μM, showed a dose-dependent effect on locomotion and VMR. Alfaxalone at 1μM completely reversed the 5mM PTZ-induced VMR phenotype (Figure 3.2.5) which, resulted in a VMR response resembling that of DMSO treated larvae. The VMR response was also reversed at a concentration of 3 μM alfaxalone, however at this concentration, the activity levels during the dark period were reduced indicating that the compound was mildly sedating at this concentration (Figure 3.2.5). The sedative effect of alfaxalone was prominent at concentrations of 10 μM and 30 μM, where larval locomotor activity was completely absent even though the larvae were still alive and displayed an active beating heart. The other neurosteroid tested, allopregnanolone, also reversed the PTZ-induced phenotype. However, the concentration required was higher than that needed when treating with alfaxalone (Figure 3.2.6)

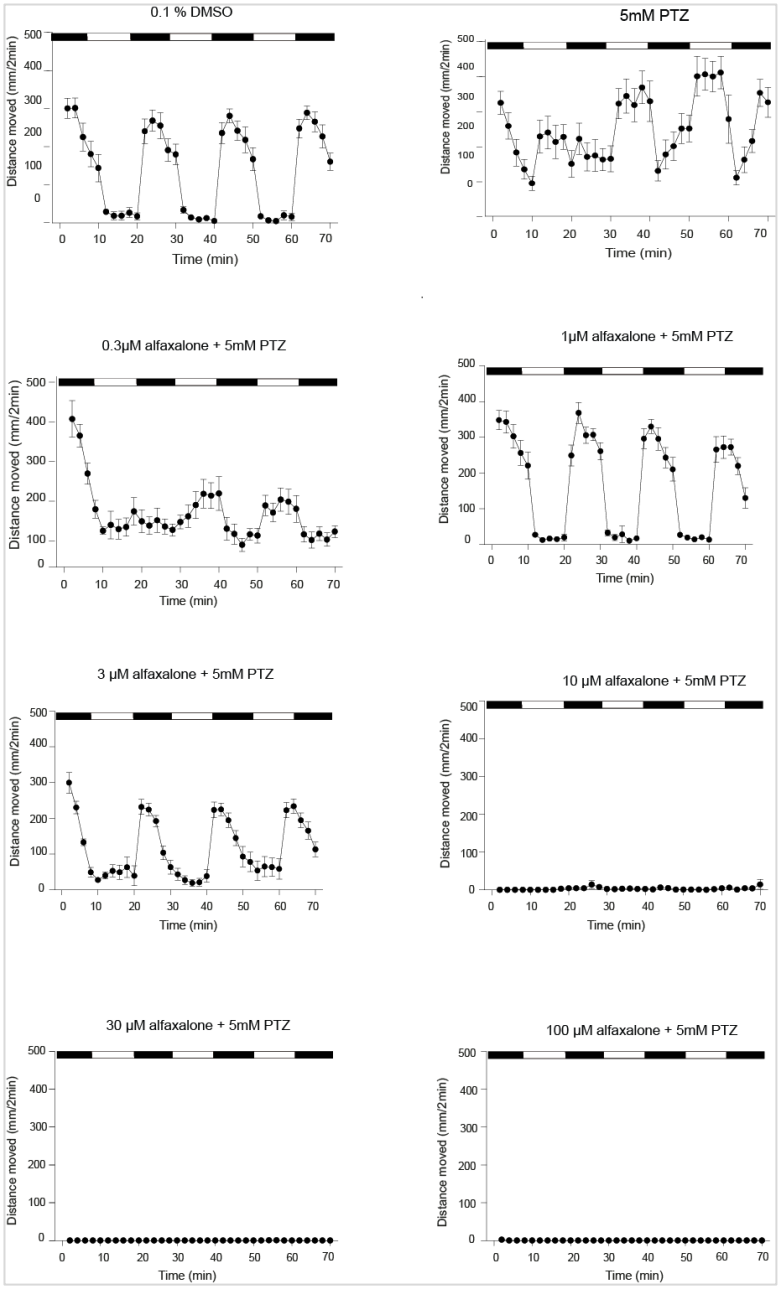

Figure 3.2.5: Alfaxalone efficiently reverse the PTZ-induced phenotype.

Graphs from a 96-well experiment, showing the effect of the synthetic neurosteroid, alfaxalone, on the swimming activity of dpf old zebrafish larvae treated with 5 mM PTZ.T The 3 highest concentrations of the compound is highly sedative, but larvae had a visual heartbeat, at the end of the experiment. Tracking performed during 10 min intervals of dark (black bar) and light (white bar) for a total of 70 min. There are 12 larvae in each treatment group; experiment was repeated 3 times (n=3).

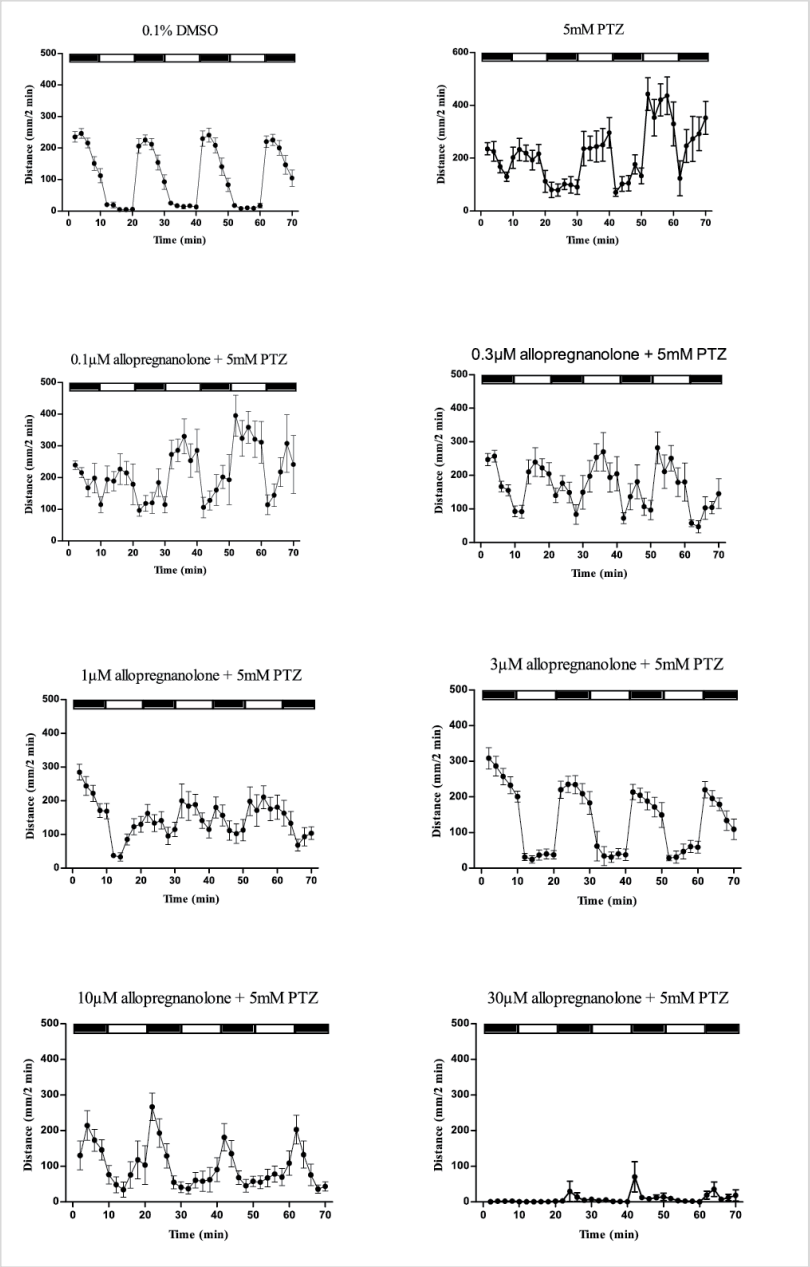

Figure 3.2.6: The neurosteroid, allopregnanolone reverses the PTZ-induced phenotype

Graphs from a 96-well experiment, showing the effect of the neurosteroid allopregnanolone, on the swimming activity of 5 dpf old zebrafish larvae treated with 5 mM PTZ.T The 3 highest concentrations of the compound is highly sedative, but larvae had a visual heartbeat, at the end of the experiment. Tracking is performed during 10 min intervals of dark (black bar) and light (white bar) for a total of 70 min. Each graph represents the average distance moved by the 12 larvae in each treatment group; experiment was repeated 3 times (n=3).

3.2.4.1 The sedative effect of diazepam can be suppressed by co-treatment with neurosteroids

Both diazepam and the neurosteroids bind to the $GABA_A$-receptor, but at different subunits. From mice it has been suggested that a combination of a neurosteroid and a benzodiazepine have a beneficial effect on seizures (Gasior et al. 1997). From the current screen, both of these compounds are effective in the PTZ-model; therefore we tested if a combination of diazepam and alfaxalone would potentiate the effects of the two compounds on reversing the PTZ-induced VMR responses. A sub-threshold concentration of 0.3 μM alfaxalone, which did not have any effect on the PTZ-induced phenotype in larvae (Figure 3.2.5), and had no visible sedative effects, was applied together with increasing concentrations of diazepam, as tested earlier. The combination with alfaxalone significantly potentiated the effect of diazepam on the PTZ-induced phenotype (Figure 3.2.7). The minimal effective concentration (MEC) of diazepam capable of reversing the effect of PTZ was lower, in combination with alfaxalone, when compared to the effective dose in the diazepam-only experiments. Hence, 0.1μM of diazepam resulted in a reversal of the PTZ induced light/dark response in combination with a low level of neurosteroid, compared to 10μM diazepam needed in the single treatment experiment (Figure 3.2.7).

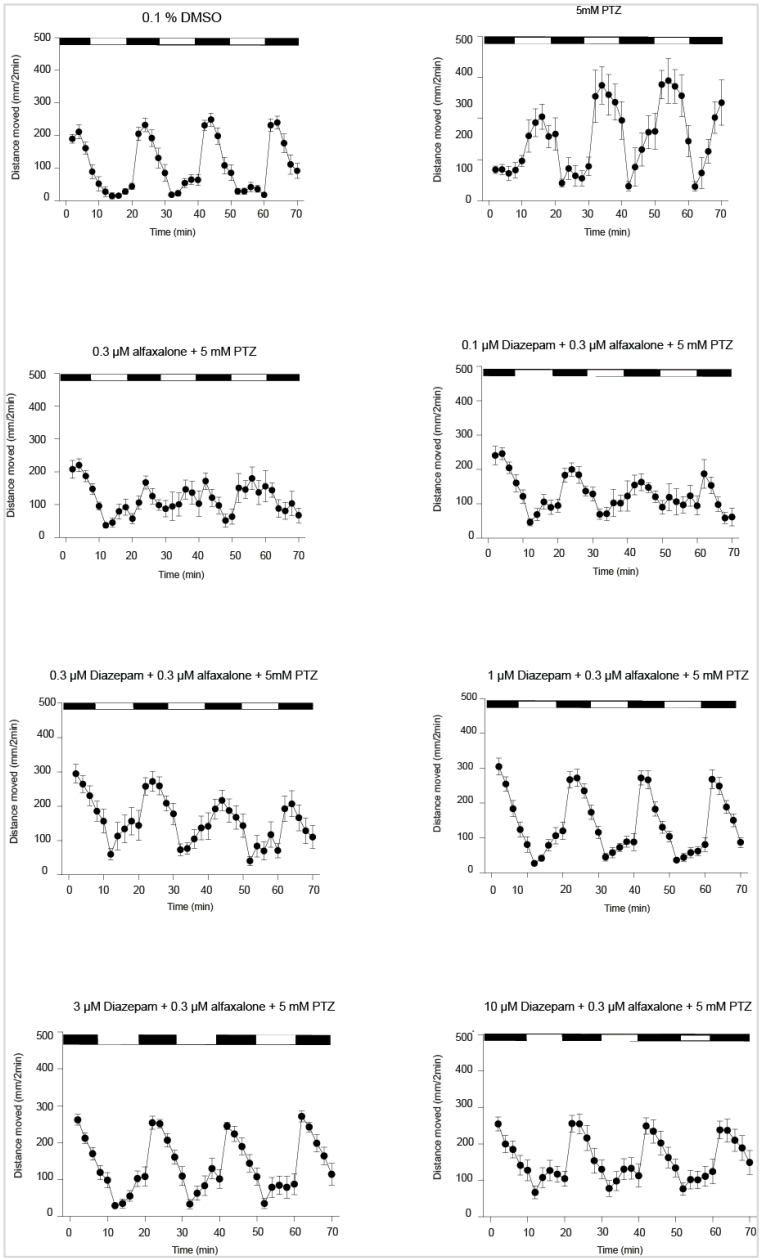

Figure 3.2.7: Combinational treatment with alfaxalone abolishes sedative effect of diazepam.

Graphs from a 96-well experiment, showing the swimming activity of 5 dpf old zebrafish larvae treated with a combination of the synthetic neurosteroid, alfaxalone, and increasing concentrations of diazepam. Tracking plotted in 10 min intervals of dark (black bar) and light (white bar) for a total of 70 min. Each graph represents the average distance moved by the 12 larvae in each treatment group; experiment was repeated 3 times (n=3).

		Fraction of control				Fraction of control	
Therapeutic	**Concentration**	**Light**	**Dark**	**Therapeutic**	**Concentration**	**Light**	**Dark**
PTZ	5 mM	2.16	0.76				
Acetazolamide	1 µM	2.34	0.62	Lorazepam	300 nM	1.86	0.67
	3 µM	2.35	0.54		1 µM	1.74	0.67
	10 µM	2.15	0.63		3 µM	1.65	0.61
	30 µM	2.23	0.58		10 µM	1.47	0.57
	100 µM	1.97	0.58		30 µM	1.15	0.42
	300 µM	1.81	0.68		100 µM	1.00	0.44
Alfaxalone	100 nM	1.84	0.43	Oxcarbazepine	1 µM	1.98	0.72
	300 nM	1.12	0.32		3 µM	2.30	0.78
	1 µM	1.20	1.02		10 µM	1.93	0.72
	3 µM	0.65	0.72		30 µM	2.12	0.81
	10 µM	0.04	0.01		100 µM	2.01	0.85
	30 µM	0.02	0.01		300 µM	2.33	1.08
Allopregnanolone	10 nM	2.45	0.77	Pentobarbital	1 µM	1.77	0.97
	30 nM	2.30	0.77		3 µM	1.74	0.89
	100 nM	1.71	0.64		10 µM	1.54	0.77
	300 nM	1.07	0.85		30 µM	1.28	0.52
	1 µM	0.64	0.54		100 µM	0.82	0.40
	3 µM	0.15	0.11		300 µM	0.01	0.05
Carbamazepine	1 µM	1.77	0.74	Phenytoin	10 µM	1.93	0.82
	3 µM	1.79	0.77		30 µM	2.01	0.81
	10 µM	1.82	0.80		100 µM	2.03	0.78
	30 µM	1.77	0.79		300 µM	1.91	0.75
	100 µM	1.66	0.84		1 mM	1.73	0.72
	300 µM	0.16	0.08		3 mM	1.51	0.63
Diazepam	30 nM	1.87	0.52	Retigabine	100 nM	1.90	0.63
	100 nM	1.65	0.50		300 nM	1.91	0.63
	300 nM	1.39	0.48		1 µM	1.23	0.26
	1 µM	1.05	0.49		3 µM	0.41	0.14
	3 µM	1.08	0.38		10 µM	0.14	0.06
	10 µM	1.01	0.39		30 µM	0.02	0.01
Ethosuximide	100 µM	2.05	0.90	Valproate	10 µM	2.50	0.48
	300 µM	1.78	0.77		30 µM	2.35	0.53
	1 mM	1.65	0.80		100 µM	2.21	0.57
	3 mM	1.37	0.66		300 µM	1.80	0.55
	10 mM	0.74	0.76		1 mM	1.00	0.50
	30 mM	0.47	0.26		3 mM	0.52	0.28
Levetieacetam	100 µM	2.26	0.87	Zonisamide	1 µM	2.40	0.83
	300 µM	2.21	0.84		3 µM	2.28	0.80
	1 mM	2.23	0.87		10 µM	2.19	0.74
	3 mM	2.09	0.78		30 µM	2.33	0.74
	10 mM	2.06	0.84		100 µM	1.90	0.66
	30 mM	1.90	0.85		300 µM	1.95	0.73

Table 3:1: Effect of compounds on the behaviour of 5dpf WT larvae treated with 5mM PTZ.

Data represented as fractions of movement in fish treated with PTZ and compounds, compared to the fraction of movement by fish treated with DMSO only. The average distance moved is calculated as a total of all the light and all the dark intervals for each individual experiment, and then the average for all three experiments are calculated. For larvae swimming more that the DMSO control in the light period, the number is represented with green, those moving less than the DMSO in the light or dark period are represented with a red number. Black numbers indicate that that value equals that of the DSMO control larvae, indicating a complete reversal of the PTZ induced light/dark response.

3.2.5 PTZ increase the expression of *c-fos*

It has been demonstrated previously that seizure-like behaviour is accompanied by an increase in expression levels of *cfos* in neurons (S. C. Baraban et al. 2005; Singewald, Salchner, and Sharp 2003). The *cfos* gene can thus act as a useful marker for elevated levels of neuronal activity following seizure, which has previously been demonstrated in larval zebrafish subjected to 15mM of PTZ (S. C. Baraban et al. 2005; Baxendale et al. 2012). To test whether the reversal of the PTZ-induced VMR response influenced c*fos*-expression, RNA was extracted from larvae at the end of a PTZ experiment and quantitative PCR was used to analyse expression levels of *cfos*. The gene expression levels of *cfos* were increased in larvae treated with both 5 mM PTZ and 15mM PTZ (Figure 3.2.8). This corresponds to what has previously been reported (S. C. Baraban et al. 2005; Ellis, Seibert, and Soanes 2012). To test if alfaxalone had any effect on *cfos* expression, RNA was extracted from larvae at the end of a VMR-assay. Larvae treated with a combination of alfaxalone and PTZ showed a lower *cfos* expression compared to larvae treated with PTZ alone.

Moreover, larvae treated with alfaxalone alone, also showed a slight increase in *c-fos* expression, compared to that of DMSO treated fish (Figure 3.2.8).

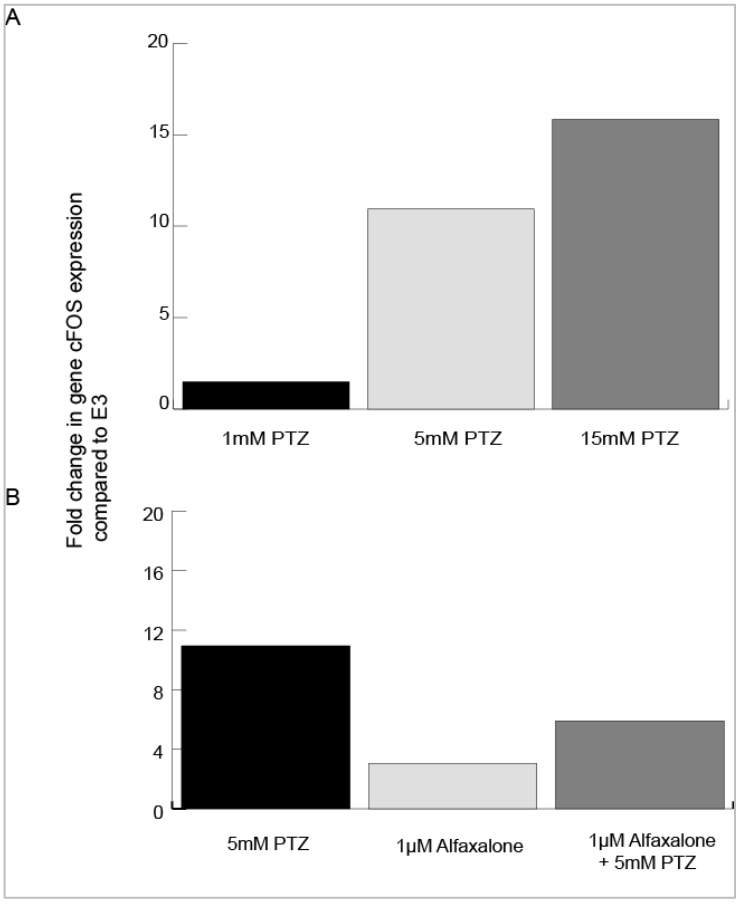

Figure 3.2.8: Preliminary quantitative PCR analysis of cfos expression in zebrafish larvae.

A: Expression of *cfos* increases in zebrafish larvae treated with increasing levels of PTZ. RNA was extracted from 12 larvae in each group. B: larvae treated with alfaxalone and PTZ showed a reduced expression of *cfos* compared to that of 5mM PTZ alone (n=2).

3.3 Discussion

Work by Afrikanova and others has demonstrated that the use of a zebrafish assay in screening compounds with anti-convulsant effects has been a valid model to use. These studies all reported the use of 15 – 20 mM PTZ and a locomotor assay performed under constant darkness (Afrikanova et al. 2013; S. C. Baraban et al. 2005). We have found that there are some disadvantages of this method, as the use of 15mM PTZ leads to a robust seizure activity, ultimately resulting in the loss of posture and hereby a decreased swimming activity. Another issue is the use of a tracking protocol performed under constant darkness as the majority of AEDs used today possess sedative effects, and the natural swimming behaviour of zebrafish larvae under tracked during constant darkness, is an initial period of high activity, followed by a steady decrease in activity (MacPhail et al. 2009; Padilla et al. 2011). Thus analysing potentially sedative compounds under constant darkness can lead to the identification of false positive compounds. In this chapter I have demonstrated the use of a different assay to study AEDs, by using a lower PTZ concentration, combined with an alternative locomotor assay, thereby allowing us to distinguish between AEDs with a general sedating effect and compounds with a specific PTZ-relating activity. Using this technique, we have determined that the majority of compounds tested were not effective in reversing the PTZ-induced phenotype, but diazepam and two neurosteroids, alfaxalone and allopregnanolone were effective.

3.3.1 Low concentration of PTZ leads to distinct behavioural phenotype in zebrafish larvae

The concentrations of PTZ used in this study to induce a behavioural change in the larvae are lower than concentrations used in other epileptic studies previously performed in zebrafish (Afrikanova et al. 2013; S. C. Baraban et al. 2005; Hortopan, Dinday, and Baraban 2010). The high levels of PTZ, 15mM - 20mM, employed in zebrafish studies by others, induce a strong convulsive phenotype. Zebrafish treated with 15mM of PTZ go through a series of behavioural stages, termed I, II and III, as part of the seizure activity (Baraban et al., 2005). The culmination of the increased swimming activity in larvae treated with 15mM PTZ is a whole-body clonus-like convulsions, closely resembling that of PTZ-induced seizures reported in rodents (Erhardt et al. 1995). The fish lose posture, and spend a lot of time on the side, only moving slightly in bursts (S. C. Baraban et al. 2005), resulting in an overall decrease in swimming activity. The increase in swimming activity caused by the high concentration of PTZ (15-20 mM) in the dark is the main quantified output from these assays (S. C. Baraban et al. 2005; Berghmans et al. 2007; Vermoesen et al. 2011). The zebrafish assay described in this chapter uses a lower concentration of PTZ and quantifies the change in larval behaviour by observing the reversed response to changes in the light-dark cycles. Using short-duration cycles of light and dark conditions, it was shown that zebrafish larval activity increased during dark phases and dropped during light phases, as it has been reported previously (MacPhail et al. 2009). Three concentrations of PTZ were tested, 1 mM, 5mM and 15mM in this

study. As reported previously, the highest concentration (15mM) showed a high increase in swimming activity in the beginning of the assay, which declined over time, most likely due the onset of clonus-like seizures, causing the fish to lose posture and inhibiting their swimming (S. C. Baraban et al. 2005). Interestingly, the 5mM concentration of PTZ induced a complete reversal of the ordinary dark/light behaviour observed in 5 dpf old larvae. This could suggest that 5mM PTZ causes a sub-threshold inhibition of GABA-signalling, which when challenged with a stressor, e.g. a sudden burst of light, triggers swimming activity (Figure 3.2.1). In a non-human primate model of photosensitivity and seizure, it has been shown that baboons with naturally occurring photosensitive seizures, have very low GABA levels (Lloyd et al. 1986). These baboons have recurrent seizures, which can be triggered by sudden light flashes.

3.3.2 Poor effect of compounds with previously reported anti-epileptic function on the light/dark phenotype

The ability of fourteen compounds to normalise the PTZ-induced phenotype in response to alternating periods of light and dark conditions was assessed. Only three of the compounds tested in this assay proved to be able to normalise the altered light/dark response, caused by low levels of PTZ treatment. However, the normalisation was partly masked by a sedating effect of the compounds. Rodent pharmacology studies have indicated that valproate and benzodiazepines are among

the most effective AEDs to inhibit PTZ-evoked seizures (Löscher 2011), and this has been shown previously in zebrafish larval PTZ models (Afrikanova et al. 2013; S. C. Baraban et al. 2005; Berghmans et al. 2007). PTZ-models in zebrafish larvae have shown that larvae treated with valproate, diazepam and ethosuximide protect against PTZ-induced seizures, both in the locomotor assay and in reducing the aberrant electrical discharges, normally associated with PTZ induced seizures (Afrikanova et al. 2013). Diazepam is a known benzodiazepine compound used in the clinic as an anti-convulsant, anxiolytic, sedative, and muscle relaxant. It exerts these effects through the modulation of the $GABA_A$-receptor, and has a strong affinity for the α1 and the α3 subunits, enhancing the association of GABA to the receptor, thereby enhancing the inhibitory neurotransmission caused by GABA activity (Olsen and Sieghart 2009). The sedative effect of diazepam became evident in the VMR at 0.3µM, which was visible as a reduced swimming activity in the light period (Figure 3.2.4). These findings are consistent with previous reports, albeit the sedative effect of diazepam reported by others occurred at higher concentrations (Afrikanova et al. 2013; Berghmans et al. 2007; Hortopan, Dinday, and Baraban 2010). An explanation for this could the different tracking conditions used when analysing the locomotor activity. When untreated larvae are tracked under constant darkness, they show an initial period of high activity, which quickly declines and become a low steady state swimming (MacPhail et al. 2009). In larvae treated with 15mM PTZ, a rapid increase in general locomotor activity is present, followed by a slow decline, due to the clonus-like convulsions induced over time (Afrikanova et al. 2013; S. C. Baraban et al. 2005). Thus a general lack of movement could be attributed to the natural

swimming behaviour of larvae under constant light conditions (MacPhail et al. 2009). A possible interpretation of this lack of movement could be that the compound has a specific anti-convulsive effect, but the observed response is the result of a sedative effect by a particular compound. By challenging the larvae with constant changes in light conditions, a sedative effect will become more obvious. Ethosuximide also show a limited effect in the VMR-reversal, but from electrographic activity data, it has been shown that ethosuximide has low or no effect on reducing PTZ induced electrical discharges (Afrikanova et al. 2013; S. C. Baraban et al. 2005). A suggested effect of Ethosuximide is through the increase in GABA-synthesis and release (Greenhill et al. 2012), which could be attributed to the partial rescue of the PTZ-induced phenotype in this assay. However this increase in GABA signalling could ultimately cause sedation as well.

Valproate is a broad-spectrum anti-epileptic drug, thought to function through a GABA related mechanism. Valproate inhibits the breakdown of GABA thereby increasing the availability of the inhibitory neurotransmitter, which is likely to be the mechanism of action resulting in the effect in the current PTZ assay (Owens and Nemeroff 2003). Only three of the tested compounds, namely diazepam, allopregnanolone and alfaxalone, demonstrated an ability to neutralize the PTZ-induced change in behaviour. This does not correlate with earlier work using a higher PTZ concentration, where the majority of standard AEDs were able to suppress PTZ-induced excessive movements in larval zebrafish (Berghmans et al. 2007). The assay used to screen the AEDs in this study found the majority of the AEDs to be sedative (see Table 3:1), which would mask a potential rescue of the phenotype. The sedative

effect is consistent with what Afrikanova and colleagues reported (2013), and is a known side effect of many of the classic AEDs (Afrikanova et al. 2013; Cavanna et al. 2010). A reason for the discrepancy between the data presented in this study and that of Berghmans and colleagues is the incubation time of AEDs. Berghmans and colleagues applied the AEDs for 24 hours, whereas this study only incubated the larvae for 1 hour with the AEDs. (Berghmans et al. 2007). The bioavailability of the compounds must be assumed different after 24 hours of incubation, compared to an incubation time of one hour, and the sedative effect could be more pronounced after longer exposure times, which could explain the discrepancy between the Berghmans study and others, the present one included (Afrikanova et al. 2013; Berghmans et al. 2007).

3.3.3 Neuroactive steroids are effective against PTZ-induced behavioural changes

Amongst the therapeutics able to neutralize the PTZ-induced phenotype in this study, the most pronounced effects were seen by treatment with two neuroactive steroids, alfaxalone and allopregnanolone. Neurosteroids are metabolites of steroid hormones known to regulate neural activity. Allopregnanolone is a neurosteroid synthesized from cholesterol in the brain, and it has an important functional association to anxiety disorders and depression. A reduction of allopregnanolone levels has been shown to correlate negatively with depression, anxiety and post-partum depression (Brot et al. 1997; Schüle, Nothdurfter, and Rupprecht 2014). Allopregnanolone and the synthetic neurosteroid, alfaxalone, are positive allosteric modulators of the

$GABA_A$-receptor. They bind to a specific motif between α and β-subunits of the α1, α3 and α4-subunit containing receptor (Chase Matthew and Doodipala Samba 2013). The binding increases the opening time of the receptor, allowing more Cl^- to pass through the channel, thereby creating a hyperpolarized membrane potential, decreasing the likelihood of another action potential to be fired (Akk et al. 2007; Twyman and Macdonald 1992). The $GABA_A$ receptors have several modulatory allosteric binding sites for benzodiazepines, general anaesthetics, barbiturates and neurosteroids (Korpi, Gründer, and Lüddens 2002). The mechanism underlying the effect of alfaxalone and allopregnanolone reported here is thus likely based on the ability of these compounds to modulate $GABA_A$ receptor-mediated transmission, working together to fine-tune GABA response, thereby reversing the exaggerated response to light.

3.3.4 Co-treatment with a neurosteroid abolishes the sedative effect of diazepam

From mice, it has been shown that a combination of neurosteroids and benzodiazepines had a combined beneficial effect on epileptic symptoms (Gasior et al. 1997). In addition, there are also several rodent studies that have demonstrated a rationale for neuroactive steroids in the pharmacological management of seizure-related disorders (Gasior et al. 1997; Kaminski, Livingood, and Rogawski 2004) and evidence suggests that circulating neurosteroids regulate seizure frequency and severity (Reddy and Jian 2010) . Whilst monotherapy is normally the preferred goal when seeking to treat various diseases, combination effects are also an important

consideration. Poly-drug therapy can be applied when the drug combination improves control of disease symptoms without increasing toxic manifestations of the pharmaceutical treatments. Ideally, such adjunct therapy permits side-effect profiles to be reduced through the lowering of individual drug dosages. From the experiments with the combined treatments, it appears that there is a good synergistic effect of diazepam and the neurosteroids, is such a way that lower concentration of both compounds can be used, and thereby avoid the sedative effect of both compounds, which is in an undesired effect in seizure treatment. In a previous study by Gasior et al. (1997), it was shown that inactive doses of neuroactive steroids markedly enhanced the anticonvulsant effects of diazepam against PTZ in mice without significantly increasing motor toxicity (Gasior et al. 1997). This is consistent with the data presented in this study, where both alfaxalone and allopregnanolone markedly enhanced the ability of diazepam to rescue the PTZ-induced phenotype. Interestingly, potentiation of the protective action of diazepam was not accompanied by an augmentation of the behavioural toxicity of diazepam. As a result, the therapeutic index of the drug combinations was markedly improved over that of diazepam itself.

3.3.5 **Other behavioural effects of PTZ**

In rodents and non-human primates several reports suggest that low level of PTZ can cause anxiety-like behaviours. This low level of $GABA_A$ antagonism have been reported to cause anxiety-like behaviours in the elevated plus maze and the open field test (Jung, Lal, og Gatch 2002). Another possible explanation for the lack of

effect of the AEDs in this PTZ-assay could be due to the fact that the primary behavioural response seen in larvae treated with low-levels of PTZ and subjected to changes in the light and dark conditions are not a seizure related. From rodent and primate studies it has been shown that low levels of PTZ has been reported to cause anxiety in these models (Jung, Lal, and Gatch 2002). This could be a possible explanation for the lack of effect of the anti-convulsant compounds, and the highly specific effect of Diazepam and the two neurosteroids, which have all known anxiolytic properties.

3.4 Conclusion

Using the zebrafish larvae as a complementary model to screen small molecules has become increasingly popular. A number of publications describe the use of larvae as a model of epilepsy and screening potential AEDs. These publications have described the behaviour of zebrafish larvae treated with high levels of PTZ, and utilized the total distance moved during constant illumination as a measure of effect of different compounds. I found by modulating zebrafish behaviour with 5mM PTZ and challenging the larvae with changes in light conditions, a different behavioural response is detected. This alternative assay could potentially serve as an alternative in-vivo screening method to screen potential AEDs, thereby help to identify compounds with or without sedative effects. About 30 % of patients with epilepsy there is no treatment because they do not respond to the current possible treatments. There is a need for new assays to find new treatments for this group of patients. Novel assays like the zebrafish assay offers the possibility that new specie might be

affected differently and hereby open the window for a different way of treating epilepsy patients.

4 Modulating behaviour through cAMP and MAPK

4.1 Introduction

Developing new treatment strategies for mental disorders is needed. No new treatments for anxiety disorders have been developed since the approval of selective serotonin reuptake inhibitors (SSRI). These were approved in 1987, and the efficacy of these compounds has not improved considerably since then (Cryan and Sweeney 2011). Another class of compounds with anxiolytic effect are the benzodiazepines. Developed in the 1950's this class of compounds targets the $GABA_A$-receptor, leading to an increased inhibitory neuro-transmission, resulting in sedation, tolerance, drowsiness and dependence. Despite these side effects, the benzodiazepine compounds are still used to treat acute anxiety and panic attacks, due to the fast acting effect of these compounds (Argyropoulos and Nutt 1999).

The need for discovering compounds targeting different pathways involved in regulating anxiety is evident. For this, the use of the zebrafish is promising. Several reports have demonstrated the translational value of the zebrafish model in anxiety research, both utilising the larvae (Richendrfer et al. 2012; Schnörr et al. 2012; Steenbergen, Richardson, and Champagne 2011b; Ellis, Seibert, and Soanes 2012) and adult animals (Levin, Bencan, and Cerutti 2007; Egan et al. 2009; Maximino et al. 2010).

A pathway known to be involved in neuronal signalling pathways is the cAMP pathway (see 1.1.4.2). The regulation of cAMP signalling is controlled by the phosphodiesterase enzymes (PDE), the sole mean of inactivating cAMP in the cell.

Indications for the importance of this pathway's involvement in mental illness have come from both genetic and pharmacological studies.

Millar et al showed that the *PDE4B* gene was completely disrupted by a chromosome translocation in two patients with psychosis (Millar et al. 2005, 1). PDE4B and PDE4D have also been shown to be interacting partners of the DISC1-gene (Disrupted in Schizophrenia 1), a schizophrenia susceptibility gene, underpinning the importance of PDE4-function in psychiatric illness (Bradshaw and Porteous 2012).

Further evidence has been provided by studies done in knockout models in mice, where mice lacking *PDE4B*- function show an anxiety-like behaviour (Zhang et al. 2008), and *PDE4D*-knock out models show antidepressant and anxiolytic-like behaviours (Zhang et al. 2002b; Schaefer et al. 2012). These data demonstrate both the importance and complexity of PDE4-signalling in regulation of cAMP, and its importance in regulating emotional behaviour. Treatment with phosphodiesterase inhibitors cause several effects indicating the importance of cAMP regulation in the brain. Rolipram is a phosphodiesterase 4 inhibitor, and is important for the hydrolysis of cAMP in the CNS and in the cells of the immune system. Much evidence point to the fact, that Rolipram has a beneficial effect on neuronal survival (Kranz et al. 2014) and regeneration of axons following spinal cord injury (Nikulina 2004). In rodents, Rolipram has been shown to improve cognitive performance when mice are tested in the Morris water maze, and moreover, treatment with phosphodiesterase inhibitors leads to increased cognitive effects (Akar et al. 2014). In a mouse model of Huntington's disease, the R6/2-mouse, Rolipram treatment can

improve the survival of striatal neurons and increase neurogenesis in brain areas affected by Huntington's Disease (Demarch et al. 2008)

These findings suggest an important role for cAMP signalling and PDE4 activity in the CNS. The majority of these findings have been attributed to the activation of the transcription factor, cAMP Response Element Binding protein (CREB) (Demarch et al. 2007; Demarch et al. 2008; Wang et al. 2012; Nakagawa et al. 2002).

How the mechanism of PDE4/cAMP signalling leads to anxiety-like behaviour is not clear. To elucidate how cAMP and PDE4 are involved in regulating behaviour, the zebrafish larvae could be a possible model organism, in the quest to identify the signalling pathways involved in anxiety, mediated through cAMP signalling. In zebrafish, Rolipram causes decreased habituation in the startle response (Best et al. 2008), and other compounds with PDE-inhibitor effect cause increased activity in adult zebrafish (Maximino, Lima, et al. 2011) and thigmotaxis in larvae (Schnörr et al. 2012).

4.1.1 Measuring anxiety in zebrafish larvae

Thigmotaxis, or wall-hugging, is one of the most reliable behavioural outputs when studying anxiety-related behaviour in rodents. Animals displaying anxiety-like behaviours tend to stay close to the wall or floor of an arena and this specific behaviour can be attenuated by treating with anxiolytic compounds, and enhanced by anxiogenic compounds (Reviewed in Steimer 2011). Zebrafish larvae display thigmotatic behaviour when they are challenged with conditions or compounds

causing anxiety or stress (Richendrfer et al. 2012; Schnörr et al. 2012). In 2011, Schnörr and colleagues demonstrated how the treatment with compounds with anxiolytic or anxiogenic effects influenced the placement of 5 dpf old zebrafish in a multi-well plate (Schnörr et al. 2012). Caffeine, a compound known to cause anxiety in rodents (El Yacoubi et al. 2000), increased the time the larvae spend at the edge of the well, and this was enhanced further when the fish were challenged with a stress-inducing stimuli, the onset of sudden darkness. This effect was attenuated by diazepam, an known anxiolytic compound (Schnörr et al. 2012). Richendrfer et al. reported an increase in thigmotatic behaviour in 7 dpf zebrafish larave treated with caffeine. Challenging the larvae using an computer-generated image of a red bouncing ball in one area of the well, further enhanced the thigmotatic behaviour (Richendrfer et al. 2012). The validation of the thigmotaxis as an anxiety-related behaviour, was further established by the fact that diazepam decreased the thigmotaxis, whereas fluoxetine, a selective serotonin re-uptake inhibitor with anti-depressant effect, had no effect on thigmotatic behaviour (Richendrfer et al. 2012).

The light dark test is another assay with translational value, that has been established for larval zebrafish. Using a tank consisting of one black and one white half, Steenbergen and colleagues demonstrated that 6 dpf old zebrafish larvae have a strong preference for the white compartment of the tank, and this preference is further enhanced when fish are treated with caffeine, wherease larvae treated with buspirone and diazepam, both known anxiolytics, have a increased exploratory behaviour in the dark comparement and a decreased latency to enter the dark compartment of the tank (Steenbergen, Richardson, and Champagne 2011b).

4.1.2 Modulation of cAMP in zebrafish larvae cause thigmotaxis

From a small molecule screen our lab identified Rolipram as causing an increased response to touch and hyper-contracted pigmentation pattern in 5 dpf old zebrafish (Richardson et al. 2008). Further work in our lab has also shown that Rolipram also cause thigmotaxis in an open-field assay in 3 and 5 dpf old larvae (N. Grant, Master thesis). The thigmotatic response is dependent on light conditions, as larvae kept in the dark do not show an increased thigmotaxis (N. Grant, Master thesis). These data suggests that Pde4-inhibition in zebrafish cause anxiety-like behaviours. However, the pathway involved in this behaviour is not yet clear. To further elucidate on this pathway, the zebrafish offers the unique potential to probe signalling pathways regulating behaviour through small molecule screens on larvae.

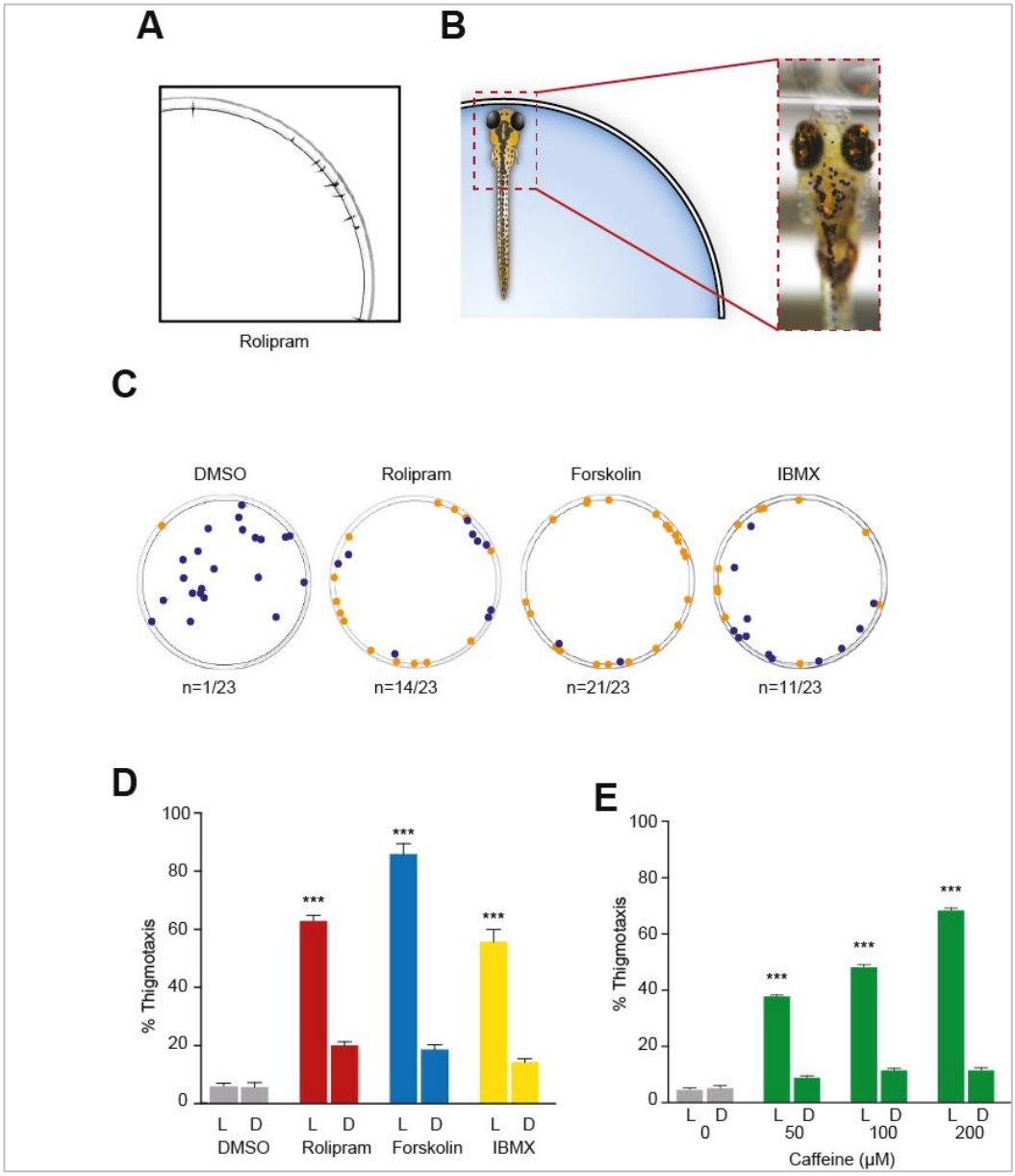

Figure 4.1.1: Cyclic AMP modulators cause thigmotaxis in zebrafish larvae.

A: The placement of 3 dpf old larvae at the edge of an arena after Rolipram treatment, B: A schematic of the placement of A 3 dpf old zebrafish at the edge. C: Pictures from a petri dish assay, counting number of fish placed at the edge of the petri dish after drug exposure (Blue dots represents larvae counted as not displaying thigmotatic behaviour, whereas orange dots mark larvae counted as displaying thigmotatic behaviour). D: The distribution of embryos at the edge is increased in the light compared to the dark. E: Caffeine causes thigmotaxis in zebrafish embryos, which is enhanced when the fish are placed in the light (*Data from K. Anastasaki, PhD thesis, 2011, figure from Lundegaard et al, 2015*).

4.1.2.1 MAPK signalling regulate thigmotaxis in zebrafish larvae

To elucidate on the possible pathways involved in regulating the anxiety-like behaviour in zebrafish larvae caused by PDE4-inhibiton, Nicola Grant, a former master student in our lab performed a small molecule screen (N.Grant, Master Thesis, 2011). Using the thigmotaxis assay, a selection of kinase inhibitors were screened for compounds capable of restoring normal orientation in the thigmotaxis assay. Eighty kinase inhibitors (Screen-Well^{TM} Kinase Inhibitor Library, Enzo Life Sciences, UK), were screened on zebrafish larvae treated with 15µM of Rolipram, displaying the thigmotaxis behaviour. From this screen inhibitors targeting the MAPK pathway was indentified as disrupting the Rolipram-induced thigmotaxis (N. Grant, Master thesis, 2011). The most potent compound capable of reversing the thigmotatic behaviour was the MEK inhibitor, PD98059. Due to the potent effect of PD98059, another MEK inhibitor, PD0325901, was also tested. PD0325901 have been in clinical development for treating several types of cancers (Zhao and Adjei 2014). Treatment with PD0325901 also abolished the Rolipram-inudced thigmotaxis in 3 and 5 dpf old zebrafish (N. Grant, Master thesis, 2011 & Lundegaard et al, manuscript in preparation).

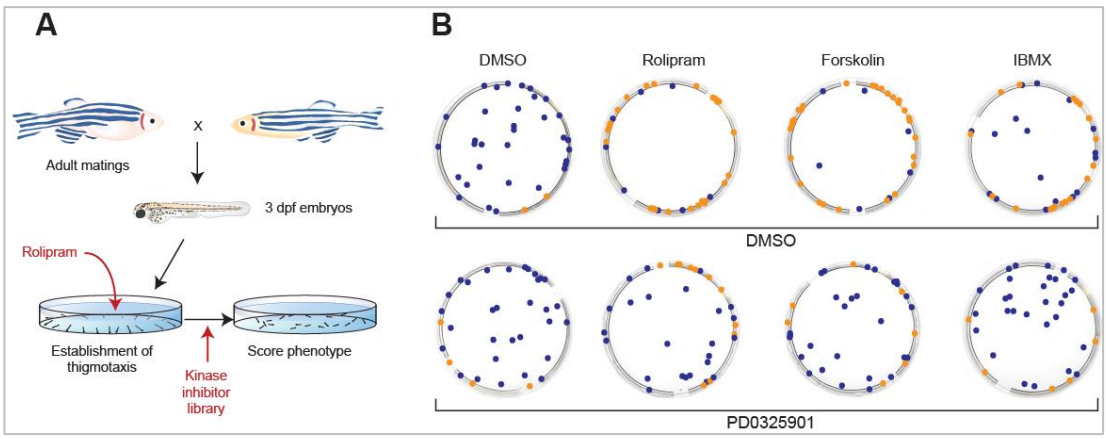

Figure 4.1.2: MEK inhibition counteracts thigmotaxis induced by cAMP-modulation in zebrafish larvae.

A: A schematic showing the setup of the screen by Nicola Grant. B: The thigmotaxis assay, showing the data with the MEK inhibitor PD0325901. Orange dots represent larvae oriented correctly, e.g. perpendicular to the edge of the dish, and thereby counted as displaying thigmotatic behaviour. Blue dots represent larvae, which do not display thigmotaxis *(Date from K. Anastasiaki 2011 Ph.D thesis and Lundegaard et al. 2015*).

4.1.2.1.1 The MAPK pathway

The data from the small molecule screen done by our lab, clear suggest an important function of mitogen-activated protein kinase pathway in the mediation of anxiety-like behaviour in zebrafish larvae (see Figure 4.1.2). The mitogen-activated protein kinase pathway is involved in the regulation of a myriad of cellular processes including cell proliferation, differentiation, gene regulation, development, and motility, among other functions (Stork and Schmitt 2002; Gerits et al. 2008). Signal transduction through this cascade can be mediated through the small G proteins, like the RAS-SOS-GRB2 complex. The activation of Ras results in the subsequent activation of several cytoplasmic kinases (Thomas and Huganir 2004).

So far, four distinct MAPK cascades have been identified. These include ERK, JNK, p38 and BMK. Of these four, ERK has been described intensively (Rubinfeld and Seger 2005). The ERK cascade consists of three levels of subsequent serine/threonine protein kinases: a RAF (C-Raf, B-Raf and Raf-1), two MEK (MEK1/MEK2). Upon activation of RAS, the ERK cascade members are recruited by scaffold proteins and the RAF protein activates its downstream targets MEK1 and MEK2 by phosphorylation. This allows the activation of MEK1/2, and MEK1/2 in turn phosphorylates ERK1 and ERK2. Phosphorylated ERK can either translocate into the nucleus or directly phosphorylate other proteins in the cytoplasm (Figure 4.1.3). In the nucleus, ERK1/2 mainly activates MSK1/2, another protein kinase, which can activate CREB1 and ELK1 (Rubinfeld and Seger 2005).

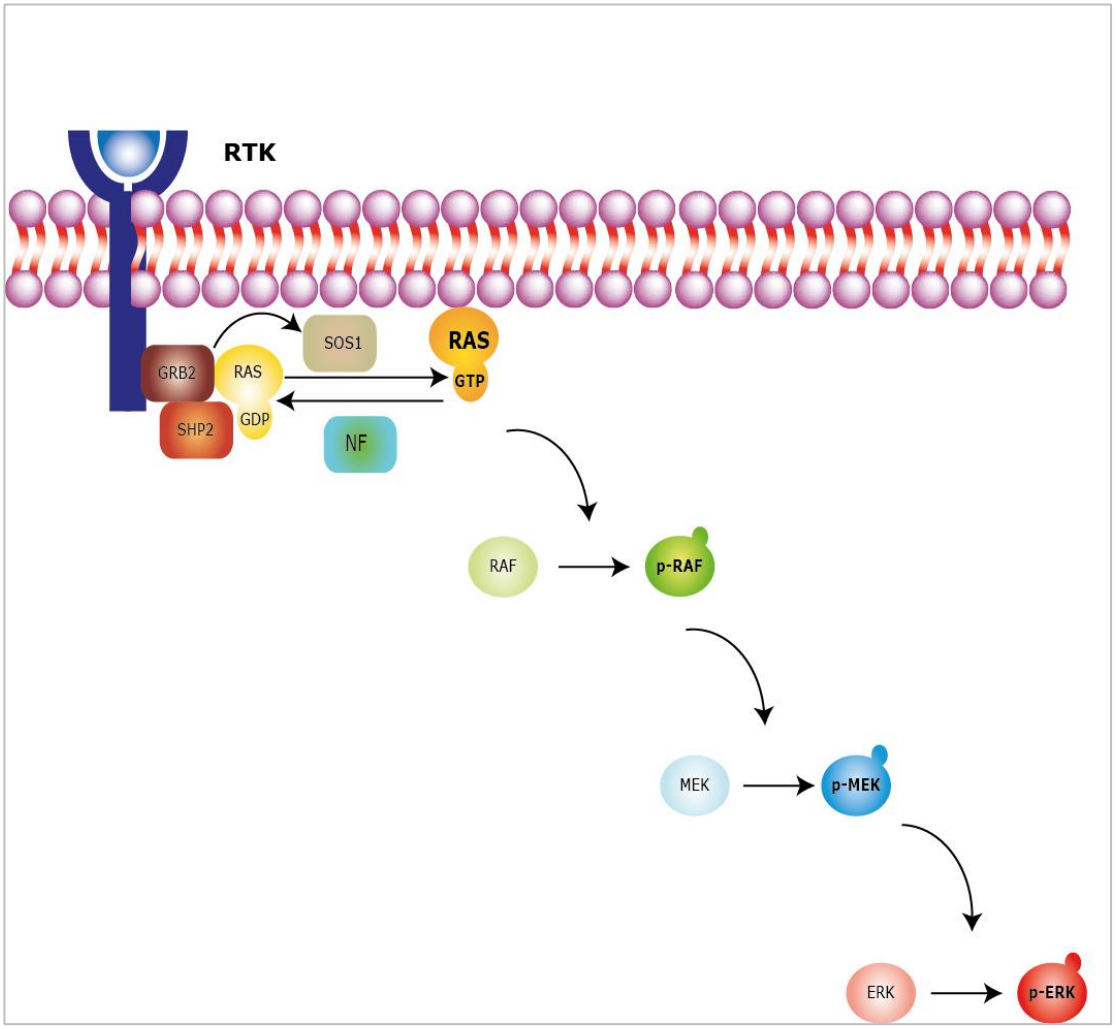

Figure 4.1.3: The RAF-MEK-ERK signalling cascade.

Schematic representation of the RAF-MEK-ERK signalling pathway. Initial activation of the pathway is through ligand binding of the extracellular receptor. This leads to a dimerization of the receptor complex, causing the G-protein to become phosphorylated and activated. The disassociation of the α-subunit allows the cascade to progress through a series of phosphorylation events, ultimately resulting in a translocation of the activated kinase, ERK, into the nucleus (*From K. Anastasaki, Ph.D. Thesis, 2011)*

4.1.2.1.2 Regulation of PDE4 isoforms by PKA and MAPK signalling

The regulation of the transcription and activity of the PDE4 isoforms and the alternative spliced variants are subject to a complex regulation (Simon J. MacKenzie et al. 2002). Crucial for regulating this activity is the UCR regions. In the long-formPDE4s, the phosphorylation of UCR1by PKA activates the long form PDE4s, thereby leading to the degradation of cAMP, ultimately leading to the inactivation of PKA signaling. However, the UCR2 negative regulate PDE4 through phosphorylation by ERK, but this inhibitory effect can be reversed by the subsequently phosphorylation of UCR1 by PKA (Lim, Pahlke, and Conti 1999; Simon J. MacKenzie et al. 2002). The UCR1 domain is absent in short and super-short forms of PDE4 (see Figure 1.1.4), suggesting that PKA cannot activate these two splice forms of PDE4 (Simon J. MacKenzie et al. 2002). However, there has been a binding site for ERK identified on all the catalytic domains of PDE4 isoforms, expect for PDE4A (Baillie et al. 2000). In addition to this complex phosphorylation pattern, the effects of PDE4 phosphorylation by ERK are different depending on isoform. Short isoforms are activated by ERK phosphorylation, whereas the long isoforms are strongly inhibited, and super-short isoforms are weakly inhibited by phosphorylation (S.J. MacKenzie et al. 2000). This elaborate phosphorylation pattern establish several layers of regulation of cAMP degradation and activation, and demonstrates the elaborate cross-talk taking place between the two signaling pathways (Figure 4.1.4).

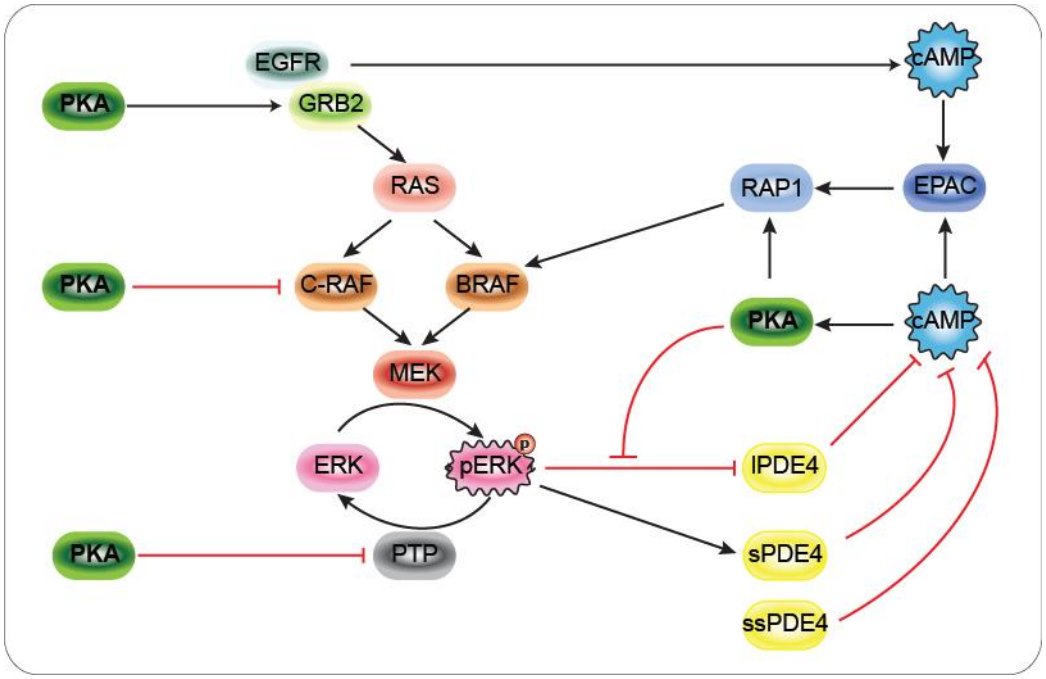

Figure 4.1.4: Schematic illustration of the cross talk between cAMP and RAF-MEK-ERK signalling pathways.

There are several points of cross talk between the MAPK and cAMP pathways. G-coupled receptor activation leads to increase in cAMP, which in turn stimulates EPAC or PKA. EPAC activation leads to phosphorylation of the small GTPase, RAP1. Rap1 activates BRAF, which is part of the MAPK signalling pathway (*Illustration from K. Anastasaki, PhD thesis, 2011*).

4.2 Aim of chapter

The aim of this chapter was to evaluate the effect of cAMP and MAPK cross talk in zebrafish larvae, and how this cross talk modulates behaviour.

4.3 Results

Our lab has previously identified Rolipram from a small molecule screen, as causing contracted melanocytes and a hyper-response to touch (Richardson et al. 2008), and additional work in the lab identified a thigmotaxis-behaviour in an open field assay in 3 and 5 dpf larvae (N. Grant, Master thesis). These findings promoted us to further study the behavioural effect of Rolipram in zebrafish larvae. The data presented in this chapter demonstrates how chemical modulation of cAMP levels control locomotor activity in zebrafish larvae. Specifically the inhibition of PDE4-enzymes leads to hyperactivity and prolonged movement. Rolipram treatment also enhanced cAMP levels in vivo and activated the MAPK pathway. This activity of the MAPK pathway could be reversed by treating larvae with a specific MEK-inhibitor, PD0325901, resulting in a reversal of hyperactivity and MAPK signalling.

4.3.1 Rolipram increase the swimming activity of zebrafish larvae

Hyperactivity has been associated with anxiety and stress in zebrafish larvae (Ellis, Seibert, and Soanes 2012; Q. Li et al. 2014), and measuring swimming activity under

aversive conditions, like constant darkness, is known to attenuate such behaviours (MacPhail et al. 2009; Schnörr et al. 2012).

Using Rolipram to activate cAMP signalling, our lab discovered that treatment with Rolipram caused thigmotaxis in 3 and 5 dpf old larvae (see Figure 4.1.1). This was further substantiated through the effects of other compounds known to modulate cAMP, like IBMX and Forskolin (Figure 4.1.1).

Analysing the behaviour of zebrafish after Rolipram treatment showed that Rolipram significantly increases the swimming activity of 5 dpf old larvae. Forskolin, an activator of the adenylate cyclases, and 3-isobutyl-1-methylxanthine (IBMX), a non-selective PDE inhibitor, were also capable of inducing hyperactivity of 5 dpf old larvae. Figure 4.3.1 show that modulation of cAMP causes a significant increase in the total distance moved by the zebrafish in both the dark and the light.

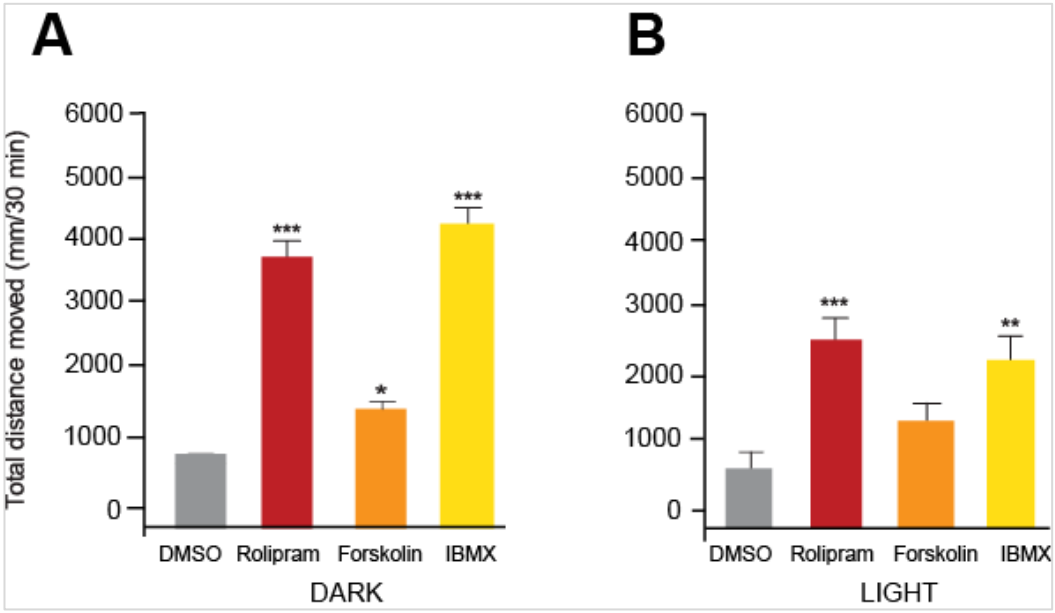

Figure 4.3.1: Compounds modulating cAMP levels cause an increase in locomotor activity.

Bar graph showing the total distance moved, tracked during constant darkness (A) or light (B) for 30 min. DMSO (n=48), Rolipram (n=48), Forskolin (n=48) and IBMX (n=48). Data analysed using One-way ANOVA, followed by Tukey's post-test. Data plotted as mean +/- SEM. $P < 0.001$ (***), $p < 0.01$ (**), $p<0.05$(*).

4.3.2 Long-term effect of Rolipram treatment on swimming activity

To determine if the Rolipram-induced hyperacitvity was due to a lack of habituation to the darkness and the small environment, I tested Rolipram on larvae under prolonged exposure to darkness (5 hours) and analysed the swimming activity for for the first 10 minutes of every hour, to test if the hyperactivity was sustained over time. Data was calcluated as total distance moved over time. Five dpf larvae were plated in a 96-well plate, 1 larvae per well, and treated with a final concentration of 15µM

Rolipram or the equivalent volume of DMSO as a control, and the plate was immediately placed in the DanioVision observation chamber with the lights off. All the larvae were screened for developmental defects prior to testing. This was done to exclude unhealthy larvae from the assay before start. The initial swimming activity was recorded for the first 10 minutes, to determine the baseline activity of the larvae after drug addition and the disturbance of being moved from the light to the darkness of the chamber. After one hour the Rolipram treated group showed a significant increase in swimming activity, compared to that of the DMSO-treated larvae. The DMSO-treated larvae showed a gradual decrease in activity over time, whereas the Rolipram treated group continued to display increased swimming activity compared to the DMSO-larvae, throughout the course of the experiment (Figure 4.3.2). These findings show that the hyperactivity caused by Rolipram treatment does not diminish over time, and the larvae do not adjust to their environment as seen in DMSO-treated fish.

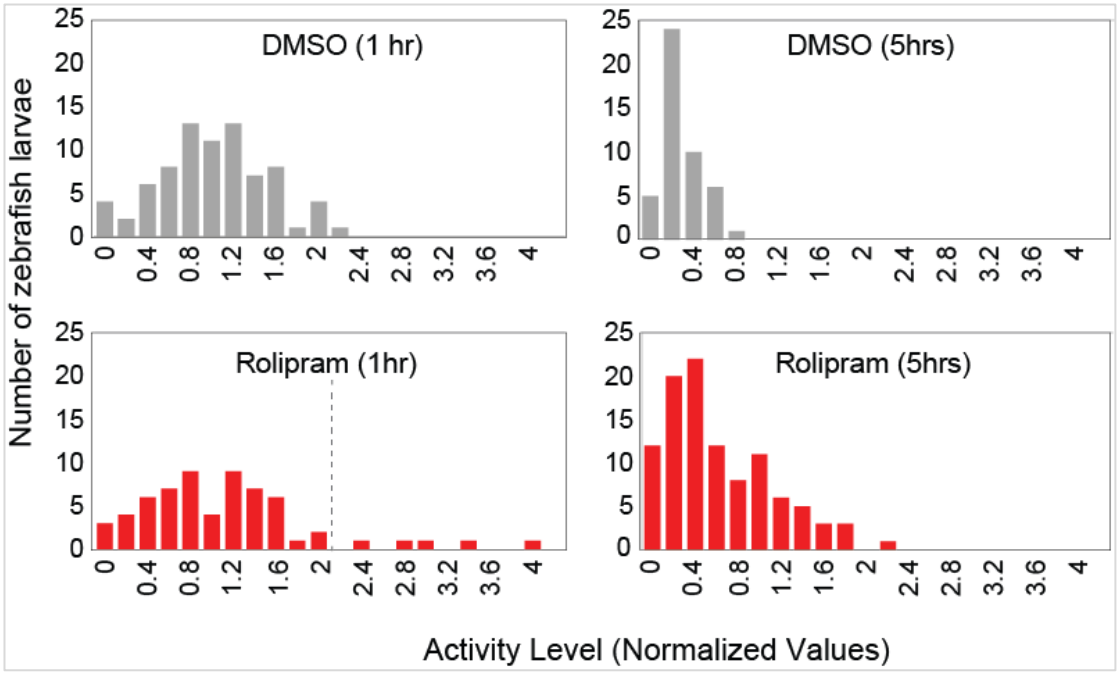

Figure 4.3.2: Long term effect of Rolipram on swimming activity on 5 dpf old larvae

Histograms showing the normalized data plotted from habituation experiment with Rolipram. Fish treated with DMSO (n=48) or Rolipram (n=48) were tracked under constant darkness for 5 hours. The distance travelled for the first 10 min of every hour were analysed. The data from individual wells were normalized and values are allocated to bins, and plotted in histograms.

4.3.3 PDE inhibitors cause an increase in locomotor activity

Modulation of cAMP in 5 dpf old zebrafish larvae cause an increased thigmotaxis and hyperacitivty in the swimming assay. To further elucidate on the function of the role of PDEs in mediating hyperactivty, I did a blind screen on 10PDE inhibitors (See Table 4: 1). Using a dose curve approach, I tested all the compounds in a concentration range from 3nM to 3µM. I screened all the compounds three times in the swimming assay. The test was performed blindly, and four compounds were identified as causing an increase in actibity of 5 dpf old larvae. Three out of the four hits targeted the PDE4 family, suggesting that the specific inhibition of PDE4 is enough to induce hyperactivity in the zebrafish larvae. One other compound, a dual-PDE-type inhibitor targeting both PDE2 and PDE10, also caused an increase in swimming activity (Figure 4.3.5).

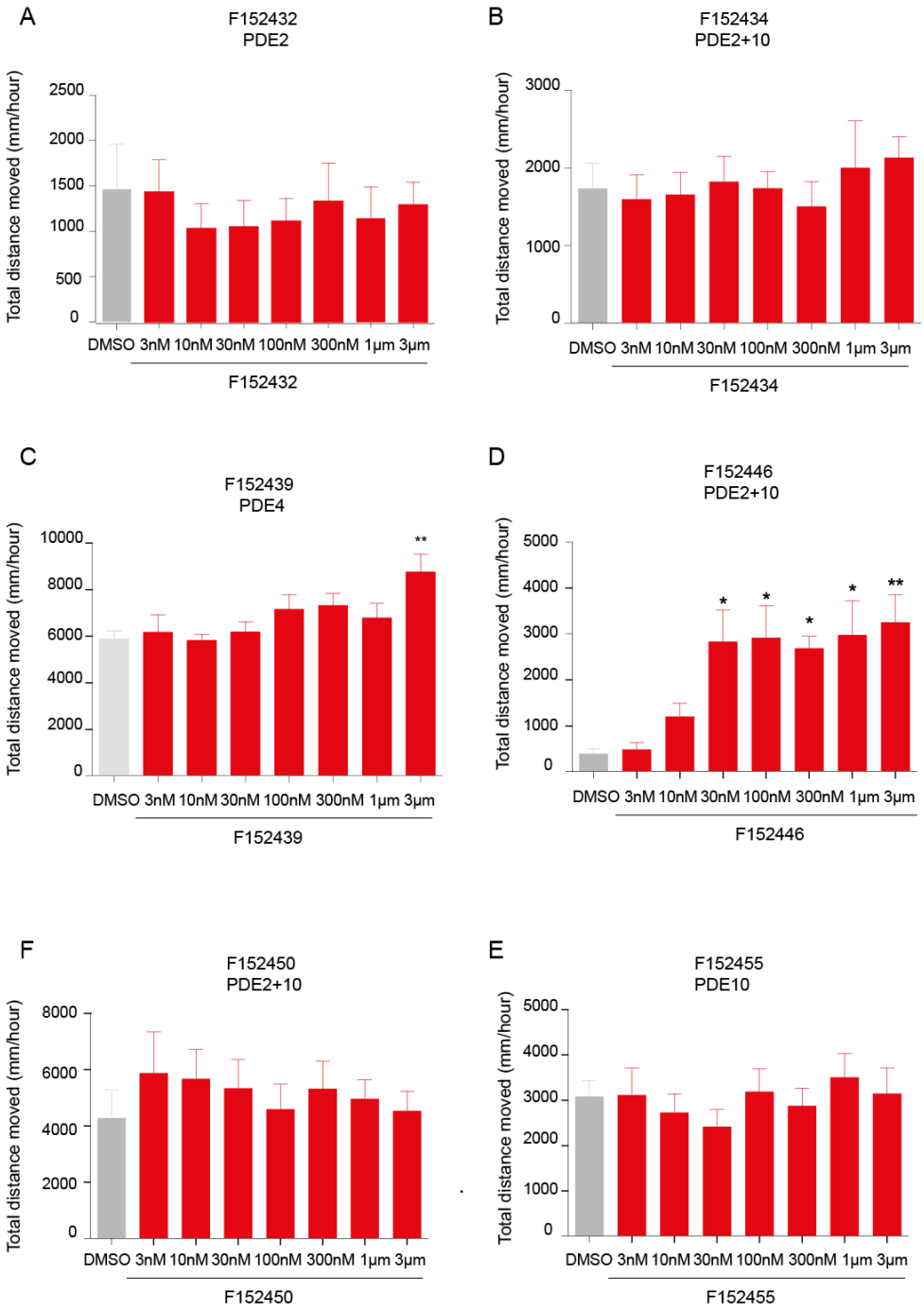

Figure 4.3.3: Graph 1 of 2: concentration response of phosphodiesterase inhibitors.

Dose response of the PDE-inhibitors from Lundbeck. The data is total distance moved over 1 hour, with 12 larvae per drug concentration. All experiments is repeated n=3. The data are analysed using One-Way ANOVA, followed by Bonferroni's post-test. $P<0.05$. * = significant,** = highly significant.

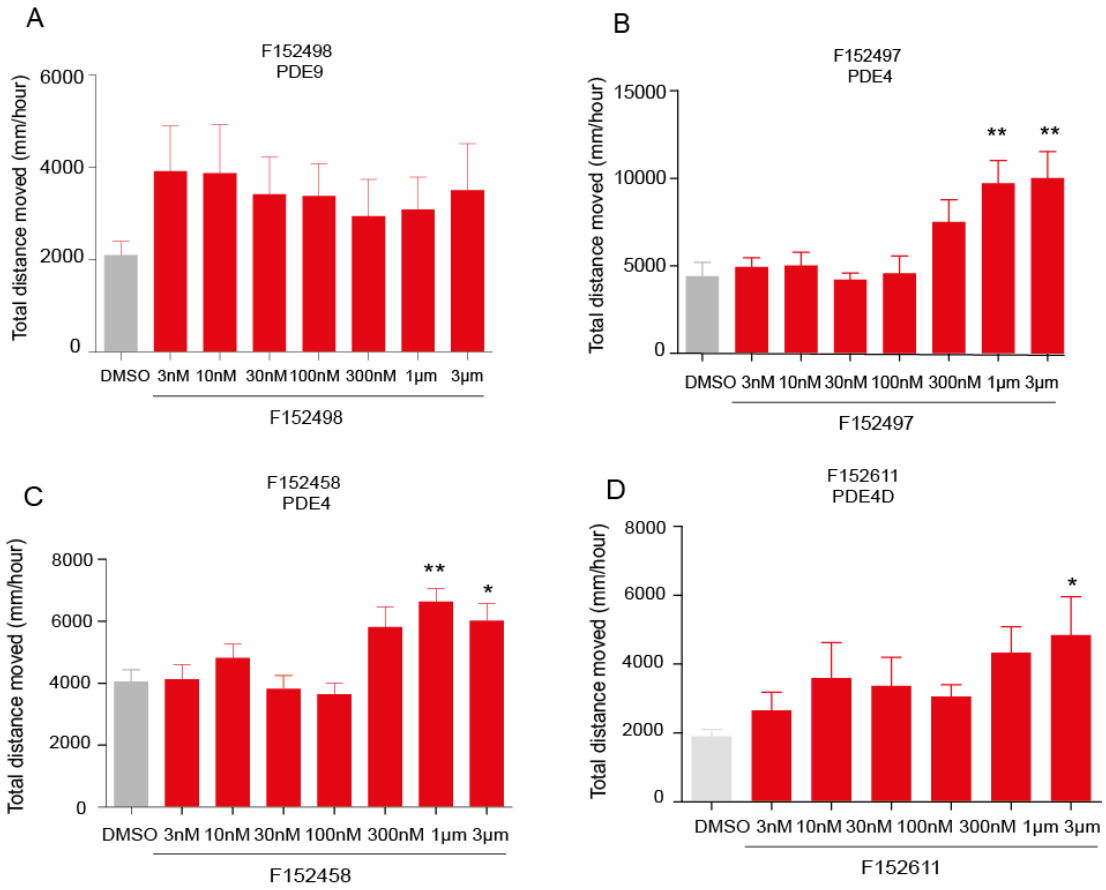

Figure 4.3.4: Graph 2 of 2: concentration response of phosphodiesterase inhibitors

Dose response of the PDE-inhibitors from Lundbeck. The data is total distance moved over 1 hour, with 12 larvae per drug concentration. All experiments is repeated n=3. The data are analysed using One-Way ANOVA, followed by Bonferroni's post-test. $P < 0.05$. * = significant, ** = highly significant.

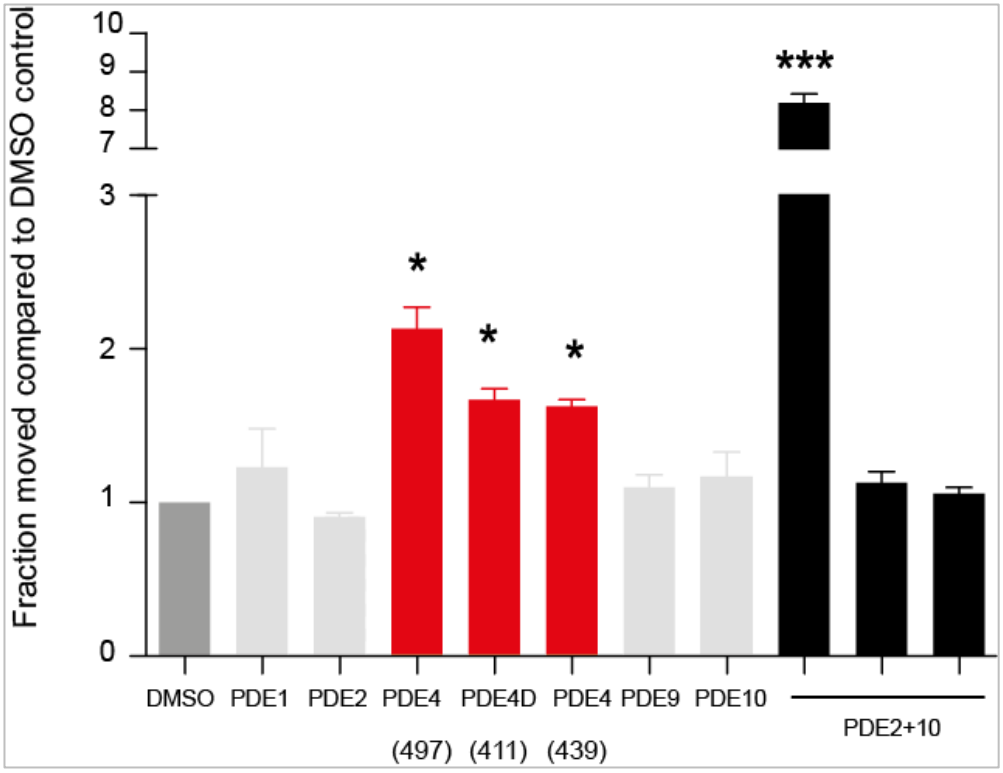

Figure 4.3.5: Summary of PDE inhibitors and their effect of swimming activity in 5 dpf larvae

The swimming activity of larvae compared to that of DMSO control. The data shown is the dose causing the maximum increase in swimming activity, or the highest dose tested, for the compounds that show no effect on swimming activity. The data are plotted as the fraction of movement of compared to that of DSMO treated fish. Data are analysed using One-Way ANOVA

Compound	Source of structure	Primary Target(s) [100% inhibition]	Concentrations tested in zebrafish	Concentration causing increased swimming activity
PDE1 (458)	Pfizer	PDE1 (83nM) PDE4 (2.6µM)	3nM – 3µM	Not significant
PDE2 (432)	Neuro3D	PDE2 (130nM)	3nM – 3µM	Not significant
PDE4 (439)	Decode	PDE4 (77%@10µM)	3nM – 10µM	3µM
PDE4D (611)	Decode	PDE4 (64%@10µM)	3nM – 3µM	3µM
PDE4 (Rolipram)	Sigma	PDE4 (1.6µM)	3nM – 3µM	3µM
PDE9 (498)	Bayer	PDE9 (140nM)	3nM – 3µM	Not significant
PDE10 (455)	Lundbeck	PDE2(4nM) PDE10 (4nM)	3nM – 3µM	Not significant
PDE2+10 (446)	Lundbeck	PDE2 (4nM) PDE7 (1.2µM) PDE10 (40nM) PDE11 (1.8µM)	3nM – 100µM	Significant at 10nM – 20µM
PDE2+10 (434)	Lundbeck	PDE2 (7nM) PDE5A (2.9µM) PDE10 (1nM) PDE11 (1.3µM)	3nM – 3µM	Not significant
PDE2+10 (450)	Lundbeck	PDE2 (13nM) PDE4 (51nM) PDE10 (3.1nM) PDE11 (3.5µM)	3nM – 3µM	Not significant

Table 4: 1: List of PDE inhibitors included in the PDE screen

List of PDE inhibitors from Lundbeck A/S. I obtained 10 PDE inhibitors, targeting different PDEs. Included in the screen was Rolipram, synthesised at Lundbeck. The table lists the Lundbeck identification number (omitting F15), patenting company (except Rolipram), the concentration of compound needed to give 100 % inhibition of the listed isoforms of PDEs, the drug concentrations tested in the swimming assay and at what concentration a significant activity increase was observed.

4.3.4 Inhibition of MEK impedes hyperactivity in PDE4 treated larvae

To test if the hyperactivity could caused by Rolipram and other cAMP modulators could be due to an activation of the MAPK-pathway, 5 dpf old larvae were treated with a combination of Rolipram and the specific MEK1/2 inhibitor, PD0325901. In the presence of 1.5µM PD0325901, the hyperactivity of Rolipram treated larvae was absent compared to those treated with Rolipram alone.

From these experiments, it was clear that hyperactivity is also regulated through the MAPK pathway. Larvae treated with Rolipram and PD0325901 are not hyperactive. Data from these experiments also revealed another interesting cavioat. When the data was plotted as bar graphs, with mean distance moved +/- SEM, there was a large degree of intre-experimental variation. From a histogram plot of normalised data, it became clear, that in the group of larvae treated with Rolipram, there is a distinct group of larvae that swim significantly more than that of the DMSO-treated larvae (Figure 4.3.6) . This could indicate that there is a subset of larvae in the group, that respond much stronger to Rolipram (Figure 4.3.6 B). The Rolipram-induced hyperactivity was absent in larvae treated with both Rolipram and 1.5µM PD0325901, and there was no affect of the treatement with 1.5 µM PD0325901 alone.

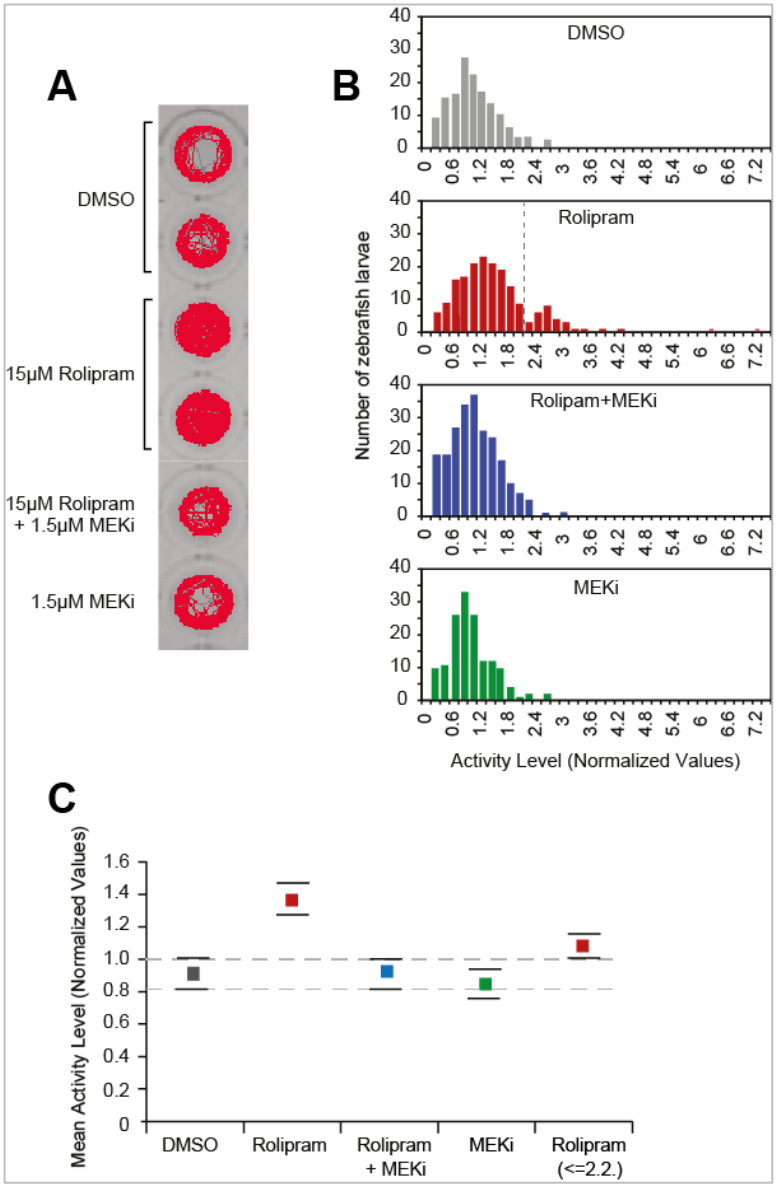

Figure 4.3.6: Rolipram induced hyperactivity can be reversed through MEK-inhibition

A: Movement plots from EthoVision XT9. B; Histograms plotted with the normalized data from a total of 7 experiments of behavioural analysis with Rolipram, PD0325901 and a combination of the two. Data are displayed as bins, which have been bootstrapped to determine the 95CI for the data. C: Bootstrapped means plotted with a 95% CI, showing a significant increase in swimming activity when larvae are treated with Rolipram. Note that when removing the group of larvae with very high swimming activity (B), the Rolipram still causes an significant increase in swimming activity, displayed by none-overlapping CI, and that 0 is not included in the 95% CI (C).

4.3.5 Increased cAMP levels in vivo following treatment with Rolipram

Rolipram targets the PDE4-proteins, which are responsible for the hydrolysis of cAMP (S J MacKenzie and Houslay 2000). Inhibition of PDE4 leads to increased cAMP signaling in vivo, but to what extent this inhibition can been seen as a measurable effect in zebrafish larvae has not been shown. To determine if the effect of Rolipram and forskolin did result in increased cAMP in vivo, cAMP was extracted from 5 dpf old larvae and treated with the same treatment regime as previously described for the hyperactivity assays. Results show that there is a significant higher level of cAMP in larvae treated with Rolipram, and more pronounced increase after treatment with forskolin, an AC activator (Figure 4.3.7).

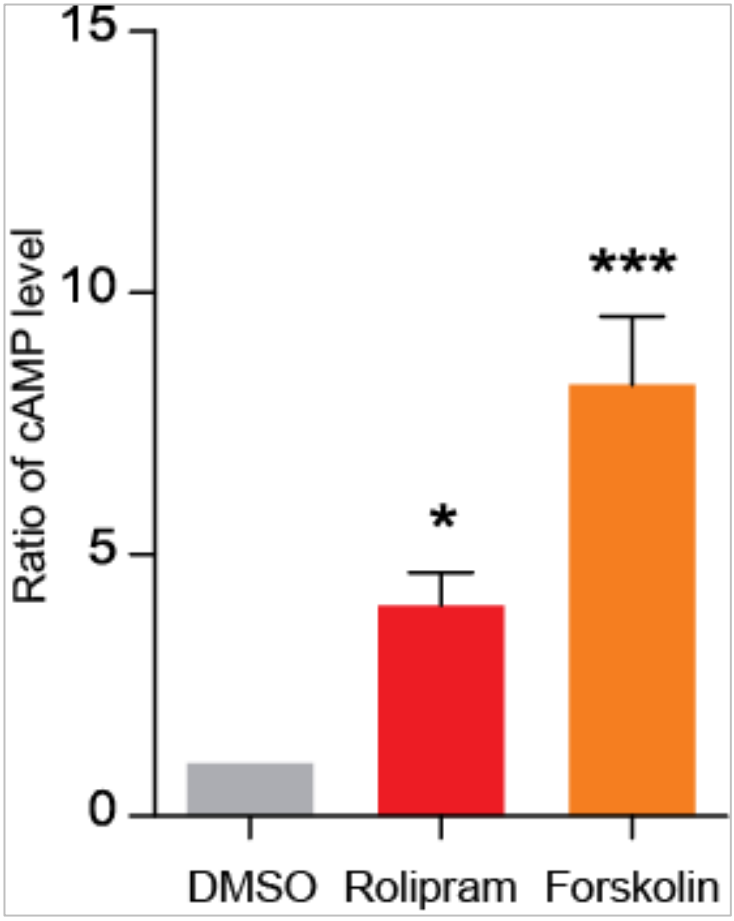

Figure 4.3.7: Increased cAMP levels in whole larvae treated with Rolipram and Forskolin.

Data from ELISA assay measuring cAMP levels. Data are represented as ration of cAMP levels compared to DMSO-treated fish. Data analysed using One-Way ANOVA, followed by Turkey's post-test. $P<0.05$. *= significant, ***= highly significant. Data from 15 larvae per extraction per treatment, experiment repeated 3 times.

4.3.5.1 MEK inhibition only cause decreased cAMP levels in forskolin treated larvae, but not in Rolipram-treated larvae

To test if the reduced hyperactivity observed in larvae subsequently treated with PD0325901 correspond with a similar decrease in cAMP levels, cAMP levels were determined from combination-treated larvae. Interestingly, in larvae treated with the combination of Rolipram and PD0325901 there was no reduction in cAMP levels, but instead a small increase, although this was not significant (n=3). When adding PD0325901 til forskolin-treated larvae, there was a significant reduction in cAMP levels, compared to forskolin-only treated larvae.

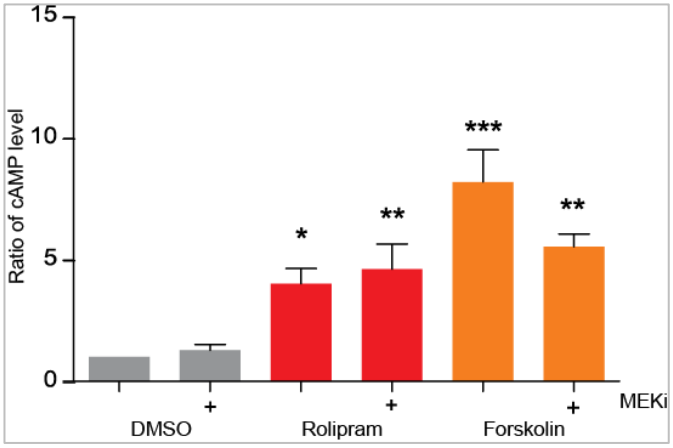

Figure 4.3.8: ELISA assay determining cAMP levels following combination treatment.

5 dpf old zebrafish were treated with Rolipram +/-PD0325901 or Forskolin +/- PD0325901. Data are plotted as ratio of cAMP compared to DMSO control, which is 1. 15 fish per treatment group, experiment repeated 3 times. Data analysed with One-way ANOVA, followed by Tukey's post-test ($p<0.05$), * = significant, ** = very significant, *** = highly significant.

4.3.6 Rolipram activates MAPK signalling

As the inhibition of MEK can rescue the Rolipram-induced hyperactivity, but does not reverse the increased levels of cAMP, I tested whether the MAPK-pathway was activated by Rolipram treatment. A total of 15 five dpf old larvae were treated with Rolipram showed increased pERK levels, determined by Western blot analysis of whole larvae. The expression of pERK in larvae treated with the combination of Rolipram and PD0325901 showed very low levels of pERK, moreover confirming that the MEK-inhibiton has worked, in the larvae treated with PD0325901 alone there was no pERK expression, where as total ERK levels were unchanged (Figure 4.3.9).

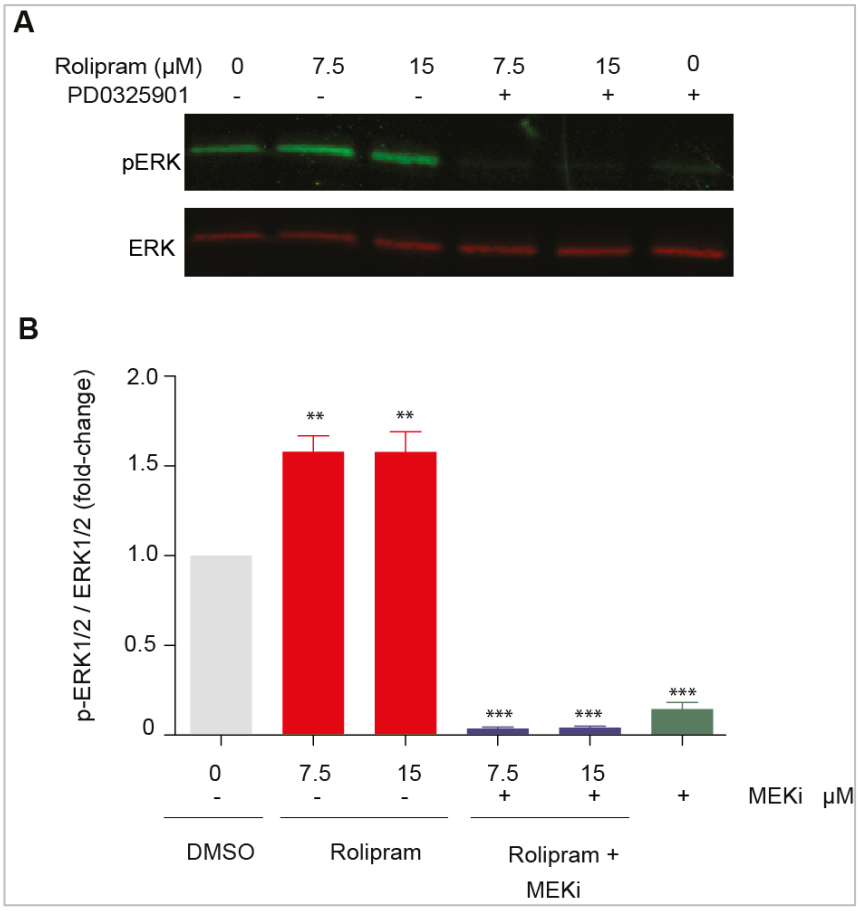

Figure 4.3.9: Rolipram treatment increases pERK in whole embryos.

A: Western blot analysis using anti-pERK (Green) (anti-phosphor p42/44, Cell Signalling Technology, 1:2000) and total ERK (red, anti-p42/44, Cell signalling Technology,1:1000). B: Quantification of protein levels; pERK levels normalized to the total ERK levels. Quantification was done using the LiCor Image Suite. Data analysed using One-Way ANOVA, followed by Tukey's Post-test. $P<0.05$. (** = very significant, *** = highly significant.

4.4 Discussion

The work presented in this chapter illustrates the use of zebrafish larvae to study the effect of PDE inhibition and cAMP manipulation on the swimming behaviour in 5 dpf zebrafish larvae. The results in this chapter established that compounds increasing cAMP levels in zebrafish larvae also induce locomotor hyperactivity, specifically through PDE4 inhibition but not through the inhibition of other PDE proteins. This hyperactivity is reversible by treatment with the MEK-inhibitor, PD0325901, which also results in decreased pERK expression, but not cAMP levels in the larvae. This suggests a cross-talk between the cAMP and MAPK pathway in regulating behaviour in zebrafish larvae. In particular, the PDE4-family appears to be of importance for this link.

4.4.1 Rolipram increases cAMP in zebrafish larvae

Larvae treated with Rolipram and forskolin have increased levels of cAMP, which correlates with previous *in vivo* and *in vitro* studies (Andersen 2002; Lelkes et al. 1998). Forskolin treatment increases activity of adenylate cyclase, and thereby directly stimulate cAMP-synthesis (Alewijnse et al. 1997), whereas Rolipram inhibits cAMP hydrolysis (Beavo, Francis, and Houslay 2006). (Miles D. Houslay, Baillie, and Maurice 2007). Increased cAMP levels are associated with improved cognition (Wiescholleck and Manahan-Vaughan 2012), mood regulation (Landgraf, McCarthy, and Welsh 2014), anti-depressive effects (Lelkes et al. 1998) and neurogenesis (Y.-F. Li et al. 2011), thus demonstrating the importance of this second

messenger in the nervous system. The data presented in this chapter shows that increased cAMP is involved in the modulation of hyperactivity and anxiety in zebrafish larvae.

4.4.2 PDE4 inhibition causes hyperactivity in zebrafish larvae

The phosphodiesterase 4 inhibitor, Rolipram, was developed as a mood stabilizer but was withdrawn due to adverse effects, like emesis in patients (Zhu, Mix, and Winblad 2001). It is still extensively used as a tool compound in research, due to its strong specificity for PDE4-enzymes (Cherry, Thompson, and Pho 2001; Dlaboga, Hajjhussein, and O'Donnell 2006; Y.-F. Li et al. 2009b). In rodent behavioural models, Rolipram causes antidepressant-like effects, with decreased immobility in the forced swim test and the tail suspension test . Furthermore, cognitive tests indicate that Rolipram improves spatial learning and cognition (Y.-F. Li et al. 2009b). As Rolipram targets all four isoforms or PDE4-proteins, it has been difficult to determine the biological role of the different isoforms. The generation of knockout mouse models lacking specific *PDE4*-genes, has helped to elucidate on the roles of the different member of genes in the family. *PDE4A and PDE4B*-knockout mice display anxiogenic-like behaviours (R. T. Hansen, Conti, and Zhang 2014; Zhang et al. 2008), whereas *PDE4D*-knockout mice show an anti-depressant phenotype (Zhang et al. 2002b).

My studies inducate , that in zebrafish larvae Rolipram cause behavioural phenotypes relating to an anxiogenic-like phenotype. The fact that PDE4 inhibition causes anxiety-like behaviours in zebrafish larvae, could indicate that at this stage of

development *Pde4a and Pde4b* are more highly expressed or more important in regulating behaviour in zebrafish. This is further supported by the fact, that a subset of larvae show an increased response to Rolipram, which could be attributed to differences in expression levels of different Pde4s. *pde4b*, resulting in a increased anxiogenic effect of Rolipram (Figure 4.3.6). The importance of *pde4*-function in mediating zebrafish larval behaviour was further supported by the data from the 11 PDE-inhibitors, where it was demonstrated that inhibition of the Pde4 proteins caused hyperactivity in zebrafish larvae. Of the 11 PDE-inhibitors tested, three specifically targeted the PDE4-family. One was Rolipram and one was a second pan-PDE4 inhibitor. Interestingly, the 611-compound is designed to be a specific Pde4d-inhibitor, as the structure is modelled on the small PDE4D-allosteric modulator described previously (Burgin et al. 2010). This compound also caused an increase in hyperactivity in zebrafish larvae. Further studies need to be performed to further substansiate these findings. However, from rodent models, it has been shown that pde4d-knockout mice have an anti-depressive behaviour, and are in-sensitive to the anti-depressive effects of Rolipram. This indicates, that Pde4d is important in the anti-depressant effects of Rolipram in zebrafish.

A PDE2/10 inhibitor (#446) was identified in this screen to also have an effect on the swimming activity of zebrafish larvae, whereas other PDE2 and 10 inhibitors tested (compound #434 and #450) had no effect. Moreover, two compounds targeting either PDE2 (#432) or PDE10 (#455) had not effect on swimming activity. These data indicate that the effect observed in larvae treated with the #446 compound could be attributed to off-targets effects. PDE2 and PDE10 are dual-substrate

phosphodiesterase. They hydrolyse both cAMP and cGMP (Beavo, Francis, and Houslay 2006), and are highly expressed in the brain. It remains to be determined whether PDE2 and PDE10 are important for this behaviour in zebrafish, but it is noteworthy that all the PDE4-selective compounds in this screen significantly modulated hyperactivity in zebrafish larvae indicating that PDE4 is an important regulator of activity in zebrafish.

4.4.3 Cyclic AMP and MAPK cross talk in zebrafish behaviour

Treatment with PD0325901 reversed the hyperactivity in larvae treated with Rolipram. Increased cAMP activates PKA, and this in turn can activate the MAPK pathway through B-raf activation (Erhardt et al. 1995; Vogt Weisenhorn et al. 2001). B-raf depletion in juvenile mouse brains cause an anxiolytic behaviour, evident on the molecular level as decreased pERK levels (Wefers et al. 2012) whereas depletion during adulthood leads to depressive behaviour in adult mice (Wefers et al. 2012). The involvement of MAPK in depression has also been demonstrated by Einat and colleagues. They treated mice with a MEK inhibitor, SL327, and subsequently tested their performance in the forced swim test, and found that mice treated with SL327 showed an increase in total swim activity and decrease in total immobility time, indicative of a less depressive behaviour (Einat et al. 2003). To further substantiate these findings, they stained for pERK in brain slices from rats subjected to condioned fear. The rats have a significantly higher expression of pERK in the amygdala, an

area of the brain known to be involved in the regulation of fear and anxiety. (Einat et al. 2003).

In the Rolipram-treated larvae, the PD0325901 treatment did not decrease the increase in cAMP levels, but it did reverse the hyperactivity of the larvae, and also the levels of pERK. It is known that pERK is involved in the regulation of PDE4-activity (M D Houslay and Baillie 2003; Waltereit and Weller 2003). In cardiac muscle cells, a signalling complex consisting of the A-Kinase Anchoring Protein (AKAP), PKA and long-form PDE4D3. This complex recruits ERK5 to the signaling module, and phosphorylated ERK5 inactivates long-form PDE4D3, which leads to increaed cAMP levels. This in turn activates PKA, leading to a second phosphorylation of long-form PDE4D3 on a different residue, resulting in an activation of PDE4D3, and hence hydrolysis of cAMP (Reviewed in Jarnaess and Taskén 2007). This indicates that increased activity of MAPK in the larvae, are important in regulating the hyperactivity.

In fear conditioned rats, pERK levels are increased ant they rats display an anxiogenic phenotype in the elevated plus maze (Ailing et al. 2008). Subsequent treatment of the fear-conditioned rats with a MEK inhibitor, PD09859, leads to an abolishment of the anxiogenic behavior, indicated by a higher degree of exploratory nature, the amount of time spent in the open arms and increased number of rearings. This performance correlated with a decreased pERK expression, thus indicating the inhibition of the MAPK-pathway can lead to anxiolytic effects (Ailing et al. 2008). In zebrafish, the regulation of cAMP through PDE4-inhibition leads to hyperactivity

and thigmotaxis. By using the zebrafish as a model system, I have shown that the cross-talk between cAMP and MAPK modulates zebrafish hyperactivity.

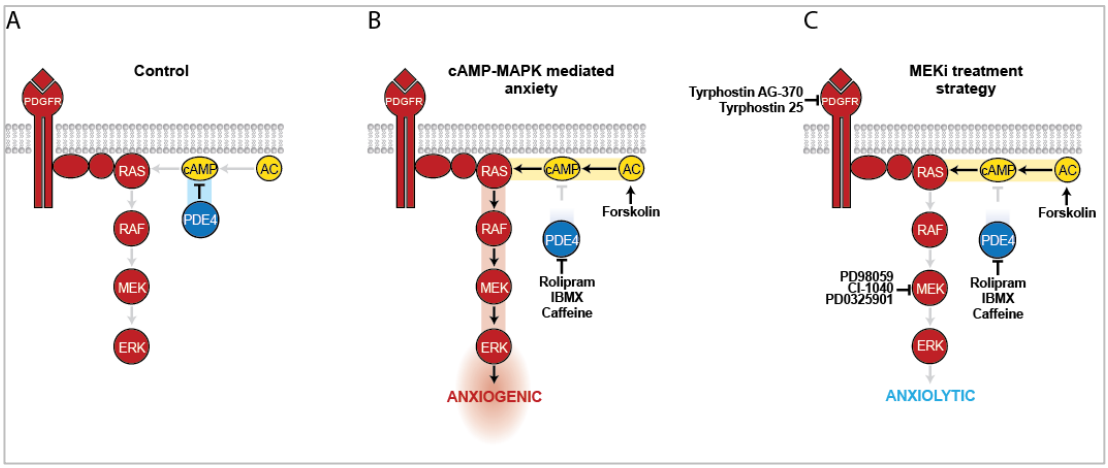

Figure 4.4.1: A novel therapeutic strategy: MEK inhibitors to treat cAMP-mediated anxiety.

A: In control fish, PDE4 is a negative regulator of cAMP activity. This regulation is obtained through hydrolysis of the second messenger. B: In zebrafish, blocking PDE4 or activating AC with small molecules leads to increased cAMP *in vivo*. This cause an activation of the MAPK signalling pathway, leading to an anxiogenic response in zebrafish. C: The activation of the MAPK signalling pathway obtained through increase cAMP levels in vivo can be perturbed by blocking the MAPK-signalling pathway at different levels. Either through direct blockage of the receptor or by blocking the downstream signalling pathway using specific inhibitors, inhibiting ERK or MEK.

4.4.4 Concluding remarks

During this study I found that inhibitors of PDE4-activity play an important role in regulating anxiogenic-like behaviour in zebrafish larvae. Using small molecules in combination with automated tracking equipment, I have shown that zebrafish larvae become hyperactive in darkness and light, when treated with cAMP modulators. This

anxiogenic-like behaviour is dependent on MAPK signalling, as cAMP modulators cause increase in pERK levels in 5 dpf old larvae, and subsequently treatment with a specific MEK inhibitor, PD0325901, leads to alleviation of this anxiogenic phenotype. These data suggests that by using the zebrafish embryo model, it is possible to elucidate on the cross-talk between cAMP and MAPK pathways in regulation of behaviour in zebrafish (Figure 4.4.1).

5 Modulation of behaviour in adult zebrafish through PDE4 inhibition

5.1 Introduction

The use of adult zebrafish in behavioural research has expanded over the recent years. In particular the modulation of behaviour using known anxiolytic or anxiogenic compounds have been well described (Egan et al. 2009; Levin, Bencan, and Cerutti 2007; Bencan, Sledge, and Levin 2009; Maximino, da Silva, et al. 2011). Work in our lab (Figure 4.1.1) and others (Richendrfer et al. 2012; Schnörr et al. 2012) have shown that modulation of the cAMP pathway can induce thigmotaxis is larvae zebrafish. Moreover, five day old zebrafish larvae treated with PDE4-inhibitors show an increase in swimming activity both in the light and in the dark (see 4.4.2). I found that this hyperactivity can be reversed by treating the larvae with a specific MEK inhibitor, PD0325901. The nervous system of the larvae at 5 dpf is less complex than that of adults, and adult fish have a more elaborate and developed behavioural repertoire (R. E. Blaser, Chadwick, and McGinnis 2010; Collier and Echevarria 2013). To gain insight into how Rolipram affects adult zebrafish behaviour and the cross talk between cAMP and MAPK in regulating behaviour, adult behavioural assays were performed. The novel tank test and the shoaling assay have both been validated as means of studying anxiety and stress in adult zebrafish (Speedie and Gerlai 2008; Stewart et al. 2011), therefore these two assays were used.

5.1.1 The shoaling assay

Zebrafish are social animals, that live in groups, and have an innate shoaling behaviour, that develops with age (C Buske and Gerlai 2011). The organization and tightness of the shoal changes in response to external stimuli, like to presence of a

predator (Speedie and Gerlai 2008), the addition of alarm pheromones to the water (Speedie and Gerlai 2008) and psychoactive drugs (Green et al. 2012).

The shoaling assay has been suggested as a potential assay to model social withdrawal, aggression and depression. Modulation of glutamate signalling by treating fish with MK-801, a NMDA-receptor antagonist, disrupts the shoal in zebrafish. Fish treated with MK-801, a NMDA receptor antagonist, show an disruption in shoaling behaviour, displayed by a larger distance between individual subjects, which has been interpreted as a display of anti-social or depressive behaviour (Maaswinkel, Zhu, and Weng 2013). An alternative version of the shoaling assay is the group performance task (Gebauer et al. 2011). This assay measures both the shoal cohesion but also the placement in the tank. In this assay, buspirone causes the fish to explore the top of the tank, but with no effect on shoal cohesion (Gebauer et al. 2011).

5.1.2 The novel tank assay

The novel tank test is one of the most widely used behavioural tests when analysing adult zebrafish behaviour (Egan et al. 2009; Levin, Bencan, and Cerutti 2007; Rachel E. Blaser and Rosemberg 2012). Like rodents, when introduced into a novel environment, fish also show an initial lack of exploration. They tend to stay close to the walls and bottom of the tank, when they are initially introduced into the tank. The bottom dwelling behaviour is sometimes referred to as geotaxis. After a habituation period the fish become more exploratory and venture up into the top of the tank (Levin, Bencan, and Cerutti 2007). Parameters measured in this test are the amounts

of time spend at the bottom of the tank, how long and how often, the fish is in the top zone of the tank, frequency of erratic movements and freezing behaviours. These parameters have all been shown to be modulated by treatment with either anxiolytic or anxiogenic compounds (Egan et al. 2009). Chronic treatment with fluoxetine, commonly known as Prozac ®, produces anxiolytic-like behaviours in adult zebrafish, when administered for two weeks. The fish showed a higher degree of exploration to the top zone of the novel tank and less erratic behaviours and freezing incidents. Furthermore, the level of whole body cortisol is significantly reduced in the fish. These data all suggests that the adult zebrafish model is suitable for studying anxiety-like behaviours.

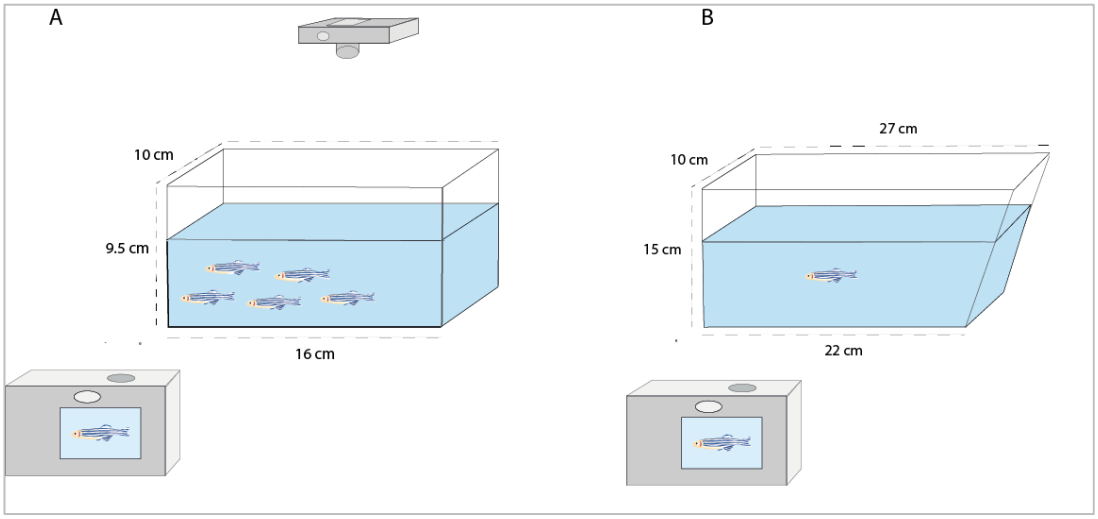

Figure 5.1.1: Schematic of the shoaling assay and the novel tank assay.

A: A schematic representation of the shoaling assay/group behaviour task. B: Schematic representation of the novel tank assay setup.

5.2 Aim of chapter

The aim of this chapter was to establish two assays to evaluate anxiety-like behaviours in adult zebrafish. After implementation, the behavioural assays will be used to evaluate the effect of PDE4-inhibition on adult zebrafish.

5.3 Results

5.3.1 Setting up the novel tank assay

In the novel tank assay, I treated single fish separately with compound or vehicle for 20 minutes prior to testing in the novel tank. After treatment the fish were carefully transferred to the novel tank and the behaviour was recorded from the side for 6 min (Figure 5.2.1). This amount of time has been shown by other to be sufficient to allow the fish to habituate to the novel tank and slowly begin exploring the top of the tank as well as the bottom (Egan et al. 2009). As the data from the larvae experiments (see 4.3.1) indicate that Rolipram causes anxiety-like behaviour, e.g. thigmotaxis and hyper activity, I wanted to test the effect of Rolipram on adult fish in the novel tank test. Before testing Rolipram, a test routine for the novel tank test was first established using a compound with a known anxiolytic effect. This enabled me to distinguish between normal behaviour and anxiolytic behaviour of the fish in the novel tank test.

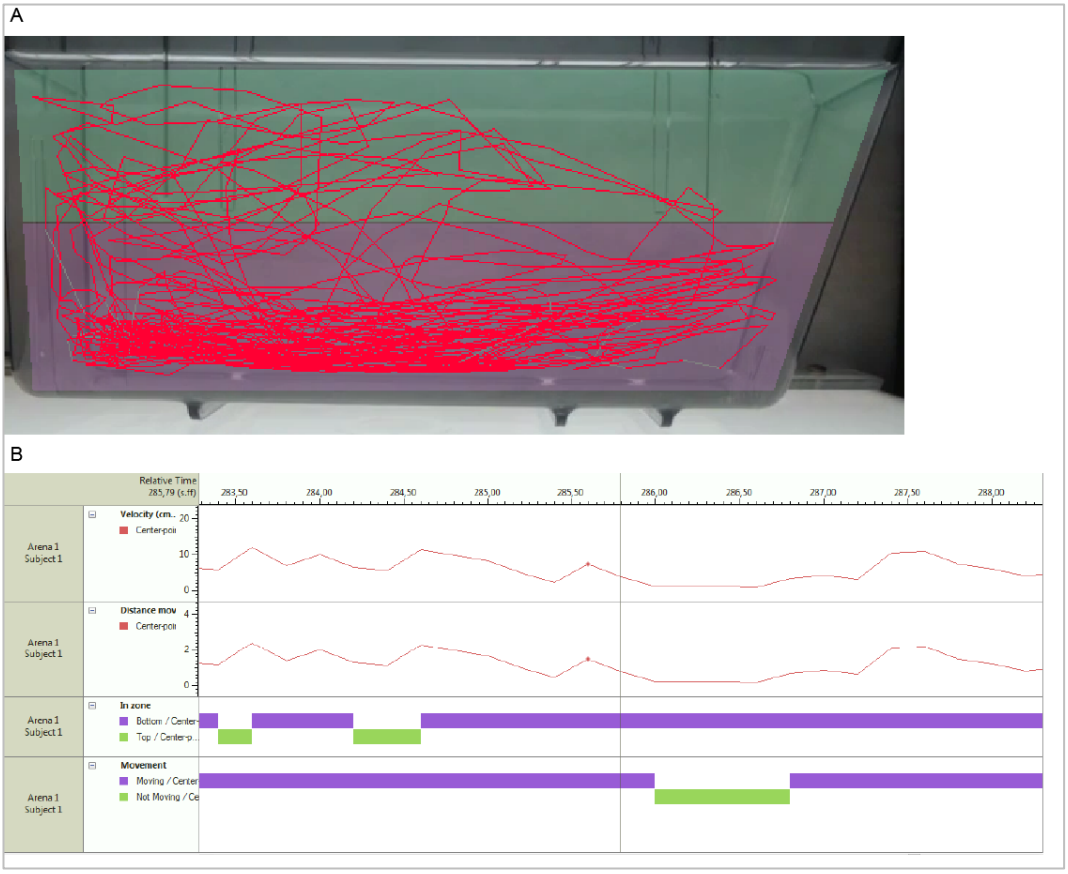

Figure 5.3.1: Visualisation of the EthoVision output from a novel tank test.

A: Traces of the swimming pattern of an adult fish tracked for 5 minutes in the novel tank test. The purple area is the bottom zone and the green is the top zone. B: Screenshot of the integrated tracking visualization of the i: swimming distance, ii: velocity, iii: zone placement and iv: movement status for a 4 second time interval. The purple bar is when the fish is active in the bottom zone and the green bar is the top zone of the tank.

5.3.1.1 Buspirone causes an anxiolytic effect on zebrafish

Buspirone is a specific serotonin 5-HT1a partial agonist, with known anxiolytic properties that has been shown to induce anxiolytic behaviour in zebrafish models in the novel tank diving test (Bencan, Sledge, and Levin 2009; Gebauer et al. 2011).

Previous reports have described several behavioural parameters as a measure of anxiety levels in adult fish tested in the novel tank test. These include time spent at the top of the tank, latency to enter the top zone, total distance moved, frequency of erratic behaviours and time spent immobile (referred to as freezing) (Egan et al. 2009; Rachel E. Blaser and Rosemberg 2012; Gebauer et al. 2011). The behaviour of adult fish treated with 0.001% DMSO, as a control, was compared to that of fish treated with 10µM buspirone, with a final DMSO content of 0.001%. The fish treated with 10µM buspirone for 20 minutes spend a significantly higher amount of time at the top of the tank, compared to untreated fish, and the latency to move into the top zone was significantly lower in the buspirone treated fish compared to untreated fish (Figure 5.3.2 A&B). Adult fish treated with 10µM of buspirone for 20 minutes showed a higher degree of top-zone entries in the novel tank test than fish treated with DMSO only, although this was not significant (Figure 5.3.2 C). There was no effect on the distance travelled between the two treatment groups, and although buspirone-treated fish showed a lower amount of erratic episodes, this was not significant. The same was true for the freezing behaviours (Figure 5.3.2 E&F). These experiments allowed me to establish the novel tank assay.

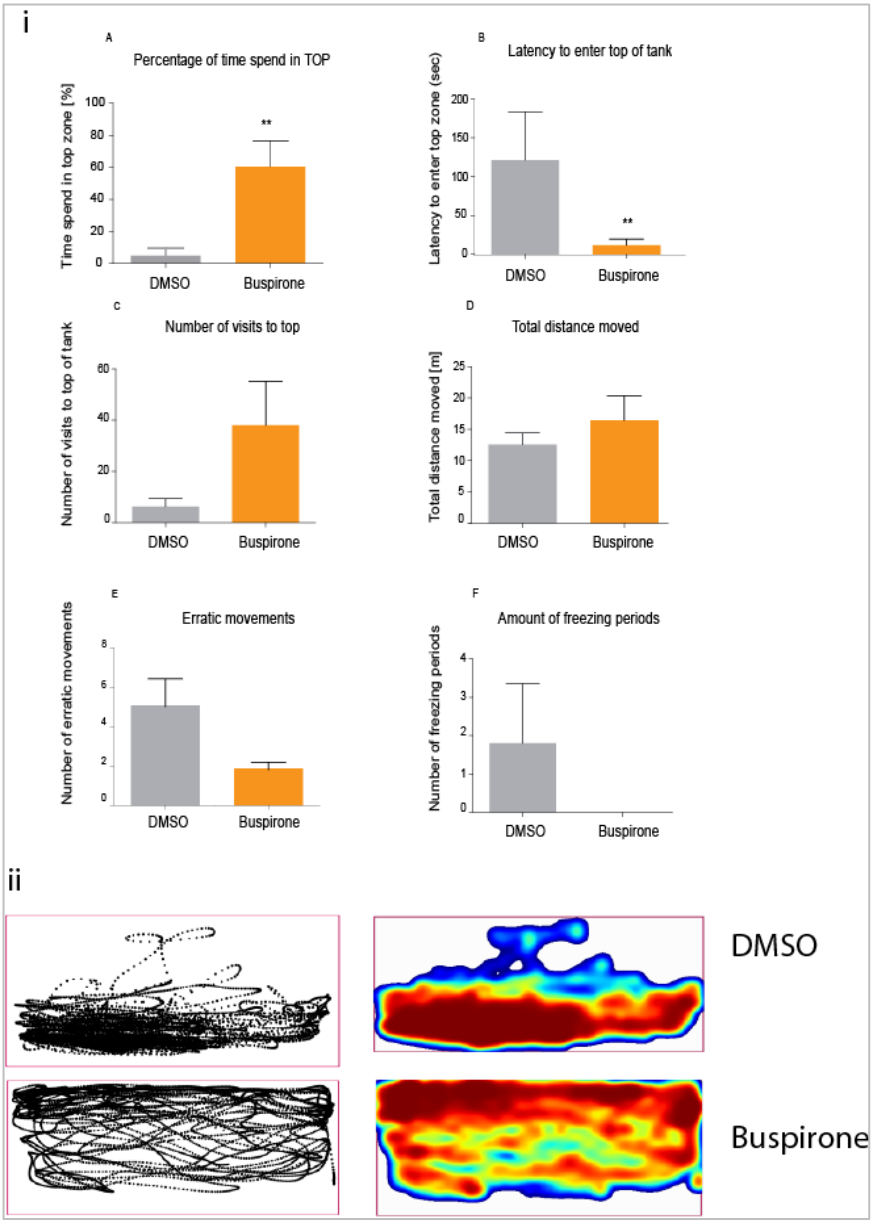

Figure 5.3.2: Buspirone causes significant increases in time spend in the top of the novel tank.

i: (A) Adult zebrafish treated with buspirone show an increase in the time spend at the top of the tank ($p<0.01$), and also a latency to enter the top of the tank ($p<0.05$) (B). There was no significant difference between the erratic movements (E) or the freezing behaviours (F) displayed by fish treated with buspirone. The experiment was repeated 2 times, with 5 fish per treatment group. All trials were 6 minutes in total, and the data was analysed using an unpaired t-test. ii: tracking plots and heat maps from the Actual Track software (Actual Analytics Ltd, UK).

5.3.2 Rolipram and its effect on the behaviour of adult zebrafish

Modulation of cAMP levels through PDE4 inhibition and adenylate cyclase activation has been shown to increase thigmotaxis and locomotor activity in zebrafish larvae (see Chapter 4). Larvae treated with caffeine, a weak PDE-inhibitor, has been shown by our lab (Figure 4.1.1) and others (Richendrfer et al. 2012) to display increased thigmotaxis in an open field test, and increased latency of larvae to enter the dark compartment in the light-dark test (Steenbergen, Richardson, and Champagne 2011b). Adult fish treated with caffeine display increased erratic behaviours, freezing and latency to enter the top zone of the tank of the novel tank (Egan et al. 2009). To test if Rolipram treatment causes similar effects in adult fish, I treated adult zebrafish with Rolipram, and implemented two different behavioural assays, the novel tank test and the shoaling assay.

5.3.2.1 Anxiogenic effect of Rolipram in the novel tank diving test

Adult zebrafish were treated with 30µM of Rolipram for 20 min and transferred to a novel tank. The activity and movement pattern of the fish was recorded from the side in a 6 minute trial (Egan et al. 2009). Movies were subsequently analysed using EthoVision XT9 (Noldus Information Technology, The Netherlands). Zebrafish treated with 30µM Rolipram showed a significant increase in the time spend at the bottom of the tank ($p=0.05$, $n=5$), and also a significantly higher number of erratic movements. There was however, no difference in the distance moved by Rolipram

treated fish or the velocity, indicating that the overall locomotor activity was not affected in fish treated with Rolipram (Figure 5.3.3).

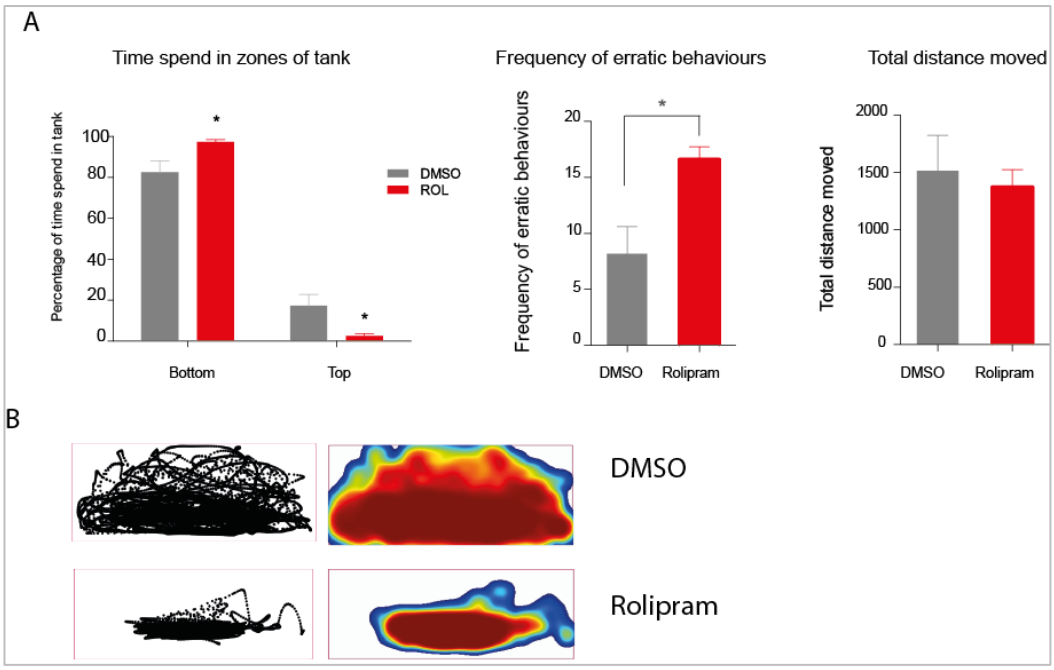

Figure 5.3.3: Behavioural analysis of the Rolipram effect on adult zebrafish.

A: Bar graph showing the behavioural data from a novel tank test. Rolipram increase time spend in the bottom zone. Zebrafish showed behaviours previously reported to be related with anxiety. A significant increase in time spend at the bottom of the tank ($p<0.05$) and the frequency of erratic behaviours ($p<0.05$) were observed in fish treated with Rolipram. Groups of 5 fish were treated with DMSO or Rolipram, 2 experimental repeats. B: Birds nest and heat maps of representative experiment of one fish treated with 0.001% DMSO (top) and one fish treated with 30µM Rolipram (bottom).

5.3.3 Inhibiting MEK activity does not cause anxiolytic behaviour in adult fish

The effect of buspirone on adult zebrafish is very pronounced, causing the fish to spend more time at the top of the tank and less time performing erratic movements and freezing bouts (Figure 5.3.2). The MAPK pathway has been implicated in anxiety and depression in rodents (Coyner et al. 2014; Wefers et al. 2012). In larval zebrafish our lab has shown that treatment with a MEK-inhibitor alleviates thigmotaxis (Figure 4.1.1) and hyper activity (Figure 4.3.6). Thus inhibiting MEK causes an anxiolytic effect. To test if inhibiting MEK in adult fish causes an anxiolytic response in the novel tank test, I treated adult fish with 3µM PD0325901 and analysed their response to introducing them into a novel environment in the novel tank assay. The treatment with PD0325901 did not result in a significant difference in anxiolytic behaviours compared to that of DMSO-treated fish (Figure 5.3.4). There was no observed difference in the time spend in the top zone of the tank, latency to enter or erratic behaviours. The freezing behaviour is the one parameter with a clear difference. However, the freezing behaviour was only displayed by one DMSO-treated fish, thereby skewing the data (Figure 5.3.2). Therefore it appears that inhibition of MEK does not cause an effect of behaviour on its own.

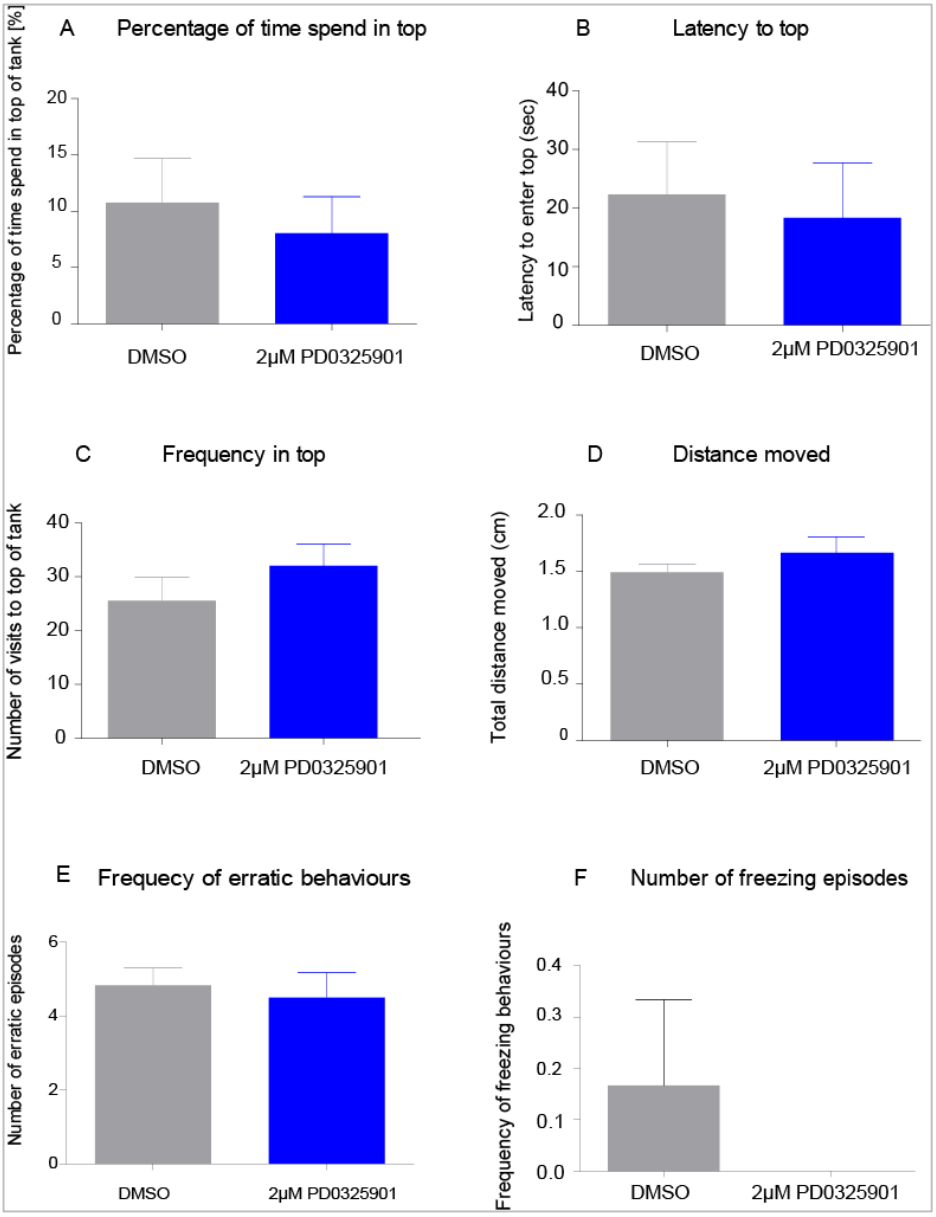

Figure 5.3.4 PD0325901 treatment does not cause an anxiolytic phenotype in the novel tank.

Adult zebrafish treated with 3µM PD0325901 for 20 min prior to testing the in the novel tank assay. The only behavioural parameter with a distinct difference between the two treatments was the frequency of freezing episodes. Only one adult fish (DMSO treated) showed a freezing period (7 seconds). None of the other fish had any freezing episodes (5 fish per treatment group, repeated 2 times). All data were analysed using an unpaired student's t-test.

5.3.3.1 Rolipram causes bottom dwelling in groups of adult fish

When fish treated with Rolipram are transferred to a novel tank, they display anxiety-like behaviours (Figure 5.3.3), thus treatment with Rolipram cause a stress or anxiety-like response in both larvae and adult zebrafish. To further establish the effect of Rolipram in adult zebrafish, I used a second behavioural assay, the shoaling assay. Shoaling is an innate behaviour in adult fish, and this behaviour has been demonstrated as another parameter that is modulated in zebrafish in response to anxiety and fear in adult fish (Green et al. 2012; Christine Buske and Gerlai 2011). The response to alarm pheromones or the presence of a predator leads to a change in the placement in the tank, causing the fish to spend more time at the bottom of the tank, and in a tighter shoal (C Buske and Gerlai 2011; Green et al. 2012). As PD0325901 can reverse the Rolipram induced hyperactivity, suggesting the importance of MAPK signalling in mediating the anxiety-like response in zebrafish larvae, I wanted to test if this regulating was conserved in adult zebrafish. I therefore tested if a potential phenotype can be reversed by co-treating the adult fish Rolipram and PD0325901.

The fish were incubated in a 1.5L tank with either DMSO, 30µM Rolipram, 3µM PD0325901or 30µM Rolipram + 3µM PD0325901 for 20 minutes. After incubation the fish were filmed for 5 minutes from above, to measure shoaling density, and from the side to evaluate the placement in the tank. I found that fish treated with Rolipram spend significantly higher percentage of their time at the bottom of the tank, with very low level of exploration to upper half of the tank (Figure 5.3.5). This bottom-

dwelling was not observed in the fish with DMSO or PD0325901 alone and thus similar to the findings from the novel tank assay. When the fish were treated with a combination of Rolipram and PD0325901, the bottom dwelling was significantly different from the Rolipram only fish, suggesting that the importance of MAPK signalling in regulating anxiety-like behaviours due to PDE4 inhibition is conserved in adult zebrafish. Interestingly, there was no effect on the shoal cohesion between the fish in any of the treatment groups.

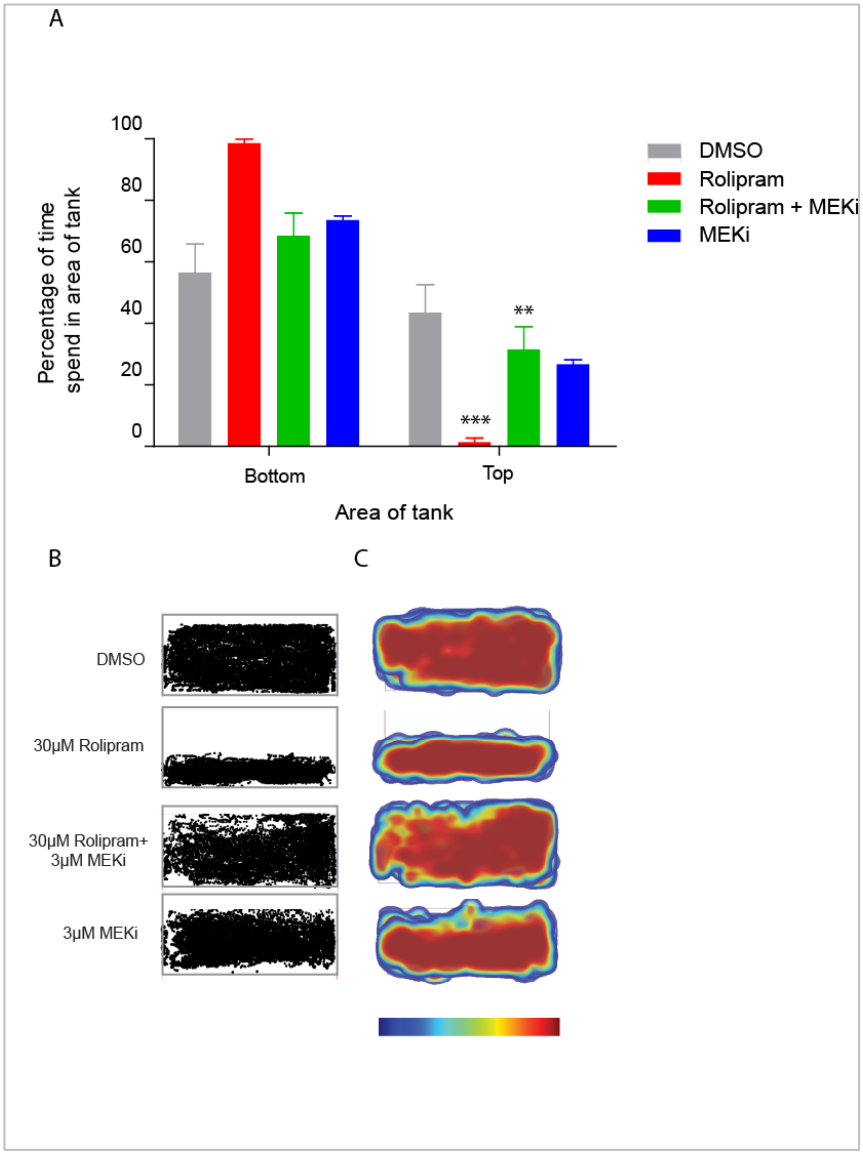

Figure 5.3.5: Placement in tank in the shoaling assay after Rolipram and PD0325901 treatment.

A: Bar graphs showing the percentage of time spend in the top or bottom of the shoaling tank, 5 fish per group. Data are presented as mean +/- SEM (experimental replicates n= 5 per treatment group). Data were analysed a Two-Way ANOVA followed by a Bonferroni's posthoc test. Rolipram treated fish spend significantly more time at the bottom of the tank ($p<0.001$) and this is reversed by co-treatment with 3µM PD0325901 ($p<0.01$). There was no effect of PD0325901 alone ($p>0.05$). B) Representative tracking plots of the swimming pattern of a group of 5 adult zebrafish from each of the 4 treatment regimes. C: Activity heat maps corresponding to the relative time the fish spent in the areas of the tank during the 5 min trial. The heat maps are 2D Kernel Density Estimates, with a custom colour map to enhance visibility; Clear/yellow = none/minimal, blue = low, red =high.

5.3.4 Increased whole-body cortisol levels in adult zebrafish in response to Rolipram treatment

The endocrine response to stress and anxiety in mammals are regulated by the HPA axis. In humans and zebrafish the stress hormone released during anxiety and stress is cortisol, and increased whole body levels of this hormone during stress or anxiety in adult zebrafish have been demonstrated previously (Barcellos et al. 2007; Egan et al. 2009). To determine if fish treated with Rolipram have increased cortisol levels, groups of five fish from a shoaling assay were snap frozen, and cortisol was extracted, using a modified version of a previously described method (Cachat et al. 2010; Ramsay et al. 2006) and cortisol levels were determined using an enzyme immunoassay kit (Salimetrics Europe Ltd, UK). Fish treated with Rolipram had a significantly higher level of cortisol (mean=6.749, SEM +/- 1.106) compared to fish treated with DMSO (Mean=1.662, SEM +/- 0.2965) (Figure 5.3.6).

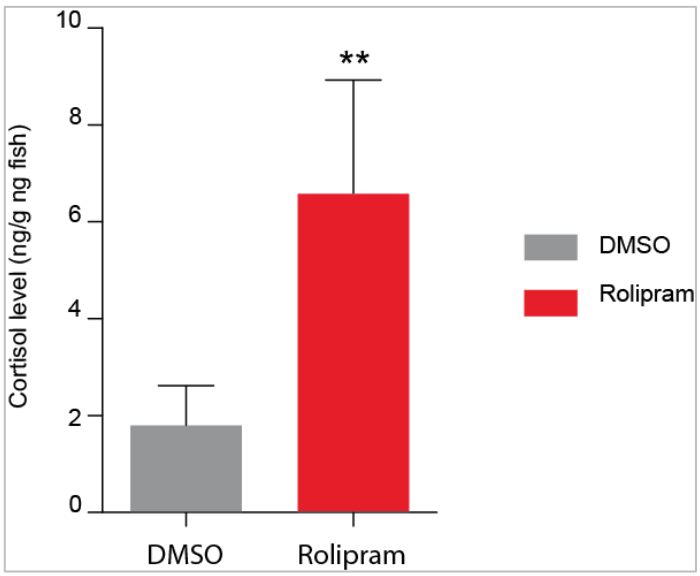

Figure 5.3.6: Increased cortisol levels in adult zebrafish treated with 30µM Rolipram.

Cortisol levels in fish treated with 30µM Rolipram or 0.001% DMSO (20 minutes) was measured. A significantly higher level of cortisol was detected in fish treated with Rolipram (Students t-test; p=0.0113). The experiment was repeated 3 times in total, with 5 fish in each treatment group per experiment.

5.3.5 Genetic modulation of Pde4d activity

The next series of experiments were to examine whether phenotypes observed through pharmacological modulation of Pde4-activity are recapitulated in a genetic model of *pde4d*-loss of function. From the Zebrafish Mutation Project (The Sanger institute, Cambridge, UK) we obtained a zebrafish mutant line with a nonsense point mutation (A to T) located at the beginning of the PDEase domain of the Pde4d-gene in the zebrafish. The nonsense mutation causes the conversion of a lysine residue to a Stop codon, with the predicted result being a loss-of-function. From rodent studies using *pde4d-/-* mice, it has been shown that homozygous knockout mice show a

decrease in immobile periods in the forced swim test and the tail suspension test, two tests commonly used to assess depression-like phenotypes in rodents (Zhang et al. 2002a). To determine if any of the phenotypes caused by Rolipram in the adult fish was recapitulated in the *Pde4d-mutants* I did a novel tank assay and a shoaling assay on adult *Pde4d-/-* and their wild-type siblings.

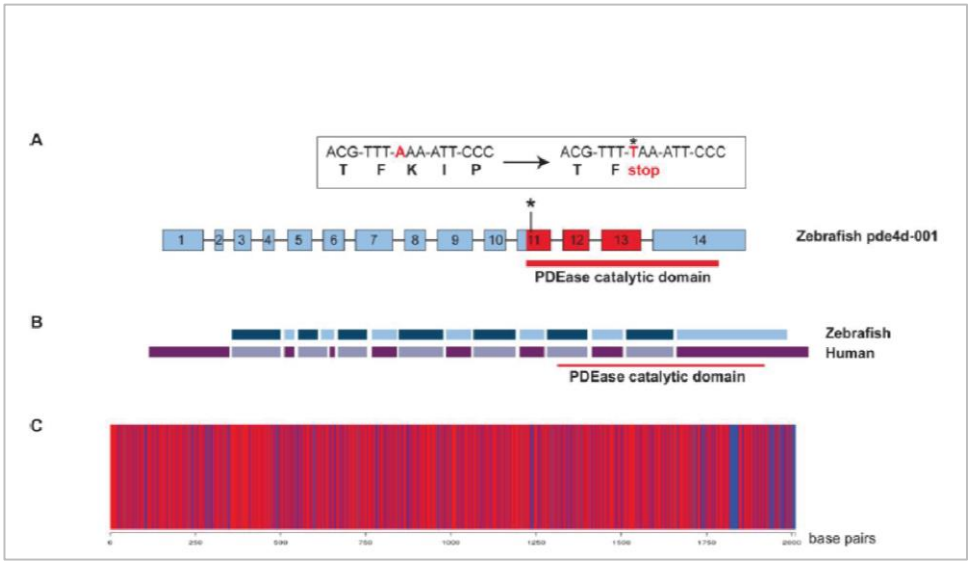

Figure 5.3.7: The PDE4D gene in humans and zebrafish

A: A schematic representation of the zebrafish transcript, pde4d-001, and the point of mutation in the pde4d-/- fish from the Sanger Institute. B: The overlap between the exon-homology between zebrafish and human PDE4D. The exons are aligned according to homology. The exons are coloured with different colours for the ease of comparison, with alternating exons in alternating dark and light colour. The dark and light blue are the zebrafish gene structure and the dark and light purple are the human gene structure. C: Sequence alignment between zebrafish and human cDNA sequences of PDE4D. The red lines represent sequence identity, blue lines mismatching sequences. There is an overall 90.5 % sequence identity (*From K. Anastasaki, PhD Thesis, 2011*).

5.3.5.1 Pde4d-loss of function cause an decrease in shoaling behaviour

Heterozygous pde4d-mutants from the Sanger institute were outcrossed and the progeny were genotyped. Heterozygous mutants were kept and in-crossed, progeny were genotyped and wild-type, heterozygous and homozygous mutants were raised in the facility. The siblings were out-crossed and progeny were genotyped. This was repeated until a F4 generation of wild-type, heterozygous and homozygous siblings were obtained. These fish were used for testing in the behavioural assays. To test for shoaling differences in the mutants compared to wild-type siblings, the behaviour of the fish were recorded from the side and the top, in the shoaling assay. The behaviour of the fish were analysed using Actual Track (Actual analytics Ltd, UK). The data analysis showed that *pde4d*-/- have a decreased shoaling behaviour, evident as a larger distance between individual fish in the group compared to that of wild-type siblings (Figure 5.3.8). There was also a significant difference between the time spend at the top of the tank, compared to that of the wild-type-siblings. The latency to enter the top tank was also decreased (Figure 5.3.8).

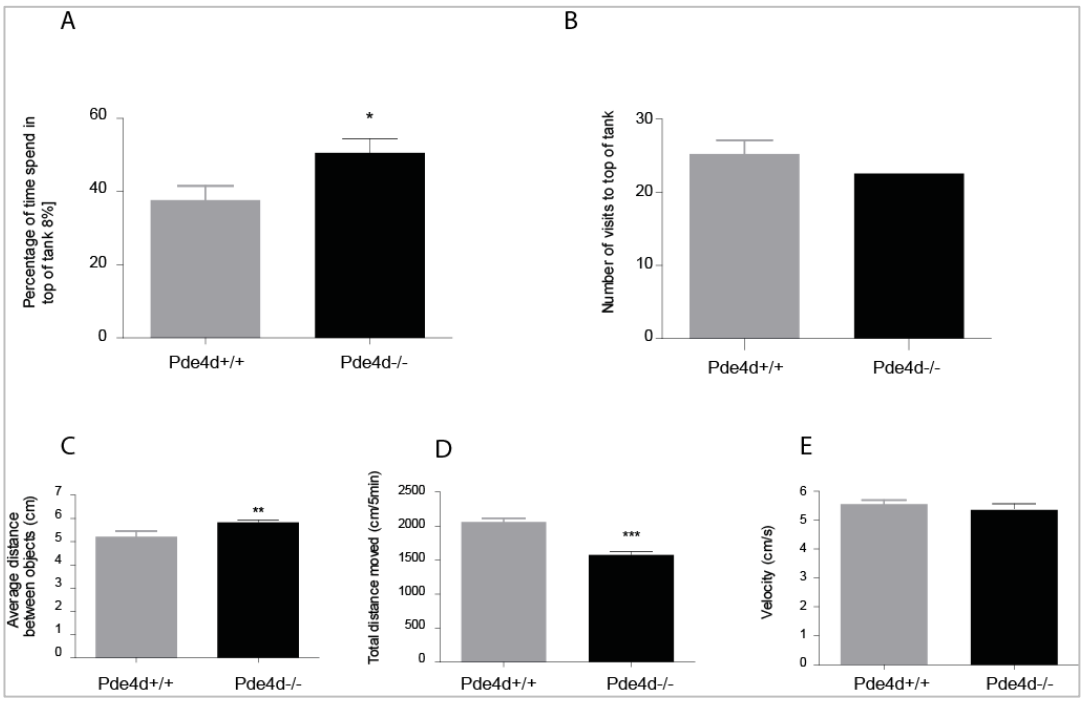

Figure 5.3.8 Adult pde4d-/- zebrafish have a decreased shoaling behaviour.

Bar graphs depicting the data from a shoaling assay from groups of 4 fish (n=4 per genotype). The fish were tracked in the shoaling assay, and data was analysed in using an independent-samples t-test to determine if there was a significant difference in the behaviours in the shoaling assay. **A&B** display data analysed from the side and **C-E** display data from the top. Groups of 4 fish were tracked and a total of 16 fish were analysed from each genotype.

5.3.5.2 A specific pde4d-inhibitor partly recapitulates the phenotype of the pde4d-mutants

From the PDE-inhibitor screen on larval zebrafish I identified a PDE4D-inhibitor that caused a significant increase in swimming activity of the zebrafish larvae. This inhibitor is designed as a specific PDE4D-inhibitor, originally published by Burgin and colleagues in 2010 (Burgin et al. 2010). This small allosteric inhibitor binds to the catalytic site of PDE4D-protein, thereby inhibiting its binding to cAMP. To test if the behavioural phenotype of observed in the *pde4d*-mutants is recapitulated by the PDE4D-specific inhibitor, I did a shoaling assay using groups of 4 fish, treated with 3µM of the PDE4D inhibitor (#611) or 0.001% DMSO. After 20 minutes of incubation, the behaviour of the fish was recorded from the side and top. The fish treated with the PDE4D-inhibitor showed a significant decrease in shoal cohesion, compared to that of DMSO treated fish, and also a significant increase in velocity (Figure 5.3.9)

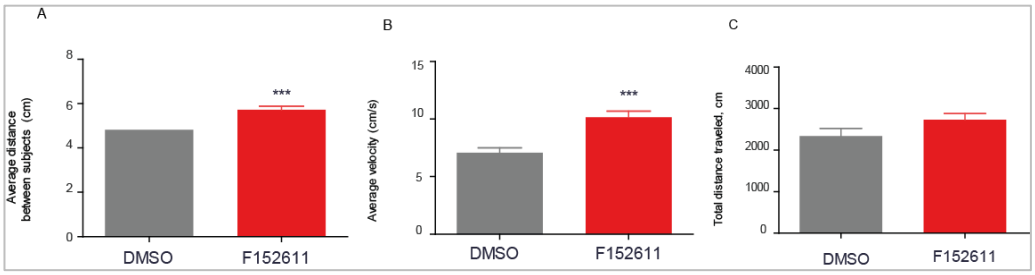

Figure 5.3.9: F152611 increases the shoaling distance in wild-type fish.

Bar graphs showing the data from the shoaling assay with adult fish treated with 3µM F152611. A) F152611 significantly increases the distance between the fish in the shoal ($p<0.0001$), and also cause an increase in velocity of the fish (B) ($p=0.001$). There was no difference in the distance travelled ($p=0.1162$). Data analysis is done using a Student's T-test.

5.4 Discussion

5.4.1 Validation of the novel tank assay using a known anxiolytic compound

The use of adult zebrafish in behavioural research has been developed during the last decade (Robert Gerlai, Fernandes, and Pereira 2009; Kalueff, Stewart, and Gerlai 2014; R. E. Blaser, Chadwick, and McGinnis 2010; Egan et al. 2009; Green et al. 2012). Using the natural behaviours of zebrafish to study the effect of compounds with known function have aided in the development of the zebrafish as a useful model in neurobiology research (Egan et al. 2009; Stewart et al. 2011). Using a 5-HT1a-recepter agonist, buspirone (Buspar®), a novel tank assay was established in the lab. Fish treated with buspirone displayed a behavioural phenotype indicating a less anxious state, visible as more time spend at the top of the tank, less erratic behaviours, decreased latency to enter the top area of the tank. These data corresponds to what others have reported on the use of buspirone in the novel tank (Bencan, Sledge, and Levin 2009; Egan et al. 2009). During the setup of this assay several challenges arose. When setting up assays to study the behaviour of adult fish, there are several considerations to make. In the novel tank assay I have used a tank that is identical in shape to the home tank the test fish are housed in in the fish room. Initially a larger, rectangular tank was tested, but fish introduced into this tank displayed a high degree of freezing and no ventures into the top of the tank (data not shown). This indicates that the fish have a heightened anxiety level, making the analysis of the behaviour in the novel tank difficult. Therefore, I used a trapezoid shaped tank (Aquatic Habitats, FL, USA) similar to the home tank the fish were

housed in. Also, the stocking density of the fish has an important impact on the anxiety and stress levels of the fish (Ramsay et al. 2006), and thus fish for behavioural assays were housed at a maximum stocking density of 4 fish per litre of water. A minimum level of handling is also recommended, as handling and netting also increase the stress levels in the fish, further adding to the bias of results (Ramsay et al. 2009), and prior to testing, fish are moved in their home tank to the testing room, and allowed to acclimatise for a minimum of two hours before the testing is done, also to minimize stress and anxiety.

5.4.1.1 Challenges when using automated behavioural analysis

The ease of capturing and analysing behavioural data in zebrafish has increased with the availability of tracking software for automated behavioural analysis. The ability to load a large amount of captured behavioural recordings into the software and allowing the software to run the analysis overnight is very useful and time-saving. However, it is very important to look through the captured data after data analysis has been completed, as mistakes in the tracking occur. The result of these mistakes can be miscalculations of animal behaviour, due to loss of subject recognition by the software. A way to validate the tracking can be done by using the Integrated Visualization-function available in EthoVision XT9 and later versions. This function allows the researcher to scan through the acquired data, and thereby detecting any time points where the software lost contact with the subject (Figure 5.4.1).

Although the use of automated tracking software is very practical and makes the analysis of behavioural parameters like the amount of time the individual fish spends

in different areas of the tank and the distance they move, fast and high-throughput, some behaviours are still difficult analyse using automated setups. The quantification of erratic behaviours and freezing behaviours is determined by manual quantification, done by the researcher. This is time consuming and labours, making the novel tank test difficult to use in a high-throughput screen setting.

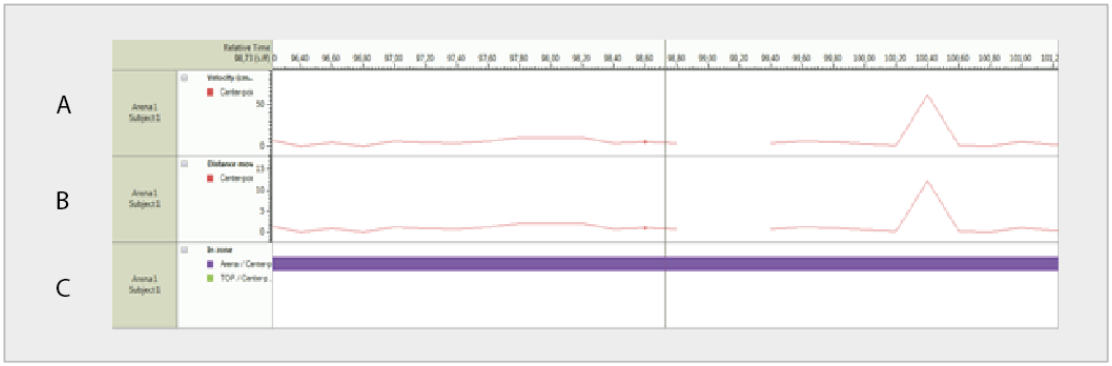

Figure 5.4.1: Screenshot depicting the errors in tracking occurring in EthoVision XT9

The data output from the integrated visualization function showing the tracked data of one fish from a shoaling assay. The tracking window displays a 4 sec time window of tracking. The visualization can be followed in real time while the data is analysed. A: The velocity of the subject is tracked throughout the trial. Notice the break in tracking (red line) which indicates that the software lost track of the fish for a short while. B: Distance moved in the entire arena. The same part of the trial is missing, due to loss of signal or bad detection settings. C show the placement in the novel tank, purple is the total arena and green the top of the tank.

5.4.2 Rolipram increase bottom dwelling and erratic behaviours in the novel tank test

Previous reports on the effect of Rolipram in animal models have indicated that Rolipram causes an anxiolytic and anti-depressant-like phenotype (Y.-F. Li et al. 2009b; Silvestre, Fernández, and Palacios 1999). However, other reports have reported the effect of Rolipram to be anxiogenic (Heaslip and Evans 1995), and data from our lab indicates that Rolipram causes anxiety-like behaviour in zebrafish larvae. Rolipram administered to adult fish cause an increase in behaviours previously shown to be associated with anxiety, namely erratic behaviours and less time spend at the top of the tank (Figure 5.3.3) (Rachel E. Blaser and Rosemberg 2012; Egan et al. 2009; Levin, Bencan, and Cerutti 2007). These behaviours are similar to the behavioural effects of caffeine, another cAMP modulator. Caffeine induce erratic behaviours and the time spend at the bottom of the tank, which is a behaviour that is thought to be in parallel to thigmotaxis in rodents (Egan et al. 2009). Caffeine is a weak PDE-inhibitor, and treatment with caffeine cause an increase in cAMP levels. Similar effects have been shown in Rolipram treated animals (Zhang et al. 2005). Thus the increased cAMP levels through PDE-inhibition can potentially be responsible for the behavioural phenotypes displayed by fish treated with Rolipram. Rolipram

5.4.2.1 Rolipram cause bottom dwelling in the shoaling assay, but has no effect on shoal cohesion

As the phenotype of Rolipram treatment corresponds to an anxiety-like behaviour of adult zebrafish in the novel tank test, I sought to further characterise the response of adult fish to Rolipram treatment. The shoaling behaviour of adult fish have previously proven to be sensitive to modulation with alarm pheromone, hallucinogenic compounds (Green et al. 2012) and anxiolytic compounds (Gebauer et al. 2011). Fish treated with Rolipram display a significant increase in the time spend at the bottom of the tank during the group-behaviour assay (Figure 5.3.5) and in the novel tank test (Figure 5.3.3), and also an increase in whole body cortisol levels (Figure 5.3.6). Bottom dwelling is thought to be a thigmotaxis-like behaviour (Bencan, Sledge, and Levin 2009), and has been reported to be increased in fish subjected to chronic unpredictable stress (Piato et al. 2011). Interestingly fish that has been subjected to unpredicted chronic stress, show an increase in bottom dwelling, without an effect on the shoal cohesion (Piato et al. 2011). Subjecting fish to treatment with alarm pheromone leads to an increase in shoal cohesion, causing the fish to swim closer together (Speedie and Gerlai 2008). This is suggested to be an anti-predatory response, functioning as a protection against predators (Miller and Gerlai 2007). Alarm pheromone does not cause the fish to spend more time at the bottom of the tank when they are in a shoal (Speedie and Gerlai 2008), but does increase bottom dwelling in the novel tank test (Egan et al. 2009). This suggests that the response displayed by shoaling zebrafish, that are being subjected to an

immediate threat, like the presence of a predator, can be distinguished from the response caused by chronic stress or being challenged by placement in a novel environment. Rolipram had no effect on the shoal cohesion in adult zebrafish, indicating that the behavioural output in Rolipram-treated fish is caused by a signalling pathway not related to the alarm pheromone response. However, this needs to be examined in more detail, preferably making use of available mutant lines.

5.4.2.2 MEK inhibition reverse the Rolipram-induced phenotype

In the larval assays we have shown that the Rolipram-induced changes in behaviour can be reversed by inhibiting the MAPK-signalling pathway through MEK-inhibition (see Chapter 4). Groups of adult fish treated with Rolipram, show an increased bottom dwelling, which can be reversed by inhibiting MEK signalling (Figure 5.3.5). Increased cAMP signalling can activate the MAPK signalling through PKA and ERK phosphorylation. From rodent studies it is known, that rats that have been subjected to fear conditioning display increased anxiety in the elevated plus maze. This increased anxiety is correlated with an increase in pERK expression in the prefrontal cortex (Ailing et al. 2008). Treating these fear conditioned rats with the MEK inhibitor PD98059 significantly improved the performance of the rats in the elevated plus maze, and down-regulated the pERK expression in vivo (Ailing et al. 2008). Furthermore, rodent studies have shown that as mice subjected to conditioned fear show an increased expression of pERK in the amygdala. Mice that have been genetically selected as low or high anxiety-like behaviour, are selected and bred for 3 generations, before they are subjected to conditioned fear test and sacrificed.

Immunohistochemistry showed that mice initially selected as having a high anxiety state showed a significant increase in pERK expressing cells in the amygdala of these mice, indicating the MAPK signalling is activated in anxiety (Coyner et al. 2014). Thus, as Rolipram activates the MAPK pathway, the increased thigmotaxis, hyperactivity and bottom dwelling is reversible by treatment with the PD0325901. This suggests that also in zebrafish, the MAPK pathway play an important role in regulating anxiety-like behaviour.

5.4.2.3 Genetic disruption of Pde4d-activity cause disruption in shoal cohesion and less bottom dwelling in zebrafish

Rodent studies using knock-out models of specific PDE4-genes have helped in the understanding of the diverse functions of the different genes in regulating behaviour (R. T. Hansen, Conti, and Zhang 2014; Zhang et al. 2008; Zhang et al. 2002a). Mice lacking PDE4A and PDE4B display anxiogenic like behaviours in the elevated plus maze and the open-field test (R. T. Hansen, Conti, and Zhang 2014; Zhang et al. 2008). Mice lacking *PDE4D* show decreased immobility time in the forced swim test (FST) and the tail suspension tests (TST). These behaviours are reminiscent of an anti-depressive effect, and treatment with classic anti-depressants, like fluoxetine (Prozac®), decrease the immobility time in these tests (Zhang et al. 2002a). Rolipram has previously been reported to induce anti-depressant effects in animal models (Reviewed in Zhu et al., 2001), but treatment with Rolipram did not enhance the anti-depressant-like behaviour in the *PDE4D-/-* mice, tested in the FST and TST. However, the wild-type siblings did show an increase in mobility in the FST and

TST following treatment with Rolipram. This suggests that the anti-depressant effect of Rolipram is mediated through PDE4D-function. In zebrafish, Rolipram induced anxiogenic-like effects, and when treating *Pde4d-/-* adults with Rolipram, they still appear to spend more time at the bottom of the tank (preliminary data, data not shown). This suggests, that the bottom-dwelling in zebrafish, is not dependent on *Pde4d*-function.

Genetic loss of *Pde4d*-function in adult zebrafish causes the fish to spend more time at the top of the tank in shoaling assay, recorded from the side (Figure 5.3.8). Moreover, they display decreased shoal cohesion, causing them to swim further apart (Figure 5.3.8). These findings suggest that loss of *Pde4d* function in zebrafish does not cause anxiety, instead it appears that the fish are less anxious, spending more time at the top of the tank, and this is reminiscent with effects seen in fish treated with known anxiolytic compounds, like fluoxetine (Bencan, Sledge, and Levin 2009) and buspirone (Figure 5.3.2). However, these phenotypes have also been indicated to be reminiscent of a disruption of social behaviour, which has been demonstrated in fish treated with MK-801, a NMDA-receptor antagonist, used to model autism and schizophrenic-like behaviour in animal models, and have shown to disrupt shoal cohesion in adult zebrafish (Maaswinkel, Zhu, and Weng 2013).

Interestingly, a PDE4D-compound identified in the PDE-screen, F152611, which has been designed as an allosteric modulator of PDE4D (Burgin et al. 2010), also disrupted the shoal cohesion when WT fish were treated with the compound. However, there was not effect on the placement in the tank. These data are

surprising, as when tested in the larvae assay, F152611 caused an increase in swimming activity, indicating that the effect on zebrafish larvae were the same as the effect seen in Rolipram treated larvae. This suggests that this compound might have another function than that of Rolipram in adult zebrafish but not in larvae. Further studies are needed to fully understand the function of Pde4d-function in zebrafish.

5.4.3 Concluding remarks

In zebrafish Rolipram caused an anxiety-like behaviour which is further supported by the increase in cortisol levels in fish treated with Rolipram. In the adult zebrafish, the cross-talk between cAMP and MAPK signalling in regulating behaviour is conserved, as the behavioural effects of Rolipram in the shoaling assay could be counteracted by inhibiting the MAPK-signalling pathway, through MEK-inhibition.

The anxiogenic effect of Rolipram in zebrafish could be speculated to be through Pde4a and Pde4b inhibition, as *PDE4A* and *PDE4B-/-* mice shown anxiogenic behaviour. To further support this is the fact that the *pde4d-/-* zebrafish did not recapitulate the behavioural phenotype of Rolipram in the shoaling assay or the novel tank assay. Instead, genetic loss of *pde4d*-function leads to phenotypes resembling an anti-depressant phenotype or antisocial phenotype. The PDE4D-inhibitor did recapitulate the decreased shoaling observed in pde4d-/- fish, but interestingly the effect on the placement in the tank resembled that of Rolipram treated fish. This suggests either that the inhibitor might have some affinity for other PDE4 isoforms,

or the genetic loss of pde4d during development cause a neurodevelopment defect, resulting in the decreased shoaling and higher placement in the tank.

6 Future directions and concluding remarks

6.1 Final comments

Some of the major challenges in drug discovery today are the low number of new drugs that are approved and made available to patients. This is especially bad for treatments for psychiatric illnesses. A reason for this is due the high research costs needed to develop new drugs from the beginning until the drug reach the market. These research costs and the loss of revenue as patents expire faster today than 20 years ago, has a major impact on the risks big pharmaceutical companies are willing to take when venturing into new drug developments (Garnier 2008).

This is a major challenge, as the number of people suffering from mental disorders is increasing, making new and effective treatments more needed than ever. One way to bring down costs of drug development and to get a head start on it, is to do drug repurposing (Fishman and Porter 2005). Drug repurposing is the use of existing drugs for new targets. When undertaking clinical trials the target and predicted effects of the drug needs to be stated prior to the trial, which often leads to failure of the drug, due to side effects, e.g. behavioural effects or toxic effects (Chong and Sullivan 2007).

The use of rodents in drug screens is slow, expensive and laborious. For this the zebrafish offers an excellent compromise between "system complexity and practical simplicity" (R. Gerlai 2003).

Therefore, the need for an alternative screen model in drug discovery is needed. In this project, I have used the zebrafish as a model organism to screen small molecules

for their effect on behavior. Using a well-known pro-convulsant drug, PTZ, I show that in this project I have employed the zebrafish in small molecule screens to modulate the behaviour of larvae and adult fish. In this project I have made use of the zebrafish larvae as a model organism to test compounds with known effect on behaviour. Using two different types of behavioural larval assays, the light/dark response and the swimming activity assay, I have shown that, mainly through the cAMP and MAPK pathway.

I first studied the effect of compounds that could induce a clear behavioural response, which would be possible to modulate chemically. I used the PTZ model, as this model had been described previously as a an epilepsy model in zebrafish larvae (S. C. Baraban et al. 2005). This model served as an excellent model to do a phenotypic small molecule screen for compounds reversing that potential effect of PTZ. From the initial studies I found an unexpected effect of PTZ, which at the time, was unreported. Using low levels of PTZ, and alternating light/dark cycles, I found that 5 dpf old larvae display a complete reversal of activity in response to changes in light or darkness. To identify compounds capable of reversing this effect, I screened 14 compounds with known anti-epileptic or $GABA_A$ binding affinity. I showed that by combining a diazepam with a neurosteroid, potentiated the effect of diazepam, and increased the concentration needed to reverse the PTZ induced phenotype. Secondly, I established that compounds known to elevate cAMP levels causes hyperactivity in zebrafish larvae. In particularly the inhibition of PDE4-isoforms produce hyperactivity that is reversible by inhibiting MEK. These data suggests that

the zebrafish larvae model could serve as a model of choice in phenotypic drug screens.

Furthermore, the involvement of cAMP and MAPK cross-talk in mediating behaviour in zebrafish, suggests that this approach would be useful in small molecule screens for drug-repurposing approaches. Screening known compounds for their potential to reverse behaviours induced by cAMP increase, could potentially lead to the identification of compounds modulating pathways important for regulating behaviour, not previously known.

Lastly, I demonstrated that chemical and genetic modulation of PDE4 activity in adult fish affected behaviour. I showed that in adult fish, Rolipram caused an anxiety-like phenotype in the novel tank assay and in the shoaling assay. That the effect was anxiogenic-like was further confirmed by the increased levels of cortisol detected in adult fish treated with Rolipram.

The behaviour of Rolipram-treated fish differs from the observed behavioural phenotype of a pde4d-/- adult zebrafish. Both in the shoaling and the novel assay do the fish lacking pde4d-activity behave differently than the Rolipram-treated fish. In contrast to a bottom seeking behaviour, these fish spend more time exploring the tank, both in a group and as an individual fish. From rodent studies, there is an overlap between the behaviours of Rolipram treated mice and mice lacking *PDE4D*. Both Rolipram treated mice and PDE4d-/- mice show increased performance in memory tests and also a decrease in immobility in the forced swim test and the tail suspension test, indicating a less depressive behaviour. Furthermore, Rolipram fails

to enhance the performance of the *PDE4d-/-* mice, but do so in the *PDE4D+/+* mice, indicating the anti-depressant and cognitive enhancing effects of Rolipram could be medicated through PDE4-inhibition. *Pde4d-/-* fish treated with Rolipram show an increased bottom seeking behaviour, suggesting that this effect of Rolipram in zebrafish is mediated through the inhibition of other isoforms of PDE4 than Pde4D.

6.2 Further directions

6.2.1 Identify specific site of MAPK activation in zebrafish brains

So far the data I have suggest that cAMP elevations increase pERK expression, and this increased activity of pERK lead to anxiety-like behaviours in zebrafish. This suggests that involvement of the amygdala. The amygdala is important in the regulation of fear and anxiety in rodents, and studies have shown that fear conditioned anxiety leads to an up-regulation of pERK in the amygdala and the medial prefrontal cortex (Ailing et al. 2008). Treatment with a specific MEK inhibitor reverses this effect, both behavioural and biochemically.

It would therefore be interesting to see what areas of the central nervous system show up-regulation of pERK after PDE4-inhibition. This will aid in the elucidation in understanding the regulation of anxiety in zebrafish.

Also a detailed gene expression profile of the different isoforms of PDE4 could aid in our understanding of how cAMP and PDE4-isoforms help regulate behaviour in zebrafish. From the Rolipram experiments on larvae, there are some indications that a subset of larvae respond with higher hyperactivity than the rest. Testing the expression level of *Pde4a, Pde4b* and *Pde4d* in larvae identified as strong responders to Rolipram treatment could perhaps help to elucidate the reason for this high activity level in a subset of larvae.

6.2.2 Translational value of the Rolipram assay in adult zebrafish

Rolipram has been shown to cause antidepressant and cognitive enhancing effects in rodents (Akar et al. 2014; Y.-F. Li et al. 2009b). In the zebrafish, Rolipram and PDE4 inhibitors cause anxiety-like effects, suggesting a different mechanism of action in mice and zebrafish. However, most of the studies done on rodents use a chronic treatment regime of Rolipram, and not an acute. Therefore, we tried to dose mice with Rolipram for 1 hour, and then test them in the open field test for 10 minutes. This time has been described as being the most effective when evaluating anxiolytic or anxiogenic behaviours, as mice might habituate to the environment if tracked for longer. Mice were dosed and allowed to rest for 1 hour after dosing.

From the preliminary data, it appears that the mice treated with Rolipram show an anxiety-like behaviour, with less exploration time in the centre of the arena, and more time spend at the edge of the arena, a thigmotatic behaviour, and more periods of freezing. To test if this behaviour was dependent on MAPK signaling, we dosed the mice with two different doses of MEK inhibitor for 1 hour after Rolipram dosing. Mice treated with Rolipram and PD0325901 showed a higher degree of exploration in the center of the arena and less periods of immobility. The data was not statistically significant, but did show a trend. These preliminary data suggests that acute treatment with Rolipram might cause anxiety-like behaviour in mice, and this can be reversed by inhibiting MAPK signalling. More work is needed, though, to establish the connection between cAMP and MAPK signalling in mice.

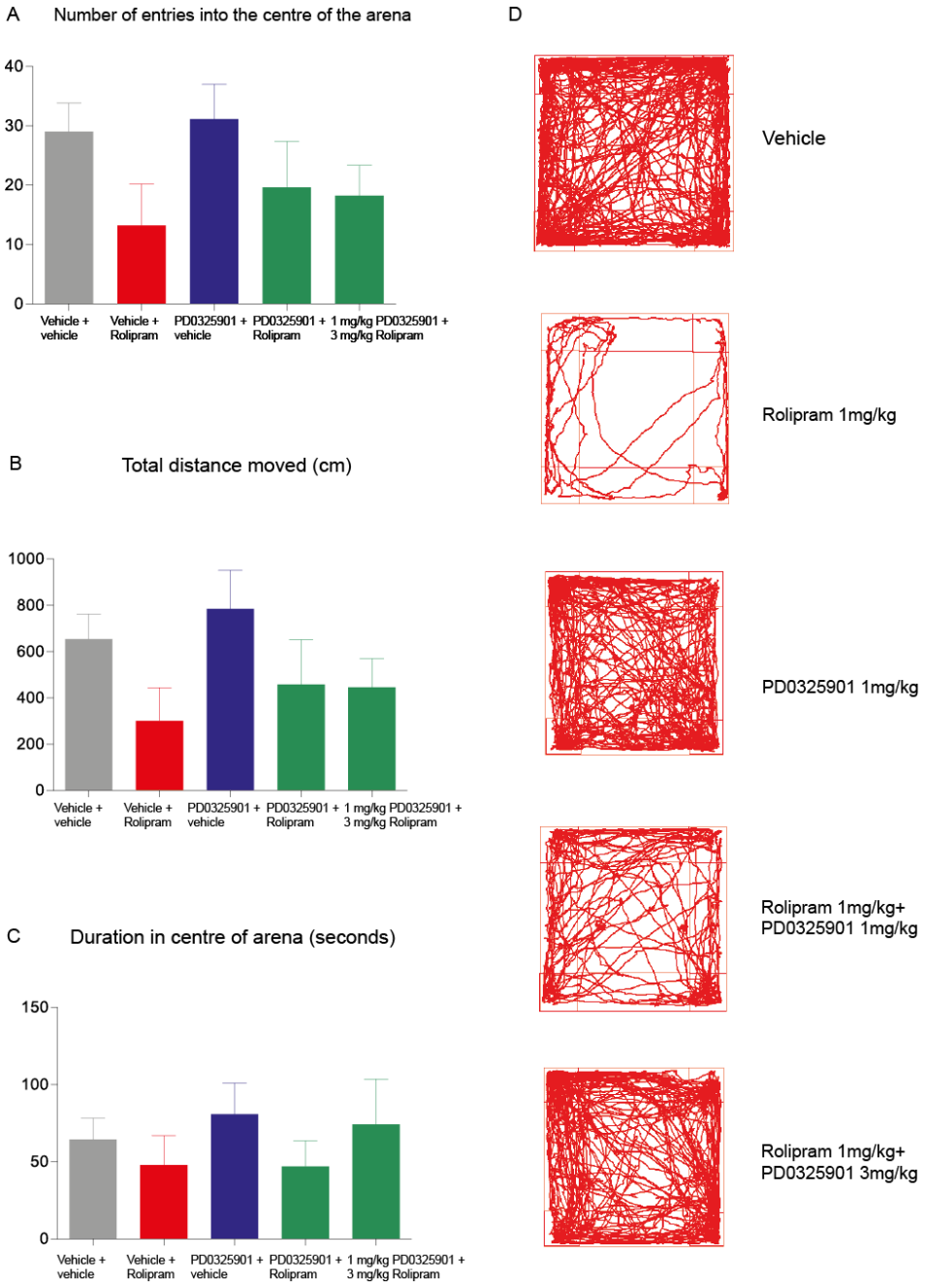

Figure 6.2.1: Data from the open field test on Rolipram and PD0325901 treated mice.

A: Quantified data from open field trials. The number of entries into the centre of the arena are calculated (n=12 mice), B: The total distance moved (n=12) and C: the time spend in the centre of the arena. D: Mice were tracked using the EthoVision XT9.5 software. The traces in figure D show the movement pattern of 1 mouse in the open field arena during an entire trial.

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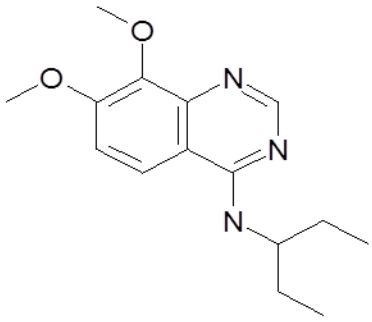
8 APPENDIX – Supplementary data

8.1 Chemical structures and synthesis methods of for the PDE inhibitors from Lundbeck A/S

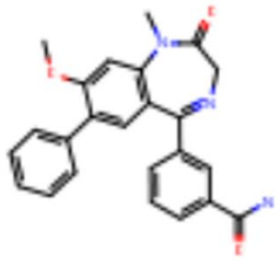
Compound 1 = F152458, PDE1 inhibitor



MW 275.35, IC50: 30nM (PDE1B), 140nM (PDE1C)

Compound 1 was synthesized as described in "Small-molecule phosphodiesterase probes: discovery of potent and selective CNS-penetrable quinazoline inhibitors of PDE1". John M. Humphrey, Eddie Yang,a Christopher W. am Ende, Eric P. Arnold, Jenna L. Head, Stephen Jenkinson, Lorraine A. Lebel, Spiros Liras, Jayvardhan Pandit, Brian Samas, Felix Vajdos, Samuel P. Simons, Artem Evdokimov, Mahmoud Mansoura and Frank S. Menniti, Med. Chem. Commun. 2014, 5, 1290.

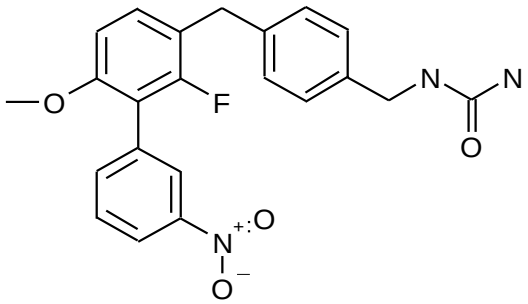
Compound 2 = F152432



MW: 399.442, IC50 PDE2: 130 nM

Compound 2 (ND7001) was synthesized as described in "Preparation of benzo[1,4]diazepin-2-one derivatives as phosphodiesterase PDE2 inhibitors", Abarghaz, Mustafa; Biondi, Stefano; Duranton, Jerome; Limanton, Emmanuelle; Mondadori, Cesare; Wagner, Patrick, Eur. Pat. Appl. (2005), EP 1548011 A1 20050629.

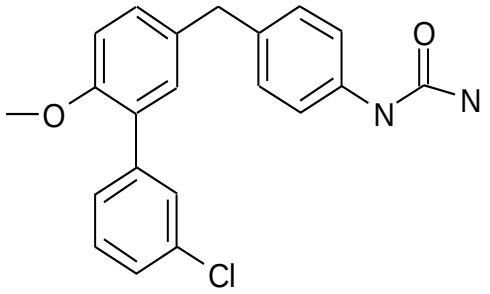
Compound 3 = F152439, PDE4 inhbitor



MW 366.84

Compound 3 was synthesized as described in “biaryl PDE4 inhibitors for treating pulmonary and cardiovascular disorders”, Singh Jasbir; Gurney Mark; Burgin Alex; Kiselyov Alexander; Rao Munagala; Hagen Timothy, wo2009067621.

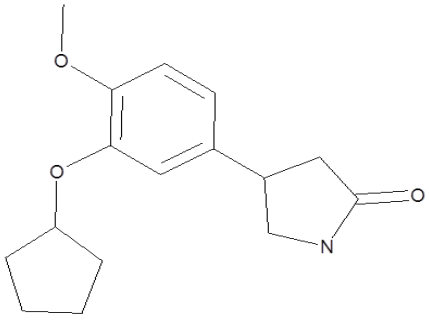
Compound 4 = F152611, PDE4D-inhibitor



MW 409.41

Compound 4 was synthesized as described in “biaryl PDE4 inhibitors for treating pulmonary and cardiovascular disorders”, Singh Jasbir; Gurney Mark; Burgin Alex; Kiselyov Alexander; Rao Munagala; Hagen Timothy, wo2009067621.

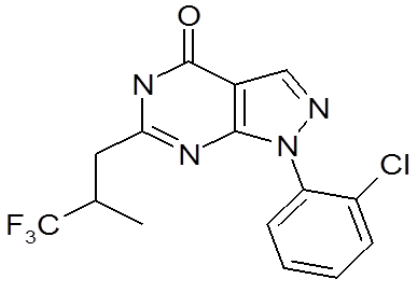
Compound 5 = F152497 = Rolipram, PDE4 inhibitor



MW: 275.40, IC50 PDE4: 1600 nM

Compound 5 (Rolipram) is commercially available from Sigma-Aldrich (catalog number R6520).

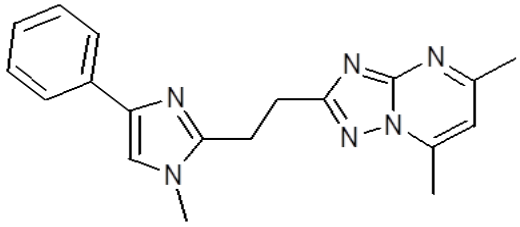
Compound 6 = F152498. PDE9 inhibitor



MW: 356.73, IC50 PDE9 = 55nM

Compound 6 (Bay 73-6691) has been described in "Characterization of the First Potent and Selective PDE9 Inhibitor Using a cGMP Reporter Cell Line", Frank Wunder, Adrian Tersteegen, Annegret Rebmann, Christina Erb, Thomas Fahrig, Martin Hendrix, Molecular Pharmacology 2005, 68, 1775.

Compound 7 : F152455, PDE10 (+2?) inhibitor



MW: 332.41, IC50 PDE10: 15 nM

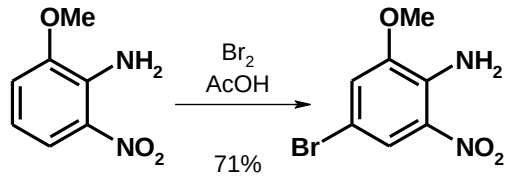
Compound 7 was synthesized as described in "NOVEL PHENYLIMIDAZOLE DERIVATIVES AS PDE10A ENZYME INHIBITORS", Andreas Ritzen, Jan Kehler, Morten Langgard, Jacob Nielsen, John Paul Kilburn, Mohamed M. Farah, patent application US 2010/0016303Al).

The compound has further been described in the: M. Jørgensen, J. Kehler, M. Langgaard, N. Svenstrup, L. Tagmose "Selective inhibitors of PDE2, PDE9, and PDE10: modulators of activity of the central nervous system" , Annual Reports in Medicinal Chemistry 2013, 48, 37 and at the 243rd ACS Meeting in San Diego, March 25-29 2012 ("4-Phenyl imidazoles: A novel class of phosphodiesterase 10A (PDE10A) inhibitors as a potential new generation of antipsychotics", Jan Kehler, Andreas Ritzén, Mauro Marigo, Ask Püschl, John Paul Kilburn, Morten Langgård, Christoffer Bundgaard, Mads Kreilgård, Claus Tornby Christoffersen, Lise Tøttrup Brennum, Anders B. Lassen, Björn Steiniger-Brach, Jacob Nielsen; oral presentation MEDI 296).

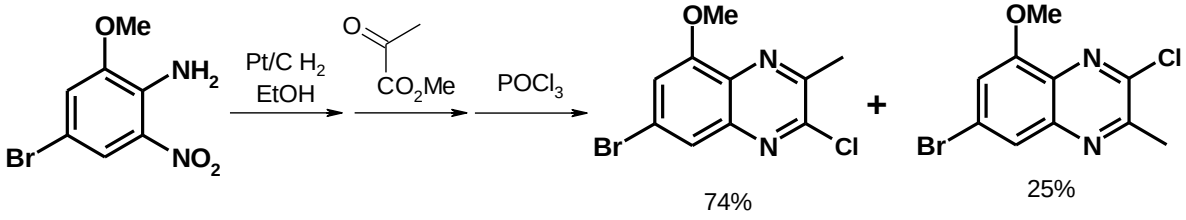
Compound 8 = F152446, PDE2 + 10 dual inhibitor

MW: 338.80, IC50 5.5nM (PDE2), 0.85nM (PDE10)

Synthesis of AF40315:

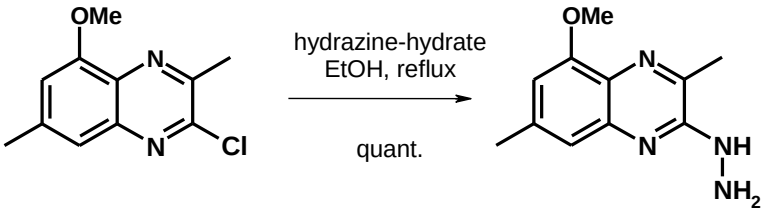


Compound 8 (4-Bromo-2-methoxy-6-nitro-phenylamine) was synthesized as follows: 2-Methoxy-6-nitro-phenylamine (34.5 g, 205 mmol) and NaOAc (27.9 g, 341 mmol) were dissolved in acetic acid (300 mL) at ambient temperature. A solution of bromine (10.6 mL, 205 mmol) in acetic acid (25 mL) was added drop-wise over 15 minutes. After 10 minutes the mixture was cooled on an ice/water bath to precipitate a solid that was washed with heptanes and dried to afford the title compound (35.8 g, 71%).

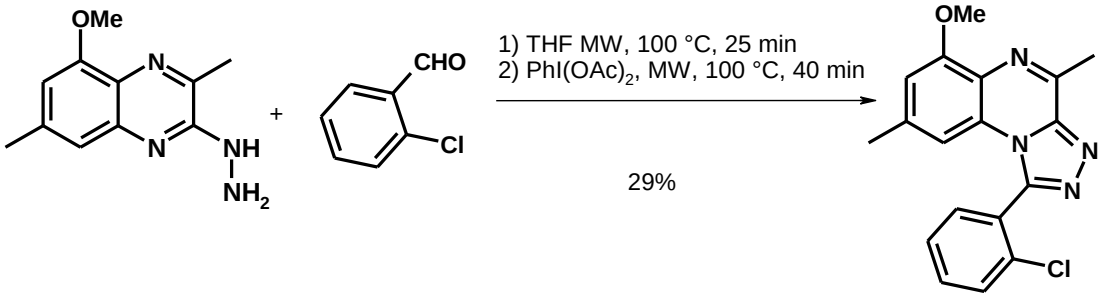


7-Bromo-2-chloro-5-methoxy-3-methyl-quinoxaline. 4-Bromo-2-methoxy-6-nitro-phenylamine (20.2 g, 82.0 mmol) was dissolved in a mixture of ethanol (100 mL)

and THF (200 mL). 5% Pt/C (4.0 g) was added, and the mixture was treated with hydrogen gas (2 bar) at ambient temperature for 2 hours. The catalyst was filtered off, and the volatiles were removed *in vacuo* to afford 5-bromo-3-methoxy-benzene-1,2-diamine in quantitative yield. This material was dissolved in methanol (750 mL) and treated with 2-oxo-propanoic acid methyl ester (7.80 mL, 86.3 mmol) at ambient temperature overnight. The crude solid was filtered off and the filtrate was partially concentrated *in vacuo* (to allow the dark brown/black mother liquer to be discarded before obtaining a second crop of solid). The combined solids were dried to afford 18.5 g of a mixture of 7-bromo-5-methoxy-3-methyl-1H-quinoxalin-2-one and 6-bromo-8-methoxy-3-methyl-1H-quinoxalin-2-one as a beige solid. This material was refluxed in phosphoryl chloride (200 mL) for 1 hour. The volatiles were removed *in vacuo.* The residue was quenched with ice/water and extracted into Et_2O/DCM. The organic layer was washed with sat. aq $NaHCO_3$ and subsequently with brine before it was dried over $MgSO_4$ and filtered. The filtrate coated onto Celite by co-concentration *in vacuo* prior to chromatographic pufication (eluent: heptanes $\rightarrow$ EtOAc). After repeated chromatography, 7-bromo-3-chloro-5-methoxy-2-methyl-quinoxaline (17.5 g, 74%) and 7-bromo-2-chloro-5-methoxy-3-methyl-quinoxaline (6.0 g, 25%) were isolated as the first and second eluting isomers, respectively.



(5-Methoxy-3,7-dimethyl-quinoxalin-2-yl)-hydrazine. Hydrazine monohydrate (4.5 mL, 92 mmol) was added to suspension of 2-chloro-5-methoxy-3,7-dimethyl-quinoxaline (1.58 g, 7.10 mmol) in ethanol (120 mL). The resulting suspension was refluxed for 15 h. The crude mixture was concentrated *in vacuo* to approximately half its original volume before toluene (70 mL) was added and the mixture was concentrated to dryness *in vacuo* to afford the title compound (1.55 g, quant.).

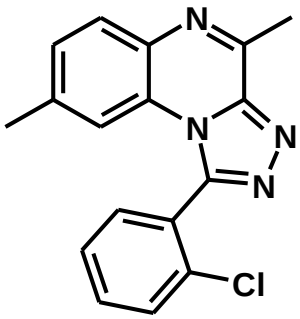


1-(2-Chloro-phenyl)-6-methoxy-4,8-dimethyl-[1,2,4]triazolo[4,3-a]quinoxaline. A mixture of (5-methoxy-3,7-dimethyl-quinoxalin-2-yl)-hydrazine (300 mg, 1.37 mmol) and 2-chlorobenzaldehyde (203 mg, 1.44 mmol) was suspended in THF (11 mL) in a MW vial. The vial was capped, and the overhead space was flushed with argon before the mixture was heated at 100 °C for 25 minutes under MW conditions. After cooling to ambient temperature, iodobenzene diacetate (593 mg, 1.84 mmol) was added. The mixture was subjected to MW irradiation at 100 °C for 40 min. The volatiles were removed *in vacuo*. The residue was purified by chromatography (eluent: heptanes → EtOAc) to afford the title compound as a yellowish solid (133 mg, 29%).

^{1}H NMR (500 MHz, CDCl3): δ 7.72-7.51 (m, 4H), 6.84 (s, 1H), 6.58 (s, 1H), 4.08 (s, 3H), 3.21 (s, 3H), 2.27 (s, 3H).

LC/MS:, retention time 0.63 min, UV-purity 99%, ELS-purity 100%, mass observed 339.1 g/mol. Analytical LC/MS data was obtained on a Waters Aquity UPLC-MS consisting of Waters Aquity including column mamager, binary solvent manager, sample organizer, PDA detector (operating at 254 nM), ELS detector, and TQ-MS equipped with APPI-source operating in positive ion mode. LC-conditions: The column was Acquity UPLC BEH C18 1.7µm ; 2.1x50mm operating at 60°C with 1.2 ml/min of a binary gradient consisting of water + 0.05 % trifluoroacetic acid (A) and acetonitrile + 5% water + 0.05 % trifluoroacetic acid. Gradient: 0.00 min 10 % B; 1.00 min 100 % B; 1.01 min 10 % B; 1.15 min 10 % B. Total run time: 1.15 min.

Compound 9 = F152434, PDE2+10 dual inhibitor



MW: 308.76, IC50 3.5nM (PDE2), 40nM (PDE10)

Compound 9 (**1-(2-chlorophenyl)-4,8-dimethyl-[1,2,4]triazolo[4,3-a]quinoxaline)** was synthesized as described in M. Jørgensen, A.T. Bruun, L.K. Rasmussen, M. Larsen PCT Int. Appl. (2013), WO 2013034755 (Example **Ij1**). Below is characterization data for Compound 9:

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.91 (d, *J* = 8.2 Hz, 1H), 7.70 – 7.65 (m, 1H), 7.65 – 7.61 (m, 2H), 7.53 (ddd, *J* = 7.5, 5.4, 3.4 Hz, 1H), 7.35 (ddd, *J* = 8.3, 1.7, 0.5 Hz, 1H), 6.95 (d, *J* = 0.5 Hz, 1H), 3.04 (s, 3H), 2.25 (s, 3H).

LC/MS:, retention time 1.56 min, UV-purity 98.1%, ELS-purity 100%, mass observed 309.0 g/mol. Analytical LC/MS data was obtained on a Sciex API150EX instrument equipped with APPI-source operating in positive ion mode. The HPLC consisted of a Shimaduzu LC10-Advp LC pump, SPD-M20A PDA detector (operating at 254 nM), and SCL-10A system controller. The autosampler was a Gilson 215, column oven was a Jones Chromatography 7990R, and ELS detector was a Sedere Sedex 85. LC conditions: the column was a Waters Symmetry C-18 4.6 x 30 mm, 3.5 microm operating at 60 °C with 3.0 mL/min of a binary gradient

consisting of water + 0.05% trifluoroacetic acid (A) and methanol + 0.05% trifluoroacetic acid (B); gradient: 0.01 min 17% B, 0.27 min 28% B, 0.53 min 39% B, 0.8 min 50%B, 1.07 min 59% B, 1.34 min 68% B, 1.6 min 78% B, 1.87 min 86% B, 2.14 min 93% B, 2.38 min 100% B, 2.4 min 17% B, 2.8 min 7% B; total run time 2.8 min.