Functioning of the endocannabinoid system in stress and anxiety in zebrafish larvae

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Contents

Chapter 1

Introduction and scope of this thesis

Introduction

The endocannabinoid system (ECS), a lipid signaling system, is primarily known from its ability to interact with d9-tetrahydrocannabinol (THC), the best known psychoactive compound of *Cannabis sativa*, or cannabis. It is known that cannabis was already used in China almost 5000 years ago, because of its healing properties. We also know that Queen Victoria's personal physician, Sir Russell Reynolds, described therapeutic effects of cannabis in the 19th century, mentioning relieve of mental, sensorial and muscular ailments (Reynolds 1890). However, the use of cannabis as a recreational drug induced fear of substance abuse, which overshadowed its medicinal properties. In the last few decades, scientists gained interesting pharmaceutical knowledge regarding the ECS, and as a result, interest in the potential healing capacity of this system has increased again. To date, most research on the ECS has been done in rodents. In this thesis, we have studied the potential of the zebrafish larval model in studying the ECS, as a complementary model to the existing rodent models. More specifically, we have looked at the role of the ECS in regulating locomotion and anxiety, and its interaction with the hypothalamic-pituitary-interrenal (HPI) axis, or stress axis. This research may help in discovering drug targets in the ECS for treatment of anxiety or stress related disorders.

The endocannabinoid system

It took many years after the discovery of THC, before the two receptors were discovered that mediate the effects of this compound. The cannabinoid receptor 1 (Cnr1) was discovered in 1990 (Matsuda et al. 1990), and cannabinoid receptor 2 (Cnr2) a few years later, in 1993 (Munro et al. 1993). Cnr1 is mostly distributed presynaptically and is expressed in several subtypes of neurons, such as glutamatergic, GABAergic and monoaminergic neurons (Freund et al. 2003). However, the density differs between types of neurons, and is for example much higher in GABAergic neurons than in glutamatergic neurons in the hippocampus (Albayram et al. 2011). In addition, the distribution of Cnr1 throughout the brain shows notable local differences (Chevaleyre et al. 2006; Van Waes et al. 2012). Cnr1 is a G protein-coupled receptor, which upon activation inhibits adenylate cyclase and N- and P/Q-type Ca^{2+} channels, and activates K^{+} channels, leading to an inhibited neurotransmitter release and a subsequent lowered excitability of the presynaptic neuron (Fig. 1). Like Cnr1, Cnr2 is a GPCR and also facilitates inhibition of adenylate cyclases (Ibsen et al. 2017). Its function is often linked to a variety of immune events and Cnr2 has anti-inflammatory effects (Cabral and Griffin-Thomas 2009). Initial research on Cnr2 did not show any expression in the central nervous system (CNS), but instead showed only expression in the periphery (Atwood and Mackie 2010). However, recently it was reported that Cnr2 is also present in the brain, where it exerts functional effects, such as modulating neuronal excitability and network synchronization (Chen et al. 2017).

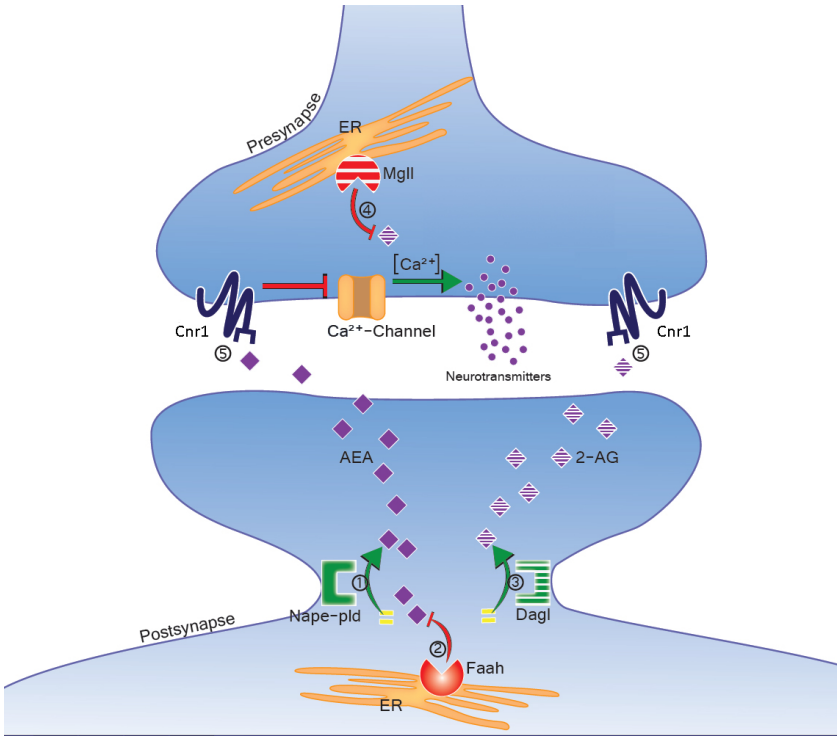

Fig. 1 The ECS consists of the Cnrs, the ligands N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) and their metabolic enzymes. **1** AEA is postsynaptically synthesized from phospholipids by *N*-acyl phosphatidylethanolamine-specific phospholipase D (Nape-pld) and **2** degraded by fatty acid amide hydrolase (Faah), while **3** 2-AG is synthesized by diacylglycerol lipase (Dagl), which is **4** presynaptically degraded by monoacylglyceride lipase (Mgll). **5** Binding of AEA or 2-AG to Cnr1 inhibits opening of Ca^{2+}-channels, which results in less intracellular Ca^{2+} and subsequently a reduced neurotransmitter release. ER = Endoplasmatic Reticulum.

At least two endogenous ligands are responsible for Cnr1 and Cnr2 activation, *N*-arachidonoylethanolamine (anandamide; AEA) (Devane et al. 1992) and 2-arachidonoylglycerol (2-AG) (Sugiura et al. 1995). These signaling lipids, also called endocannabinoids (eCBs), are postsynaptically synthesized and released in a retrograde fashion. Upon release into the synaptic cleft, eCBs can activate Cnrs or can be taken up by transporters into synaptic terminals or glia cells for rapid degradation (De Petrocellis et al. 2004; Pazos et al. 2005). AEA is primarily degraded by the enzyme fatty acid amide hydrolase (Faah), while 2-AG is hydrolyzed by monoacylglyceride lipase (Mgll). Interestingly, the biosynthesis, secretion and metabolism of AEA and 2-AG are differently regulated. As a result, levels of 2-AG and AEA can vary greatly in the same organ, tissue or cell and

can even undergo opposite changes (Di Marzo and De Petrocellis 2012). It has been suggested that AEA represents a tonic signal, whereas 2-AG represents a phasic signal (Hill and Tasker 2012). A tonic signal would regulate neurotransmitter release under steady-state conditions, while a phasic signal is needed for (acute) synaptic plasticity. This idea may be further supported by the fact that Faah is expressed postsynaptically (Egertová et al. 2003), whereas Magl is located presynaptically (Dinh et al. 2002). Degradation of AEA and 2-AG thus takes place at different levels of the signaling pathway and therefore AEA and 2-AG may have different lifetimes (Steiner and Wotjak 2008). Especially 2-AG would require a short lifetime, since its potential role in regulating acute synaptic plasticity. However, functional interpretation of eCB levels is complicated, since 2-AG can also serve as an intermediate in several lipid metabolic pathways. For example, 2-AG can function as a source of arachidonic acid for biosynthesis of prostaglandins (Nomura et al. 2011), which may be the reason why 2-AG is far more abundant than AEA (Buczynski and Parsons 2010). Furthermore, it has been suggested that eCBs do not only regulate Cnr activity, but may also fine-tune cell homeostasis via interactions with other targets, such as the transient receptor potential vanilloid type-1 channel (Di Marzo and De Petrocellis 2012).

The HPA axis

Anxiety disorders are often associated with a dysfunctional hypothalamic pituitary adrenal (HPA) axis (Carlo et al. 2012). The HPA axis is activated upon stress, which results in an increase of circulating glucocorticoids and a subsequent changed activity of multiple target systems in our body. Stressful stimuli cause the activation of neural inputs of corticotropin releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus (PVN), which leads to the release of CRH, but also of vasopressin in the basal hypothalamus. CRH is transported to the anterior pituitary and vasopressin to the posterior pituitary. They both stimulate the secretion of stored adrenocorticotropic hormone (ACTH) from corticotrope cells (Steiner and Wotjak 2008). The secreted ACTH is transported by the blood to the adrenal glands where they stimulate the synthesis of glucocorticoids in the adrenal cortex, leading to an increased secretion of glucocorticoids into the blood. Generally glucocorticoids act on the mineralocorticoid receptor (MR) and the glucocorticoid receptors (GR), and these receptors are expressed by many different types of neurons, but also other cell types. These receptors act as ligand-activated transcription factors, and many glucocorticoid-evoked actions in different organs have been described (Pecoraro et al. 2006; Tasker and Herman 2011). Besides their stress-related effects, such as energy release, glucocorticoids control the negative feedback of the HPA axis (Tasker and Herman 2011). This negative feedback loop regulates HPA axis activity at the level of the hypothalamus (Evanson et al. 2010) and the pituitary (Russell et al. 2010),

but also other regions have been reported, such as the thalamus (Furay et al. 2008; Hill et al. 2011; Jaferi and Bhatnagar 2006). One of the main functions of the fast glucocorticoid negative feedback loop is termination of the neuroendocrine stress response which prevents from depletion of stress hormones in order to maintain stress responses (Sapolsky et al. 2000). The other important function of glucocorticoid mediated feedback is to modulate long-term stress-related memory consolidation (McGaugh and Roozendaal 2002).

Interaction of the ECS and HPA Axis

The ECS has been shown to be involved in the regulation of the HPA axis. It is thought that glucocorticoids induce eCB synthesis, which subsequently inhibits HPA axis activity. In an interesting study it was shown that the glucocorticoid feedback on the hypothalamic secretion of CRH is eCB-dependent (Di et al. 2003). In this study, the effect of dexamethasone, a GR agonist, on glucocorticoid-mediated inhibition of glutamate release was blocked by Cnr antagonists AM251 and AM281. This was confirmed by two other studies (Campolongo et al. 2009; Coddington et al. 2007), in which it was shown that rapid behavioral responses to corticosterone administration are diminished by blockade of the Cnr1, suggesting that glucocorticoids function via a Cnr1-dependent mechanism. It has been proposed that corticosterone triggers the synthesis of AEA and 2-AG in the PVN of the hypothalamus, which subsequently activate local Cnr1s to reduce glutamate release from these neurons (Di et al. 2003). Indeed, it has been shown that administration of corticosterone increases the AEA content within the amygdala and hippocampus in rats (Hill et al. 2010), which indicates that glucocorticoids indeed regulate eCB signaling. It is thought that the rapid negative feedback loop of the HPA axis goes via an enhanced eCB synthesis, which subsequently results in the inhibition of the HPA axis (Fig. 2) (Hill and Tasker 2012).

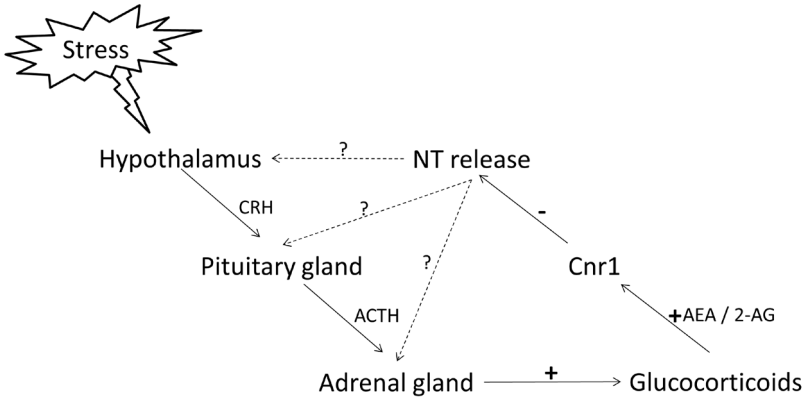

Fig. 2 A proposed mechanism for the involvement of the ECS in the negative feedback loop of the HPA axis. The HPA axis is activated upon stress, which eventually results in the production and secretion of glucocorticoids. These glucocorticoids activate the GR which through unknown signaling results in the release of eCBs (AEA and/or 2-AG). The eCBs in turn activate Cnr1, which results in less neurotransmitter (NT) release and thereby less activation of HPA axis involved brain regions. These regions could be the hypothalamus, the pituitary gland or the adrenal gland, but it could also be an area upstream of the hypothalamus.

The zebrafish as an animal model in CNS research

In this thesis, we have studied the ECS in the zebrafish larval model. The zebrafish (*Danio rerio*) is a freshwater fish which naturally occurs in Southeast Asia, and belongs to the family of *Cyprinidae* (also called the carp family). Over the last decade, it has emerged as a popular animal model in biomedical research. This can be attributed to the many advantages this model brings, such as: high fecundity, external fertilization, rapid development, optical transparency of embryos and larvae, low maintenance costs and the ease of genetic manipulation (Stewart et al. 2014). Together with its easy breeding and relatively small housing, these characteristics make this model ideal for *in vivo* high-throughput screening (HTS). The zebrafish shares a similar central nervous system (CNS) morphology with humans (Kalueff et al. 2014) and is extensively used in CNS research (Stewart et al. 2014). The zebrafish model is highly suitable for translational neuroscience, especially for identification of genes involved in brain disorders (Kalueff et al. 2014).

Since zebrafish are optically transparent and have a relatively small brain, several imaging techniques can be applied to study its CNS. Based on magnetic resonance imaging (MRI), a three-dimensional atlas of the zebrafish brain has recently become available, which has a resolution comparable to conventional

histology (Ullmann et al. 2010). Others have, for example, applied optical projection tomography (OPT) for visualizing cell populations in the adult zebrafish brain (Lindsey and Kaslin 2017). To image neuronal activity *in vivo* using fluorescence microscopy techniques, so-called genetically encoded calcium indicators (GECIs) have been developed (Nakai et al. 2001). These fluorescent calcium indicators are fluorescent molecules which change their fluorescence properties upon chelation with calcium, a reporter for neural activity. These GECIs have been improved, resulting in a new calcium indicator called GCaMP. Recently, these molecules have been modified to become photoconvertible, making temporal analysis possible (Fosque et al. 2015; Hoi et al. 2013). Furthermore, GCaMPs can now also be analyzed in a freely swimming zebrafish larva (Kim et al. 2017).

The possibilities for HTS of behavior is another advantage of the zebrafish as an animal model for CNS research. Automated observations allow for detailed measuring of locomotor responses (distance moved, velocity, turning angle, startle, freezing) and are commercially available. Noldus (Netherlands) has developed DanioVision, while ViewPoint (France) has made ZebraLab, both automated systems specifically designed for HTS of zebrafish larval behavior. These systems, but also custom-made systems, are often applied to study basal locomotion (Girdhar et al. 2015; Marques et al. 2018), optokinetic responses (Mueller and Neuhauss 2010; Portugues et al. 2014), behavioral profiling (Baker et al. 2018; Thornqvist et al. 2019) or neuropsychiatric disorders (Khan et al. 2017; Levitas-Djerbi and Appelbaum 2017; Stewart et al. 2012). It is possible to measure multiple fish simultaneously (96well plates for larvae for example) and screen multiple drugs at different doses at the same time. This led to a new direction in neuroscientific research, behavioral phenomics, where small molecules and genetic variations are tested in HTS of behavior.

Research on the ECS in zebrafish and comparison with other models

The ECS has not often been studied in the zebrafish model. A PubMed search on 'zebrafish' and 'cannabinoid' yielded 51 results, whereas 'rodents' and 'cannabinoid' resulted in 9863 items. Luckily, the basic characteristics, such as the expression profile of the Cnrs, their ligands and the metabolic enzymes are known. Expression of *cnr1* was consistently detected throughout larval development in the dorsal telencephalon, pretectum, torus longitudinalis (specific ray-finned fish structure) and periventricular hypothalamus (Lam et al. 2006). The expression pattern of *cnr2* has been identified as well, and *cnr2* appeared to be expressed mainly in peripheral tissues (Rodriguez-Martin et al. 2007). Analysis of the zebrafish genome revealed that most ECS genes are present in zebrafish (McPartland et al. 2007), although no homolog was found for the gene responsible for *N*-acylethanolamine acid amidase, while some other genes have two zebrafish homologs

each (Demin et al. 2018). Other, more functional, ECS research in zebrafish has mainly focused on development, metabolism, memory and anxiety.

Development

The ECS seems to play an important role in CNS development. Knockdown of the *cnr1* gene revealed that Cnr1 is involved embryonic axonal growth and fasciculation (Watson et al. 2008), which is consistent with data from similar studies in rodents (Mulder et al. 2008; Wu et al. 2010). Axonal outgrowth was also impaired in a knockdown of the gene responsible for diacylglycerol lipase (Daglα), specifically in retinotectal, cerebellar and facial nerves (Martella et al. 2016a), which affected the control of motion, vision, and spontaneous movement. Since the enzyme Daglα is involved in the synthesis of 2-AG, it can be speculated that 2-AG is important for the development of a functional visual system. Exposure to phytocannabinoids (plant derived cannabinoids) THC and cannabidiol (CBD) during gastrulation, a developmental stage between 5.25 hours post fertilization (hpf) and 10.75 hpf, affected axial development of motor neurons, and reduced the number of startle responses to sound stimuli, but not to touch stimuli (Ahmed et al. 2018). The teratogenic brain effects of ECS manipulation have also been reported in other animal studies (Fernandez-Ruiz et al. 2000). For example, prenatal exposure to Cnr agonist WIN55,212-2 alters migration of glutamatergic neurons and GABAergic interneurons in rats (Saez et al. 2014) and CP55,940 affects facial, visual and neuronal development in mice (Gilbert et al. 2016). A recent study done in humans corroborates the results from animal studies, showing a volume reduction in regions rich in Cnr1 receptors in young, regular cannabis users, which correlates with the amount and duration of cannabis exposure (Battistella et al. 2014).

The ECS also plays a role in morphological development. For example, activation of Cnrs by THC exposure in zebrafish, resulted in morphological defects during embryogenesis (Ahmed et al. 2018; Akhtar et al. 2013; Carty et al. 2018; Thomas 1975), including pericardial edema, yolk sac edema, and a curvature of the rostro-caudal axis. Synthetic cannabinoids WIN55,212-2 and CP55,940 had no developmental effect (Akhtar et al. 2013). Contradictory findings were presented for CBD, a phytocannabinoid with low binding affinity for Cnrs. In one study, CBD exposure caused morphological abnormalities at 96hpf, such as edemas (yolk sac and pericardial), curved axis, fin deformities and swim bladder distention (Carty et al. 2018). However, in another study where the same concentrations of CBD were applied, no morphological malformations at 96hpf were found (Valim Brigante et al. 2018). It should be noted that the only morphological readout both authors had in common was the size of the pericardial area. Others have studied the effect of CBD exposure during gastrulation, and noted malformations, such as curved tails and cardiac edema, already at 48hpf (Ahmed

et al. 2018). Knocking out Cnr1 or Cnr2 does not produce any malformations, and the knockout fish are viable and fertile (Liu et al. 2016). Knocking out cannabinoid receptor interacting protein 1 (Cnrip1), a protein interacting with the intracellular region of Cnr1, does not affect development, viability or fertility either (Fin et al. 2017). Blocking Cnr1 by administration of the Cnr1 antagonist AM251 does not cause morphological effects, but reduces the hatching rate at 72 hpf (Migliarini and Carnevali 2009). Interestingly, the hatching rate was also reduced (by about 20%) upon exposure to CBD (Valim Brigante et al. 2018).

Metabolism

From rodent studies it is known that the ECS is involved in lipid metabolism (Di-Patrizio and Piomelli 2012). A study done in zebrafish larvae and adults showed that AEA modulates lipid metabolism, as AEA administration modulates transcription of sterol regulator element binding protein (*srebp*) and insulin-like growth factors (*igf-1* and *igf-2*) (Migliarini and Carnevali 2008), genes involved in lipid metabolism. Overexpression of the hepatic *cnr1* gene induces upregulation of important lipogenic genes, such as *srebp*, which eventually results in hepatic steatosis or steatohepatitis in zebrafish (Pai et al. 2013). This is in agreement with rodent literature, where antagonizing Cnr1 with rimonabant has a hepatoprotective effect (Gary-Bobo et al. 2007) and steatosis is absent in *cnr1* knockout mice (Osei-Hyiaman et al. 2005).

Embryos treated with rimonabant also showed less lipid accumulation in the head, while the Cnr agonist WIN55,212-2 increased this lipid accumulation (Nishio et al. 2012). This is in agreement with another study done in zebrafish embryos, where fat accumulation is decreased in rimonabant-treated embryos while exposure to the CB1 agonist WIN 55,212-2 increases fat accumulation (Fraher et al. 2015). Other compounds tested in this study were the Cnr1 agonist oleamide (which increases lipid levels), the Cnr2 agonist HU308 (which increases lipid levels) and the Cnr2 inverse agonist AM630 (which decreases lipid levels). Others have tested the cannabinoids d9-tetrahydrocannabivarin (THCV) and CBD in different models of hepatosteatosis. In zebrafish, these compounds increased yolk lipid mobilization (Silvestri et al. 2015), although it should be noted that the reduction of intracellular lipid levels was also present in a *cnr1* knockdown human cell line (Silvestri et al. 2015). Since the applied cannabinoids have low binding affinity to the Cnrs in general, the effects may have been non-ECS specific. In obese mice, the same compounds inhibited the development of hepatosteatosis (Wargent et al. 2013), suggesting a similar lipid reducing functioning of the ECS.

The role of the zebrafish ECS in metabolic disruption has also been studied. The xenoestrogen bisphenol A (BPA) is considered a metabolic disruptor and

triggers hepatosteatosis in adult zebrafish (Martella et al. 2016b). BPA-treated zebrafish also showed an increase of 2-AG and AEA levels in the liver, an increase in the expression of *cnr1*, and an aberrant profile of metabolic gene expression (Martella et al. 2016b). Another commonly studied metabolic disruptor, di-isononyl phthalate (DiNP), has also been tested on the effects on the ECS in female adult zebrafish brain and liver (Forner-Piquer et al. 2017). The results showed that three week exposure to DiNP decreased AEA levels in the brain, but increased AEA levels in the liver. Furthermore, the expression of various ECS metabolic enzymes was altered in both the brain and in the liver.

Another measure of energy homeostasis in zebrafish is the size of the yolk sac, which is the primary source of energy for zebrafish embryos and larvae. Exposure to the Cnr antagonist rimonabant increases yolk sac size (Nishio et al. 2012). This effect is blocked in *cnr1* morpholino knockdown fish, but not in *cnr2* morpholino knockdown fish, suggesting that this yolk sac size increase is Cnr1-dependent (Nishio et al. 2012). Treatment with rimonabant also decreased food (paramecia) intake in young zebrafish (Shimada et al. 2012), although it was not investigated whether this was an ECS-specific effect (the Cnr antagonist rimonabant is known to have off-target effects).

Memory

From rodent and human studies it is known that the ECS modulates cognitive processes, including acquisition, consolidation, retrieval and extinction of memory (Morena and Campolongo 2014). In adult zebrafish, THC impairs spatial but not emotional associative memory functioning (Ruhl et al. 2014). The impairment of spatial memory could be related to aberrant signaling in the zebrafish telencephalon. Since these results have also been found in the mammalian striatum (Valjent et al. 2001), this suggests a similar effect of the ECS in zebrafish forebrain as in the striatum of mammals. The lack of effect of THC on associative memory is also in agreement with rodent studies, where THC does not affect performance of rats in a black-white discrimination task (Jentsch et al. 1997), nor in a visiual discrimination task with two figures (Mishima et al. 2001). THC also inhibits acquisition of fear learning in zebrafish (Ruhl et al. 2017), possibly by inhibiting activity in the medial and lateral pallium of the dorsal telencephalon. These regions are, like the hippocampus of mammals, indeed involved in spatial cognition, trace memories and emotional and fear conditioning (Broglio et al. 2005). In another test, the cannabinoid CBD induces memory impairment in an inhibitory avoidance task, which is a paradigm of associative learning (Nazario et al. 2015). Finally, as in rodents, food reward reduces avoidance learning behavior in zebrafish (Manuel et al. 2015), which was accompanied by a decreased telencephalic gene expression of *cnr1*.

Anxiety

Anxiety, an excessive feeling of unease which can appear without any particular reason or cause, can become a disorder when it gets chronic and unjustified. The ECS may exert an important role in modulating emotional states by changing eCB signaling (Hill and Gorzalka 2009; Viveros et al. 2005). Many studies, both in humans and rodents, have shown that eCBs are involved in anxiety (Lisboa et al. 2017). In general, the effects produced by cannabinoids are biphasic, meaning that low doses are anxiolytic whereas high doses are anxiogenic (Viveros et al. 2005). For example, mice display no response in a light-dark box anxiety test at low concentrations of THC (0.03 mg/kg), an anxiolytic response at moderate concentrations (0.3 mg/kg) and an anxiogenic response at high concentrations (5 mg/kg) (Valjent et al. 2002).

In zebrafish, research on anxiety and the ECS has thus far been done only in adult fish. Taken together, the effects of zebrafish ECS manipulation on anxiety are generally corresponding with studies done in rodents. In a social interaction test, WIN-treated fish spend relatively more time in the chamber with an unknown fish compared with an empty chamber (Barba-Escobedo and Gould 2012), which is considered an anxiolytic effect. In another approach, both acute and long-term exposure to WIN in an light-dark plus maze (a cross maze with two bright and two dark arms) was tested (Connors et al. 2013). Acute exposure to WIN results in fewer entries into the light arm at all concentrations tested, but the total number of entries is reduced as well. This suggests that larvae were less mobile and more research is needed to determine whether this is related to anxiety-like behavior. Interestingly, the long-term exposure results in an increased number of total entries, also an increased number of light entries, more time spent in the light, and a decreased latency to move out of starting position, all characteristics which suggest an anxiolytic effect (Connors et al. 2013).

In contrast, acute THC exposure (20 min) results in anxiogenic-like behavior in a novel tank test (Stewart and Kalueff 2014). The two concentrations tested, 30 mg/L (100μM) and 50 mg/L (160μM), both produced a decrease of swimming in the top layer and an increase in slow bottom dwelling. Furthermore, the latency to the top layer was increased as well as the number of transitions to upper half of the tank. At 30 mg/L both the velocity and the traveled distance were lower, while these parameters were unaffected at 50 mg/L. Although this study strongly suggests an anxiogenic effect of THC, this was not confirmed in another study (Ruhl et al. 2014). In this study, THC was tested in an escape response test, in which fish are placed in a center-closed arena surrounded by a white paper drum with one black segment. This paper drum turns around the arena and the black segment is considered a threatening object. In this test, the percentage of escape responses is not different between the THC-treated group and the

vehicle-treated group. It should be noted however, that both the concentration (100nM) and exposure time (1hr) tested in this study are very different when comparing with the study mentioned above (Stewart and Kalueff 2014).

One study done in zebrafish showed the effects of blocking Cnr1 (Tran et al. 2016). In this study, the Cnr1 antagonist AM251 was administered for 1 hour followed by a novel tank test. Fish treated with the highest concentration of AM251 (1 mg/L) showed an increase in anxiety-like behavior, including freezing, increased bottom dwelling, decreased locomotor activity and elevated erratic movements. At a concentration of 0.1 mg/L, AM251 had no effect.

Aim of this study

The ECS is involved in numerous physiological and pathological conditions (Pacher et al. 2006), among which are mood disorders (Hill and Gorzalka 2009). Understanding the functioning of the ECS can thus be highly valuable in the search for new drug targets. To fully utilize the potential of the ECS as a drug target, more research is needed. The zebrafish larva is a promising animal research model, but its application in ECS research has remained limited thus far. The research described in this thesis was designed to get a basic understanding of the ECS in zebrafish , with a specific focus on the effects of ECS activity on anxiety-related behavior and HPI-axis functioning during the larval stage. In this study both the effects of the endogenous activity and of pharmacological activation of Cnrs will be studied. The results will help determining the feasibility of zebrafish larvae as animal models for biomedical research on the ECS.

1. Characterize the effect of exogenous ECS activation on zebra fish larval behavior
2. Gain insight in the role of the endocannabinoids in (anxiety-related) behavior
3. Confirm whether the ECS plays a role in cortisol secretion
4. Characterize where potential ECS involvement in cortisol secretion takes place

Outline

In this thesis, research on the effects of ECS modulation on locomotion, anxiety-like behavior and HPI axis activity in zebrafish larvae is described.

Chapter 2 describes the effect of ECS manipulation on zebrafish larval locomotion in a visual motor response test. Several treatments have been applied, such as administration of Cnr agonists and a Cnr1 antagonist. In addition, a *cnr1* knockout fish was used. Finally, desensitization of Cnr1 was examined using behavior as a readout. Exogenous Cnr1 activation resulted in a reduced locomotion, whereas eCBs seemed not to have an effect on locomotion.

Chapter 3 explains the effect of ECS manipulation on anxiety-like behavior in zebrafish larvae. Using a light/dark preference test, the effect of Cnr1 activation and knocking out *cnr1* on several parameters were studied, including time spent and distance moved in dark zone and latency to visit dark zone. The activation of Cnr1 by the agonist WIN55,212-2 had an anxiolytic effect, which was abrogated in a *cnr1*$^{-/-}$ mutant line. Endogenous activation or blocking Cnr1 with a Cnr1 antagonist did not affect anxiety-like behavior.

Chapter 4 investigates the effect of Cnr1 activation and blockade on cortisol production and at what level of the HPI axis these effects are mediated. We found that activation of Cnr1 by treatment with a Cnr agonist increased basal cortisol levels. This increase in basal cortisol could be blocked with antalarmin, a Crh-receptor 1 antagonist, indicating that increased Crh levels are associated with the Cnr1-induced cortisol increase.

Chapter 5 summarizes the research chapters and puts the data in a bigger context. In addition, the future direction of ECS research using the zebrafish model is discussed.

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Chapter 2

Functional characterization of the cannabinoid receptors 1 and 2 in zebrafish larvae using behavioral analysis

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Abstract

The endocannabinoid system (ECS) comprises the cannabinoids anandamide and 2-arachidonoylglycerol and the cannabinoid receptors 1 and 2 (Cnr1 and Cnr2). In this study, we have characterized the function of Cnr1 and Cnr2 in relation to behavior in zebrafish, which has become a versatile animal model in biomedical research. Behavioral analysis of zebrafish larvae was performed using a visual motor response (VMR) test, which allows locomotor activity to be determined under basal conditions and upon a dark challenge. Treatment with the non-specific Cnr agonists WIN55,212-2 and CP55,940 resulted in a decrease in locomotion. This was observed for both basal and challenge-induced locomotion, although the potency for these two effects was different, which suggests different mechanisms of action. In addition, WIN55,212-2 increased the reaction time of the startle response after the dark challenge. Using the Cnr1 antagonist AM251 and a *cnr1*$^{-/-}$ mutant line it was shown that the effects were mediated by Cnr1 and not Cnr2. Interestingly, administration of the antagonist AM251 alone does not have an effect on locomotion, which indicates that endogenous cannabinoid activity does not affect locomotor activity of zebrafish larvae. Upon repeated dark challenges, the WIN55,212-2-effect on the locomotor activity decreased, probably due to desensitization of Cnr1. Taken together, these results show that Cnr1 activation by exogenous endocannabinoids modulates both basal and challenge-induced locomotor activity in zebrafish larvae, and that these behavioral effects can be used as a readout to monitor the Cnr1 responsiveness in the zebrafish larva model system.

Introduction

The endocannabinoid system (ECS) is a neuromodulatory system that consists of the cannabinoid receptors 1 and 2 (Cnr1 and Cnr2 respectively), the endogenous ligands anandamide and 2-arachidonoylglycerol (AEA and 2-AG respectively) and the metabolic enzymes involved in synthesis or degradation of those ligands. The Cnr1 is a presynaptic G-protein-coupled receptor (GPCR), which upon activation inhibits adenylate cyclase and N- and P/Q-type Ca^{2+}-channels, and activates K^+ channels, leading to inhibition of neurotransmitter release. The Cnr1 can regulate synaptic neurotransmission of excitatory and inhibitory circuits throughout the central nervous system (CNS). As a result, the ECS is important in regulating aspects of brain function, including mood, anxiety, appetite, memory consolidation and the control of locomotor activity. Like Cnr1, Cnr2 is a GPCR and also mediates its action via inhibition of adenylate cyclases (Ibsen et al. 2017). It is most abundantly present on cells of the immune system and has anti-inflammatory effects (Cabral and Griffin-Thomas 2009). Atwood and Mackie suggested that it might be the more peripherally located cannabinoid receptor, because initial research on the Cnr2 did not show any expression in the CNS (Atwood and Mackie 2010). However, recent data have shown both expression and functional effects of the Cnr2 in the brain (Atwood and Mackie 2010; Chen et al. 2017).

The psychoactive component of the cannabis plant (*Cannabis, marijuana*), Δ9-tetrahydrocannabinol (THC), has been known for many years to affect animal behavior, such as aggressiveness, memory, dominance and locomotion (Grunfeld and Edery 1969). The role of the ECS on locomotion led to an increased interest for cannabinoids as a potential (symptomatic) treatment against locomotor-related diseases, such as Parkinson's disease, Huntington's disease or spasticity (Romero et al. 2002). After the discovery of Cnr1 in 1990 (Matsuda et al. 1990), it was shown in rodents that several agonists for this receptor have an inhibitory effect on locomotion (Anderson et al. 1996; Richter and Loscher 1994). However, there have sometimes been ambiguities in the behavioral data (Drews et al. 2005; McGregor et al. 1996; Polissidis et al. 2013), possibly due to differences among genetic strains of experimental animal, or differences in protocols such as the route of administration or dosage and exposure time.

In the present study we have used zebrafish larvae to investigate the effects of Cnr1 and Cnr2 activation on locomotion. This model is a well-developed animal model for biomedical research and can be used as a complementary model to rodents (Ahmad et al. 2012; Kalueff et al. 2014; Khan et al. 2017; Stewart et al. 2014). Several features have made the zebrafish larval model increasingly popular. Zebrafish larvae can easily be obtained in large numbers, and their small size, rapid development, and optical transparency allow for phenotypic screening in

relatively large numbers of replicates (Kimmel et al. 1995). In addition, the availability of tools for genetic manipulation and the availability of the entire genomic sequence enables genetic studies in this model (MacRae and Peterson 2015; Varshney et al. 2015).

Over the last decade, the ECS of zebrafish has been characterized and it was shown that it contains the same receptors, ligands and metabolic enzymes as its mammalian equivalent (Krug and Clark 2015; McPartland et al. 2007). Interestingly, the metabolic enzyme Faah2 is absent in mice, but is conserved in both humans and zebrafish (Krug et al. 2018). In 2006, the expression of the *Cnr1* gene was analyzed in zebrafish larvae and adults by *in situ* hybridization (Lam et al. 2006). This was followed by spatial analysis of *cnr2*, the gene responsible for encoding Cnr2 (Rodriguez-Martin et al. 2007), and developmental analysis of *dagl*α, the gene encoding the metabolic enzyme Daglα (Watson et al. 2008). Oltrabella *et al*. recently presented an expression profile of zebrafish ECS genes during embryogenesis (Oltrabella et al. 2017). Most of the investigated genes were stably transcribed after 48 hours post fertilization (hpf), such as *cnr1, cnr2, mgll, dagl, faah, faah2*, and *napepld*.

Only a few functional studies have been done on the role of the ECS on behavior in zebrafish larvae. Chronic exposure to Cnr1 antagonist AM251 resulted in a lower hatching rate at 72 hpf and a dramatic decrease of motility at 96 hpf, while the developmental morphologic stages stayed the same (Migliarini and Carnevali 2009). Embryonic exposure to THC resulted in a reduced number of spontaneous muscle twitches while the embryos appeared morphologically normal (Thomas 1975).

Other subjects on the ECS in zebrafish larvae have been investigated as well, such as lipid metabolism (Nishio et al. 2012), leukocyte migration (Liu et al. 2013) and development (Akhtar et al. 2013; Migliarini and Carnevali 2009), and a number of studies have been performed on adult zebrafish (for a recent overview of work on the ECS in zebrafish, see Krug and Clark 2015).

Here, we aim to use zebrafish larvae to determine the role of Cnr1 and Cnr2 on locomotion. For this purpose, we have analyzed behavior of zebrafish larvae using a visual motor response (VMR) test, which includes both a bright phase and a dark phase or 'challenge' of 4 min. Zebrafish larvae display escape and avoidance behavior in response to threatening tactile, acoustic or visual stimuli (Colwill and Creton 2011). Because zebrafish larvae are scotophobic (averse to darkness) (Maximino et al. 2010; Steenbergen et al. 2011), the VMR assay allows for analyzing anxiety-like behavior such as hyperactivity and a startle response when the lights are turned off (Burgess and Granato 2007; Ellis et al. 2012; Peng et al. 2016), in addition to basal locomotion when the lights are on.

In order to determine the role of Cnr1 and Cnr2 in mediating the observed locomotor effects, we exposed the larvae to specific cannabinoid receptor agonists and antagonists and we utilized a *cnr1*$^{-/-}$ mutant line (Liu et al. 2016). Studies done in other animal models showed that activation of Cnr1 affects motor behavior (Rodriguez de Fonseca et al. 1998; Wiley et al. 2014), whereas Cnr2 is generally considered to be psychoinactive (Fernandez-Ruiz et al. 2007). It can therefore be hypothesized that only modulation of Cnr1 affects locomotion, but it should be noted that receptor specificity may vary between species (Atwood and Mackie 2010). Our data show that activation of Cnr1 by exogenous cannabinoids results in a strong dose-dependent inhibition of both basal and dark challenge-induced locomotion in zebrafish larvae. Interestingly, inactivation of Cnr1 does not have an effect on locomotion, suggesting that endogenous cannabinoids are not involved in the regulation of locomotor activity at this stage of development.

Materials and methods

Embryo care

Fish were maintained and handled according to the guidelines on the ZFIN website (ZFIN, http://zfin.org). Fertilization was performed by natural spawning (group crossings), and eggs were initially raised in 10 cm Petri dishes containing 50 mL of 10% Hanks' balanced salt solution (HBSS; for specifications see (Ali et al. 2011), on a 14h light:10h dark cycle at 28°C. At 1 day post fertilization (dpf) the eggs were put individually in a 96 well plate (Costar 3599, Corning Inc., NY, USA) with 250 µL 10% HBSS. The larvae were left until 5 dpf. All analyses were performed at 5 dpf between 11:00 and 15:00. Tubingen (Tu) wild type fish were used, as well as the cannabinoid receptor 1 mutant line *cnr1*$^{-/-}$ (Liu et al. 2016), kindly provided by Prof. Wolfram Goessling of Harvard Medical School.

Test compounds

The following compounds were used: WIN55,212-2, HU-910 and AM251 (Hoffmann-La Roche, Switzerland); CP55,940, (−)-nicotine (Sigma-Aldrich, MO, USA); JWH-133 (Tocris Bioscience, UK) and ethanol (98% purity; Boom, The Netherlands). All compounds were dissolved in 10% HBSS, and dimethylsulfoxide (DMSO) was used as a carrier (final concentration of 0.08% DMSO). Larvae treated with vehicle (0.08% DMSO in 10% HBSS) showed no difference in activity compared to the control group (10% HBSS). The applied concentrations were based on pilot experiments; lower concentrations were ineffective while higher concentrations were toxic. When the treatment consisted of exposure to 1 compound, 50 µL of this compound was added to a total volume of 300 µL. When the treatment consisted of exposure to 2 compounds, 25 µL of the first compound was added, 15 min later followed by addition of 25 µL of the second compound.

Behavioral analysis

After addition of the compound(s), the 96 well plate was transferred to the recording apparatus (ZebraBox, Viewpoint S.A., France) and the recording started immediately after. The experimental recording consisted of three steps. First, larvae were acclimated to the behavioral setup with lights on for 4 min. This period was kept short since the Cnr1 is known to become rapidly desensitized upon prolonged activation (Hsieh et al. 1999). Second, a dark challenge of 4 min lights off was applied, which results in hyperactive behavior. Third, the larvae were left to recover for 30 min with the lights on. To investigate the effect of desensitization of the Cnr1, a different protocol was introduced. In this protocol the 4 min lights on acclimatization phase was followed by 3 rounds of alternating 4 min lights off and 30 min lights on periods. Videos were recorded using FlyCapture software (Point Grey, Canada) at 24 frames per second, and were analyzed using EthoVision 10 XT (Noldus, The Netherlands). Larvae that were dead at the beginning of the experiment were excluded from the analysis. The activity of each larva was assessed by determining the distance moved during 1 min periods, and is presented as average velocity (mm/min). We defined the startle response as a movement with a minimum velocity of 15 mm/s during the first 5 seconds after the lights went off. Using these thresholds we excluded non-startle behavior. This approach was validated by analyzing our videos for embryos with a C- or O-shaped body flexure (Burgess and Granato 2007; Eaton et al. 1977), which is a startle characteristic. Each experiment was performed three times, using a different clutch of eggs each time. Data shown are means of all larvae ± standard error of the mean (SEM).

Statistics

The experimental data were analyzed with a one-way analysis of variance (ANOVA) with the concentration or compound as variable. A Dunnett's post-hoc test was performed to analyze multiple comparisons and statistical significance was reported at $p \leq 0.05$. All analyses were done, and all graphs created with, GraphPad Prism 7 (GraphPad Software Inc., CA, USA).

Results

The visual motor response (VMR) test

In the present study, a behavioral assay often referred to as the visual motor response (VMR) test (Emran et al. 2008) has been used to investigate the role of the ECS on swimming kinematics in zebrafish larvae. In this assay, the larvae are first allowed to acclimatize to the setup, and then anxiety-like behavior is induced by turning off the light (Ellis et al. 2012; Peng et al. 2016). There is an increased swimming velocity during the dark period (Fig. 1a). When the lights are turned on again, the fish recover and locomotion rapidly returns to basal levels. The graph presented in Fig. 1b shows the average velocities during all three phases of the experiment, and in the following figures data will be presented like this.

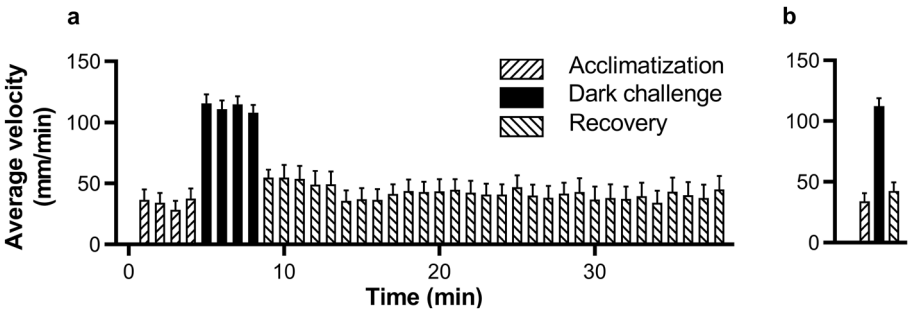

Fig. 1 Behavior of zebrafish larvae assayed using the VMR test. **a** Average swimming velocities of vehicle-treated larvae per one-minute interval. In the first 4 min the larvae acclimatize, with the lights on ('Acclimatization'). This period is followed by a 4 min dark challenge, which is associated with increased locomotor activity, reflecting anxiety-like behavior ('Dark challenge'). In the final phase the fish are allowed to recover for 30 min with the lights on again ('Recovery'). **b** The average velocities from each phase were determined, and these average values are presented in a bar graph. This type of graph is used also in Figures 2-8. Data shown are means ± SEM

Dual Cnr agonists, but not Cnr2 agonists, decrease locomotor activity

The VMR test was used to investigate the behavioral effects of activation of Cnr1 and Cnr2. First, we administered the Cnr1 and 2 dual agonists WIN55,212-2 and CP55,940. These compounds are the most commonly applied and well-characterized Cnr agonists available. We found that WIN55,212-2 produced a dose-dependent reduction of locomotor activity in both the light and dark phases (Fig. 2a). In the concentration range 32-8000 nM there was a significant, dose-dependent suppression of the average swimming velocity in both the light and dark phases compared to controls (vehicle only). Treatment with another dual Cnr agonist, CP55,940, also resulted in inhibitory effects on locomotion (at 500 nM and higher, Fig. 2b). The maximum inhibitory effect for the dark phase is reached at 2000 nM for WIN55,212-2. However, the maximum inhibitory effect for the acclimatization and recovery phase is reached at lower concentrations (125 nM and 32 nM, respectively).

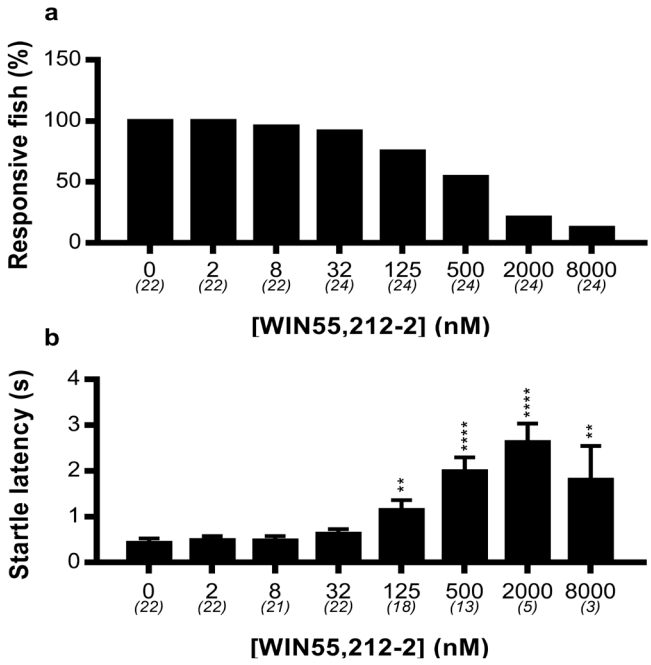

Fig. 2 Effect of dual Cnr agonists on the average swimming velocity in the VMR test. **a** The effect of WIN55,212-2. This agonist causes a dose-dependent inhibition of swimming velocity in both the light and dark phases. **b** The effect of CP55,940. This agonist also inhibits locomotion in the light and in the dark phase in a dose-dependent way. Group-sizes are reported in parentheses. Data shown are means ± SEM. Significant differences compared to the corresponding vehicle-treated control group are indicated.* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$

The dark challenge induces a strong startle response, which is often observed as an immediate reaction to a threatening stimulus (Peng et al. 2016). We analyzed the effect of WIN55,212-2 on the startle response. The number of fish showing a startle response to the dark-challenge showed a dose-dependent decrease after administration of WIN55,212-2 (Fig. 3a). Of 24 fish exposed to the concentrations of 2000 and 8000 nM, only 5 and 3 fish, respectively showed a startle response to the dark challenge. The startle latency (reaction time) of the responsive fish increased two-fold at a concentration of 125 nM and four-fold at a concentration of 500 nM (Fig. 3b).

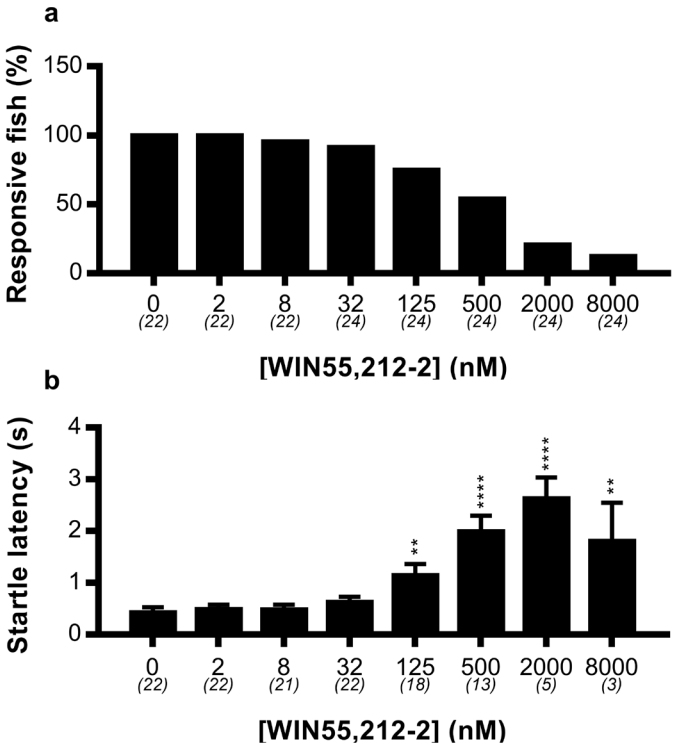

Fig. 3 The effect of WIN55,212-2 on the startle response after a dark challenge. The behavior of the larvae during the first 5 s of the dark challenge was analyzed. **a** Percentage of larvae responding to the dark challenge by showing increased swimming velocity. From 125 nM and higher, a strong decrease of responsive fish can be noticed. **b** From the responsive fish, the reaction time was calculated. The latency was strongly reduced at concentrations of 125 nM and higher. Group-sizes are reported in parentheses. Data shown are means ± SEM. Significant differences compared to the corresponding vehicle-treated control group are indicated. *** $P \leq 0.001$; **** $P \leq 0.0001$

To investigate whether the inhibiting effect of WIN55,212-2 and CP55,940 was Cnr1 or Cnr2 mediated, we applied the specific Cnr2 agonists HU-910 and JWH-133. Administration of these two compounds did not result in any effect on locomotion, either during the basal phase or dark-challenge phase (Fig. 4). To validate if this inhibiting effect on locomotion was thus Cnr1-mediated, we used a Cnr1 mutant line (Liu et al. 2016). In these *cnr1*$^{-/-}$ larvae we found no inhibitory effect of WIN55-212,2 or CP55,940 on the average swimming velocity in either the light or dark phases (Fig. 5). In fact, there was an opposite off-target effect: the velocity in the dark phase was increased by WIN55-212,2.

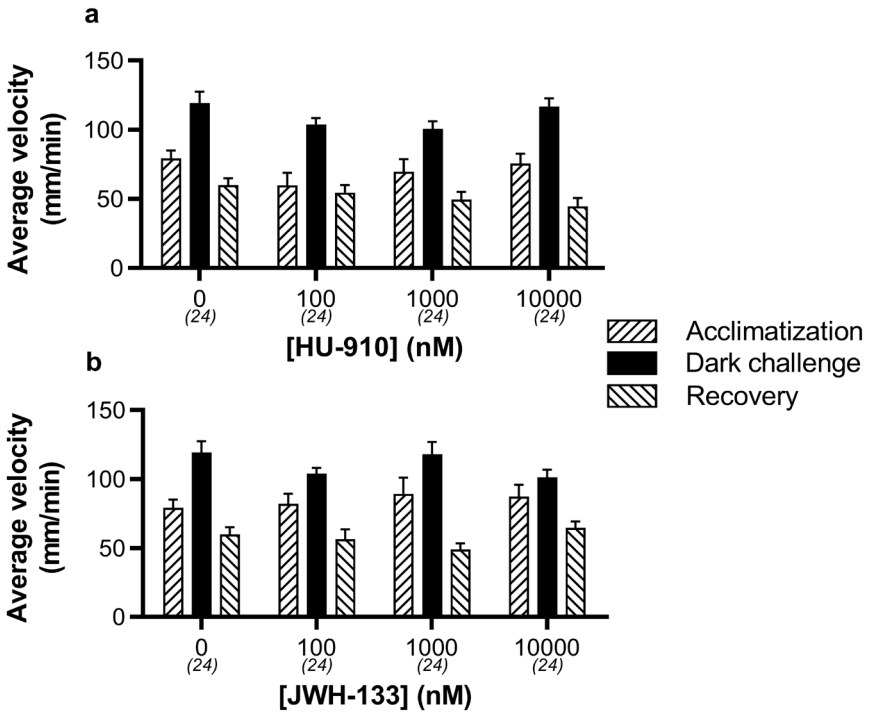

Fig. 4 Effect of Cnr2 agonists on the average swimming velocity in the VMR test. **a** HU-910 and **b** JWH-133 have no effect on locomotion, in contrast to Cnr agonists WIN55,212-2 and CP55,940. Group-sizes are reported in parentheses. Data shown are means ± SEM

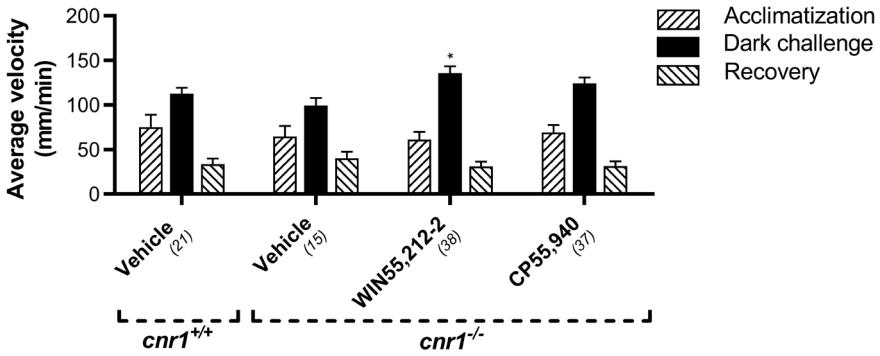

Fig. 5 The effect of WIN55,212-2 and CP55,940 on locomotion in *cnr1*$^{-/-}$ zebrafish larvae. The inhibitory effect of WIN55,212-2 (0.5 μM) and CP55,940 (2 μM) on locomotion in both the dark and the light phase, as observed in wild type larvae (Fig. 2), was absent in the *cnr1*$^{-/-}$ larvae. In fact, a slight increase in mobility during the dark phase was observed in the WIN55,212-2-treated larvae, as compared to the vehicle-treated larvae. No differences were found between the vehicle-treated *cnr1*$^{-/-}$ and *cnr1*$^{+/+}$ larvae. Group-sizes are reported in parentheses. Data shown are means ± SEM. A significant difference compared to the corresponding vehicle-treated control group is seen. * $P \leq 0.05$

The Cnr1 antagonist AM251 does not affect locomotor activity, but blocks the effect of WIN55,212-2

To investigate the effect of a pharmacological inhibition of Cnr1, we applied the Cnr1 antagonist AM251 in our assay. Treatment with AM251 (0.5 μM) did not affect locomotor activity in either the light or the dark phase, suggesting that endogenous Cnr1 agonists (AEA and 2-AG) do not affect locomotor behavior under these conditions. However, AM251 pre-treatment blocked the inhibitory effect of WIN55,212-2 (125 nM) treatment on locomotion. The antagonist abolished the WIN55,212-2 effects on the average velocity in both the light and the dark phase (Fig. 6).

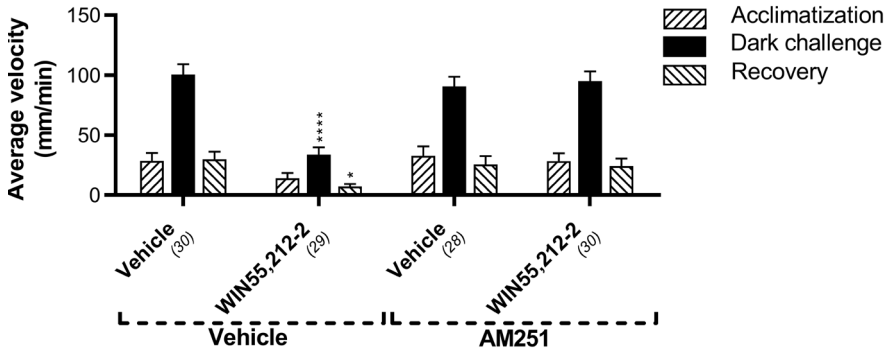

Fig. 6 The effect of AM251 on locomotion of WIN55,212-2-treated zebrafish larvae. Administration of the Cnr1 antagonist AM251 (0.5 µM) showed no effect on swimming velocity, but abolished the effect of WIN55,212-2 (125 nM) on locomotion. Group-sizes are reported in parentheses. Data shown are means ± SEM. Significant differences compared to the corresponding phase of the vehicle/vehicle-treated control group are indicated. * $P \leq 0.05$; **** $P \leq 0.0001$

Ethanol and nicotine can increase locomotor activity in the presence of WIN55,212-2

To study whether the inhibitory effect of WIN55,212-2 on locomotor activity is an effect of a decreased ability to move, we administered ethanol (1% v/v) 15 min after treatment with WIN55,212-2 (125 nM). Acute ethanol exposure is known to increase the locomotor activity of zebrafish larvae (Guo et al. 2015; MacPhail et al. 2009). In our assay, ethanol indeed increased the swimming velocity of the larvae. Interestingly, ethanol administration also increased the locomotor activity in the presence of WIN55,212-2 in both the light and the dark phase (Fig. 7).

A similar experiment was performed using nicotine, which has also been shown to increase the locomotor activity of zebrafish larvae as well (Petzold et al. 2009). Similarly to ethanol treatment, nicotine treatment (10 µM) increased the swimming velocity of the larvae. This treatment also increased the locomotor activity in the presence of WIN55,212-2 in both the light and dark phase.

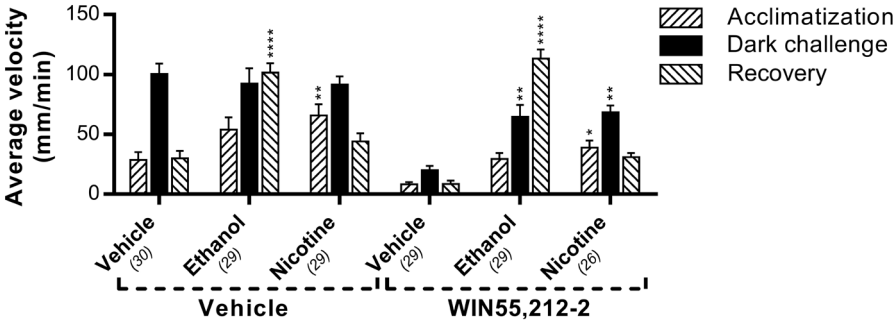

Fig. 7 The effect of ethanol and nicotine on locomotion of WIN55,212-2-treated larvae. Administration of ethanol (1% v/v) and nicotine (10 μM) to WIN55,212-2-pre-treated (125 nM) larvae increases the locomotion in both the light and dark phase, indicating that the immobility induced by the Cnr1 agonist is not due to a physical limitation. Group-sizes are reported in parentheses. Data shown are means ± SEM. Significant differences compared to the corresponding phase of the vehicle/vehicle or WIN55,212-2/vehicle-treated control group are indicated. * $P \leq 0.05$; ** $P \leq 0.01$; **** $P \leq 0.0001$

Desensitization of Cnr1

G protein-coupled receptors (GPCRs) can become desensitized upon prolonged activation (Gainetdinov et al. 2004). Therefore, we tested the desensitization of the Cnr1 upon activation by WIN55,212-2 (2000 nM). Larvae were exposed to three subsequent dark challenges, separated by 30 min with lights on (Fig. 8). In the vehicle-treated larvae all three dark challenges elicited a similar locomotor response, and no differences between the locomotor activity of the light phases was detected. As observed before, WIN55,212-2 treatment abolished the behavioral response in the first dark challenge. However, the second dark challenge did elicit a response and this was increased in the third dark period (17.0 ± 4.8 and 48.4 ± 7.5 mm/min respectively), although it was still decreased compared to the vehicle-treated larvae. The decreased locomotor activity in the light phases did not change over time. These data demonstrate a desensitization of the Cnr1, which is reflected in a decreased inhibition of the behavioral response to a dark challenge, but not in a decreased inhibition of the mobility under light conditions.

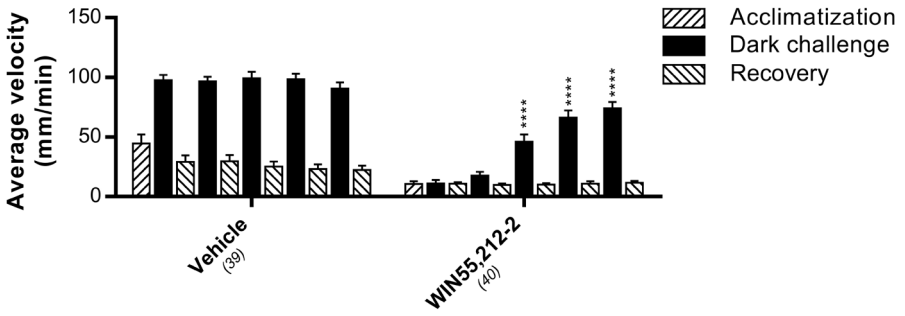

Fig. 8 The effect of WIN55,212-2 on average swimming velocity upon repeated dark challenges. The response to repeated dark challenges did not significantly change in vehicle-treated larvae. WIN55,212-2 (2 μM) abolished the response to the first two dark challenges, but a reduced effect of this Cnr1 agonist was observed on the response to the third, fourth and fifth dark challenge, indicating receptor desensitization. Group-sizes are reported in parentheses. Data shown are means ± SEM. A significant difference compared to the first dark challenge is indicated. **** $P \leq 0.0001$

Discussion

In this study, we have functionally characterized the Cnrs in zebrafish larvae using a behavioral assay with pharmacological interventions. We have shown that the dual Cnr agonists WIN55,212-2 and CP55,940 have a pronounced dose-dependent inhibitory effect on zebrafish larval locomotion in the VMR test, both under basal conditions and after a dark challenge. These effects were not observed upon treatment with the Cnr2 agonists HU-910 and JWH-133. This shows that the inhibitory effects of WIN55,212-2 and CP55,940 on locomotion were Cnr1-mediated, which was also demonstrated using the Cnr1 antagonist AM251 and a *cnr1*$^{-/-}$ mutant. Administration of the Cnr1 antagonist AM251 alone does not affect locomotion in our assay, which suggests that the endogenous cannabinoids are not active in regulating locomotor activity in the zebrafish larvae at the developmental stage studied here.

The maximum inhibitory effect of WIN55,212-2 is reached at lower concentrations in the light phase (125 nM) compared to the dark phase (2000 nM), which means these compounds show a higher potency in the light than in the dark (Fig. 2). This might be explained by the locomotor activity being higher in the dark than in the light. Complete inhibition of the locomotion may thus require more Cnr1 activation in the dark than in the light. Interestingly, WIN55,212-2 dose-dependently decreases locomotion in the acclimatization phase, whereas CP55,940 does not. This discrepancy may be due to differences in the pharmacokinetics of these compounds, due to differences in for example skin adherence, absorption through the skin and distribution through the body. Previously, it has been shown that WIN55,212-2 diffuses across human skin faster than CP55,940 (Valiveti et al. 2004).

To determine the specificity of the inhibitory effect of WIN55,212-2 in our zebrafish model, we applied the Cnr2 agonists HU-910 and JWH-133. These highly selective Cnr2 agonists do not inhibit locomotor activity, which is in line with the results obtained with the *cnr1*$^{-/-}$ mutant in our study and data from other studies (Hanus et al. 1999; Malan et al. 2001). However, in other publications inhibition of locomotion after Cnr2 agonist exposure has been shown (Kruk-Slomka et al. 2017; Onaivi et al. 2008; Xi et al. 2011). Exposure of *cnr1*$^{-/-}$ mutant larvae to WIN55,212-2 and CP55,940 did not result in any inhibitory effect on locomotion in these larvae. This indicates that the inhibitory effects are indeed Cnr1-mediated. In the same *cnr1*$^{-/-}$ larvae we found an increase in locomotor activity during the dark challenge (WIN55,212-2). This off-target effect may be due to developmental changes as a result of the Cnr1 deficiency, since it was not observed after co-administration with the Cnr1 specific antagonist AM251 (Fig. 6).

Different processes may be involved between the inhibition of locomotion upon

Cnr1 activation in the dark phase and in the light phase. Zebrafish larvae are scotophobic (Maximino et al. 2010; Steenbergen et al. 2011) and show anxiety-like behaviors in the dark (Ellis et al. 2012; Peng et al. 2016). Because cannabinoids have anxiolytic properties (Korem et al. 2016; Morena et al. 2016; Patel et al. 2017), it could be that, next to the inhibition of motor functioning, a second, anxiety-related, component is playing a role in the dark challenge. The locomotion is indeed lowered in the dark phase, but locomotion is also inhibited under basal circumstances (lights on). This suggests that locomotion itself is impaired due to the treatment, and with this test we are thus not able to distinguish anxiety-related effects from locomotion-related effects in the dark phase. A more specific anxiety-assay, such as the light-dark preference test (Steenbergen et al. 2011), should be used to study the potential anxiolytic properties of cannabinoids in zebrafish. Administration of WIN55,212-2 not only inhibits locomotion, but also impairs the startle response. The number of larvae responding with a startle was reduced and the startle latency was increased. However, since the locomotion is reduced in the light phase as well, we cannot determine whether the inhibitory effect on the startle response is caused by an impaired motor system or if the startle reflex itself is affected. Using our images, we were not able to discriminate between different types of previously described startle responses of zebrafish. These responses include the C-bend that has been observed upon acoustic/vibrational stimuli and is mediated by Mauthner cells (Eaton et al. 1977), and the O-bend that has been described in response to a sudden decrement in light intensity (Burgess and Granato 2007). This latter response is independent of the Mauthner circuitry and considered to be primarily navigational. We suggest that the observed startle responses in our experiments most likely involve O-bends, since they are elicited by a dark stimulus (although it should be noted that the stimulus used in our study slightly differed from the dark flash demonstrated to elicit O-bends (Burgess and Granato 2007)).

Interestingly, AM251 alone does not alter the swimming kinematics, a finding consistent with the study of Akhtar et al., who found no effect of 0.5 mg/L (0.9 μM) AM251 on locomotion in 5 dpf zebrafish larvae (Akhtar et al. 2013). Higher concentrations (> 4 μM) resulted in toxic effects, which could explain the reduced locomotion found by Akhtar *et al*. at concentrations of 4 mg/L (7.2 μM) or higher. Our data indicate that at the early larval stages of development the endocannabinoid levels are insufficient to modulate locomotion, or that the system is not sensitive enough yet to be modulated by these endogenous levels, even though a complete ECS (including the metabolic enzymes and endogenous ligands) is present in the developing zebrafish larvae (Martella et al. 2016; Oltrabella et al. 2017). Studies done on rodents showed different outcomes upon modulating endogenous signaling, with some researchers reporting inhibition of locomotion (Cosenza et al. 2000; Long et al. 2009; Mallet et al. 2008), while others found no effect (Celorrio et al. 2016; Komaki et al. 2015).

When we looked at the effect of the Cnr1 agonists on locomotion upon prolonged exposure, we found that the inhibitory effect decreases in the dark phase, while it remains in the light phase. We think that this reduction in the dark phase can be attributed to a mechanism commonly referred to as desensitization, which is a well-known effect for GPCRs (Rajagopal and Shenoy 2018).

In a previous study the effect of longer exposure (1-96 h) of cannabinoids on larval zebrafish locomotion was investigated (Akhtar et al. 2013). In that study, 1 h exposure to relatively high concentrations (1.1-3.4 μM for WIN55,212-2, and 6-48 μM for CP55,940) were used, which must have resulted in desensitization of the receptors, according to our results. Using a light and dark protocol, it was found that cannabinoids THC, WIN55,212-2 and CP55,940 cause hyperlocomotion in the dark, and hypolocomotion in the basal light phase. The hypolocomotion under basal conditions is in line with our data, whereas the hyperlocomotion in the dark phase is opposite to our results. Different mechanisms of action could play a role here. The relatively high concentrations combined with a relatively long exposure time may result in desensitization of the receptors and potential off-target effects (Hajos and Freund 2002; Hudson et al. 2016). We found desensitization after exposure to 2000 nM WIN (Fig. 8, third dark challenge), but also for 500 nM (third dark challenge, data not shown) and even faster for 8000 nM (second dark challenge, data not shown). Furthermore, the way of administration of the compounds could affect the behavior. Akthar *et al*. replaced 175 μL of the 250 μL of swimming water, whereas we added 50 μL of compound resulting in a final volume of 300 μL. Finally, different strains of zebrafish were used, which may show different behavior. Recently it was shown for example that the AB and TL strain differ in baseline HPI-axis activity, habituation to acoustic stimuli and motor behavior (van den Bos et al. 2017). These differences could contribute to the apparent discrepancies between their study and ours.

The cannabinoid-induced effect on locomotion is often associated with the modulating function of the ECS on dopamine transmission (Fernandez-Ruiz et al. 2010). Interestingly, Lam *et al*. reported co-localization of the *cnr1* mRNA and tyrosine-hydroxylase, the rate-limiting enzyme for dopamine synthesis, in the caudal region of the zebrafish hypothalamus (Lam et al. 2006). The authors suggest that this particular region may be involved in regulating locomotion. Another study found that dopamine receptors do indeed have a pronounced effect on locomotor development and motor activity in zebrafish larvae, although this was not related to any specific brain region (Lambert et al. 2012). In mice it was shown that dopamine receptor (D)1 agonist quinelorane and D2 agonist 6-Br-APB were both able to attenuate motor dysfunction caused by Cnr agonist levonantradol (Meschler et al. 2000). In rats, the Cnr1 antagonist rimonabant blocked the cataleptic effect of CP55,940, but was not able to block the

catalepsy elicited by D1 and D2 receptor antagonists (SCH 23390 and raclopride respectively) (Anderson et al. 1996). Interestingly, CP55,940 did potentiate the catalepsy induced by the D1 and D2 receptor antagonists. This suggests that the ECS plays a role upstream of the dopamine receptors and may be able to modulate the endogenous dopamine tone, which is an interesting subject for future study.

Since at high concentrations of dual Cnr agonist WIN55,212-2 the fish in our study were completely immobile, we hypothesized that they were unable to swim. Therefore we tried to induce recovery of the fish by administering ethanol or nicotine. It has been shown previously that both ethanol (1%) and nicotine (10 μM) strongly induce locomotor activity (MacPhail et al. 2009; Petzold et al. 2009). Our results confirm that both ethanol and nicotine induce hyperlocomotion (in the recovery phase and acclimatization phase respectively), but do not in the dark phase. The locomotion in the dark phase may have reached its ceiling level and therefore cannot go any higher. The delayed response to ethanol compared with nicotine can probably be explained by a slower uptake rate of ethanol. Administration of either ethanol or nicotine increased locomotor activity even in the presence of WIN55,212-2, which shows that the ECS does not limit physical ability to swim and does not directly affect the motor neurons of the somatic nervous system. Since the locomotion-modulating effects of ethanol and nicotine are regulated by altering dopaminergic signaling (Arias et al. 2010; King et al. 2004), it is reasonable to assume that ethanol and nicotine overrule the effect of the ECS on the dopamine receptor. This suggests that the inhibiting effect of the ECS on locomotion is solely mediated by the dopamine receptor, and is not caused by a direct effect on motor neurons.

In conclusion, we have shown that activation of Cnr1 in zebrafish larvae suppresses locomotion both in basal conditions and during a dark challenge. As a result, this study provides an assay which can be used to determine the sensitivity of the Cnr1 *in vivo*, using its behavioral effects as a readout. The activity of endogenous ligands for the Cnr1 do not affect the outcome of our assay, which makes it very suitable for studying the effects of exogenous manipulation. Therefore, this assay can be used as a tool for genetic and chemical screening to unravel novel pathways involved in the modulation of Cnr1 signaling and the link between Cnr1 activity, dopamine signaling and locomotion.

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Chapter 3

The effect of cannabinoid receptor 1 activation on anxiety-like behavior in zebrafish larvae

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Abstract
Anxiety disorders are among the most common mental disorders. Pharmacological treatment is intensive and close supervision is required to secure a balance between benefits and adverse effects; the latter including tolerance and dependence. The endocannabinoid system (ECS) has emerged as a potential drug target for the treatment of anxiety disorders. The ECS is a signaling system which comprises the endocannabinoids anandamide and 2-arachidonoylglycerol and the cannabinoid receptors 1 and 2 (Cnr1 and Cnr2), which regulate neurotransmitter release and thus modulate neuronal excitability. The zebrafish larva is a promising model for the screening of psychoactive drugs, and its ECS is highly homologous to that of rodents and other mammals. In the present study, we have investigated the effect of Cnr1 activation on anxiety-like behavior in zebrafish larvae, using a light/dark preference test. The activation of Cnr1 by the agonist WIN55,212-2 had an anxiolytic effect, which was abrogated in a *cnr1*$^{-/-}$ mutant line, and by co-administration of the Cnr1 antagonist AM251. Mutation of the cnr1 gene, administration of AM251 alone, or increasing levels of the endocannabinoid anandamide by chemical inhibition of the enzyme fatty acid amide hydrolase (FAAH), did not change anxiety-like behavior. These results show that in zebrafish larvae the endogenous activity of the ECS is insufficient to modulate anxiety-like behavior, but that administration of an exogenous Cnr1 agonists reduces anxiety-like behavior. Therefore, zebrafish larvae represent an excellent model to study the behavioral effects of pharmacological Cnr1 activation and screen for novel anxiolytic drugs.

Introduction

Anxiety is a feeling of apprehension, fear or worry, often without a specific threat and often out of proportion to the danger anticipated. Generalized anxiety disorder (GAD) is one of the most common mental disorders and leads to functional impairment and disability. Patients have high rates of absenteeism from work and are frequently hospitalized, and GAD has high rates of comorbidity with major depressive disorder and other mood disorders (Revicki et al. 2012). Current first-line pharmacotherapy for GAD consists of selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), along with benzodiazepines such as valium (Koen and Stein 2011). Although SSRIs and SNRIs have proven efficacy in both the short-term and long-term treatment of GAD, they are both associated with side effects such as insomnia, nausea, headache, fatigue and withdrawal effects on discontinuation (Bandelow et al. 2017). Benzodiazepines are not recommended for use as routine treatments of anxiety because of their addictive potential and because of adverse effects, including sedation and cognitive impairment (Baldwin et al. 2014). As a result, currently there is a great demand for novel anxiolytic drugs.

The endocannabinoid system (ECS) constitutes a lipid signaling system which can modulate both neuroendocrine and inflammatory pathways. It comprises the cannabinoid receptors 1 and 2 (Cnr1 and Cnr2), the endocannabinoids (eCBs) anandamide and 2-arachidonoylglycerol (AEA and 2-AG) and the metabolic enzymes involved in synthesis or degradation of those ligands. Cnr1 and Cnr2 are presynaptic G-protein-coupled receptors (GPCR). Cnr1 is mainly expressed in the central nervous system and activation results in inhibition of neurotransmitter release by inhibiting adenylate cyclases and N- and P/Q-type Ca^{2+}-channels, and by activating K^{+} channels (Freund et al. 2003). By regulating excitatory and inhibitory synaptic neurotransmission, Cnr1 mediates several cognitive functions, such as memory, mood, stress and anxiety. Cnr2 is abundantly expressed on immune cells, including lymphocytes and macrophages, and plays an important role in immune regulation (Cabral and Griffin-Thomas 2009). Recently it was shown that this receptor also has neuronal effects, as both expression and functional effects of Cnr2 were shown in the brain (Chen et al. 2017).

Several researchers have indicated that the ECS could be a potential drug target for the treatment of anxiety disorders (Chhatwal and Ressler 2007; Gaetani et al. 2009; Hill and Gorzalka 2009; Patel et al. 2017; Ruehle et al. 2012). The ECS has been mentioned as one of the top therapeutic targets for posttraumatic stress disorder (Krystal et al. 2017), a highly prevalent major depressive disorder in people following a major traumatic experience such as warfare that is often comorbid with GAD. However, relatively few studies have been conducted on the relationship between anxiety and the ECS in humans. Most of these studies indi-

cate that eCBs are indeed involved in anxiety modulation, although most clinical studies to date have not used objective or clinician-rated measures of anxiety, or included only very small sample sizes. For these reasons, based on existing clinical studies, no robust conclusion on the effect of modulation of the human ECS on anxiety can be drawn (Lisboa et al. 2017; Mandolini et al. 2018; Turna et al. 2017). In studies done on the ECS of experimental animals, both acute and chronic anxiety tests have been applied (Lisboa et al. 2017). In general, pharmacological and genetic manipulation of the ECS show that cannabinoids are involved in the regulation of anxiety. Most of these animal studies have been done on rodents, and these studies can be time-consuming and costly. Furthermore, rodents lack expression of fatty acid hydrolase subtype 2 (Faah2), an enzyme that metabolizes AEA by hydrolysis, which is present in other mammals and fish. Another animal model, to be used complementary to the common rodent models, could thus be advantageous in studying the ECS *in vivo*.

The zebrafish larva is a popular animal model which is well-developed for biomedical research and is widely used to complement data from rodent models (Ahmad et al. 2012; Khan et al. 2017). Easy maintenance, rapid development and high fecundity are features which have further increased interest in this model. The zebrafish genome has been sequenced and is well characterized. Genetic manipulation is relatively easy, and zebrafish are physiologically and genetically highly homologous to humans. Zebrafish have also been mentioned as a promising animal model for studying complex brain disorders (Kalueff et al. 2014), and the zebrafish neurotransmitter system is highly comparable to the mammalian neurotransmitter system (Gomez-Canela et al. 2018). Several interesting tools have emerged over the last few years. The optical transparency of zebrafish larvae allow for *in vivo* mapping of neuronal circuits in behaving fish (Feierstein et al. 2015), using for example calcium indicators, such as GCaMPs (Fosque et al. 2015; Turrini et al. 2017).

Most zebrafish ECS genes show an orthologous relationship with the human ECS genes (McPartland et al. 2007), and there is a high degree of conservation between the zebrafish and mammalian ECS receptors and metabolic enzymes (Demin et al. 2018). These properties make the zebrafish a very interesting complementary model to study the ECS. Most research on this subject has focused on the developmental effects of cannabinoids (Ahmed et al. 2018; Akhtar et al. 2013; Carty et al. 2019; Carty et al. 2018; Migliarini and Carnevali 2009; Thomas 1975; Valim Brigante et al. 2018; Watson et al. 2008), but effects on metabolism (Liu et al. 2016; Migliarini and Carnevali 2008; Nishio et al. 2012; Pai et al. 2013; Silvestri et al. 2015), memory (Ruhl et al. 2015; Ruhl et al. 2014; Ruhl et al. 2017) and anxiety (Barba-Escobedo and Gould 2012; Connors et al. 2013; Ruhl et al. 2014; Stewart and Kalueff 2014) have been studied as well. The research

on the role of the ECS in anxiety-like behavior has until now mainly focused on adult zebrafish.

In a previous study (Chapter 2), we have analyzed zebrafish larval behavior using a visual motor response (VMR) test. Although this assay usually allows for analyzing anxiety-like behavior, we were not able to distinguish anxiety-related effects of tested cannabinoids, due to coinciding locomotor-related effects. In the present study we have investigated potential modulation of anxiety-like behavior by the ECS in zebrafish larvae using a different assay. For this purpose, we have utilized a light/dark preference test, which is commonly used to study anxiety-like phenotypes (Steenbergen et al. 2011). This test consists of a plastic box, which is divided into a bright and a dark compartment. Since zebrafish larvae are scotophobic (Maximino et al. 2010), they incline to move or stay more in the bright zone compared to the dark zone. Our data show that upon treatment with Cnr1 agonist WIN55,212-2, zebrafish larvae move relatively more in this zone dark zone, spend relatively more time in the dark zone and move sooner into the dark zone. This effect is Cnr1-specific, since neither pharmacological inhibition with the Cnr1 antagonist AM251, nor genetic knockout of the *cnr1* gene abolished the effect of WIN55,212-2. Furthermore, our results suggest that endogenous cannabinoids are not involved in regulating anxiety during this developmental phase.

Materials and methods

Zebrafish strains and husbandry

Zebrafish (*Danio rerio*) were handled and maintained according to the ZFIN guidelines (ZFIN, http://zfin.org). Group crossings were set up to stimulate natural spawning and fertilization. Eggs were raised in 10 cm Petri dishes containing 50 mL of 10% Hanks' balanced salt solution (HBSS; for specifications see (Ali et al. 2011)), on a 14h light:10h dark cycle at 28°C. The behavioral analyses were performed at 5 dpf between the times of 11:00 and 15:00. Wild-type Tubingen (Tu) fish were used, and a cannabinoid receptor 1 knockout line (*cnr1*$^{-/-}$) (Liu et al. 2016)), was kindly provided by Prof. Wolfram Goessling of Harvard Medical School.

Test compounds

The following compounds were used: WIN55,212-2 and AM251 (MedChemExpress, Sweden), and PF-004457845 (Sigma-Aldrich, MO, USA). All compounds were dissolved in 10% Hanks Balanced Salt Solution (HBSS), and dimethylsulfoxide (DMSO) was used as a solvent (final concentration of 0.08% DMSO). The compounds and dosage selected were based on a previous study (Chapter 2 of this thesis) and pilot experiments. In the case of co-exposure (AM251 and WIN55,212-2), fish were first treated with AM251 for 15 minutes, after which fish were exposed to the combination of AM251 and WIN55,212-2.

Behavioral analysis

The behavioral test that was used to monitor anxiety-like behavior, was the so-called light/dark preference test, which has been characterized and validated before (Steenbergen et al. 2011). The light/dark preference test consists of a plastic box (dimensions: L, 45 mm x W, 30 mm x H, 21 mm), divided into an equally-sized bright and dark compartment (Fig. 1). A total of 4 boxes were placed in a frame, which was placed in the DanioVisionTM recording apparatus (Noldus, The Netherlands). A single larva was then carefully transferred from a Petri dish to the center of the bright compartment of each box, containing 5 mL of water containing the vehicle or test compound. We applied removable separators between the compartments, to prevent larvae from swimming into the dark zone before the start of the analysis. When each box contained one larva, the separators were removed and video recording was carried out for 15 minutes using an infrared camera. The camera recorded at 60 frames per second. Each treatment group consisted of at least 25 larvae.

Videos were analyzed in EthoVision® XT v. 12 software (Noldus, The Netherlands). The following parameters were measured: distance moved in the dark zone, as a percentage of total distance moved, to assess zone preference; time spent in the dark zone, as a percentage of total testing time, to correct for individual differences in swimming activity; latency to visit the dark zone, to assess anxiety for the dark compartment.

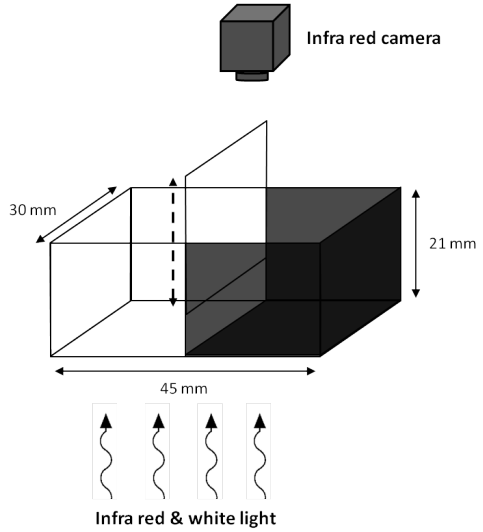

Fig. 1 Schematic overview of the light/dark preference test apparatus. It consists of an infrared camera and a plastic box, which is divided into two equally-sized compartments. Both compartments, one bright and one dark, are matte and opaque. A physical barrier (or separator) was placed between the two compartments, and this barrier was removed at the start of video recording.

Statistics

The experimental data were analyzed with a one-way ANOVA test with the concentration or compound as independent variable. Dunnett's post-hoc test was performed to analyze multiple comparisons. All analyses were done and all graphs were created using GraphPad Prism 7 (GraphPad Software Inc., CA, USA). Data shown are means ± standard error of the mean (SEM). Statistical significance was reported at $p \leq 0.05$.

Results

A light/dark preference test was used in order to screen for anxiolytic effects of ECS manipulation in zebrafish larvae. In this test, larvae are placed in a box containing a light and a dark compartment, and the distance moved and time spent in the dark compartment (as a percentage of the total distance and time), and the latency to enter the dark compartment for the first time are considered a read-out for anxiety. Since zebrafish larvae prefer the light compartment, high values for the first two parameters and low levels for the third parameter are considered to reflect low levels of anxiety (Steenbergen et al. 2011).

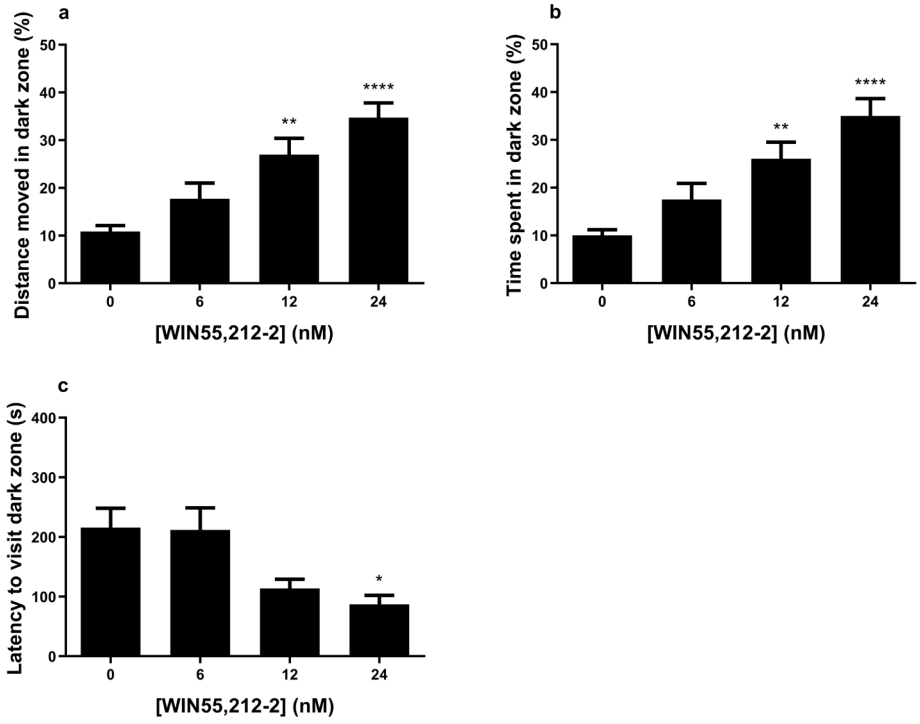

Fig. 2 The effect of WIN55,212 on anxiety-related behavior in the light/dark preference test. WIN55,212 dose dependently increases **a** the distance moved in the dark zone, **b** the time spent in the dark zone and **c** decreases the latency to visit the dark zone. Data shown are means ± SEM. Significant differences compared to the corresponding vehicle-treated control group are indicated.* $P \leq 0.05$; ** $P \leq 0.01$; **** $P \leq 0.0001$

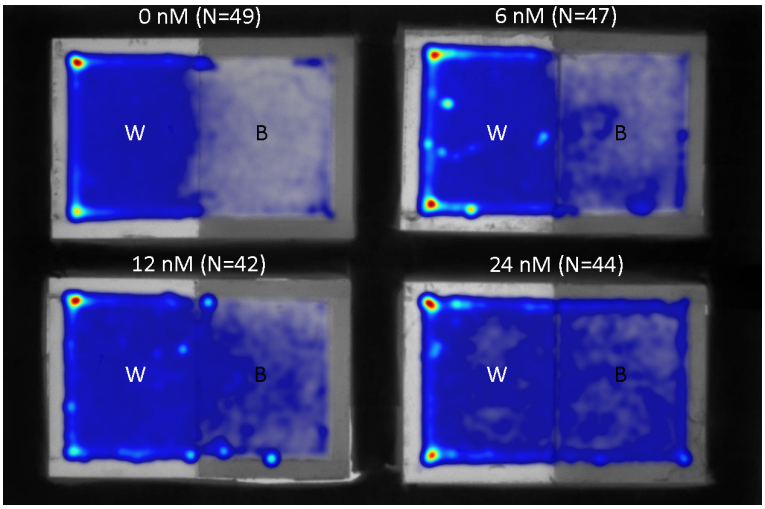

Fig. 3 General distribution of larvae in the light/dark preference test at different concentrations of WIN55,212-2. This heatmap was generated by EthoVision® XT v. 12 software (Noldus, The Netherlands) and shows the distribution of the larvae as represented by the data in Fig. 2.

Treatment with WIN55,212-2 results in an anxiolytic phenotypic

First, the Cnr1 agonist WIN55,212-2 was administered to study the effect of Cnr1 activation. The results showed that that with increasing concentration of WIN55,212-2, the relative distance moved and time spent in the dark zone increased. The relative distance moved in the dark was significantly higher in WIN55,212-2-treated larvae (12 and 24 nM, Fig. 2a) compared to the vehicle-treated group. WIN55,212-2-treated larvae not only moved more in the dark compartment, but also spent relatively more time in it (12 and 24 nM, Fig. 2b). The heat map in Fig. 3 confirms that with increasing concentrations of WIN55,212-2, larvae spent more time in the dark compartment. Furthermore, larvae treated with WIN55,212-2 (24 nM) had a lower latency to enter the dark compartment (Fig. 2c).

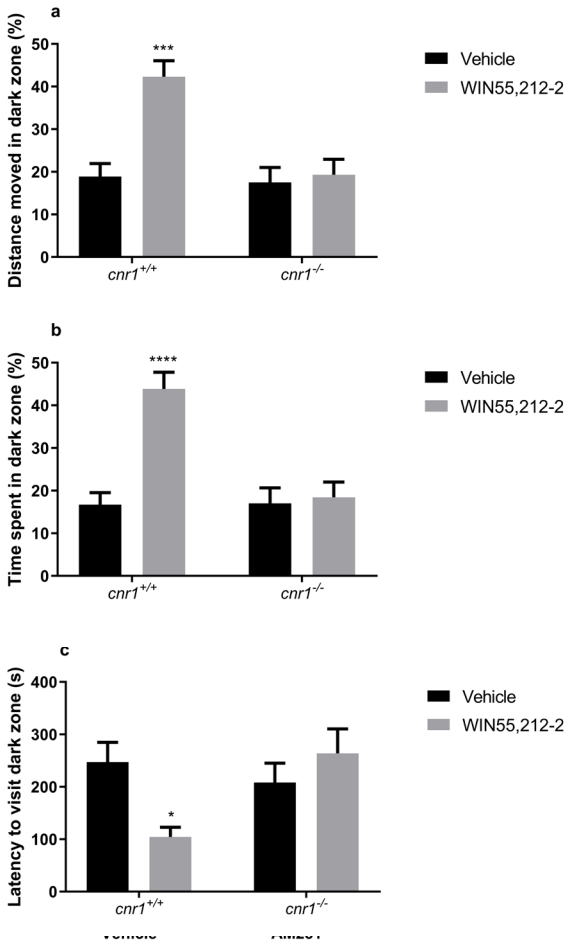

Fig. 4 Cnr1 specificity of the anxiolytic effect of WIN55,212-2. The anxiolytic effect of WIN55,212-2 (24nM), as shown by **a** increased distance moved in the dark zone, **b** increased time spent in the dark zone and **c** decreased latency to visit the dark zone, was abolished in the *cnr1*$^{-/-}$ mutant line. Data shown are means ± SEM. Significant differences compared to the corresponding vehicle-treated control group are indicated.* P ≤ 0.05; *** P ≤ 0.001; **** P ≤ 0.0001

The anxiolytic effects of WIN55,212-2 are Cnr1 specific

Subsequently, to investigate the Cnr1 specificity of the anxiolytic effect of WIN55,212-2, the compound was applied at a 24 nM concentration to a *cnr1*$^{-/-}$ mutant line (Fig. 4). No differences were found between the *cnr1*$^{+/+}$ and *cnr1*$^{-/-}$ line in the vehicle-treated groups. However, the anxiolytic effects of WIN55,212-

2, as shown by relative distance moved in dark zone (Fig. 4a), relative time spent in dark zone (Fig. 4b) and latency to visit the dark zone (Fig. 4c) were present in the *cnr1*$^{+/+}$ larvae, but absent in the *cnr1*$^{-/-}$ mutants.

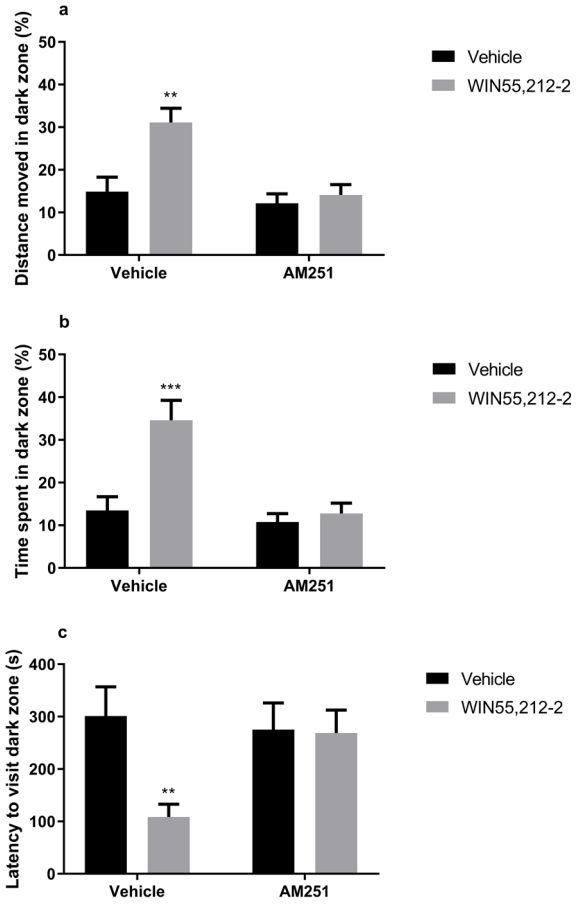

Fig. 5 Pharmacological verification of the cnr1 specificity of the anxiolytic effect of WIN55,212-2. The anxiolytic effect of WIN55,212-2 (24nM), as shown by **a** increased distance moved in the dark zone, **b** increased time spent in the dark zone and **c** decreased latency to visit the dark zone, was blocked by pretreatment with AM251 (0.5μM). Data shown are means ± SEM. Significant differences compared to the corresponding vehicle-treated control group are indicated. ** $P \leq 0.01$; *** $P \leq 0.001$

Treatment with AM251 or Faah inhibition does not affect anxiety-related behavior

We also tested Cnr1 specificity of the anxiolytic effect of WIN55,212-2 by using a specific Cnr1 antagonist. We found that pretreatment with the antagonist AM251 completely blocked the anxiolytic effects of WIN55,212-2 on the relative distance moved in dark zone (Fig. 5a), the relative time spent in dark zone (Fig. 5b) and the latency to visit the dark zone (Fig. 5c). Similarly to the effect of mutation of the *cnr1* gene, treatment with AM251 alone did not cause any effect in this assay.

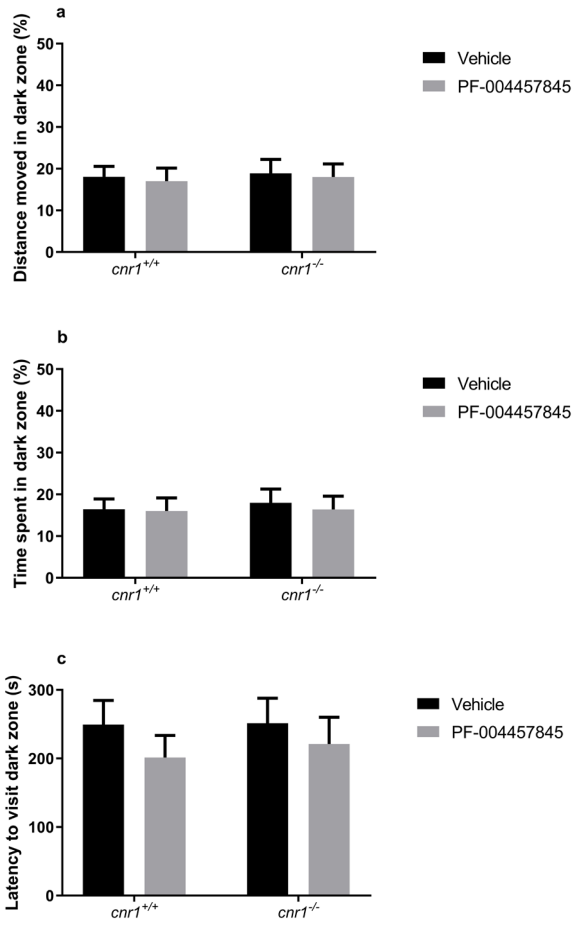

Fig. 6 Effect of PF-004457845 treatment (1μM) on behavioral parameters **a** distance moved in the dark zone, **b** time spent in the dark zone and **c** latency to visit the dark zone, in the light/dark preference test. Treatment with this FAAH inhibitor increases AEA-concentration by a 5-fold, but does not affect anxiety-related behavior in this assay. Data shown are means ± SEM.

Apparently, blocking Cnr1 activity, either genetically or pharmacologically, does not affect anxiety-related behavior (in vehicle-treated larvae), suggesting that there is little or no endogenous activity of the ECS under the conditions of this assay. To further investigate the endogenous ECS activity, we administered PF-004457845 (1 μM), an inhibitor of the metabolic enzyme Faah, to decrease the degradation of AEA. With the concentration and exposure time applied here, this results in a 5-fold increase of AEA (unpublished data, dr. V. Kantae). Our results show that this treatment, in contrast to exogenous activation by WIN55,212-2, does not affect the relative distance moved in the dark zone (Fig. 6a), the relative time spent in the dark zone (Fig. 6b), or the latency to visit the dark zone (Fig. 6c). Thus, even an increased activity of the endogenous ECS activity in zebrafish larvae does not affect the behavior in the light/dark preference test.

Discussion

In the present study, we have investigated the effects of Cnr1 activation on anxiety-related behavior using a light/dark preference test. We show that activation of Cnr1 by treatment with the Cnr1 agonist WIN55,212 has pronounced anxiolytic effects. WIN55,212-2-treated fish spent more time in the dark zone, they move relatively more in this zone, and they enter the dark zone sooner. These effects were not observed in a *cnr1*$^{-/-}$ mutant line, nor after pretreatment with the Cnr1 antagonist AM251. This implies that the anxiolytic effects of WIN55,212-2, observed in in our assay, are mediated specifically by Cnr1. Administration of AM251 alone or mutation of the *cnr1* gene does not affect anxiety-related behavior in our assay, which suggests that endogenous cannabinoids do not modulate anxiety in zebrafish larvae.

These data are in agreement with previous results (Chapter 2), in which we used a VMR test and showed that Cnr1-specific activation reduced the mobility of that larvae, and we could not distinguish between effects on locomotor activity and anxiety. In order to develop a simple assay that can be used to screen specific effects on anxiety-related behavior in a vertebrate organism, we studied the behavior of zebrafish larvae in the light/dark preference test. The light/dark preference test was initially developed for mice (Crawley and Goodwin 1980), and is based on the aversion of rodents to bright areas (Bourin and Hascoët 2003). It has been adapted for use in adult zebrafish (Maximino et al. 2010), and has subsequently been adjusted, characterized and validated for zebrafish larvae (Steenbergen et al. 2011). The latter test was developed to assess anxiety responses in zebrafish larvae, which, in contrast to rodents and adult zebrafish, prefer the light zone over the dark zone; anxiolytic compounds increase the time they spend in the dark. Using this test, we managed to show that WIN55,212-2 had an anxiolytic effect, causing a dose-dependent increase of the relative time spent and the relative distance moved in the dark zone, but also a lower initial latency to move into the dark.

In adult zebrafish, a few studies have been performed on the effects of cannabinoids on anxiety-related behavior, and most of them show anxiolytic effects. In one study, a comparable approach to ours, a light/dark cross maze, was used (Connors et al. 2013). Acute exposure to WIN55,212-2 had no effect on anxiety, but prolonged exposure (daily feeding with WIN55,212-2-containing dried food for 1 week) had an anxiolytic effect.

In another study, WIN55,212-2 was shown to have anxiolytic properties in a social interaction test (Barba-Escobedo and Gould 2012), and Stewart and Kalueff (Stewart and Kalueff 2014) found that acute exposure to delta (9)-tetrahydrocannabinol (THC), a Cnr1 agonist, reduced anxiety-related behavior (latency to

move to top, number of top transitions and time spent in the top) in a novel tank test. Interestingly, Ruhl *et al*. (2014) found no effect of THC exposure in an escape-response test (Ruhl et al. 2014), in which the fish were confronted with an approaching object.

In others species the picture is more complicated. Several studies, performed using various animals and protocols, showed biphasic effects (Bellocchio et al. 2010; Genn et al. 2004; Haring et al. 2011) upon cannabinoid exposure, with low doses inducing anxiolytic effects and high doses anxiogenic effects. In addition, the anxiety-related effects of cannabinoids differ between species. For example, in mice WIN55,212-2 has an anxiolytic effect, whereas it increases anxiety in rats (Haller 2007). This variation might be explained by differences in the balance of GABAergic and glutamatergic signaling, which has been shown to be different between species (Haller et al. 2007). Anxiety processing by cannabinoids may be dependent on this signaling. For example, anxiolytic effects after a low dose of Cnr1 agonist CP-55,940 were blocked in mice with a *cnr1* knockout in cortical glutamatergic neurons specifically, whereas the anxiogenic effects after a high dose were blocked in a *cnr1* knockout in forebrain GABAergic neurons only (Rey et al. 2012). Thus, data on the effects of cannabinoids on anxiety-like behavior are highly inconsistent between and within different species. Our model system using zebrafish larvae may in future studies help to reveal the factors that cause this variation in behavioral response to Cnr1 activation.

In our study, Cnr1 activation has an anxiolytic effect, but in which brain regions does Cnr1 act to mediate these effects? Interestingly, Lau *et al*. (2011) exposed adult zebrafish to the light/dark preference test and mapped *c-fos* neuronal activity in their brain (Lau et al. 2011). It was shown that dorsal telencephalon (Dm) activity predicates choice in anxiety-like behavior in zebrafish. It has been shown that *cnr1* is expressed in the (medial zone of the) Dm in both larval (Watson et al. 2008) and adult zebrafish (Lam et al. 2006), which suggests that Cnr1 directly manipulates anxiety-like behavior. In the adult zebrafish brain, several regions are involved in the regulation of anxiety, including the habenula (Fontana et al. 2018; Lau et al. 2011) and the medial zone of the Dm (Lau et al. 2011; von Trotha et al. 2014). Finally, it was shown that reduced avoidance behavior was associated with lower telencephalic gene expression levels of *cnr1* (Manuel et al. 2015). It is interesting to note that it has been suggested that the Dm in fish is homologous to the cortical amygdala in mammals (Friedrich et al. 2010), which is also the brain area which is often associated with anxiety in humans (Babaev et al. 2018; Shin and Liberzon 2010).

Consistent with our previous results from the VMR test, our results of the light/dark preference test show that blocking endogenous activation of Cnr1 in zebrafish larvae

by the antagonist AM251 does not have any effect on the zebrafish larval behavior. However, in adult zebrafish, treatment with AM251 increased anxiety-like responses, such as freezing and bottom dwelling (Tran et al. 2016). This result indicates that the endogenous activity at the larval stages of development is insufficient to modulate anxiety-like behavior. Even though a complete ECS is present at these stages (Martella et al. 2016; Oltrabella et al. 2017), the levels and/or release of endocannabinoids seem to be insufficient to result in Cnr1-mediated behavioral effects. In addition, the results obtained using the *cnr1*$^{-/-}$ mutant line confirmed that endogenous ligands are weakly active or inactive in affecting anxiety-like behavior in zebrafish larvae. Increasing the concentration of AEA by treatment with Faah-inhibitor PF-004457845 did not change anxiety-related behavior either. Again, the levels of endogenous ligands may be insufficient to have a measurable effect on behavior in our assay. Another explanation could be that the available AEA remains inactive as it needs to be secreted to activate Cnr1 (Gabrielli et al. 2015).

In conclusion, we have shown that the exogenous activation of Cnr1 in zebrafish larvae reduces anxiety-like behavior in a light/dark preference test. Since endogenous ligands do not appear to sufficiently activate Cnr1 to affect anxiety in zebrafish larvae, the absence of endogenous stimulation makes the zebrafish larval model highly suitable to investigate effects of pharmacological Cnr1 activation on anxiety-related behavior, and screen for novel anxiolytic cannabinoid drugs. This assay may additionally be used to study factors that determine the behavioral response to Cnr1 agonists.

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Chapter 4

The effect of cannabinoid receptor 1 activation on cortisol production in zebrafish larvae

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Abstract

The teleost hypothalamic-pituirary-interrenal (HPI) axis (hypothalamic-pituirary-adrenal (HPA) axis in mammals), is a feedback loop of the hypothalamus, pituitary and interrenal gland. Upon stress, the HPI axis is activated, which results in the production and secretion of the glucocorticoid hormone cortisol. It has been suggested that HPI axis activation can be modulated by the endocannabinoid system (ECS). The ECS is a neuromodulatory system which consists of cannabinoid receptor 1 and 2 (Cnr1 and Cnr2 respectively) and their ligands anandamide (AEA) and 2-arachidonoylglycerol (2-AG). In this study, we have used zebrafish larvae as an animal model to study the interaction of the ECS and the HPI axis. We found that activation of Cnr1 by treatment with the agonist WIN55,212-2 increased basal cortisol levels. Blocking Cnr1 with the antagonist AM251, or mutation of *cnr1* had no effect on basal cortisol levels, but the mutation did abolish the WIN55,212-2-induced increase in basal cortisol level. This suggests that the WIN55,212-2 effect was mediated by Cnr1, but that endogenous cannabinoid levels are insufficient to activate this receptor at this stage of development. Similar increasing effects of WIN55,212-2 were observed for the stress-induced cortisol concentrations. The WIN55,212-2-induced increase in basal cortisol could be blocked with antalarmin, a Crh-R1 antagonist, indicating that increased Crh levels are associated with the WIN55,212-2 effect. Taken together, these results show that Cnr1 activation in zebrafish larvae increases cortisol production, probably by (directly or indirectly) acting on the hypothalamus and increasing Crh secretion. Since endogenous cannabinoids do not play a role in regulating HPI axis acivity at this stage of development, this model is suitable for studying the interaction between the ECS and the HPI axis through pharmacological Cnr1 modulation.

Introduction

The endocannabinoid system (ECS) is a central regulatory system that affects a wide range of biological processes. It consists of a group of molecules known as endocannabinoids (eCBs) as well as the two cannabinoid receptors that they bind to. These two receptors, named cannabinoid receptor 1 and 2 (Cnr1 and Cnr2) are mainly expressed in the brain (Matsuda et al. 1990) or in the periphery (Munro et al. 1993) respectively. The ECS, particularly through the action of Cnr1, is a major neuromodulatory system of the brain, which has a strong influence on the balance of excitatory and inhibitory neurotransmitters. Cnr1, which is a G protein-coupled presynaptical receptor, inhibits, upon activation, adenylate cyclase and N- and P/Q-type Ca^{2+}-channels, and activates K^{+}-channels, leading to an inhibited neurotransmitter release and a subsequent lowered excitability of the presynaptical neuron (Ameri 1999). This mechanism allows for regulating several brain functions, such as appetite, memory, pain tolerance and mood (Pacher et al. 2006). Cnr1 can be activated by two eCBs: anandamide (AEA) or 2-arachidonoylglycerol (2-AG). These endogenous ligands are synthesized and secreted post-synaptically, cross the synapse and subsequently activate Cnrs (Lovinger 2007). This signal can be terminated by re-uptake and enzymatic degradation of these eCBs (Basavarajappa 2007).

The ECS has been shown to be affected by the action of the steroid hormone cortisol (Hill and Tasker 2012), which is a product of the hypothalamic-pituitary-adrenal axis (HPA axis). The HPA axis is a major part of the neuroendocrine system, which regulates different body processes, especially upon stress-induced activation (Pecoraro et al. 2006). Stressful stimuli cause activation of specific neurons in the the hypothalamus, which leads to the release of corticotropin releasing hormone (Crh) from these neurons. Crh stimulates the secretion of stored adrenocorticotropic hormone (ACTH) from corticotrope cells. The secreted ACTH is transported by the blood to the adrenal gland where it stimulates both the synthesis of glucocorticoids and its release into the blood. The main glucocorticoid in humans is cortisol, whereas rodents mainly secrete corticosterone. Generally, glucocorticoids act on the mineralocorticoid receptor (MR) and the glucocorticoid receptors (GR), and these receptors are expressed by many different cell types. Upon activation, these receptors act as transcription factors and their transcriptional activity leads to changes in a wide variety of physiological processes, allowing for homeostatic recovery after stress (Oakley and Cidlowski 2013). Besides their stress-related effects, glucocorticoids control the negative feedback of the HPA axis and thereby their own production (Oakley and Cidlowski 2013).

There is a bidirectional interplay between the HPA axis and the ECS. It is thought that glucocorticoids induce secretion of eCBs (Di et al. 2003), which subsequently inhibit HPA axis activity. This is considered particularly important in

regulating the negative feedback of the HPA axis (Di et al. 2003). Administration of corticosterone to rats increases 2-AG contents in several brain regions, such as the hypothalamus (Hill et al. 2010a) and the hippocampus (Wang et al. 2012). This 2-AG increase also occurs when rats or mice are exposed to stress (Hill et al. 2011; Rademacher et al. 2008; Wang et al. 2012). Upon corticosterone exposure, brain AEA levels are also elevated, although these changes are more transient (Hill et al. 2010a). Other, long-term effects have also been described, such as glucocorticoid repression of fatty acid amide hydrolase (FAAH; the enzyme responsible for AEA degradation) expression in mice (Waleh et al. 2002) and lowering of FAAH expression upon isolation stress in rats (Robinson et al. 2010), resulting in increased eCB levels. However, other studies showed contradictory results. For example, chronic corticosterone administration lowered AEA levels, which was CRH receptor 1 (CRH-R1)-dependent, and exerted through increased FAAH activity (Gray et al. 2016). Furthermore, chronic corticosterone exposure has been shown to increase FAAH activity in one study (Bowles et al. 2012). Finally, the effects of stress exposure on the ECS are brain region-specific, since stress lowers AEA levels in the amygdala and prefrontal cortex (PFC) in both rats and mice (Hill et al. 2009; McLaughlin et al. 2012; Patel et al. 2005; Rademacher et al. 2008), but increases AEA levels in the mouse ventral striatum (Rademacher et al. 2008) and has no effect on AEA levels in the mouse forebrain and cerebellum (Patel et al. 2005). It has been suggested that the stress-induced AEA decrease in the amygdala, but not the PFC, is caused by an acute increase in CRH, which through CRH-R1 activation increases FAAH activity (Gray et al. 2015). This rapid decline of AEA levels thus results in disinhibition of HPA axis activity and subsequently increased glucocorticoids secretion (Hill and Tasker 2012).

In turn, the ECS modulates the activity of the HPA axis. CNR1 activation generally results in biphasic activation of the HPA axis, with high doses increasing circulating glucocorticoid concentrations, while low doses decrease these concentrations (Hillard et al. 2016). Blocking or removing CNR1, by administration of an antagonist or by generating a receptor knockout respectively, increases glucocorticoid release upon stress (Cota et al. 2007; Hill et al. 2011; Manzanares et al. 1999; Patel et al. 2004; Wade et al. 2006), which suggests that CNR1 activation during stress suppresses the HPA axis. This was supported by data which show that inhibition of FAAH (Patel et al. 2004) and monoacylglycerol lipase (MAGL; the enzyme responsible for 2-AG degradation) (Roberts et al. 2014), resulting in increased AEA and 2-AG levels respectively, reduced the release of glucocorticoids upon stress in mice. Interestingly, CNR1 is expressed throughout the whole HPA axis (Hillard et al. 2016), thus making regulation possible at every level.

In human pathology, dysregulation of the HPA axis, resulting in prolonged excessive exposure to cortisol, as happens in Cushing's syndrome, is associated

with a variety of signs, which can be gynecologic, dermatologic, orthopedic, metabolic or neurologic disease symptoms (Nieman 2015). The HPA axis is also overactive in major depressive disorder (MDD) (Murphy 1991), although this could differ between specific depression subtypes (Keller et al. 2017). Evidence for a link between chronic stress or elevated cortisol levels and neurodegenerative disorders is growing (Vyas et al. 2016). For example, patients with Alzheimer's, Parkinson's or Huntington's disease show elevated basal cortisol levels (Vyas and Maatouk 2013). Since the ECS is involved in HPA axis regulation, it could be a therapeutic target for treating these diseases which are linked to aberrant cortisol production.

Although the ECS could be a promising drug target for HPA axis-related pathologies, its potential is largely unmet (Hillard et al. 2016), and to better understand the relationship between the ECS and the HPA axis, more research is required. In the present study, we have used the zebrafish as an animal model, which brings several interesting features, such as easy maintenance, high fertility and possibilities for high throughput phenotypic screening (Kalueff et al. 2014; Khan et al. 2017). Additionally, the sequence of the entire genome is available, along with convenient tools for genetic manipulation (MacRae and Peterson 2015; Varshney et al. 2015). The ECS in zebrafish is highly comparable to the mammalian ECS and most zebrafish ECS genes show an orthologous relationship with human ECS genes (Krug and Clark 2015; McPartland et al. 2007). The sequencing of the zebrafish Cnr1 showed a 69% nucleotide identity and a 73.6% amino acid identity with the human CNR1 (Lam et al. 2006). The expression of Cnr1 starts by the 3 somite stage of development and is expressed throughout distinct regions in the CNS, including the preoptic area, dorsal telencephalon, periventricular hypothalamus, tegmentum and anterior hindbrain (Migliarini and Carnevali 2009; Oltrabella et al. 2017). It appears that the general pattern of expression for the adult Cnr1 is homologous to that of mammals. Up to date, research on the ECS in zebrafish has shown the involvement of the ECS in development, feeding, lipid metabolism, learning, memory, immune responses, addiction, anxiety and stress (Krug and Clark 2015).

Zebrafish also possess a comparable neuroendocrine system, which is the hypothalamic-pituitary-interrenal axis (HPI axis) (Alsop and Vijayan 2008). Instead of an adrenal gland, fish have an interrenal gland, which, like humans, produces cortisol instead of corticosterone. Fish cortisol controls similar processes to mammals, which are linked to cognition and behavior (Clark et al. 2011; Griffiths et al. 2012; Stewart and Kalueff 2012), metabolism (Mommsen et al. 1999) and the immune system (Chatzopoulou et al. 2015). Additionally, fish cortisol is involved in osmoregulation of the aqueous environment (Kwong and Perry 2013; McCormick and Farrell 2011).

In this study we aim to understand how ECS manipulation affects HPI axis functioning, under basal conditions and after exposure to stress. We have analyzed cortisol production upon exposure to (ant)agonists of Cnr1, and a Faah-inhibitor, and we have used a *cnr1*$^{-/-}$ mutant. Our results show that Cnr1 activation increases basal cortisol production and elevates the stress-induced cortisol response. Interestingly, this increase could be blocked with antalarmin, a Crh-R1 antagonist, which shows that the ECS affects HPI axis-mediated cortisol production, probably by acting at the level of the hypothalamus.

Materials and methods

Zebrafish maintenance and care

Adult zebrafish (*Danio rerio*) were maintained according to the ZFIN guidelines (ZFIN, http://zfin.org). Natural spawning occurred by group crossings. Eggs were raised in 10 cm Petri dishes containing 50 mL of 10% Hanks' balanced salt solution (HBSS; for specifications see (Ali et al. 2011), on a 14h light:10h dark cycle at 28°C. At 1 day post fertilization (dpf), eggs were placed in groups of 15 in a netted insert (Corning, NY, USA) in a 12-wells plate (Corning, NY, USA) and were cleaned daily. Each well contained 3mL HBSS, and plates were stored in a box filled with wet tissue paper, to prevent evaporation of the swimming water. All experimental procedures were conducted in compliance with the directives of the animal welfare committee of Leiden University.

Test compounds

The following compounds were applied: WIN55,212-2 and AM251 (MedChemExpress, Sweden), PF-04457845 (Sigma-Aldrich, MO, USA) and antalarmin (Cayman Chemical, MI, USA). All compounds were dissolved in 10% HBSS and dimethylsulfoxide (DMSO, final concentration of 0.08%). The compounds and dosage selected were based on previous studies (Chapter 2 and 3). In the case of co-exposure of AM251 and WIN55,212-2, fish were first exposed to AM251 for 15 minutes, after which fish were transferred to a solution of AM251 and WIN55,212-2 combined.

Treatment

At 5 dpf, larvae were treated with the test compounds. A volume of 1mL test compound was added, to a final volume of 4mL. The reported concentrations are final concentrations. If applicable, netting stress was applied as described earlier (Tudorache et al. 2015). In brief, netted inserts were lifted 3 times for 1 minute, separated by a 30 second rest (submerged) phase. After this treatment, larvae were rapidly (40-60 seconds) collected into an Eppendorf tube and snap frozen in liquid nitrogen. The samples were stored at -80°C. Each treatment group consisted of 4 samples, and each sample was created from 15 lysed larvae.

Cortisol extraction
A total of 6 glass beads (2mm diameter) were added to the sample, together with 200 uL PBS. The tube was placed into a bullet shaker (TissueLyser 2, Qiagen, Germany) and the tissue was homogenized (30 times/sec, 1.5 min). The homogenized tissue was vortexed for 1 minute and then centrifuged at 4000 rpm for 5 min. The supernatant was moved to a new tube and the cortisol concentration was measured using a cortisol ELISA.

ELISA
An ELISA kit was used to measure cortisol concentrations (Demeditec, Germany), according to the manufacturer's instructions. Absorbance was determined using a Tecan Spark 10M (Tecan, Switzerland) at 450 nM, with 620nM as a reference wavelength. The absorbance values were converted into concentrations using a calibration curve in combination with a 4 parameter logistics curve. For each sample, the concentration was then converted to absolute mass per larva.

Statistics
All data shown are means ± standard error of mean (SEM). Each mean is generated from 4 samples, and each sample came from a different experiment (technical replicate). The experimental data were analyzed with a one-way or two-way ANOVA test with the concentration, treatment or compound as variable. Dunnett's multiple comparisons test was performed to analyze multiple comparisons in figure 1-5 and Holm-Šídák's multiple comparisons test in figure 6. Statistical significance was reported at $p \leq 0.05$. All analyses were done, and all graphs created with, GraphPad Prism 7 (GraphPad Software Inc., CA, USA). Significance was defined as a p value less than 0.05.

Results

WIN55,212-2 treatment increases Cnr1 dependently cortisol concentrations

To investigate the effect of the ECS on the activity of the HPI-axis, whole body cortisol concentrations were measured in zebrafish larvae. The Cnr agonist WIN55,212-2 was added at different concentrations (125, 500 and 2000 nM) to both *cnr1*$^{+/+}$ and *cnr1*$^{-/-}$ larvae (Fig. 1a and 1b respectively), and cortisol levels were determined after different time points (5, 10, 20 and 30 min) after the start of the exposure to WIN55,212-2.

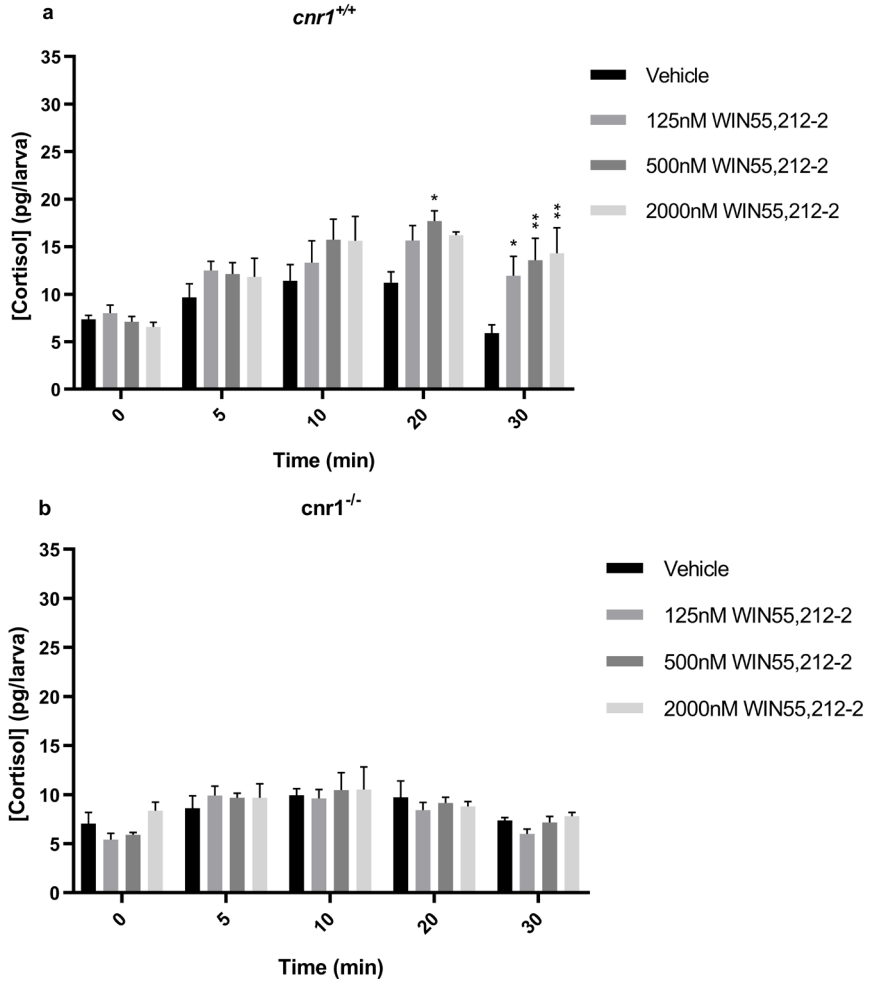

Fig. 1 Effect of WIN55,212-2 exposure on cortisol production in **a** *cnr1*$^{+/+}$ larvae and **b** *cnr1*$^{-/-}$ larvae. WIN55,212-2 increases basal cortisol levels at all concentrations tested in *cnr1*$^{+/+}$ larvae, but has no effect in *cnr1*$^{-/-}$ larvae. Data shown are means ± SEM. Significant differences compared to the corresponding vehicle-treated time group are indicated. * $P \leq 0.05$; ** $P \leq 0.01$;

The data were analyzed using a two-way ANOVA. For the *cnr1*$^{+/+}$ larvae, this analysis revealed a significant effect of time ($F(4,60)=14.41$; $P<0.0001$) and treatment ($F(3.60 = 6.916)$; $P=0.0004$). The multiple comparisons test showed a significant increase in cortisol levels (between 2 and 3 fold), after exposure to WIN55,212-2 for 20 min (500 nM) and 30 min (125, 500 and 2000 nM, compared to the vehicle-treated group). These effects of WIN55,212-2 treatment were not observed in the *cnr1*$^{-/-}$ larvae (Fig 1b). However, in these larvae the vehicle treatment did increase cortisol levels, indicated by d a significant time effect ($F(4, 60 = 8.381)$; $P<0.0001$). Taken together, these data demonstrate that exogenous activation of Cnr1 increases basal cortisol levels in zebrafish larvae.

Antagonizing Cnr1 with AM251 does not affect cortisol concentration

Subsequently, we studied the effect of treatment with a Cnr1 antagonist. Larvae were exposed to the Cnr1 antagonist AM251 for different times (5, 10, 20 and 30 min) and at different concentrations (1, 2 and 4 μM, Fig. 2). It should be noted that at these concentrations, AM251 blocks Cnr1-mediated WIN55,212-2 effects on behavior, as we have shown previously (Chapter 2 and 3).

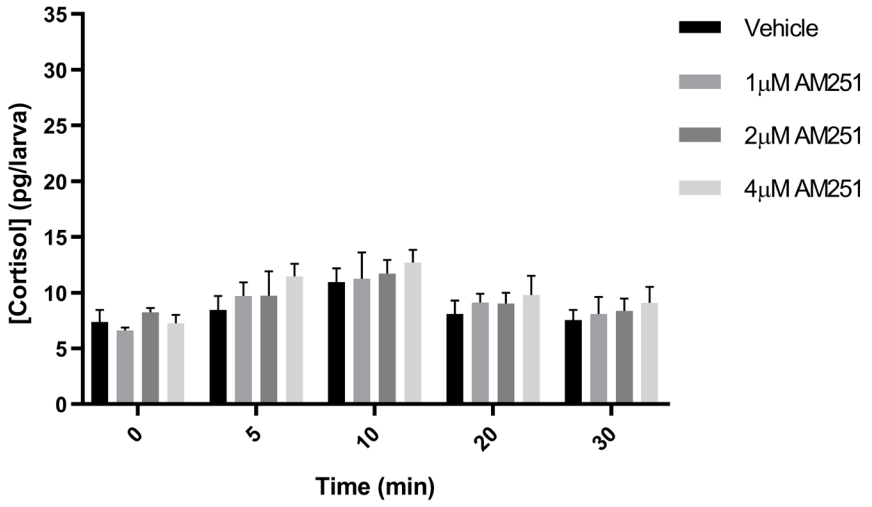

Fig. 2 AM251 exposure has no effect on cortisol production. Data shown are means ± SEM. No significant differences were found when comparing the different concentrations within the same time group.

Two-way ANOVA showed no treatment or interaction effect, but the time-effect was significant ($F(4,60)=6.306$; $P=0.0003$), similar to the effect observed in the *cnr1*$^{-/-}$ larvae.

WIN55,212-2 treatment does not affect the stress induced cortisol response

Cortisol is known to be released upon stress, so we investigated the effect of ECS manipulation on the stress-induced cortisol levels. To study the effect of Cnr1 activation on the stress-induced cortisol response, the cortisol levels were measured at different time points after stress (5, 10, 15 min), with or without exposure to WIN55,212-2 (2 μM).

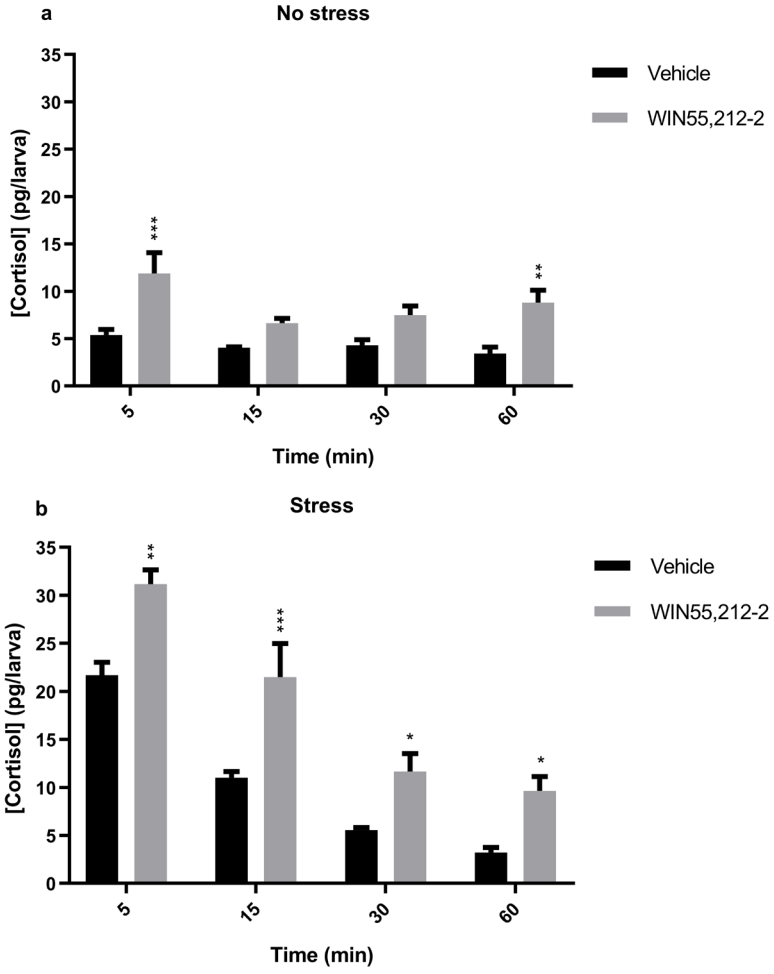

Fig. 3 The effect of netting stress and WIN55,212-2 exposure on cortisol production. Larvae were first treated with 2 μM WIN55,212-2, 20 min later followed by **a** no stress or **b** 4 min netting stress. WIN55,212-2 exposure increases both basal (at 5 and 60min) and post-stress (all time points) cortisol levels. Time is 0 min at end of stressor. Data shown are means ± SEM. Significant differences compared to the corresponding vehicle-treated time group are indicated.* P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

Similarly to the results shown in Fig. 1, the non-stressed larvae showed a significant time effect ($F(3,24)=3.927$; $P=0.0206$) and WIN55,212-2 treatment effect ($F(1,24)=35.71$; $P<0.0001$), but no interaction effect in the two-way ANOVA (Fig. 4a). Again, WIN55,212-2 treatment without stress significantly raised basal cortisol levels, at time points 5 and 60 min. In the stressed larvae, two-way ANOVA yielded a significant time effect ($F(3,24)=57.12$; $P<0.0001$) and treatment effect ($F(1,24)=45.98$; $P<0.0001$), while an interaction effect was absent (Fig. 4b). A significant increase in the cortisol concentration was observed at all time points. Interestingly, the stressed WIN55,212-2-treated larvae do not recover to the same baseline (time point 60 min) as their stressed vehicle-treated counterparts do. These data indicate that WIN55,212-2 increases the stress-induced cortisol levels, similarly to its effect on the basal levels.

cnr1$^{-/-}$ and cnr1$^{+/+}$ larvae show a similar cortisol stress response

Subsequently, we exposed *cnr1$^{-/-}$* and *cnr1$^{+/+}$* larvae to a netting stress protocol. Two-way ANOVA showed a significant time effect ($F(3,24)=49.19$; $P<0.0001$), and no significant effect of genotype or interaction between genotype and stress, indicating that there is no difference in the stress-induced cortisol response between *cnr1$^{-/-}$* and *cnr1$^{+/+}$* larvae. Apparently, endogenous cannabinoids do not affect this response in our assay. Stressed animals (Fig. 4), both *cnr1$^{-/-}$* and cnr1$^{+/+}$, showed a significant 3 fold increase in whole body cortisol levels 5 min after the stressor, which returned back to baseline after 30 min.

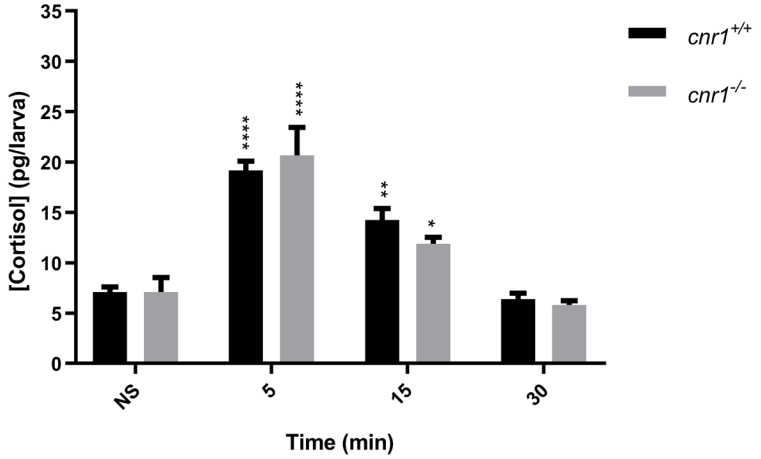

Fig. 4 The cortisol response to stress is not different between *cnr1$^{+/+}$* and *cnr1$^{-/-}$* larvae. Time is 0 min at end of stressor. Data shown are means ± SEM. No significant differences were found when comparing the different strains within the same time group. Netting stress causes a significant increase in [cortisol] in both lines, as shown by the asterisks. Significant differences compared to non-stressed fish are reported as follows * $P \leq 0.05$; ** $P \leq 0.01$; **** $P \leq 0.0001$

Faah inhibition does not affect the cortisol stress response

Next, we studied whether elevating endogenous AEA levels by administration of an inhibitor of Faah, the enzyme responsible for AEA degradation, would increase cortisol levels. The Faah inhibitor PF-04457845 was administered for 8 hours at various concentrations (0.1, 0.5 and 2.5 μM), which had previously been shown to increase AEA levels by a 5 fold (Kantae and Hankemeier, unpublished), and subsequently larvae were stressed and basal and post-stress (5, 15 and 30 min) cortisol levels were measured (Fig. 5). Two-way ANOVA for the PF-04457845-treatment in *cnr1*$^{+/+}$ larvae showed a significant time effect ($F(3,48)=54.89$; $P<0.0001$), but no concentration or interaction effect (Fig. 5a). This was similar for the PF-04457845-treatment in *cnr1*$^{-/-}$ larvae (Fig. 5b), where again only a time effect was found ($F(3,48)=44.44$; $P<0.0001$). The results showed that elevating endogenous AEA levels by PF-04457845-treatment did not result in altered cortisol concentrations, and this was observed for both basal and post-stress cortisol levels.

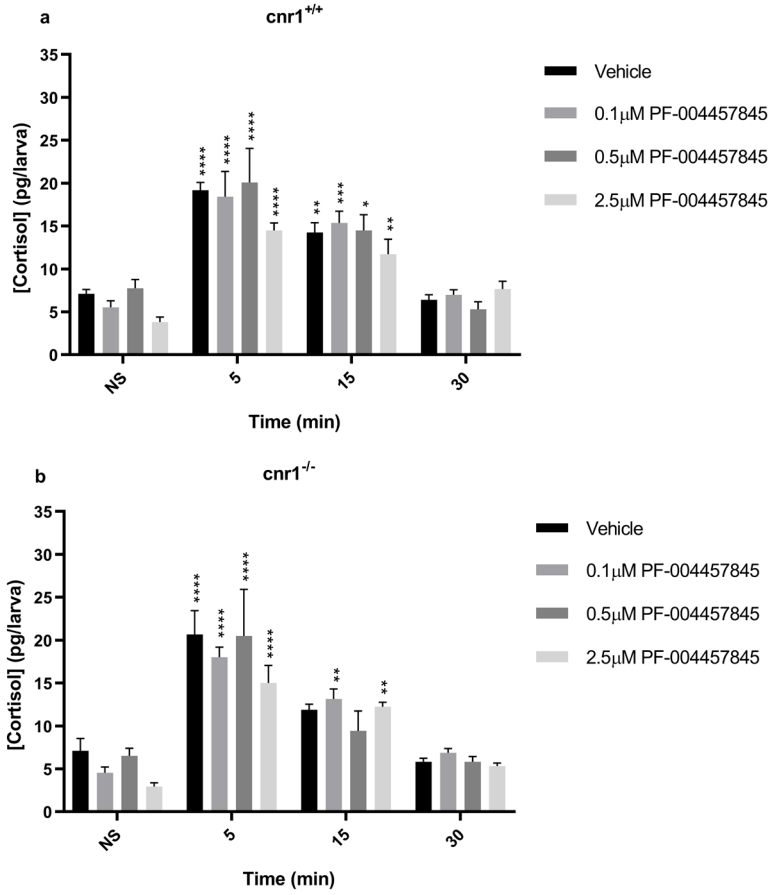

Fig. 5 The Faah-inhibitor PF-04457845 does not have an effect on cortisol production in both **a** *cnr1*$^{+/+}$ larvae and **b** *cnr1*$^{-/-}$ larvae. Time is 0 min at end of stressor. Data shown are means ± SEM. No significant differences were found when comparing the different concentrations within the same time group. Netting stress causes a significant increase in [cortisol] at all concentrations, as shown by the asterisks. Significant differences compared to non-stressed fish are reported as follows * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$

The WIN55,212-2 induced cortisol increase is Crh-R1 dependent

The observation that WIN55,212-2-induced Cnr1 activation leads to increased cortisol levels indicates that WIN55,212-2 modulates the regulation of the HPI axis. We used the Crh-R1 antagonist antalarmin to study at what level of the HPI axis this modulation occurs. First, we exposed larvae to two different concentrations of antalarmin (1.25 and 2.50 μM) for 60 min. Subsequently, larvae were stressed and their cortisol levels were measured under basal conditions and

5 min after netting stress (Fig. 6a). Two-way ANOVA showed a significant interaction ($F(2,18)=10.78$; $P=0.0008$) and stress ($F(1,18)=50.47$; $P<0.0001$) effect, but no concentration effect. Exposure to 2.50 μM antalarmin increases basal cortisol levels. At this concentration of antalarmin, stress does not increase cortisol levels anymore, whereas in the vehicle and 1.25 μM antalarmin-treated group it does. In fact, treatment with 2.50 μM antalarmin lowers the stress-induced cortisol response. Thus, blocking Crh-R1 by treatment of larvae with 2.50 μM antalarmin reduces the cortisol response to stress.

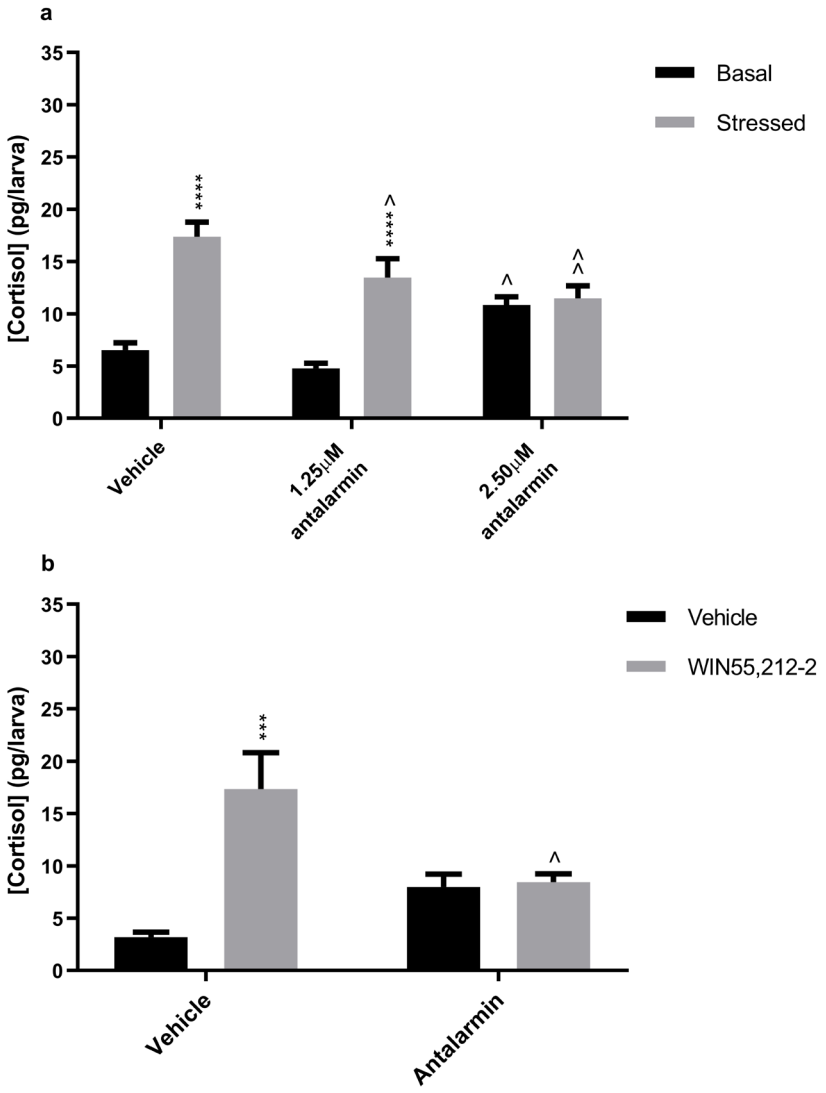

Fig. 6 The involvement of Crh-R1 on WIN55,212-2 or stress-induced cortisol response. a The effect of antalarmin on the cortisol response on 5 min netting stress. At **a** concentration of 2.50 μM, antalarmin abolishes this stress response. **b** The effect of antalarmin on the WIN55,212-2-induced cortisol increase. Antalarmin (2.50 μM) blocks the cortisol response which is induced by WIN55,212-2 exposure (2 μM), while also increasing basal cortisol levels. Data shown are means ± SEM. Significant differences compared to the corresponding (a) basal or (b) vehicle control group are indicated with asterisks as follows *** $P \leq 0.001$; **** $P \leq 0.0001$ (black vs grey). Significant differences compared to the corresponding (a) vehicle or (b) vehicle/WIN55,212-2 control group are indicated with a circumflex as follows ^ $P \leq 0.05$; ^^ $P \leq 0.01$ (black vs black and grey vs grey)

Subsequently, to see whether blocking Crh-R1 also abolishes the WIN55,212-2-induced increase in cortisol levels, we pre-exposed larvae to 2.50 μM antalarmin for 60 min, before adding 2 μM WIN55,212-2, and measured cortisol levels at 20 min after the start of the WIN55,212-2 exposure (Fig. 6b). Two-way ANOVA showed a significant interaction ($F(1,12)=12.85$; $P=0.0038$) and WIN55,212-2 ($F(1,12)=14.65$; $=<0.0024$) effect, but no antalarmin effect. Treatment with WIN55,212-2 increases basal cortisol levels, but after pre-treatment with antalarmin, WIN55,212-2-treatment does not increase basal cortisol levels anymore. These data indicate that WIN55,212-2 increases cortisol levels by acting upstream of the Crh-R1 activation.

Discussion

In the present study, we have demonstrated that exogenous Cnr1 activation by WIN55,212-2 increased basal cortisol levels in zebrafish larvae. Antagonizing Cnr1 using AM251 did not affect cortisol levels, and mutation of the receptor showed no effect either. We also investigated the effect of Cnr1 activation on the stress-induced cortisol levels. Stressed larvae showed an increase in their cortisol concentration, which was even enhanced after WIN55,212-2 exposure. This additional WIN55,212-2-induced increase was comparable to the basal increase caused by WIN55,212-2. Interestingly, we were able to block this WIN55,212-2-induced cortisol response by pretreating larvae with the Crh-R1 antagonist antalarmin, which indicates that Cnr1 acts upstream of the Crh-R1 activity.

The observed increase in cortisol levels after Cnr1 activation is in line with previous studies done in rodents. For example, exposure to the Cnr1 agonists HU-210 (Finn et al. 2004; Martín-Calderón et al. 1998; Roche et al. 2006; Rodriguez de Fonseca et al. 1996) or CP-55,940 (Marco et al. 2006; Romero et al. 2002) increased corticosterone levels in mice or rats. Studies done on the effect of WIN55,212-2 on corticosteroid concentrations are scarce, but it was found that WIN55,212-2 increases cortisol levels in castrated male calves (Zenor et al. 1999) and also increases corticosterone levels in rats (Ganon-Elazar and Akirav 2009; Steiner and Wotjak 2008). In contrast, others have reported a biphasic effect with low concentrations of Cnr agonist CP55940 resulting in a decrease of cortisol whereas high concentrations induced a cortisol increase (Patel et al. 2004). Based on the present study and our previous work (Chapter 2 and 3), we suggests that at this developmental stage WIN55,212-2 has no biphasic effect in zebrafish. Similarly to the findings of this study, our previous studies on behavioral effects of WIN55,212-2 in zebrafish larvae (in which we studied a concentration range of 2-2000 nM) showed no biphasic effect, but a dose-dependent reduction in locomotion and anxiety-related behavior. It has been hypothesized that biphasicity can be explained by a changing balance of glutamatergic and GABAergic neuronal signaling (Haller et al. 2007). Since the brain is still in development at this stage, and a developing brain may act differently compared to an adult brain (Horzmann and Freeman 2016), we think that the lack of biphasicity can be attributed to the developmental stage. To rule out potential non-Cnr1 mediated effects, such as binding to other receptors (Lowin et al. 2016), we repeated our WIN55,212-2 treatment in a *cnr1*$^{-/-}$ fish line. Indeed, the effect of WIN55,212-2 on cortisol secretion was absent in the mutant larvae, which demonstrates that the cortisol increase induced by WIN55,212-2 is specifically Cnr1-mediated.

Since Cnr1 activation results in increased basal cortisol levels, we decided to study whether Cnr1 activation affects stress-induced cortisol production as

well. When we exposed fish to WIN55,212-2 prior to netting stress, we noticed a significant additional increase of cortisol production after stress compared with the vehicle-treated controls. The WIN55,212-2-induced basal cortisol increase adds up to the stress-induced cortisol increase, and does not seem to affect the stress-induced cortisol response. However, in a study done in mice, Cnr1 activation produced a dose-dependent biphasic effect where the low dose of Cnr agonist CP55,940 inhibited stress-induced cortisol release and a high dose increased cortisol levels (Patel et al. 2004).

Exposing larvae to the Cnr1 antagonist AM251 did not result in any change in cortisol levels, which is in line with most other studies performed in rodent systems in which no effect on cortisol levels was observed upon AM251 exposure (Evanson et al. 2010; Hill et al. 2006; Hill et al. 2010b; Newsom et al. 2012; Vahatalo et al. 2015). Like antagonizing Cnr1 with AM251, knocking out Cnr1 did not result in basal cortisol level changes. In research on rodents, Cnr1 knockout has been shown not to not affect cortisol levels in most studies (Cota et al. 2007; Fride et al. 2005; Wade et al. 2006; Wenger et al. 2003), although some researchers have found an increase (Barna et al. 2004) or a decrease (Uriguen et al. 2004) in basal cortisol levels of knockout animals. In line with our data on basal cortisol levels, no significant differences at any time point were found when comparing stress-induced cortisol levels in *cnr1*$^{+/+}$ and *cnr1*$^{-/-}$ larvae. In these lines a similar increase of cortisol levels was observed after the stressor. Most other studies have shown an enhanced cortisol response upon stress in Cnr1 knockout mice (Aso et al. 2008; Barna et al. 2004; Derks et al. 2012; Roberts et al. 2014; Steiner and Wotjak 2008). However, in some studies no response was observed (Rabasa et al. 2015) or even a decreased cortisol response (Fride et al. 2005). It has been hypothesized that removal of Cnr1 abrogates endogenous tonic activation of the ECS, which reduces inhibition of HPA axis activity, thus leading to increased cortisol levels (Hill and Tasker 2012).

We also increased Cnr1 activation in our larvae by elevating endogenous AEA levels. This was performed by inhibiting the AEA-degrading enzyme Faah with PF-04457845, a highly potent and selective Faah inhibitor (Ahn et al. 2011). Basal cortisol levels were not different in PF-04457845-treated larvae compared with vehicle-treated larvae. This is in line with our hypothesis that the eCB levels are insufficient to affect cortisol-production, even though Faah inhibition at these concentrations of PF-04457845 increase AEA levels by a 5-fold (Kantae et al, unpublished). PF-04457845 did not have an effect on the stress-induced cortisol response either. In rodents, several researchers have found a similar outcome (Bedse et al. 2014; Roberts et al. 2014; Steiner and Wotjak 2008), although others found a decrease of the stress-induced cortisol response upon Faah inhibition (Carnevali et al. 2015; McLaughlin et al. 2016).

Since WIN55,212-2 affects basal and stress-induced cortisol levels, whereas AM251, Cnr1 knockout and Faah inhibition do not, we conclude that at the developmental stage of the larvae used in our study, the eCB levels are insufficient to modulate HPI axis activity. Even though a complete ECS (including the metabolic enzymes and endogenous ligands) is present in the developing zebrafish larvae (Martella et al. 2016; Oltrabella et al. 2017), the levels or release of eCBs seem to be too low to modulate the activity of the HPI-axis. This makes the zebrafish larva highly suitable for studying pharmacological manipulation of the ECS, which is an interesting drug target for stress-related disorders. Using this model, we can study the effect of exogenous Cnr1 activation on HPI-axis functioning, without interfering endogenous signaling. This may help unraveling the interaction of the ECS and the HPI-axis.

Pre-exposure of larvae with antalarmin, a Crh-R1 antagonist, before WIN55,212-2 treatment reduced the increase of cortisol caused by WIN55,212-2. This indicates that this WIN55,212-2 effect is mediated by increased Crh signaling, and that WIN55,212-2 acts either directly on the hypothalamic Crh neurons or on cells that modulate the activity of these neurons. This is in agreement with previous research in rodents, which shows that the Cnr1-induced cortisol increase coincides with increased ACTH levels (Barna et al. 2004; Manzanares et al. 1999; Steiner and Wotjak 2008), indicating there is no direct effect of Cnr1 on cortisol production.

At the highest concentration of antalarmin tested in our study (2.50 μM), antalarmin increased cortisol production under basal conditions. Studies done in rodents (Arranz et al. 2010; Cheng et al. 2007; Dong et al. 2018; Lutfy et al. 2012) and monkeys (Broadbear et al. 2002; Broadbear et al. 2004) did not show any effect of antalarmin on basal cortisol secretion. The difference could be explained by the difference in species, but also by the difference in cortisol measurement. Since we homogenize the zebrafish larvae, we measure cortisol production rather than secretion, whereas the blood samples generally taken from larger animals are a measure for secretion. One explanation for the increased basal cortisol level could be that under basal conditions compensatory mechanisms may play a role via Crh-R2 activation. It should also be noted that non-peptide ligands, such as antalarmin, may differentially modulate different signaling pathways controlled by the Crh-R1. For example, it has been shown that in human cells, antalarmin antagonizes Crh-R1 coupling to the Gs subunit competitively but to the Gi subunit noncompetitively (Berger et al. 2006). The antalarmin-induced suppression of increased cortisol levels upon stress as shown in this study is in line with previous studies done in rats (Dong et al. 2018; Traslavina and Franci 2011), although others found no effect in monkeys (Broadbear et al. 2004) or cell cultures (Arranz et al. 2010).

In summary, we have shown that WIN55,212-2 increases basal cortisol levels in zebrafish larvae. This increase is Cnr1-dependent and is probably a result of increased Crh signaling. Mutation of Cnr1, or manipulation of endogenous Cnr1 signaling by the antagonist AM251 or Faah inhibitor PF-04457845 does not affect cortisol production, which shows that the Cnr1 activation by endogenous ligands does not play a role in the regulation of the cortisol biosynthesis. The absence of endogenous signaling brings us an interesting model in which the interaction of exogenous Cnr 1 activation and HPI axis signaling can be studied.

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Chapter 5

Discussion and summary

Discussion

The endocannabinoid system (ECS) affects a wide range of systems in our body and components of the ECS are therefore considered promising drug targets for several diseases (Di Marzo 2018), including metabolic disorders, cardiovascular and respiratory disorders and central nervous system diseases (Pacher et al. 2006). Unfortunately, targeting the ECS may result in severe side effects, such as psychiatric adverse effects (Moreira and Lutz 2008) or brain damage (van Esbroeck et al. 2017). To fully exploit the ECS potential, more research is needed. In the present study, we have used the zebrafish larva to study the ECS, to investigate its potential as a complementary animal model in ECS research, next to the existing rodent models.

The zebrafish larva is an upcoming animal model, and features several interesting advantages, such as low cost, easy maintenance and small housing. It is becoming more popular in central nervous system (CNS) research, due to the availability of transgenic lines and *in vivo* microscopic analysis of brain activity in combination with screening of behavior. However, knowledge regarding the ECS in zebrafish remains limited. The aim of this research was to get a better understanding of the ECS in zebrafish larvae. The most important findings are discussed below.

The zebrafish larval model can be used complementary to rodent models, but to be able to compare data, a proper basic understanding of the model is required. From genetic and bioinformatics studies we know a complete ECS is available in the zebrafish, including cannabinoid receptor (Cnr) 1 and 2, their ligands anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and the metabolic enzymes, including fatty acid amide hydrolase (Faah) (Lam et al. 2006; McPartland et al. 2007; Rodriguez-Martin et al. 2007). Furthermore, the zebrafish ECS is highly homologous to both the rodent and human ECS (Demin et al. 2018; Klee et al. 2012; McPartland et al. 2007). A few studies of the ECS have been done using zebrafish, but most of them were done on adult fish, and not larvae (Chapter 1). In general, data from this limited number of functional studies on the ECS in zebrafish are consistent with data from rodent studies (Krug and Clark 2015). Based on the results described in this thesis, we think the zebrafish larva can be considered a good model for studying the ECS, complementary to the rodent models.

Summary of experimental chapters (2 – 4)

In **Chapter 2** we have investigated the effect of modulation of the ECS on locomotion in 5 days post fertilization (dpf) zebrafish larvae, using a visual motor response test. In this assay, the larvae were first allowed to acclimatize to the setup, and then anxiety-like behavior was induced by turning off the light (Ellis et al. 2012; Peng et al. 2016). We found that treatment with the non-specific Cnr agonists WIN55,212-2 and CP55,940 resulted in decreased locomotion, when

concentrations higher than 32nM WIN55,212-2 or 500nM CP55,940 were used. To verify whether this effect was mediated by Cnr1 or Cnr2, we co-administered a specific Cnr1 antagonist. This treatment, which abolished the effect of WIN55,212-2, indicates that activation of Cnr1 reduces the mobility of the larvae. This was confirmed by the observation that WIN55,212-2 had no effect on locomotion in a *cnr1* knockout line. The immobility of the WIN55,212-2-treated larvae was not due to paralysis, and could be rescued by treatment with stimulating compounds like ethanol and nicotine. Apparently, exogenous activation of Cnr1 results in a less mobile phenotype, but due to the decreased locomotion in both the light and dark phase, we were not able to conclude whether the reduced locomotion in the dark phase was an anxiolytic effect of the Cnr1 agonists. Interestingly, administration of AM251 alone had no effect on locomotion and the *cnr1* knockout line behaved similarly compared to wild-type animals, indicating that endogenous cannabinoid activity does not affect the motor response in zebrafish larvae.

In **Chapter 3** we have studied the effect of modulation of the ECS in 5 dpf zebrafish larvae on anxiety-like behavior in a light-dark box. The light-dark box consists of a light and a dark compartment, and the distance moved and time spent in the dark compartment (as a percentage of the total distance and time), and the latency to the first enter of the dark compartment are considered readouts for anxiety. Zebrafish larvae were exposed to WIN55,212-2. WIN55,212-2-treated zebrafish spent more time in the dark, compared to vehicle-treated larvae. Also, their latency to move into the dark for the first time was lowered. This effect was mediated by Cnr1, as it was absent in *cnr1* knockout larvae and upon cotreatment with the Cnr1 antagonist AM251. These data clearly demonstrate that exogenous activation of Cnr1 through WIN55,212-2 administration results in a less anxious phenotype. Again, both AM251 treatment and knockout of *cnr1* had no effect on larval behavior, indicating that endogenous cannabinoids do not affect the behavior of larvae in the light-dark box, similarly to the results observed in the visual motor response test in Chapter 2. This was further investigated by inhibiting degradation of AEA, one of the endogenous cannabinoids, by administration of the FAAH inhibitor PF-04457845. This treatment resulted in a 5-fold increase of AEA levels, but did not affect anxiety-related behavior of the larvae.

In **Chapter 4**, the effect of ECS modulation on cortisol production has been investigated in 5 dpf zebrafish larvae. Exposure to the Cnr agonist WIN55,212-2 resulted in increased basal cortisol levels, which increased dose-dependently, already after 20 min. exposure. Stress also increases cortisol levels, as was shown by the application of netting stress. Administration of WIN55,212-2 also resulted in a further increase of stress-increased cortisol levels.. This effect was absent in

cnr1 knockout larvae, which suggests that the basal cortisol increase is Cnr1 dependent. Interestingly, the WIN55,212-2-induced increase of basal cortisol levels could be blocked by pre-administering antalarmin, a CRH-R1 antagonist, which implies that WIN55,212-2 enhances HPI-axis activity by increasing CRH levels in the hypothalamus. The Cnr1 antagonist AM251 had no effect on basal cortisol levels, and in *cnr1* knockout larvae the basal cortisol levels were comparable to those of wild-type larvae. This demonstrates that endogenous cannabinoids are not actively involved in the regulation of basal cortisol levels in zebrafish at this stage of development. In addition, *cnr1* knockout larvae show a similar cortisol response upon netting stress compared with wild-type fish, and elevating AEA levels by administrating the FAAH inhibitor PF-04457845 does not alter the stress-induced cortisol response either, which shows that endogenous cannabinoids have no effect on stress-induced cortisol levels either.

Key findings

One of the key findings of this research is that exogenous activation of Cnr1 in zebrafish larvae resulted in various strong effects: inhibition of locomotion (Chapter 2), a less anxious phenotype (Chapter 3) and HPI axis activation (Chapter 4). These effects were all Cnr1-mediated, and this makes the zebrafish larva an interesting model to study drugs that activate Cnr1. Another interesting outcome is the apparent lack of endogenous ECS activity in 5 dpf zebrafish larvae. In all our studies, we did not observe an effect of *cnr1* knockout or blocking Cnr1 with the antagonist AM251, whereas exogenous activation of Cnr1 did have an effect which was abolished by *cnr1* knockout or AM251. Moreover, increasing endogenous ECS activity (enhanced by Faah inhibition, Faah being the AEA catabolic enzyme) did not result in altered behavior or HPI axis activity either. Taken together, we conclude that zebrafish larvae have a functional Cnr1 which mediates effects similar to those observed in other animal models, but that this receptor is not activated by endogenous cannabinoids at this stage of development.

We found only a few studies in which Cnr1 antagonism was shown to have an effect in zebrafish larvae. In one study (Akhtar et al. 2013), AM251 had an acute effect on larval locomotion (at 7.2 μM), but in our studies these concentrations of AM251 appeared to be toxic. In another study, done on 6 dpf zebrafish larvae, liver-specific *cnr1* overexpression resulted in hepatic steatosis, which was blocked by 72h AM251 exposure (Pai et al. 2013). It has also been reported that AM251 exposure significantly down-regulates *cnr1* transcription in larvae and in adult livers (Migliarini and Carnevali 2008). Furthermore, AM251 exposure affected the hatching rate and number of swimming larvae (4 dpf) in a study (Migliarini and Carnevali 2009). A study in which a *cnr1* knockout zebrafish line was applied, showed smaller livers, fewer hepatocytes and reduced liver-specific gene expression, compared with wild-type embryos at 72 hours post fertilization

(Liu et al. 2016). It has also been reported that knocking out *cnr1* or *faah2a* in zebrafish larvae (5 dpf) does not have an effect on basal locomotion, but does have an effect on the osmotic shock locomotion (Krug et al. 2018). Overall, based on the fact that a complete ECS is present in zebrafish larvae (Martella et al. 2016; Oltrabella et al. 2017) and findings from us and other groups described above, we suggest that eCBs may play a role in zebrafish larval development, but that levels of eCBs in zebrafish larvae are generally not sufficient to be involved in the modulation of behavior or HPI axis activity. In adult zebrafish, eCBs do play a role in behavioral regulation, indicating that later in development the role of eCBs changes. It was shown that acute treatment with AM251 increased anxiety-like behavior, including freezing, increased bottom dwelling, decreased locomotor activity and elevated erratic movements (Tran et al. 2016). In rodent studies, blocking Cnr1 with AM251 and knocking out *cnr1* affects behavior (Chhatwal and Ressler 2007), which has also been shown for Faah inhibitors (Chhatwal and Ressler 2007; Lau and Vaughan 2014; Scherma et al. 2008).

Using the visual motor response test, a reduction of locomotion was observed in the dark phase upon WIN55,212-2 treatment, which is considered an anxiolytic effect (Chapter 2). However, since fish also moved less in the light phase, we could not discriminate between a motor effect and an anxiety-related effect. This was addressed in the light/dark preference test (Chapter 3), which clearly showed an anxiolytic effect of Cnr1 activation, as reflected by fish spending relatively more time in the dark zone, moving more in the dark zone, and moving sooner into the dark zone. Data on the effect of Cnr1 activation on anxiety in adult zebrafish is ambiguous, showing an anxiogenic effect (Stewart and Kalueff 2014) or no effect (Ruhl et al. 2014) for delta (9)-tetrahydrocannabinol and depending on administration, no effect or an anxiolytic effect for WIN55,212-2 (Barba-Escobedo and Gould 2012; Connors et al. 2013). Also in rodents, the effect of CNR1 activation on anxiety-like behavior is complicated. In general, low doses of cannabinoids produce anxiolytic-like responses, whereas higher doses result in anxiogenic-like responses (Rubino et al. 2007). It has also been suggested that the anxiety response might be species-dependent. In mice WIN55,212-2 has an anxiolytic effect, whereas it increases anxiety in rats (Haller et al. 2007). These variations between different species or different concentrations might be explained by differences in the balance of GABAergic and glutamatergic signaling (Haller et al. 2007). Cannabinoids may act via Cnr1s located on GABAergic or glutamatergic neurons. The anxiety-related effects of cannabinoids thus depend on the relative cannabinoid responsiveness of GABAergic and glutamatergic neurotransmission (Haller et al. 2007).

Exogenous Cnr1 activation by WIN55,212-2 increased basal cortisol levels in zebrafish larvae. Pre-exposure with antalarmin, a Crh-R1 antagonist, reduced this

increase in cortisol concentration. This shows that the increased basal cortisol level upon Cnr1 activation is mediated by increased Crh signaling. Furthermore, it suggests that WIN55,212-2 acts either directly on the hypothalamic Crh neurons or upstream of the hypothalamus. This is in agreement with studies done on rodents, in which it was shown that Cnr1 activation resulted in increased ACTH levels (Barna et al. 2004; Manzanares et al. 1999; Steiner and Wotjak 2008), indicating there is no direct effect of Cnr1 on cortisol biosynthesis in the adrenal glands.

Future prospects

In this thesis it is shown that the zebrafish larva is highly suitable as a model for screening of ECS modulating compounds. First, a complete ECS is present. Second, the lack of endogenous activity of the ECS is lacking. Third, the general similarity of the observed effects to those observed in other, mammalian, animal models. And lastly, the zebrafish larva model brings several interesting features, such as optical transparency and possibilities for high-throughput screening, complementary to the advantages of rodent models. Newly synthesized Cnr1 agonists can be screened using the readouts described in this thesis, either under basal or stressed conditions, for potential beneficial effects, but also toxicity. For example, new compounds can be screened on their effects on stress-induced cortisol responses or anxiety responses. Also, both chemical and genetic approaches can be applied to investigate their effect on the responsiveness of Cnr1 to ligand activation. It will also be interesting to investigate whether there are readouts to examine Cnr2 activity in a similar way, studying for example its effect on outputs of the immune system or on metabolism. If there is no endogenous interference of eCBs with Cnr2 function either, it may be interesting to develop screening models for exogenous Cnr2 activation.

In our assays, no evidence was found for endogenous activity of the ECS. Since this is probably related to the developmental stage of the larvae, it will be interesting to study the functional development of the ECS in zebrafish. From which stage does the endogenous signaling become active and does this correlate with eCB levels and metabolic enzyme activity? It will be interesting to perform the experiments described in this thesis at different stages of development, using antagonists for blocking Cnr1 and mutant lines lacking a functional *cnr1*. Finally, different brain areas may have a different ECS functioning, as reflected by different AEA/2-AG ratios or spatial differences in Cnr1 expression. Spatial analysis is thus of great importance for understanding the ECS. The optical transparency of zebrafish larvae, combined with the availability of fluorescent reporters allows for spatial analysis of ECS metabolic activity, Cnr1 signaling or the effect of the ECS on neural activity.

Concluding remarks

This study has provided us with an interesting animal model which allows for pharmacological screening of Cnr1 agonists, and their involvement in the CNS, as shown by a change in locomotion, anxiety-like behavior and HPI axis activity. The zebrafish larval model can be used as a complementary model to the existing rodent animal models, to study the ECS. The zebrafish larval model brings several interesting features, such as optical transparency and possibilities for high-throughput screening. Furthermore, a complete ECS is present, there is lack of endogenous activity, allowing for exogenous compound screening, and zebrafish data is generally in line with rodent literature. Since the ECS is involved in many diseases, more research of this system may result in the discovery of novel drugs and drug targets.

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Addendum

Nederlandse samenvatting
Curriculum vitae

Nederlandse samenvatting

Introductie

Het endocannabinoïd systeem (ECS) beïnvloedt een breed scala aan processen in ons lichaam. Componenten van het ECS worden daarom beschouwd als veelbelovende mogelijke aangrijpingspunten voor geneesmiddelen tegen verschillende ziekten (Di Marzo 2018), waaronder cardiovasculaire, stofwisselings- en ademhalingsstoornissen en ziekten van het centrale zenuwstelsel (Pacher et al. 2006). Helaas kan het moduleren van het ECS leiden tot ernstige bijwerkingen, zoals psychiatrische klachten (Moreira en Lutz 2008) of hersenbeschadiging (van Esbroeck et al. 2017). Om de mogelijkheden van het ECS volledig te benutten, is meer onderzoek nodig. In de huidige studie hebben we zebravislarven gebruikt om het ECS te bestuderen, om het potentieel te inventariseren van de zebravis als complementair diermodel in het ECS-onderzoek, naast de bestaande knaagdiermodellen.

De interesse voor de zebravis als diermodel neemt toe. Het zebravismodel heeft, met name wanneer embryo's en larven worden gebruikt, een aantal interessante voordelen, zoals lage kosten, eenvoudig onderhoud en kleine benodigde ruimte voor huisvesting. Het model wordt steeds populairder in het onderzoek naar het centraal zenuwstelsel, vanwege de beschikbaarheid van mutanten en transgene lijnen, en *in vivo* microscopische analyse van hersenactiviteit in combinatie met screening van gedrag. Onze kennis over het ECS bij zebravissen is echter nog beperkt. Het doel van het onderzoek beschreven in dit proefschrift was daarom om een beter begrip te krijgen van het ECS bij zebravislarven. De belangrijkste bevindingen van dit onderzoek worden hieronder besproken.

Zebravislarven kunnen als kunnen als model complementair aan knaagdiermodellen worden gebruikt, maar om gegevens tussen de modellen te kunnen vergelijken is een goede basiskennis van het zebravismodel vereist. Uit genetische en bio-informatica-onderzoeken weten we dat er een compleet ECS aanwezig is in de zebravis, inclusief cannabinoïd receptor (Cnr) 1 en 2, hun liganden anandamide (AEA) en 2-arachidonoylglycerol (2-AG), en metabole enzymen zoals fatty acid amide hydrolase (Faah) (Lam et al. 2006; McPartland et al. 2007; Rodriguez-Martin et al. 2007). Bovendien is het ECS van de zebravis zeer homoloog aan zowel het ECS van knaagdieren als mensen (Demin et al. 2018; Klee et al. 2012; McPartland et al. 2007). Er zijn een paar studies van het ECS gedaan met zebravissen (Hoofdstuk 1 geeft hiervan een overzicht), maar de meeste zijn gedaan met volwassen vissen en niet met larven. Over het algemeen zijn gegevens van dit beperkte aantal functionele onderzoeken naar het ECS bij zebravissen consistent met de resultaten van onderzoek bij knaagdieren (Krug en Clark 2015). Op basis van de resultaten beschreven in dit proefschrift, denken

we dat zebravislarven kunnen worden beschouwd als een goed model voor het bestuderen van het ECS, complementair aan de knaagdiermodellen.

Samenvatting van de experimentele hoofdstukken (2 – 4)

In **Hoofdstuk 2** hebben we het effect van de activiteit van het ECS op het gedrag van vijf dagen oude zebravislarven onderzocht met behulp van een visuele motorische respons test. In deze test kregen larven eerst de tijd om te acclimatiseren in de opstelling en vervolgens werd angst-gerelateerd gedrag veroorzaakt door het licht uit te doen (Ellis et al. 2012; Peng et al. 2016). We ontdekten dat behandeling met de niet-specifieke Cnr-agonisten WIN55,212-2 en CP55,940 resulteerde in een afname van hoe veel de larven bewogen in zowel de lichte als de donkere fases van de test. Om vast te stellen of dit effect werd gemedieerd door Cnr1 of Cnr2, hebben we voorafgaand aan de test een specifieke Cnr1-antagonist toegediend. Deze behandeling deed het effect van de agonist WIN55,212-2 teniet, wat aangeeft dat het de activering van Cnr1 is die de mobiliteit van de larven vermindert. Dit werd bevestigd door de waarneming dat WIN55,212-2 geen effect had op de beweging van larven van een *cnr1* knock-out lijn. De immobiliteit van de met WIN55,212-2 behandelde larven was niet het gevolg van (gedeeltelijke) verlamming, want de mobiliteit van de larven kon worden hersteld door behandeling met stimulerende verbindingen zoals ethanol en nicotine. Blijkbaar resulteert exogene activering van Cnr1 in een minder mobiel fenotype, maar door de verminderde mobiliteit in zowel de lichte als donkere fase konden we niet concluderen of de verminderde voortbeweging in de donkere fase een anxiolytisch effect was van de Cnr1-agonisten. Interessant is dat toediening van alleen de antagonist AM251 geen effect had op de mobiliteit en dat de *cnr1* knock-out lijn zich vergelijkbaar gedroeg in vergelijking met wild type dieren, wat aangeeft dat de werking van endogene cannabinoïden de motorische respons bij zebravislarven niet beïnvloedt.

In **Hoofdstuk 3** hebben we het effect bestudeerd van de activiteit van het ECS in van vijf dagen oude zebravislarven op angst-gerelateerd gedrag in een zogenaamde licht-donker opstelling. Deze opstelling bestaat uit een bakje met een licht en een donker compartiment, en de afgelegde afstand en de tijd doorgebracht in het donkere compartiment (als een percentage van de totale afstand en tijd), en de tijd tot de eerste binnenkomst van het donkere compartiment worden beschouwd als parameters voor angst-gerelateerd gedrag. In ons onderzoek werden zebravislarven blootgesteld aan WIN55,212-2. De resultaten lieten zien dat de met WIN55,212-2 behandelde larven meer tijd in het donker doorbrachten, vergeleken met larven die een controle-behandeling hadden gekregen. Ook werd de tijd totdat ze voor het eerst naar het donkere compartiment gingen, verlaagd. Deze effecten van WIN55,212-2 werden gemedieerd door Cnr1, aangezien ze afwezig waren in *cnr1* knock-out larven en bij

gelijktijdige behandeling met de Cnr1 antagonist AM251. Deze gegevens tonen duidelijk aan dat exogene activering van Cnr1 door toediening van WIN55,212-2 resulteert in een minder angstig fenotype in zebravislarven. Opnieuw hadden zowel AM251-behandeling als knock-out van *cnr1* geen effect op het gedrag van de larven, wat aangeeft dat endogene cannabinoïden het gedrag van larven in deze licht-donker opstelling niet beïnvloeden, vergelijkbaar met de resultaten die zijn waargenomen in de visuele motorische respons test in Hoofdstuk 2. Dit werd nog eens bevestigd in een experiment waarin we de afbraak van AEA, een van de endogene cannabinoïden, verminderden door toediening van PF-04457845, een stof die het enzym FAAH remt. Deze behandeling resulteerde in een vijfvoudige toename van AEA-niveaus, maar had geen invloed op het angst-gerelateerde gedrag van de larven.

In **Hoofdstuk 4** is het effect van ECS-modulatie op de productie van het hormoon cortisol onderzocht bij vijf dagen oude zebravislarven. Blootstelling aan de Cnr-agonist WIN55,212-2 resulteerde al na twintig minuten in verhoogde basale cortisolspiegels, die afhankelijk van de WIN55,212-2-dosis toenamen. Dit effect was afwezig bij *cnr1* knock-out larven, wat suggereert dat deze basale cortisolverhoging Cnr1-afhankelijk is. Stress verhoogt ook het cortisolniveau, en dit hebben wij laten zien door larven te onderwerpen aan zogenaamde 'netting stress', waarbij ze een aantal maal uit het water worden getild in een netje. Toediening van WIN55,212-2 bleek ook de door stress verhoogde cortisolniveaus te laten stijgen. Interessant is dat de door WIN55,212-2 geïnduceerde toename van basale cortisolspiegels kan worden geblokkeerd door vooraf antalarmin toe te dienen, dat een antagonist is van de Corticotropin-Releasing Hormone-Receptor1 (CRH-R1). Dit suggereert dat WIN55,212-2 de Hypothalamus-Pituitary-Intrerenal (HPI)-as activiteit verhoogt door de Corticotropin-Releasing Hormone (CRH)-spiegels in de hypothalamus te verhogen. De Cnr1-antagonist AM251 had geen effect op de basale cortisolspiegels en in *cnr1* knock-out larven waren de basale cortisolspiegels vergelijkbaar met die van wild type larven. Dit toont aan dat endogene cannabinoïden in dit stadium van ontwikkeling niet actief betrokken zijn bij de regulatie van basale cortisolniveaus bij zebravissen. Bovendien vertonen *cnr1* knock-outlarven een vergelijkbare cortisolrespons op netting stress in vergelijking met wild type larven, en verandert het verhogen van de AEA-niveaus door toediening van de FAAH-remmer PF-04457845 ook de door stress geïnduceerde cortisolrespons niet, wat aantoont dat endogene cannabinoïden ook geen effect hebben op de door stress verhoogde cortisolspiegels.

Belangrijkste bevindingen

Een van de belangrijkste bevindingen van dit onderzoek is dat exogene activering van Cnr1 in zebravislarven resulteerde in verschillende sterke effecten:

remming van de mobiliteit (Hoofdstuk 2), een minder angstig fenotype (Hoofdstuk 3) en activering van de HPI-as (Hoofdstuk 4). Deze effecten waren allemaal Cnr1-gemedieerd, en dit maakt de zebravislarve een interessant model om medicijnen te bestuderen die Cnr1 activeren. Een ander interessant resultaat is het schijnbare gebrek aan endogene ECS-activiteit in vijf dagen oude zebravislarven. In geen van onze studies hebben we een effect waargenomen van *cnr1* knock-out of van het blokkeren van Cnr1 met de antagonist AM251, terwijl exogene activering van Cnr1 wel een effect had dat werd opgeheven door *cnr1* knock-out of AM251. Bovendien resulteerde verhoogde endogene ECS-activiteit door Faah-remming (Faah is het AEA-katabole enzym) ook niet in een verandering van angst-gerelateerd gedrag of de activiteit van de HPI-as. Samengevat concluderen we dat zebravislarven een functionele Cnr1 bevatten, die een vergelijkbare werking heeft als deze receptor heeft in andere diermodellen, maar dat deze receptor in dit stadium van ontwikkeling niet wordt geactiveerd door endogene cannabinoïden.

We vonden in de literatuur slechts enkele studies waarin het gebruik van Cnr1 antagonistene of *cnr1* knock-out enig effect bleek te hebben bij zebravislarven. In één studie (Akhtar et al. 2013) had AM251 een acuut effect op de mobiliteit (bij 7,2 µM), maar deze concentraties van AM251 bleken in onze studies toxisch te zijn. In een andere studie, uitgevoerd op zes dagen oude zebravislarven, resulteerde lever-specifieke overexpressie van het *cnr1*-gen in hepatische steatose, die werd geblokkeerd door 72 uur AM251-blootstelling (Pai et al. 2013). Er is ook gerapporteerd dat toediening van AM251 de *cnr1*-transcriptie in de levers van larven en volwassen zebravissen omlaag reguleert (Migliarini en Carnevali 2008). Bovendien had in een andere studie blootstelling aan AM251 invloed op het percentage zebraviseieren dat uitkwam en het aantal zwemmende larven (vier dagen oud) (Migliarini en Carnevali 2009). Een studie waarin een *cnr1* knock-out zebravislijn werd gebruikt, toonde kleinere levers, minder hepatocyten en verminderde lever-specifieke genexpressie, in 72 uur oude embryo's van deze lijn, vergeleken met wild type embryo's (Liu et al. 2016). Ook is aangetoond dat het uitschakelen van het *cnr1* of *faah2a* gen bij zebravislarven (vijf dagen oud) geen effect heeft op hun basale mobiliteit, maar wel op hun beweging na osmotische stress (Krug et al. 2018). Op basis van het feit dat een volledig ECS aanwezig is in zebravislarven (Martella et al. 2016; Oltrabella et al. 2017) en bevindingen van ons en andere groepen die hierboven zijn beschreven, suggereren we dat eCB's een rol kunnen spelen tijdens de ontwikkeling van zebravislarven, maar dat de concentraties van eCB's in zebravislarven over het algemeen niet voldoende zijn om betrokken te kunnen zijn bij de modulatie van systemen zoals het gedrag of de HPI-as. Bij volwassen zebravissen spelen eCB's een duidelijke rol bij gedragsregulatie. Zo is aangetoond dat acute behandeling met AM251 angst-gerelateerd gedrag verhoogde, waaronder verstijving (dood houden), meer bodembe-

woning, verminderde mobiliteit en meer grillige bewegingen (Tran et al. 2016)., Dit geeft aan dat later tijdens de ontwikkeling van zebravissen de rol van eCB's groter wordt. In knaagdieren zien we vergelijkbare effecten als in volwassen vissen, en beïnvloedt zowel het blokkeren van CNR1 met AM251 als knock-out van *cnr1* het gedrag (Chhatwal en Ressler 2007), en dit is ook aangetoond voor FAAH-remmers (Chhatwal en Ressler 2007; Lau en Vaughan 2014; Scherma et al. 2008).
In ons onderzoek werd, met behulp van de visuele motorische respons test, na behandeling met WIN55,212-2 een vermindering van de mobiliteit in de donkere fasewaargenomen , en deze vermindering wordt beschouwd als een anxiolytisch effect (Hoofdstuk 2). Omdat vissen echter ook minder bewogen in de lichte fase, konden we geen onderscheid maken tussen een motorisch effect en een angst-gerelateerd effect. Dit punt hebben we uitgewerkt in de licht-donker test (Hoofdstuk 3), waarin een anxiolytisch effect van Cnr1-activering was te zien. Dit werd duidelijk doordat de larven relatief meer tijd in de donkere zone doorbrachten, meer in de donkere zone bewogen en eerder de donkere zone in gingen, na WIN55,212-2 toediening. Gegevens over het effect van Cnr1-activering op angst bij volwassen zebravissen zijn tegenstrijdig en laten een anxiogeen effect zien (Stewart en Kalueff 2014), of geen effect (Ruhl et al. 2014), voor delta(9)-tetrahydrocannabinol en, afhankelijk van de toediening, geen effect of een anxiolytisch effect voor WIN55,212-2 (Barba-Escobedo en Gould 2012; Connors et al.2013). Ook bij knaagdieren is het effect van CNR1-activering op angst-gerelateerd gedrag gecompliceerd. Over het algemeen verminderen lage doses cannabinoïden angst-gerelateerde reacties, terwijl hogere doses leiden tot meer angstige reacties (Rubino et al. 2007). Er is ook gesuggereerd dat de angstrespons soortafhankelijk kan zijn. WIN55,212-2 heeft namelijk bij muizen een anxiolytisch effect, terwijl het bij ratten de angst verhoogt (Haller et al. 2007). Deze variaties tussen verschillende soorten of verschillende concentraties kunnen worden verklaard door verschillen in de balans van GABA en glutamaat (Haller et al. 2007). Cannabinoïden kunnen werken via CNR1's die zich op GABAerge of glutamaterge neuronen bevinden. De angst-gerelateerde effecten van cannabinoïden hangen daarom af van de relatieve cannabinoïd-responsiviteit van GABAerge en glutamaterge neurotransmissie (Haller et al. 2007), die kunnen variëren tussen individuen, soorten en bij verschillende cannabinoïd concentraties.

Tenslotte vonden we dat exogene Cnr1-activering door WIN55,212-2 de basale cortisolspiegels in zebravislarven verhoogde (Hoofdstuk 4). Wanneer de WIN55,212-2 toediening wordt voorafgegaan door blootstelling aan antalarmin, een Crh-R1-antagonist, verminderde deze verhoging van de cortisolconcentratie. Dit toont aan dat de verhoogde basale cortisolspiegel bij Cnr1-activering wordt gemedieerd door verhoogde Crh-activiteit, en suggereert dat WIN55,212-2 of-

wel direct inwerkt op de hypothalamische Crh-neuronen of andere hersengebieden die signalen naar de hypothalamus sturen. Dit is in overeenstemming met studies uitgevoerd bij ratten en muizen, waarin werd aangetoond dat Cnr1-activering resulteerde in verhoogde niveaus van het hypofyse-hormoon Adrenocorticotropic hormone (ACTH) (Barna et al. 2004; Manzanares et al. 1999; Steiner en Wotjak 2008), wat aangeeft dat er geen direct effect is van Cnr1 op de biosynthese van cortisol in de bijnieren.

Verwachting voor de toekomst

In dit proefschrift wordt aangetoond dat zebravislarven zeer geschikt zijn als model voor screening van geneesmiddelen die werken op het ECS. Ten eerste is er een compleet ECS aanwezig in de larven. Ten tweede ontbreekt het in deze fase van de ontwikkeling aan endogene activiteit van het ECS. Ten derde is er de algemene gelijkenis van de waargenomen effecten met andere (zoog)diermodellen. En ten vierde hebben zebravislarven een aantal interessante kenmerken, zoals optische transparantie en mogelijkheden voor high-throughput screening, wat ze complementair maakt aan knaagdiermodellen. Nieuw gesynthetiseerde Cnr1-agonisten kunnen worden gescreend met behulp van de tests die in dit proefschrift worden beschreven, onder basale of gestreste omstandigheden, op mogelijke gunstige effecten, maar ook op toxiciteit. Nieuwe stoffen kunnen bijvoorbeeld worden gescreend op hun effecten op door stress geïnduceerde cortisolresponsen of gedragsveranderingen. Ook kunnen zowel chemische als genetische manipulaties worden toegepast om het effect van bepaalde systemen of processen op de Cnr1-activiteit te onderzoeken. Het is ook interessant om te bestuderen of er methoden zijn om de Cnr2-activiteit op een vergelijkbare manier te onderzoeken, bijvoorbeeld door het effect ervan op het immuunsysteem of op het metabolisme te bestuderen. Als er ook geen endogene interferentie is van eCB's met de Cnr2-functie, kan het interessant zijn om vergelijkbare screeningsmodellen te ontwikkelen voor exogene Cnr2-activering.

In onze experimenten hebben wij geen bewijs gevonden voor endogene activiteit van het ECS. Aangezien dit waarschijnlijk verband houdt met het ontwikkelingsstadium van de larven, zal het interessant zijn om de functionele ontwikkeling van het ECS bij zebravissen te bestuderen. Vanaf welk stadium wordt de endogene signalering actief en correleert dit met eCB-niveaus en metabole enzymactiviteit? Het zal interessant zijn om de experimenten, zoals beschreven in dit proefschrift, uit te voeren tijdens verschillende stadia van ontwikkeling van de zebravis. Daarbij kan dan gebruik worden gemaakt van dezelfde Cnr1 antagonisten en mutante lijnen die een functionele *cnr1* missen, die wij in de hier beschreven studies hebben gebruikt. Ten slotte kunnen verschillende hersengebieden een verschillende ECS-werking hebben, zoals weerspiegeld door verschillende AEA / 2-AG-verhoudingen of plaatselijke verschillen in Cnr1-expressie. Lokale analyse van verschillende hersengebieden is dus van groot belang

voor het begrijpen van het ECS. De optische transparantie van zebravislarven, gecombineerd met de beschikbaarheid van fluorescerende reporters, maakt deze lokale analyse mogelijk van metabole activiteit van het ECS, Cnr1 activiteit en het effect van het ECS op neuronale activiteit.

Concluderende opmerkingen

Deze studie heeft ons een interessant diermodel opgeleverd, het zebravislarve model, dat farmacologische screening van Cnr1-agonisten en hun rol binnen het centrale zenuwstelsel mogelijk maakt. Dit is vastgesteld aan de hand van Cnr1-gemedieerde verandering in mobiliteit, angst-gerelateerd gedrag en HPI-as activiteit. Het zebravislarve model kan gebruikt worden als complementair model aan de bestaande knaagdierdiermodellen, om het ECS te bestuderen. Het biedt verschillende interessante kenmerken, zoals optische transparantie en mogelijkheden voor screening met grote aantallen. Bovendien is er een compleet ECS aanwezig en is endogene activiteit min of meer afwezig, waardoor exogene stoffen eenvoudig kunnen worden gescreend. De data van zebravisonderzoek zijn over het algemeen in overeenstemming met de resultaten in de knaagdierliteratuur. Aangezien het ECS bij veel ziekten betrokken is, kan meer onderzoek van dit systeem resulteren in de ontdekking van nieuwe (aangrijpingspunten voor) geneesmiddelen tegen deze aandoeningen.

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Curriculum vitae

Floris Johannes Luchtenburg was born on June 28th 1987 in Veenendaal. In 2006 he graduated from Ichthus College in Veenendaal. He obtained a Bachelor's degree in Life Science and Technology at Leiden University and Delft University of Technology in 2011, and a Master's degree in Life Science and Technology at Leiden University in 2014. During his Master's studies, he performed a research project in the group of Prof. dr. Overkleeft (Leiden Institute of Chemistry), where he investigated the synthesis of a near-IR BODIPY dye. This was followed by a research internship in the group of Prof. dr. Van den Maagdenberg at the Leiden University Medical Center, where he studied the chronification of migraine. In 2014, he started his PhD project at the Institute of Biology of Leiden University, under supervision of Prof. dr. Michael Richardson and Dr. Marcel Schaaf. Following his PhD research, in 2018 he started working as medical science liaison neuroscience at Janssen-Cilag. Currently he is working as healthcare purchaser (zorginkoper) at Zorgkantoor Zorg & Zekerheid.