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

Luchtenburg, F.J.

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).

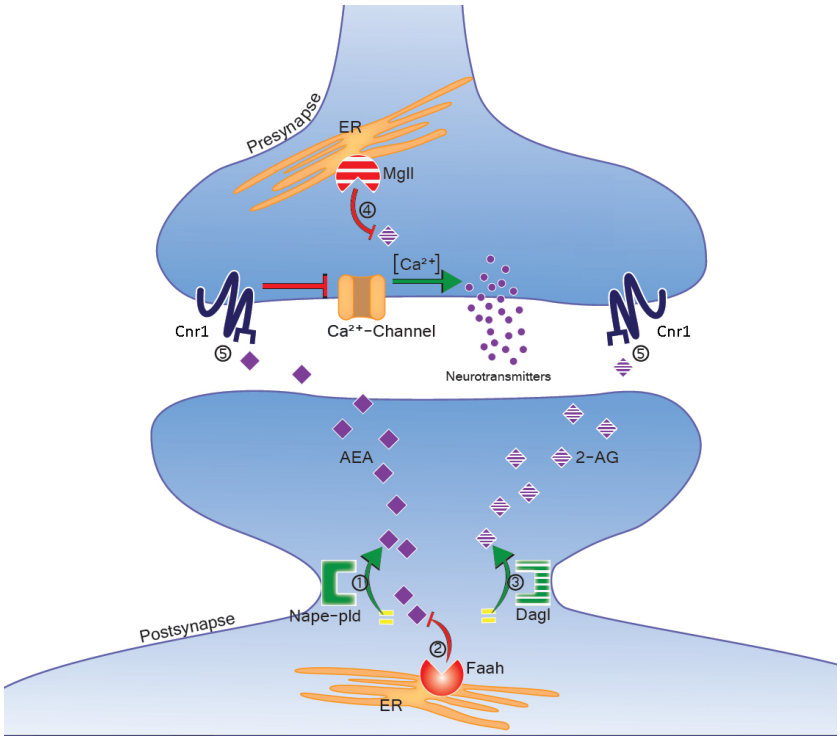

Fig. 1 The ECS consists of the Cnrs, the ligands N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) and their metabolic enzymes. **1** AEA is postsynaptically synthesized from phospholipids by *N*-acyl phosphatidylethanolamine-specific phospholipase D (Nape-pld) and **2** degraded by 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).

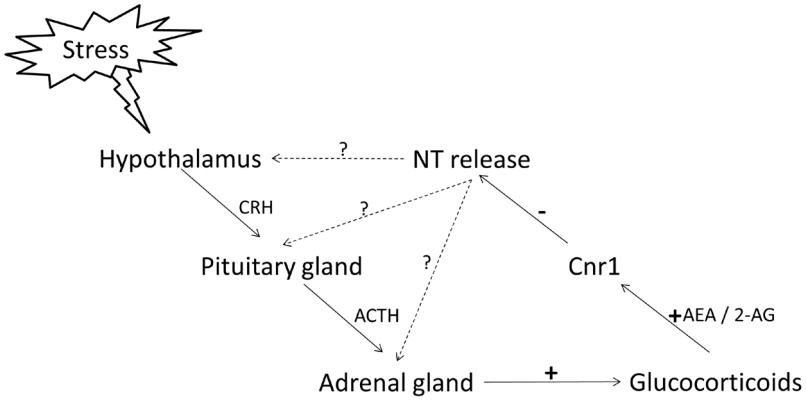

Fig. 2 A proposed mechanism for the involvement of the ECS in the negative feedback loop of the HPA axis. The HPA axis is activated upon stress, which eventually results in the production and secretion of glucocorticoids. These glucocorticoids activate the GR which through unknown signaling results in 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.

References

Ahmed KT, Amin MR, Shah P, Ali DW (2018) Motor neuron development in zebrafish is altered by brief (5-hr) exposures to THC ((9)-tetrahydrocannabinol) or CBD (cannabidiol) during gastrulation. Sci Rep 8: 10518.

Akhtar MT, Ali S, Rashidi H, van der Kooy F, Verpoorte R, Richardson MK (2013) Developmental effects of cannabinoids on zebrafish larvae. Zebrafish 10: 283-93.

Albayram O, Alferink J, Pitsch J, Piyanova A, Neitzert K, Poppensieker K, Mauer D, Michel K, Legler A, Becker A, Monory K, Lutz B, Zimmer A, Bilkei-Gorzo A (2011) Role of CB1 cannabinoid receptors on GABAergic neurons in brain aging. Proceedings of the National Academy of Sciences 108: 11256-11261.

Atwood BK, Mackie K (2010) CB2: a cannabinoid receptor with an identity crisis. Br J Pharmacol 160: 467-79.

Baker MR, Goodman AC, Santo JB, Wong RY (2018) Repeatability and reliability of exploratory behavior in proactive and reactive zebrafish, Danio rerio. Sci Rep 8: 12114.

Barba-Escobedo PA, Gould GG (2012) Visual social preferences of lone zebrafish in a novel environment: strain and anxiolytic effects. Genes, brain, and behavior 11: 366-73.

Battistella G, Fornari E, Annoni JM, Chtioui H, Dao K, Fabritius M, Favrat B, Mall JF, Maeder P, Giroud C (2014) Long-term effects of cannabis on brain structure. Neuropsychopharmacology 39: 2041-8.

Broglio C, Gomez A, Duran E, Ocana FM, Jimenez-Moya F, Rodriguez F, Salas C (2005) Hallmarks of a common forebrain vertebrate plan: specialized pallial areas for spatial, temporal and emotional memory in actinopterygian fish. Brain Res Bull 66: 277-81.

Buczynski MW, Parsons LH (2010) Quantification of brain endocannabinoid levels: methods, interpretations and pitfalls. Br J Pharmacol 160: 423-42.

Cabral GA, Griffin-Thomas L (2009) Emerging role of the cannabinoid receptor CB2 in immune regulation: therapeutic prospects for neuroinflammation. Expert Rev Mol Med 11: e3.

Campolongo P, Roozendaal B, Trezza V, Hauer D, Schelling G, McGaugh JL, Cuomo V (2009) Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. Proceedings of the National Academy of Sciences of the United States of America 106: 4888-93.

Carlo F, Carolina Lo S, Lorenzo L, Francesco P, Lisa L, Lucia G, Laura B, Giulia F, Gabriela Alina T, Giovanni C, Valdo R (2012) The Role of Life Events and HPA Axis in Anxiety Disorders: A Review. Current Pharmaceutical Design 18: 5663-5674.

Carty DR, Thornton C, Gledhill JH, Willett KL (2018) Developmental Effects of Cannabidiol and Delta9-Tetrahydrocannabinol in Zebrafish. Toxicol Sci 162: 137-145.

Chen DJ, Gao M, Gao FF, Su QX, Wu J (2017) Brain cannabinoid receptor 2: expression, function and modulation. Acta Pharmacol Sin 38: 312-316.

Chevaleyre V, Takahashi KA, Castillo PE (2006) Endocannabinoid-mediated synaptic plasticity in the CNS. Annu Rev Neurosci 29: 37-76.

Coddington E, Lewis C, Rose JD, Moore FL (2007) Endocannabinoids mediate the effects of acute stress and corticosterone on sex behavior. Endocrinology 148: 493-500.

Connors Ka, Valenti TW, Lawless K, Sackerman J, Onaivi ES, Brooks BW, Gould GG (2013) Similar anxiolytic effects of agonists targeting serotonin 5-HT1A or cannabinoid CB receptors on zebrafish behavior in novel environments. Aquatic toxicology (Amsterdam, Netherlands): 1-9.

De Petrocellis L, Cascio MG, Di Marzo V (2004) The endocannabinoid system: a general view and latest additions. Br J Pharmacol 141: 765-74.

Demin KA, Meshalkina DA, Kysil EV, Antonova KA, Volgin AD, Yakovlev OA, Alekseeva PA, Firuleva MM, Lakstygal AM, de Abreu MS, Barcellos LJG, Bao W, Friend AJ, Amstislavskaya TG, Rosemberg DB, Musienko PE, Song C, Kalueff AV (2018) Zebrafish models relevant to studying central opioid and endocannabinoid systems. Prog Neuropsychopharmacol Biol Psychiatry 86: 301-312.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science (New York, NY) 258: 1946-9.

Di Marzo V, De Petrocellis L (2012) Why do cannabinoid receptors have more than one endogenous ligand? Philos Trans R Soc Lond B Biol Sci 367: 3216-28.

Di S, Malcher-Lopes R, Halmos KC, Tasker JG (2003) Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. The Journal of neuroscience : the official journal of the Society for Neuroscience 23: 4850-7.

Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D (2002) Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci U S A 99: 10819-24.

DiPatrizio NV, Piomelli D (2012) The thrifty lipids: endocannabinoids and the neural control of energy conservation. Trends Neurosci 35: 403-11.

Egertová M, Cravatt BF, Elphick MR (2003) Comparative analysis of fatty acid amide hydrolase and cb1 cannabinoid receptor expression in the mouse brain: evidence of a widespread role for fatty acid amide hydrolase in regulation of endocannabinoid signaling. Neuroscience 119: 481-496.

Evanson NK, Tasker JG, Hill MN, Hillard CJ, Herman JP (2010) Fast feedback inhibition of the HPA axis by glucocorticoids is mediated by endocannabinoid signaling. Endocrinology 151: 4811-9.

Fernandez-Ruiz J, Berrendero F, Hernandez ML, Ramos JA (2000) The endogenous cannabinoid system and brain development. Trends Neurosci 23: 14-20.

Fin L, Bergamin G, Steiner RA, Hughes SM (2017) The Cannabinoid Receptor Interacting Proteins 1 of zebrafish are not required for morphological development, viability or fertility. Sci Rep 7: 4858.

Forner-Piquer I, Maradonna F, Gioacchini G, Santangeli S, Allara M, Piscitelli F, Habibi HR, Di Marzo V, Carnevali O (2017) Dose-Specific Effects of Di-Isononyl Phthalate on the Endocannabinoid System and on Liver of Female Zebrafish. Endocrinology 158: 3462-3476.

Fosque BF, Sun Y, Dana H, Yang CT, Ohyama T, Tadross MR, Patel R, Zlatic M, Kim DS, Ahrens MB, Jayaraman V, Looger LL, Schreiter ER (2015) Neural circuits. Labeling of active neural circuits in vivo with designed calcium integrators. Science 347: 755-60.

Fraher D, Ellis MK, Morrison S, McGee SL, Ward AC, Walder K, Gibert Y (2015) Lipid Abundance in Zebrafish Embryos Is Regulated by Complementary Actions of the Endocannabinoid System and Retinoic Acid Pathway. Endocrinology 156: 3596-609.

Freund TF, Katona I, Piomelli D (2003) Role of endogenous cannabinoids in synaptic signaling. Physiol Rev 83: 1017-66.

Furay AR, Bruestle AE, Herman JP (2008) The role of the forebrain glucocorticoid receptor in acute and chronic stress. Endocrinology 149: 5482-90.

Gary-Bobo M, Elachouri G, Gallas JF, Janiak P, Marini P, Ravinet-Trillou C, Chabbert M, Cruccioli N, Pfersdorff C, Roque C, Arnone M, Croci T, Soubrie P, Oury-Donat F, Maffrand JP, Scatton B, Lacheretz F, Le Fur G, Herbert JM, Bensaid M (2007) Rimonabant reduces obesity-associated hepatic steatosis and features of metabolic syndrome in obese Zucker fa/fa rats. Hepatology 46: 122-9.

Gilbert MT, Sulik KK, Fish EW, Baker LK, Dehart DB, Parnell SE (2016) Dose-dependent teratogenicity of the synthetic cannabinoid CP-55,940 in mice. Neurotoxicol Teratol 58: 15-22.

Girdhar K, Gruebele M, Chemla YR (2015) The Behavioral Space of Zebrafish Locomotion and Its Neural Network Analog. PLoS One 10: e0128668.

Hill MN, Gorzalka BB (2009) The Endocannabinoid System and the Treatment of Mood and Anxiety Disorders. Cns Neurol Disord-Dr 8: 451-458.

Hill MN, Karatsoreos IN, Hillard CJ, McEwen BS (2010) Rapid elevations in limbic endocannabinoid content by glucocorticoid hormones in vivo. Psychoneuroendocrinology 35: 1333-8.

Hill MN, McLaughlin RJ, Pan B, Fitzgerald ML, Roberts CJ, Lee TTY, Karatsoreos IN, Mackie K, Viau V, Pickel VM, McEwen BS, Liu Q-s, Gorzalka BB, Hillard CJ (2011) Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. The Journal of neuroscience : the official journal of the Society for Neuroscience 31: 10506-15.

Hill MN, Tasker JG (2012) Endocannabinoid signaling, glucocorticoid-mediated negative feedback, and regulation of the hypothalamic-pituitary-adrenal axis. Neuroscience 204: 5-16.

Hoi H, Matsuda T, Nagai T, Campbell RE (2013) Highlightable Ca^{2+} indicators for live cell imaging. J Am Chem Soc 135: 46-9.

Ibsen MS, Connor M, Glass M (2017) Cannabinoid CB1 and CB2 Receptor Signaling and Bias. Cannabis and Cannabinoid Research 2: 48-60.

Jaferi A, Bhatnagar S (2006) Corticosterone can act at the posterior paraventricular thalamus to inhibit hypothalamic-pituitary-adrenal activity in animals that habituate to repeated stress. Endocrinology 147: 4917-30.

Jentsch JD, Andrusiak E, Tran A, Bowers MB, Jr., Roth RH (1997) Delta 9-tetrahydrocannabinol increases prefrontal cortical catecholaminergic utilization and impairs spatial working memory in the rat: blockade of dopaminergic effects with HA966. Neuropsychopharmacology 16: 426-32.

Kalueff AV, Stewart AM, Gerlai R (2014) Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol Sci 35: 63-75.

Khan KM, Collier AD, Meshalkina DA, Kysil EV, Khatsko SL, Kolesnikova T, Morzherin YY, Warnick JE, Kalueff AV, Echevarria DJ (2017) Zebrafish models in neuropsychopharmacology and CNS drug discovery. Br J Pharmacol.

Kim DH, Kim J, Marques JC, Grama A, Hildebrand DGC, Gu W, Li JM, Robson DN (2017) Pan-neuronal calcium imaging with cellular resolution in freely swimming zebrafish. Nat Methods 14: 1107-1114.

Lam CS, Rastegar S, Strähle U (2006) Distribution of cannabinoid receptor 1 in the CNS of zebrafish. Neuroscience 138: 83-95.

Levitas-Djerbi T, Appelbaum L (2017) Modeling sleep and neuropsychiatric disorders in zebrafish. Curr Opin Neurobiol 44: 89-93.

Lindsey BW, Kaslin J (2017) Optical Projection Tomography as a Novel Method to Visualize and Quantitate Whole-Brain Patterns of Cell Proliferation in the Adult Zebrafish Brain. Zebrafish 14: 574-577.

Lisboa SF, Gomes FV, Terzian AL, Aguiar DC, Moreira FA, Resstel LB, Guimaraes FS (2017) The Endocannabinoid System and Anxiety. Vitam Horm 103: 193-279.

Liu LY, Alexa K, Cortes M, Schatzman-Bone S, Kim AJ, Mukhopadhyay B, Cinar R, Kunos G, North TE, Goessling W (2016) Cannabinoid receptor signaling regulates liver development and metabolism. Development 143:

609-22.
Manuel R, Zethof J, Flik G, van den Bos R (2015) Providing a food reward reduces inhibitory avoidance learning in zebrafish. Behav Processes 120: 69-72.
Marques JC, Lackner S, Felix R, Orger MB (2018) Structure of the Zebrafish Locomotor Repertoire Revealed with Unsupervised Behavioral Clustering. Curr Biol 28: 181-195 e5.
Martella A, Sepe RM, Silvestri C, Zang J, Fasano G, Carnevali O, De Girolamo P, Neuhauss SC, Sordino P, Di Marzo V (2016a) Important role of endocannabinoid signaling in the development of functional vision and locomotion in zebrafish. FASEB J 30: 4275-4288.
Martella A, Silvestri C, Maradonna F, Gioacchini G, Allara M, Radaelli G, Overby DR, Di Marzo V, Carnevali O (2016b) Bisphenol A Induces Fatty Liver by an Endocannabinoid-Mediated Positive Feedback Loop. Endocrinology 157: 1751-63.
Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346: 561-4.
McGaugh JL, Roozendaal B (2002) Role of adrenal stress hormones in forming lasting memories in the brain. Current opinion in neurobiology 12: 205-10.
McPartland JM, Glass M, Matias I, Norris RW, Kilpatrick CW (2007) A shifted repertoire of endocannabinoid genes in the zebrafish (Danio rerio). Molecular genetics and genomics : MGG 277: 555-70.
Migliarini B, Carnevali O (2008) Anandamide modulates growth and lipid metabolism in the zebrafish Danio rerio. Molecular and cellular endocrinology 286: S12-6.
Migliarini B, Carnevali O (2009) A novel role for the endocannabinoid system during zebrafish development. Mol Cell Endocrinol 299: 172-7.
Mishima K, Egashira N, Hirosawa N, Fujii M, Matsumoto Y, Iwasaki K, Fujiwara M (2001) Characteristics of learning and memory impairment induced by Delta(9)-tetrahydrocannabinol in rats. Jpn J Pharmacol 87: 297-308.
Morena M, Campolongo P (2014) The endocannabinoid system: an emotional buffer in the modulation of memory function. Neurobiol Learn Mem 112: 30-43.
Mueller KP, Neuhauss SC (2010) Quantitative measurements of the optokinetic response in adult fish. J Neurosci Methods 186: 29-34.
Mulder J, Aguado T, Keimpema E, Barabas K, Ballester Rosado CJ, Nguyen L, Monory K, Marsicano G, Di Marzo V, Hurd YL, Guillemot F, Mackie K, Lutz B, Guzman M, Lu HC, Galve-Roperh I, Harkany T (2008) Endocannabinoid signaling controls pyramidal cell specification and long-range axon patterning. Proc Natl Acad Sci U S A 105: 8760-5.
Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a pe-

ripheral receptor for cannabinoids. Nature 365: 61-5.
Nakai J, Ohkura M, Imoto K (2001) A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. Nat Biotechnol 19: 137-41.
Nazario LR, Antonioli R, Jr., Capiotti KM, Hallak JE, Zuardi AW, Crippa JA, Bonan CD, da Silva RS (2015) Caffeine protects against memory loss induced by high and non-anxiolytic dose of cannabidiol in adult zebrafish (Danio rerio). Pharmacol Biochem Behav 135: 210-6.
Nishio S-I, Gibert Y, Berekelya L, Bernard L, Brunet F, Guillot E, Le Bail J-C, Sánchez JA, Galzin AM, Triqueneaux G, Laudet V (2012) Fasting induces CART down-regulation in the zebrafish nervous system in a cannabinoid receptor 1-dependent manner. Molecular endocrinology (Baltimore, Md) 26: 1316-26.
Nomura DK, Morrison BE, Blankman JL, Long JZ, Kinsey SG, Marcondes MC, Ward AM, Hahn YK, Lichtman AH, Conti B, Cravatt BF (2011) Endocannabinoid hydrolysis generates brain prostaglandins that promote neuroinflammation. Science 334: 809-13.
Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Batkai S, Harvey-White J, Mackie K, Offertaler L, Wang L, Kunos G (2005) Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. J Clin Invest 115: 1298-305.
Pacher P, Batkai S, Kunos G (2006) The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol Rev 58: 389-462.
Pai W-Y, Hsu C-C, Lai C-Y, Chang T-Z, Tsai Y-L, Her GM (2013) Cannabinoid receptor 1 promotes hepatic lipid accumulation and lipotoxicity through the induction of SREBP-1c expression in zebrafish. Transgenic research 22: 823-38.
Pazos MR, Nunez E, Benito C, Tolon RM, Romero J (2005) Functional neuroanatomy of the endocannabinoid system. Pharmacol Biochem Behav 81: 239-47.
Pecoraro N, Dallman MF, Warne JP, Ginsberg AB, Laugero KD, la Fleur SE, Houshyar H, Gomez F, Bhargava A, Akana SF (2006) From Malthus to motive: how the HPA axis engineers the phenotype, yoking needs to wants. Prog Neurobiol 79: 247-340.
Portugues R, Feierstein CE, Engert F, Orger MB (2014) Whole-brain activity maps reveal stereotyped, distributed networks for visuomotor behavior. Neuron 81: 1328-1343.
Reynolds JR (1890) On the Therapeutical Uses and Toxic Effects of Cannabis Indica. The Lancet 135: 637-638.
Rodriguez-Martin I, Herrero-Turrion MJ, Marron Fdez de Velasco E, Gonzalez-Sarmiento R, Rodriguez RE (2007) Characterization of two duplicate zebrafish Cb2-like cannabinoid receptors. Gene 389: 36-44.

Ruhl T, Prinz N, Oellers N, Seidel NI, Jonas A, Albayram O, Bilkei-Gorzo A, von der Emde G (2014) Acute administration of THC impairs spatial but not associative memory function in zebrafish. Psychopharmacology.

Ruhl T, Zeymer M, von der Emde G (2017) Cannabinoid modulation of zebrafish fear learning and its functional analysis investigated by c-Fos expression. Pharmacol Biochem Behav 153: 18-31.

Russell GM, Henley DE, Leendertz J, Douthwaite JA, Wood SA, Stevens A, Woltersdorf WW, Peeters BWMM, Ruigt GSF, White A, Veldhuis JD, Lightman SL (2010) Rapid glucocorticoid receptor-mediated inhibition of hypothalamic-pituitary-adrenal ultradian activity in healthy males. The Journal of neuroscience : the official journal of the Society for Neuroscience 30: 6106-15.

Saez TM, Aronne MP, Caltana L, Brusco AH (2014) Prenatal exposure to the CB1 and CB2 cannabinoid receptor agonist WIN 55,212-2 alters migration of early-born glutamatergic neurons and GABAergic interneurons in the rat cerebral cortex. J Neurochem 129: 637-48.

Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine reviews 21: 55-89.

Shimada Y, Hirano M, Nishimura Y, Tanaka T (2012) A high-throughput fluorescence-based assay system for appetite-regulating gene and drug screening. PloS one 7: e52549-e52549.

Silvestri C, Paris D, Martella A, Melck D, Guadagnino I, Cawthorne M, Motta A, Marzo VD (2015) Two non-psychoactive cannabinoids reduce intra-cellular lipid levels and inhibit hepatosteatosis. Journal of Hepatology.

Steiner Ma, Wotjak CT (2008) Role of the endocannabinoid system in regulation of the hypothalamic-pituitary-adrenocortical axis. Progress in brain research 170: 397-432.

Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, Kalueff AV (2012) Modeling anxiety using adult zebrafish: a conceptual review. Neuropharmacology 62: 135-43.

Stewart AM, Braubach O, Spitsbergen J, Gerlai R, Kalueff AV (2014) Zebrafish models for translational neuroscience research: from tank to bedside. Trends Neurosci 37: 264-78.

Stewart AM, Kalueff AV (2014) The behavioral effects of acute Δ?-tetrahydrocannabinol and heroin (diacetylmorphine) exposure in adult zebrafish. Brain research 1543: 109-19.

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochemical and biophysical research communications 215: 89-97.

Tasker JG, Herman JP (2011) Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic-pituitary-adrenal axis. Stress (Amsterdam, Netherlands) 14: 398-406.

Thomas RJ (1975) The toxicologic and teratologic effects of delta-9-tetrahydrocannabinol in the zebrafish embryo. Toxicology and applied pharmacology 32: 184-90.

Thornqvist PO, McCarrick S, Ericsson M, Roman E, Winberg S (2019) Bold zebrafish (Danio rerio) express higher levels of delta opioid and dopamine D2 receptors in the brain compared to shy fish. Behav Brain Res 359: 927-934.

Tran S, Chatterjee D, Facciol A, Gerlai R (2016) Concentration, population, and context-dependent effects of AM251 in zebrafish. Psychopharmacology (Berl) 233: 1445-54.

Ullmann JF, Cowin G, Kurniawan ND, Collin SP (2010) A three-dimensional digital atlas of the zebrafish brain. Neuroimage 51: 76-82.

Valim Brigante TA, Abe FR, Zuardi AW, Hallak JEC, Crippa JAS, de Oliveira DP (2018) Cannabidiol did not induce teratogenicity or neurotoxicity in exposed zebrafish embryos. Chem Biol Interact 291: 81-86.

Valjent E, Mitchell JM, Besson MJ, Caboche J, Maldonado R (2002) Behavioural and biochemical evidence for interactions between Delta 9-tetrahydrocannabinol and nicotine. Br J Pharmacol 135: 564-78.

Valjent E, Pages C, Rogard M, Besson MJ, Maldonado R, Caboche J (2001) Delta 9-tetrahydrocannabinol-induced MAPK/ERK and Elk-1 activation in vivo depends on dopaminergic transmission. Eur J Neurosci 14: 342-52.

Van Waes V, Beverley JA, Siman H, Tseng KY, Steiner H (2012) CB1 Cannabinoid Receptor Expression in the Striatum: Association with Corticostriatal Circuits and Developmental Regulation. Front Pharmacol 3: 21.

Viveros MP, Marco EM, File SE (2005) Endocannabinoid system and stress and anxiety responses. Pharmacology, biochemistry, and behavior 81: 331-42.

Wargent ET, Zaibi MS, Silvestri C, Hislop DC, Stocker CJ, Stott CG, Guy GW, Duncan M, Di Marzo V, Cawthorne MA (2013) The cannabinoid Delta(9)-tetrahydrocannabivarin (THCV) ameliorates insulin sensitivity in two mouse models of obesity. Nutr Diabetes 3: e68.

Watson S, Chambers D, Hobbs C, Doherty P, Graham A (2008) The endocannabinoid receptor, CB1, is required for normal axonal growth and fasciculation. Molecular and cellular neurosciences 38: 89-97.

Wu CS, Zhu J, Wager-Miller J, Wang S, O'Leary D, Monory K, Lutz B, Mackie K, Lu HC (2010) Requirement of cannabinoid CB(1) receptors in cortical pyramidal neurons for appropriate development of corticothalamic and thalamocortical projections. Eur J Neurosci 32: 693-706.