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

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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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