Universiteit Leiden
The Netherlands

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

Luchtenburg, F.J.

Citation

Luchtenburg, F. J. (2020, October 7). *Functioning of the endocannabinoid system in stress and anxiety in zebrafish larvae*. Retrieved from https://hdl.handle.net/1887/137310

Version: Publisher's Version

License: Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden

Downloaded from: https://hdl.handle.net/1887/137310

Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/137310 holds various files of this Leiden University dissertation.

Author: Luchtenburg, F.J.
Title: Functioning of the endocannabinoid system in stress and anxiety in zebrafish larvae
Issue date: 2020-10-07

Chapter 3

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

Floris J. Luchtenburg, Marcel J.M. Schaaf, Michael K. Richardson
Institute of Biology
Leiden University
Leiden
The Netherlands

Manuscript in preparation

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.

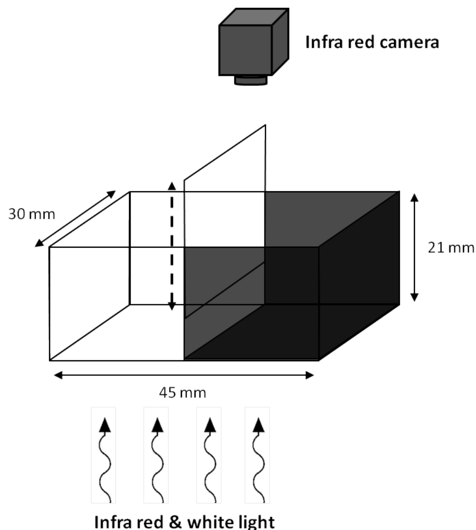

Fig. 1 Schematic overview of the light/dark preference test apparatus. It consists of an infrared camera and a plastic box, which is divided into two equally-sized compartments. Both compartments, one bright and one dark, are matte and opaque. A physical barrier (or separator) was placed between the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

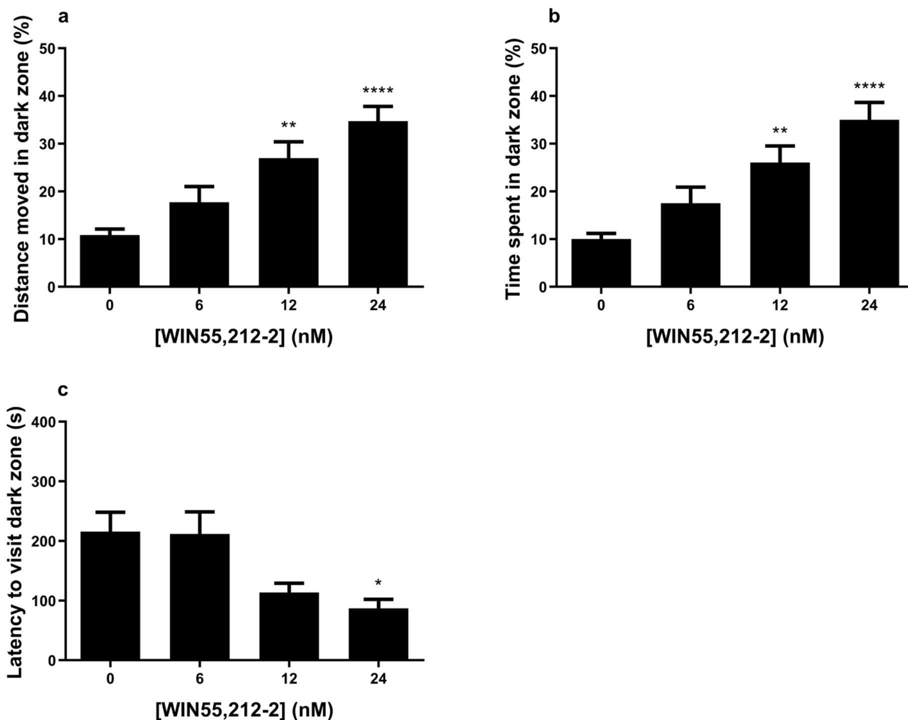

Fig. 2 The effect of WIN55,212 on anxiety-related behavior in the light/dark preference test. WIN55,212 dose dependently increases **a** the distance moved in the dark zone, **b** the time spent in the dark zone and **c** decreases the latency to visit the dark zone. Data shown are means ± SEM. Significant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

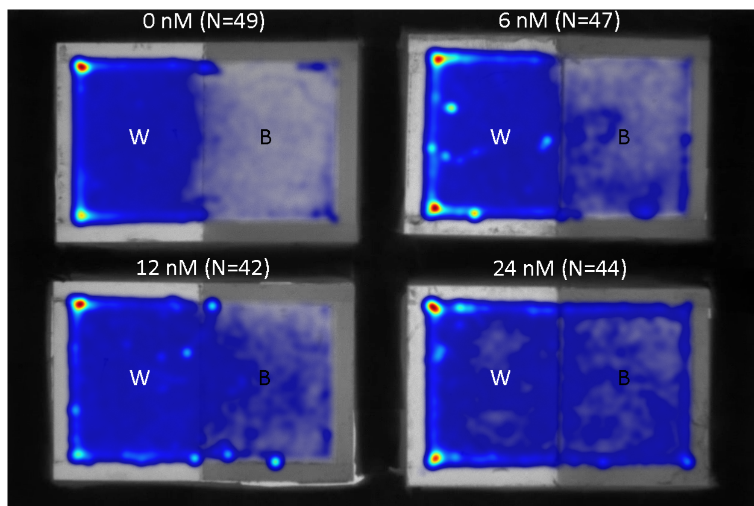

Fig. 3 General distribution of larvae in the light/dark preference test at different concentrations of WIN55,212-2. This heatmap was generated by EthoVision® XT v. 12 software (Noldus, The Netherlands) and shows the distribution of the larvae as represented by the data in Fig. 2.

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).

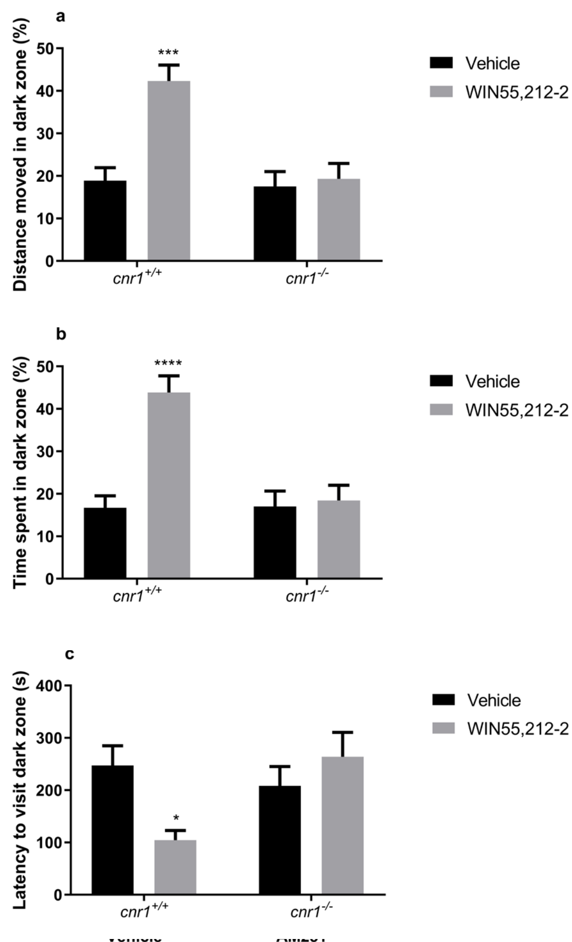

Fig. 4 Cnr1 specificity of the anxiolytic effect of WIN55,212-2. The anxiolytic effect of WIN55,212-2 (24nM), as shown by **a** increased distance moved in the dark zone, **b** increased time spent in the dark zone and **c** decreased latency to visit the dark zone, was abolished in the *cnr1*$^{-/-}$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

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.

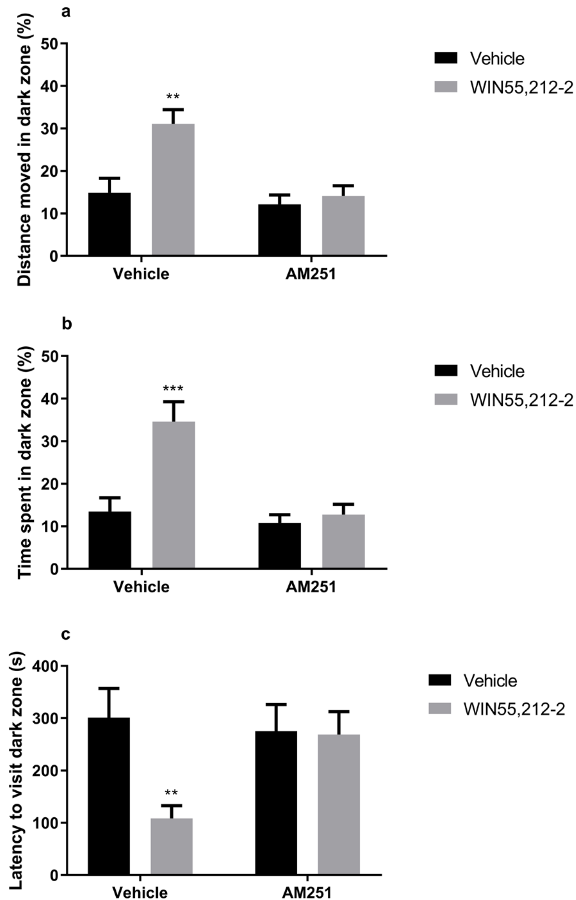

Fig. 5 Pharmacological verification of the cnr1 specificity of the anxiolytic effect of WIN55,212-2. The anxiolytic effect of WIN55,212-2 (24nM), as shown by **a** increased distance moved in the dark zone, **b** increased time spent in the dark zone and **c** decreased latency to visit the dark zone, 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.

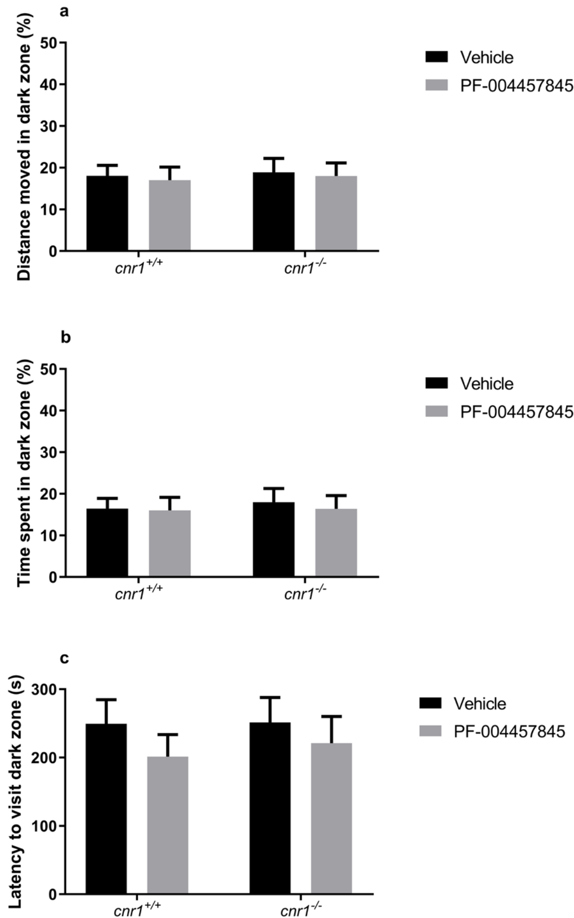

Fig. 6 Effect of PF-004457845 treatment (1μM) on behavioral parameters **a** distance moved in the dark zone, **b** time spent in the dark zone and **c** latency to visit the dark zone, in the light/dark preference test. Treatment with this FAAH inhibitor increases AEA-concentration by a 5-fold, but 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.

References

Ahmad F, Tegelenbosch RAJ, Noldus LPJJ, Richardson MK (2012) Zebrafish embryos and larvae in behavioural assays. Behaviour 149: 1241-1281.

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.

Ali S, Champagne DL, Alia A, Richardson MK (2011) Large-scale analysis of acute ethanol exposure in zebrafish development: a critical time window and resilience. PloS one 6: e20037-e20037.

Arenos JD, Musty RE, Bucci DJ (2006) Blockade of cannabinoid CB1 receptors alters contextual learning and memory. Eur J Pharmacol 539: 177-83.

Babaev O, Piletti Chatain C, Krueger-Burg D (2018) Inhibition in the amygdala anxiety circuitry. Exp Mol Med 50: 18.

Baldwin DS, Anderson IM, Nutt DJ, Allgulander C, Bandelow B, den Boer JA, Christmas DM, Davies S, Fineberg N, Lidbetter N, Malizia A, McCrone P, Nabarro D, O'Neill C, Scott J, van der Wee N, Wittchen HU (2014) Evidence-based pharmacological treatment of anxiety disorders, post-traumatic stress disorder and obsessive-compulsive disorder: a revision of the 2005 guidelines from the British Association for Psychopharmacology. J Psychopharmacol 28: 403-39.

Bandelow B, Michaelis S, Wedekind D (2017) Treatment of anxiety disorders. Dialogues Clin Neurosci 19: 93-107.

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.

Bellocchio L, Lafenetre P, Cannich A, Cota D, Puente N, Grandes P, Chaouloff F, Piazza PV, Marsicano G (2010) Bimodal control of stimulated food intake by the endocannabinoid system. Nat Neurosci 13: 281-3.

Bialuk I, Winnicka MM (2011) AM251, cannabinoids receptors ligand, improves recognition memory in rats. Pharmacol Rep 63: 670-9.

Bourin M, Hascoët M (2003) The mouse light/dark box test. European Journal of Pharmacology 463: 55-65.

Butler AB, Hodos W (2005) Comparative Vertebrate Neuroanatomy: Evolution and Adaptation. Wiley

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.

Carty DR, Miller ZS, Thornton C, Pandelides Z, Kutchma ML, Willett KL (2019) Multigenerational consequences of early-life cannabinoid exposure in zebrafish. Toxicol Appl Pharmacol 364: 133-143.

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.

Chhatwal JP, Ressler KJ (2007) Modulation of fear and anxiety by the endogenous cannabinoid system. CNS Spectr 12: 211-20.

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.

Crawley J, Goodwin FK (1980) Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav 13: 167-70.

Degroot A, Nomikos GG (2004) Genetic deletion and pharmacological blockade of CB1 receptors modulates anxiety in the shock-probe burying test. Eur J Neurosci 20: 1059-64.

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.

Feierstein CE, Portugues R, Orger MB (2015) Seeing the whole picture: A comprehensive imaging approach to functional mapping of circuits in behaving zebrafish. Neuroscience 296: 26-38.

Fontana BD, Mezzomo NJ, Kalueff AV, Rosemberg DB (2018) The developing utility of zebrafish models of neurological and neuropsychiatric disorders: A critical review. Exp Neurol 299: 157-171.

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.

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

Friedrich RW, Jacobson GA, Zhu P (2010) Circuit neuroscience in zebrafish. Curr Biol 20: R371-81.

Gabrielli M, Battista N, Riganti L, Prada I, Antonucci F, Cantone L, Matteoli M, Maccarrone M, Verderio C (2015) Active endocannabinoids are secreted on extracellular membrane vesicles. 16: 213-220.
Gaetani S, Dipasquale P, Romano A, Righetti L, Cassano T, Piomelli D, Cuomo V (2009) Chapter 5 The Endocannabinoid System as A Target for Novel Anxiolytic and Antidepressant Drugs, 1 edn. Elsevier Inc.
Garcia-Gutierrez MS, Manzanares J (2010) The cannabinoid CB1 receptor is involved in the anxiolytic, sedative and amnesic actions of benzodiazepines. J Psychopharmacol 24: 757-65.
Genn RF, Tucci S, Marco EM, Viveros MP, File SE (2004) Unconditioned and conditioned anxiogenic effects of the cannabinoid receptor agonist CP 55,940 in the social interaction test. Pharmacol Biochem Behav 77: 567-73.
Gomez-Canela C, Tornero-Canadas D, Prats E, Pina B, Tauler R, Raldua D (2018) Comprehensive characterization of neurochemicals in three zebrafish chemical models of human acute organophosphorus poisoning using liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 410: 1735-1748.
Haller J, Bakos N, Szirmay M, Ledent C, Freund TF (2002) The effects of genetic and pharmacological blockade of the CB1 cannabinoid receptor on anxiety. Eur J Neurosci 16: 1395-8.
Haller J, Mátyás F, Soproni K, Varga B, Barsy B, Németh B, Mikics E, Freund TF, Hájos N (2007) Correlated species differences in the effects of cannabinoid ligands on anxiety and on GABAergic and glutamatergic synaptic transmission. The European journal of neuroscience 25: 2445-56.
Haring M, Kaiser N, Monory K, Lutz B (2011) Circuit specific functions of cannabinoid CB1 receptor in the balance of investigatory drive and exploration. PLoS One 6: e26617.
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, 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.
Houchi H, Babovic D, Pierrefiche O, Ledent C, Daoust M, Naassila M (2005) CB1 receptor knockout mice display reduced ethanol-induced conditioned place preference and increased striatal dopamine D2 receptors. Neuropsychopharmacology 30: 339-49.

Jacob W, Yassouridis A, Marsicano G, Monory K, Lutz B, Wotjak CT (2009) Endocannabinoids render exploratory behaviour largely independent of the test aversiveness: role of glutamatergic transmission. Genes Brain Behav 8: 685-98.

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.

Koen N, Stein DJ (2011) Pharmacotherapy of anxiety disorders: a critical review. Dialogues Clin Neurosci 13: 423-37.

Komaki A, Hashemi-Firouzi N, Shojaei S, Souri Z, Heidari S, Shahidi S (2015) Study the Effect of Endocannabinoid System on Rat Behavior in Elevated Plus-Maze. Basic Clin Neurosci 6: 147-53.

Krystal JH, Davis LL, Neylan TC, M AR, Schnurr PP, Stein MB, Vessicchio J, Shiner B, Gleason TD, Huang GD (2017) It Is Time to Address the Crisis in the Pharmacotherapy of Posttraumatic Stress Disorder: A Consensus Statement of the PTSD Psychopharmacology Working Group. Biol Psychiatry 82: e51-e59.

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

Lau BY, Mathur P, Gould GG, Guo S (2011) Identification of a brain center whose activity discriminates a choice behavior in zebrafish. Proc Natl Acad Sci U S A 108: 2581-6.

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.

Litvin Y, Phan A, Hill MN, Pfaff DW, McEwen BS (2013) CB1 receptor signaling regulates social anxiety and memory. Genes Brain Behav 12: 479-89.

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.

Maccarrone M, Valverde O, Barbaccia ML, Castane A, Maldonado R, Ledent C, Parmentier M, Finazzi-Agro A (2002) Age-related changes of anandamide metabolism in CB1 cannabinoid receptor knockout mice: correlation with behaviour. Eur J Neurosci 15: 1178-86.

Mandolini GM, Lazzaretti M, Pigoni A, Oldani L, Delvecchio G, Brambilla P (2018) Pharmacological properties of cannabidiol in the treatment of psychiatric disorders: a critical overview. Epidemiol Psychiatr Sci 27: 327-335.

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.
Martella A, Sepe RM, Silvestri C, Zang J, Fasano G, Carnevali O, De Girolamo P, Neuhauss SC, Sordino P, Di Marzo V (2016) Important role of endocannabinoid signaling in the development of functional vision and locomotion in zebrafish. FASEB J 30: 4275-4288.
Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O (2002) Involvement of CB1 cannabinoid receptors in emotional behaviour. Psychopharmacology (Berl) 159: 379-87.
Maximino C, Marques de Brito T, Dias CA, Gouveia A, Jr., Morato S (2010) Scotaxis as anxiety-like behavior in fish. Nat Protoc 5: 209-16.
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.
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.
Oltrabella F, Melgoza A, Nguyen B, Guo S (2017) Role of the endocannabinoid system in vertebrates: Emphasis on the zebrafish model. Dev Growth Differ.
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.
Patel S, Hill MN, Cheer JF, Wotjak CT, Holmes A (2017) The endocannabinoid system as a target for novel anxiolytic drugs. Neurosci Biobehav Rev 76: 56-66.
Patel S, Hillard CJ (2006) Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling. J Pharmacol Exp Ther 318: 304-11.
Reich CG, Mohammadi MH, Alger BE (2008) Endocannabinoid modulation of fear responses: learning and state-dependent performance effects. J Psychopharmacol 22: 769-77.
Revicki DA, Travers K, Wyrwich KW, Svedsater H, Locklear J, Mattera MS, Shee-

han DV, Montgomery S (2012) Humanistic and economic burden of generalized anxiety disorder in North America and Europe. J Affect Disord 140: 103-12.

Rey AA, Purrio M, Viveros M-P, Lutz B (2012) Biphasic effects of cannabinoids in anxiety responses: CB1 and GABA(B) receptors in the balance of GABAergic and glutamatergic neurotransmission. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 37: 2624-34.

Ruehle S, Rey AA, Remmers F, Lutz B (2012) The endocannabinoid system in anxiety, fear memory and habituation. J Psychopharmacol 26: 23-39.

Ruhl T, Moesbauer K, Oellers N, von der Emde G (2015) The endocannabinoid system and associative learning and memory in zebrafish. Behav Brain Res 290: 61-9.

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.

Rutkowska M, Jamontt J, Gliniak H (2006) Effects of cannabinoids on the anxiety-like response in mice. Pharmacol Rep 58: 200-6.

Shin LM, Liberzon I (2010) The neurocircuitry of fear, stress, and anxiety disorders. Neuropsychopharmacology 35: 169-91.

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.

Sink KS, Segovia KN, Sink J, Randall PA, Collins LE, Correa M, Markus EJ, Vemuri VK, Makriyannis A, Salamone JD (2010) Potential anxiogenic effects of cannabinoid CB1 receptor antagonists/inverse agonists in rats: comparisons between AM4113, AM251, and the benzodiazepine inverse agonist FG-7142. Eur Neuropsychopharmacol 20: 112-22.

Steenbergen PJ, Richardson MK, Champagne DL (2011) Patterns of avoidance behaviours in the light/dark preference test in young juvenile zebrafish: a pharmacological study. Behavioural brain research 222: 15-25.

Steiner MA, Marsicano G, Nestler EJ, Holsboer F, Lutz B, Wotjak CT (2008a) Antidepressant-like behavioral effects of impaired cannabinoid receptor type 1 signaling coincide with exaggerated corticosterone secretion in mice. Psychoneuroendocrinology 33: 54-67.

Steiner MA, Marsicano G, Wotjak CT, Lutz B (2008b) Conditional cannabinoid receptor type 1 mutants reveal neuron subpopulation-specific effects on behavioral and neuroendocrine stress responses. Psychoneuroendocrinology 33: 1165-70.

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

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

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

Turna J, Patterson B, Van Ameringen M (2017) Is cannabis treatment for anxiety, mood, and related disorders ready for prime time? Depress Anxiety 34: 1006-1017.

Turrini L, Fornetto C, Marchetto G, Mullenbroich MC, Tiso N, Vettori A, Resta F, Masi A, Mannaioni G, Pavone FS, Vanzi F (2017) Optical mapping of neuronal activity during seizures in zebrafish. Sci Rep 7: 3025.

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.

von Trotha JW, Vernier P, Bally-Cuif L (2014) Emotions and motivated behavior converge on an amygdala-like structure in the zebrafish. Eur J Neurosci 40: 3302-15.

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.