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

Luchtenburg, F.J.

Chapter 4

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

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

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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

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

Materials and methods

Zebrafish maintenance and care

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

Test compounds

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

Treatment

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

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

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

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

Results

WIN55,212-2 treatment increases Cnr1 dependently cortisol concentrations

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

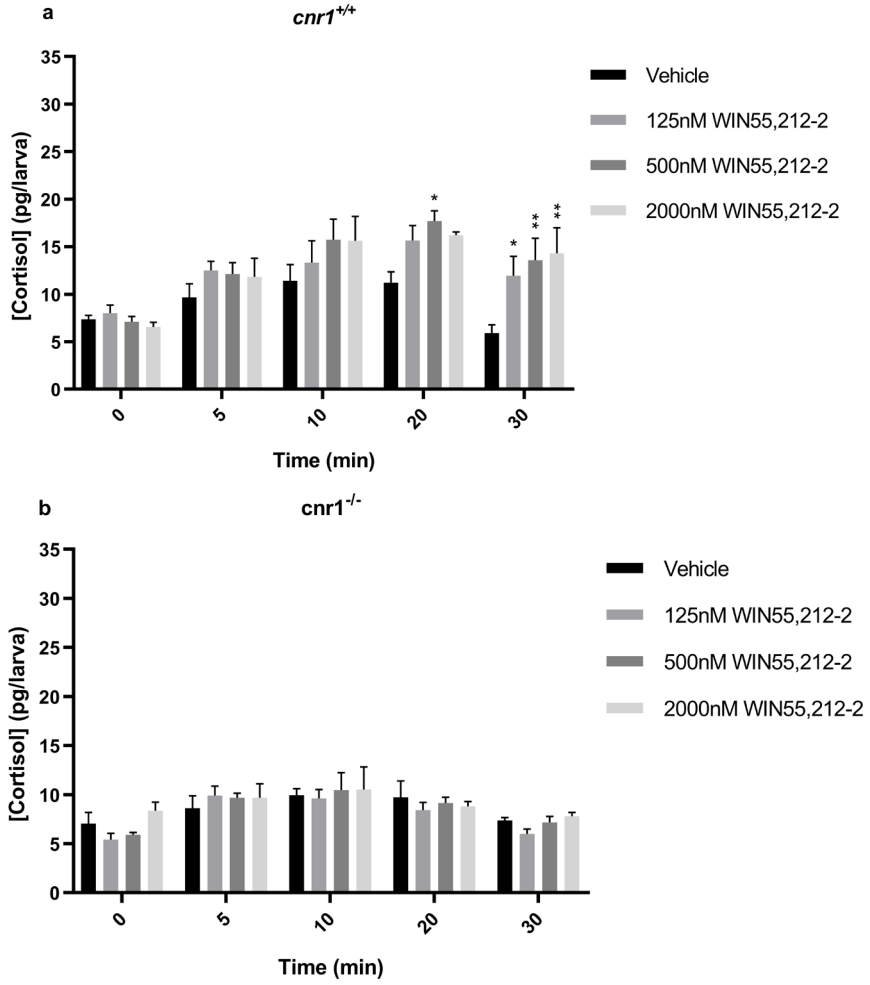

Fig. 1 Effect of WIN55,212-2 exposure on cortisol production in **a** *cnr1*$^{+/+}$ larvae and **b** *cnr1*$^{-/-}$ larvae. WIN55,212-2 increases basal cortisol levels at all concentrations tested in *cnr1*$^{+/+}$ larvae, but has no effect in *cnr1*$^{-/-}$ larvae. Data shown are means ± SEM. Significant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

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

Antagonizing Cnr1 with AM251 does not affect cortisol concentration

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

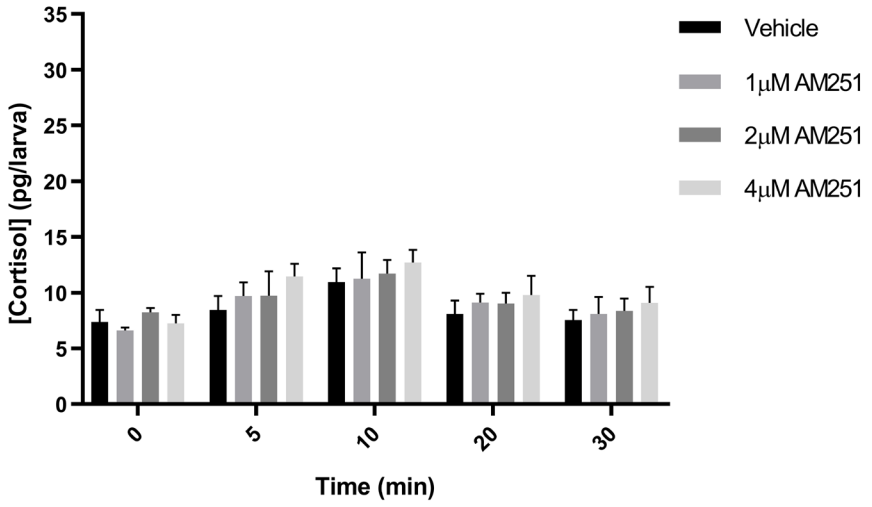

Fig. 2 AM251 exposure has no effect on cortisol production. Data shown are means ± SEM. No significant differences were found when comparing the different concentrations within the same time group.

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

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

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

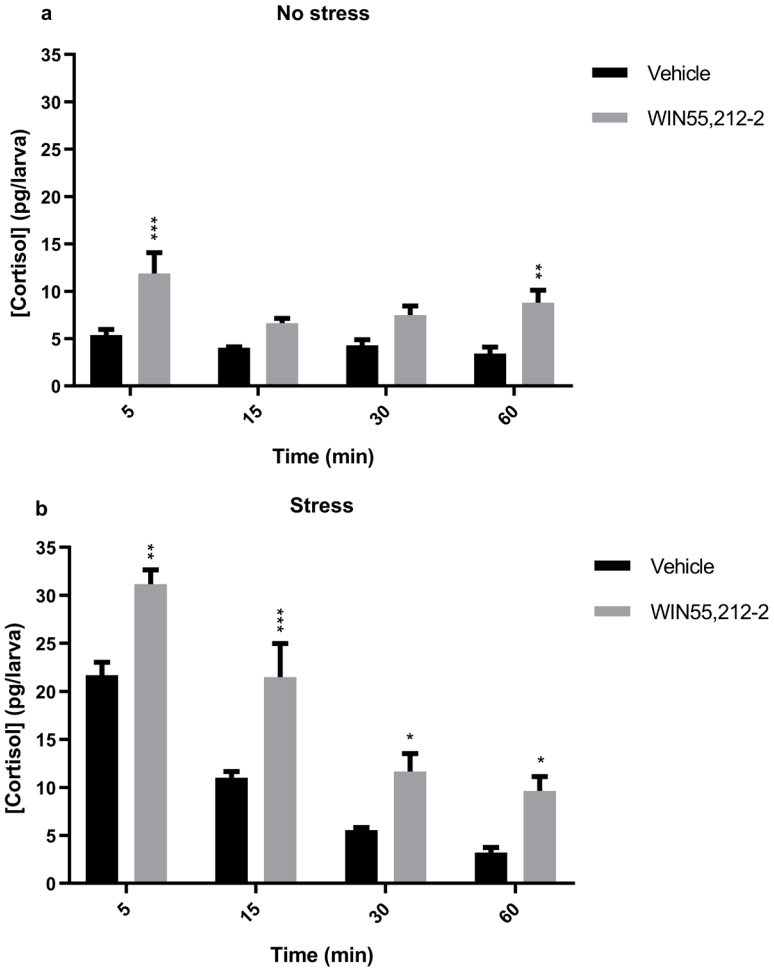

Fig. 3 The effect of netting stress and WIN55,212-2 exposure on cortisol production. Larvae were first treated with 2 μM WIN55,212-2, 20 min later followed by **a** no stress or **b** 4 min netting stress. WIN55,212-2 exposure increases both basal (at 5 and 60min) and post-stress (all time points) cortisol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

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

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

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

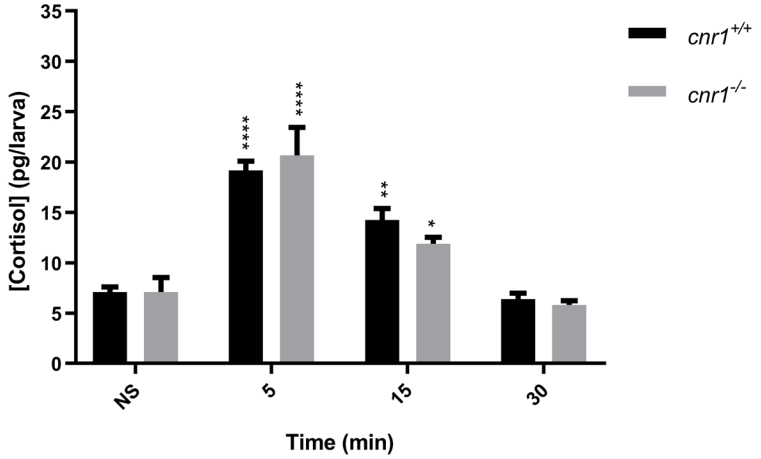

Fig. 4 The cortisol response to stress is not different between *cnr1$^{+/+}$* and *cnr1$^{-/-}$* larvae. Time is 0 min at end of stressor. Data shown are means ± SEM. No significant differences were found when comparing the different strains within the same time group. Netting stress causes a significant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

Faah inhibition does not affect the cortisol stress response

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

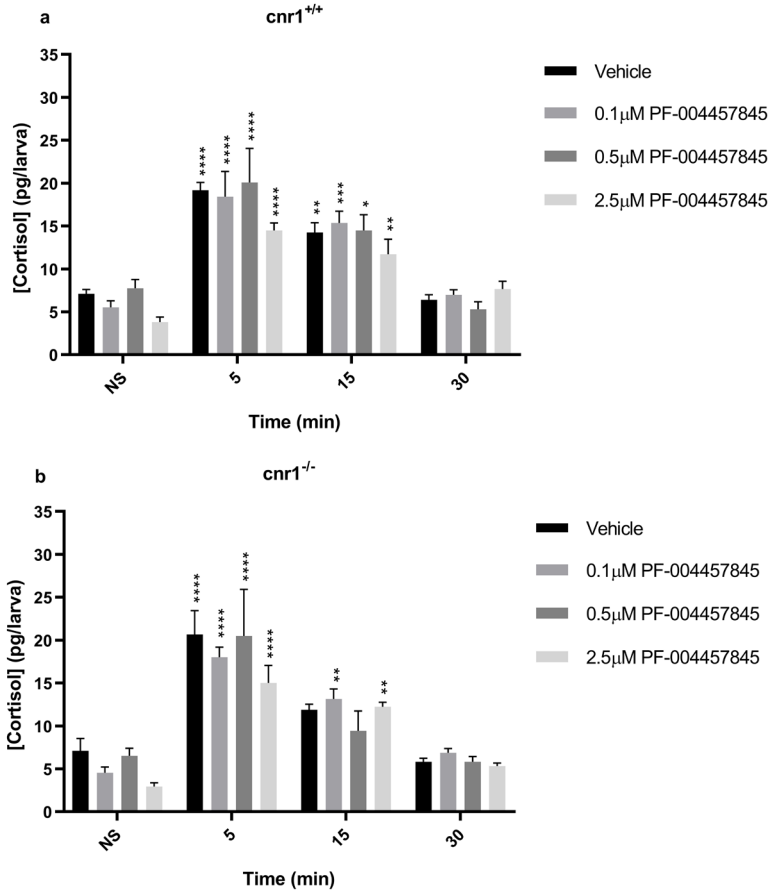

Fig. 5 The Faah-inhibitor PF-04457845 does not have an effect on cortisol production in both **a** *cnr1*$^{+/+}$ larvae and **b** *cnr1*$^{-/-}$ larvae. Time is 0 min at end of stressor. Data shown are means ± SEM. No significant differences were found when comparing the different concentrations within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

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

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

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

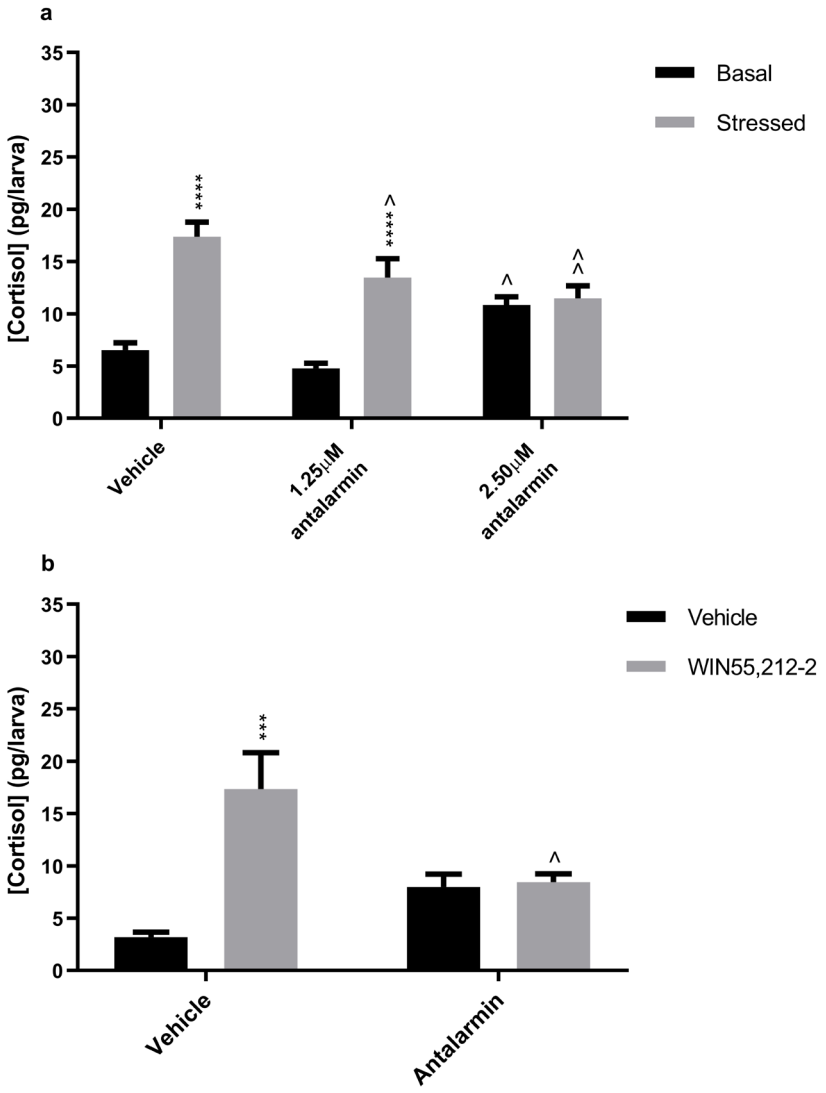

Fig. 6 The involvement of Crh-R1 on WIN55,212-2 or stress-induced cortisol response. a The effect of antalarmin on the cortisol response on 5 min netting stress. At **a** concentration of 2.50 μM, antalarmin abolishes this stress response. **b** The effect of antalarmin on the WIN55,212-2-induced cortisol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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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