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CB_1 and CB_2 receptors play differential roles in early zebrafish locomotor development

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Abstract

Endocannabinoids (eCBs) mediate their effects through actions on several receptors including the cannabinoid receptors CB_1R and CB_2R. The role played by eCBs in the development of locomotor systems is not fully understood. In this study we investigated the roles of the eCB system in zebrafish development by pharmacologically inhibiting the CB_1R and CB_2Rs (with AM251 and AM630 respectively) in either the first or second day of development. We examined the morphology of motor neurons and we determined neuromuscular outputs by quantifying the amount of swimming in 5 dpf larva. Blocking CB_2R during the first day of development resulted in gross morphological deficits and reductions in heart rate that were greater than that following treatment with the CB_1R blocker AM251. Blocking CB_1Rs from 0 to 24 hpf resulted in an increase in the number of secondary and tertiary branches of primary motor neurons, whereas blocking CB_2Rs had the opposite effect. Both treatments manifested in reduced levels of swimming. Additionally, blocking CB_1Rs resulted in greater instances of non-inflated and partially inflated swim bladders compared with AM630, suggesting that at least some of the deficits in locomotion may result from an inability to adjust buoyancy. Together these findings indicate that the endocannabinoid system is pivotal to the development of the locomotor system in zebrafish, and that perturbations of the eCB system early in life may have detrimental effects.

Introduction

The eCBs, N-Arachidonoylethanolamine (anandamide or AEA) and 2-Arachidonoylglycerol (2-AG), are highly lipophilic molecules that bind to and interact with the G-protein coupled receptors, CB_1R and CB_2R. Both CB_1R and CB_2R are negatively coupled to adenylate cyclase and are expressed in very different regions of the body. Within the CNS, CB_1Rs are highly expressed in regions of the basal ganglia such as the substantia nigra pars reticulata and globus pallidus, as well as in the hippocampus and cerebellum (Herkenham et al., 1990). CB_1Rs appear to be localized to presynaptic regions where they play neuromodulatory roles and have been implicated in homeostasis (Oltrabella et al., 2017; Ruginsk et al., 2015). CB_2Rs were first thought to be located outside of the CNS, associated with the immune system, the reproductive system and the digestive system (Howlett and Abood, 2017; Mouslech and Valla, 2009), but recent findings point to a clear distribution within the CNS of various organisms (Jordan and Xi, 2019; Liu et al., 2016). The eCB system is involved in events as diverse as oocyte maturation (Lopez-Cardona et al., 2017), liver development (Liu et al., 2016), cardiovascular function (Pacher et al., 2018) and differentiation of hematopoietic cells (Alger, 2012). Activation of CB_1Rs and CB_2Rs initiates a signalling cascade that requires the Gi/o subset of G-proteins and that results in the downregulation of cAMP levels (Herkenham et al., 1991; Onaivi et al., 2012). Ligand binding studies show that anandamide is capable of inhibiting adenylate cyclase activity in membranes possessing CB_1Rs (Childers et al 1994; Howlett and Mukhopadhyay 200), but it shows significantly less efficacy at CB_2Rs, suggesting that anandamide has differential effects on CB_1 vs CB_2 receptors. In contrast, 2-Arachidonoylglycerol (2-AG), appears to be a full agonist at CB_1R and CB_2Rs (Sugiura et al., 2006). Anandamide and 2-AG are metabolized by

the enzymes, fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MGL) respectively.

In the developing CNS endocannabinoids are involved in neuronal proliferation (Diaz-Alonso et al., 2012b; Harkany et al., 2007; Palazuelos et al., 2012), axonal growth and fasciculation (Mulder et al., 2008), neuronal chemoattraction and migration, synaptic formation and shaping neuronal connectivity (Berghuis et al., 2007). They also play roles in neurogenesis in both embryos and adults (de Oliveira et al., 2018). In humans the eCB system may contribute to the maturation of corticolimbic neuronal populations in adolescents (Meyer et al., 2018), and in chicks and mice, CB_1R protein expression first occurs before neuronal development (Psychoyos et al., 2012) and increases in a region-specific manner (Buckley et al., 1998).

Previous findings point to a role of the endocannabinoid system in zebrafish development (Akhtar et al., 2013; Akhtar et al., 2016; Carty et al., 2017; Oltrabella et al., 2017; Watson et al., 2008). For instance, reduced gene expression for the CB_1R results in a number of deficits in axonal growth, neuronal branching and fasciculation of hindbrain neurons that are known to express the CB_1R (Watson et al., 2008). When embryos are exposed to Δ^9-tetrahydrocannabinol (THC) after the first 24 hours of development, they exhibit a biphasic locomotor activity that is prevented by the CB_1R antagonist AM251(Akhtar et al., 2013). Manipulation of the eCB system through morpholino knockdown of the main catabolic enzyme for 2-AG, Daglα, leads to altered axonal growth in the midbrain-hindbrain region and abnormal movement and motion perception (Martella et al., 2016). Therefore, these studies implicate a role for the CB_1R in early development, but a role for the CB_2R in CNS development remains to be examined.

Because the eCB receptors are expressed in a region-specific manner, we set out to determine if inhibition of the prototypical eCB receptors, CB_1R and CB_2R during the early stages of zebrafish development would alter normal development of cells in the locomotor system in a fashion that was receptor specific. We treated embryos with CB_1R and CB_2R antagonists during either the first 24 hours (0-24 hpf) or the second 24 hours (24-48 hpf) of development and examined a range of features associated with locomotion. Our findings indicate that the endocannabinoid system plays a role in motor neuron pathfinding and branching, and in the development of normal locomotor activities.

Materials & methods

Animal care and CB receptors antagonist treatment

We used the TL (Tubingen Longfin) strain of wild type zebrafish that are maintained in the University of Alberta aquatic facility. All procedures were approved by the Animal Care and Use Committee at the University of Alberta (AUP#00000816). A 12 hr light/dark cycle and 28.5ºC temperature was maintained for embryos and larvae housing in incubators.

Embryos were exposed to the CB receptor antagonists AM251 0.5 µM (Selleck, USA) or AM630 5 µM (Adooq Bioscience, Irvine, CA) diluted in egg water (EW; 60µg/ml Instant Ocean) either from 0-24 hour or 24-48 hour post fertilization (hpf). The experimental dose was selected from a range of concentrations of AM251 (0.05 µM - 5µM) and AM630 (0.2 µM - 10 µM). After antagonist exposure, eggs were washed several times to remove the drugs and were then kept in normal egg water. The egg water was changed every morning until further experimentation. AM251 and AM630 were dissolved in 0.1% DMSO and a vehicle control

was run throughout the study. For immunohistochemical studies, pigmentation was blocked using 0.003% PTU (Phenylthiourea) with egg water normally from 24-hour post fertilization.

Embryo imaging and morphological observations

Photographs of embryos and larvae were taken using a Lumenera Infinity2-1R color camera mounted on a Leica DM2500 stereomicroscope under 2.5x (embryos or larvae full length images) or 5x (swim bladder at 5 dpf) magnification. Embryos were placed in a 16-well plate with a single embryo per well and were anesthetized using 0.02% MS222. To obtain body lengths, embryos were imaged with a Leica dissecting microscope and the images were analysed offline using Proanalyst software (XCITEX, USA). For heart rates, quantification was done offline using video recordings of embryonic heart activity for a continuous 30 seconds and then multiplying it by a factor of 2.

Locomotor activity measurements

To track locomotor activities, individual 5 dpf larvae were placed in a single well of a 96-well plate and then video-taped and the data analysed according to previously published procedures (Baraban et al., 2005; Leighton et al., 2018). Larvae were gently positioned in the centre of wells containing 150 µl egg water, pH 7.0 and 48 wells were used each time from a 96 well plate in our study (Costar #3599). Prior to video recording, larvae were acclimated in the well plate for 60 minutes. Plates were placed on top of an infrared backlight source and a Basler GenlCaM (Basler acA 1300-60) scanning camera with a 75 mm f2.8 C-mount lens, provided by Noldus (Wageningen, Netherlands) was used for individual larval movement tracking.

EthoVision ® XT-11.5 software (Noldus) was used to quantify activity (%), velocity (mm/s), swim bouts frequency and cumulative duration of swim bouts for one hour. To exclude

background noise, ≥0.2 mm was defined as active movement. Activity was defined as % pixel change within a corresponding well between samples (motion was captured by taking 25 samples/frames per second) as reported previously (Leighton et al., 2018).

Immunohistochemistry

Embryos at 2 dpf were dechorionated and fixed in 2% paraformaldehyde for 2 hours. After fixation, preparations were washed in 0.1M PBS (Phosphate buffer saline; in mM: 150 NaCl, 8 Na_2HPO_4, 2 $NaH_2PO_4.2H_2O$ and pH 7.2) every 15 minutes for 2 hours. Preparations were permeabilized in 4% Triton X-100 containing 2% BSA and 10% goat serum for 30 minutes, and were incubated for 48 hours at 4ºC in mouse monoclonal anti-znp1 or anti-zn8 (Developmental Studies Hybridoma Bank; DSHB) antibodies. Anti-znp1 (1:250, DSHB, University of Iowa, deposited by B. Trevarrow) identifies an isoform of synaptotagmin 2, a protein that is highly localized in primary motor axons (Fox and Sanes, 2007; Trevarrow et al., 1990). Whereas anti-zn8 (1:250, DSHB, University of Iowa, deposited by B. Trevarrow) targets DM-GRASP, a protein localized on the surface of secondary motor axons (Fashena and Westerfield, 1999; Sylvain et al., 2010). Primary antibodies were diluted at 1:250 in PBS. After incubation in the primary antibodies tissues were washed in PBS every 15 min for 3 hours and then incubated in the secondary antibody, Alexa Fluor® 488 goat anti-mouse IgG (1:1000 dilution) (Molecular Probes, Life Technologies) at room temperature for 4 hours.

The samples were further washed in PBS every 30 min for 7 hours before mounting into MOWIOL mounting media. Immunofluorescent images were taken with a Zeiss LSM confocal microscope, photographed under a 40x (primary motor axon) or 20x (secondary motor axon) objective lens. Multiple image stacks were collected using 1µm z-steps through the entire thickness of the embryo samples. Image compilations were done using Zeiss LSM image browser software and are shown as maximum intensity of z-stack compilations. The number

of primary, secondary and tertiary axon branches emanating from the primary motor axon were tracked and counted using simple neurite tracer FIJI (ImageJ). The numbers of lateral and ventral branches projecting from secondary motor axons were counted from 3 axons per sample.

Statistics

All values were reported as mean± SEM (Standard error of the mean). Statistical analysis was performed to determine significance using a one-way ANOVA followed by a Tukey post-hoc multiple comparisons test where appropriate ($p<0.05$). GraphPad Prism Software (Version 7, GraphPad, San Diego, CA) was used to carry out statistical analysis. During locomotor activity analysis, outliers due to off-tracking (when tracking software wasn't able to detect larval tracing) were rejected objectively using ROUT at Q=0.1.

Results

Morphology and Cardiac Activity

In this study we attempted to delineate the effects of the endocannabinoid system in zebrafish early development by blocking the cannabinoid receptors with the CB_1R antagonist AM251 or the CB_2R antagonist AM630 for the first 24 hours of development or the second 24 hours of development as shown in figure 1 (Fig 1A, B). Previous studies indicated that blocking the CB_1R and CB_2Rs for a full 48 hours before hatching resulted in morphological and locomotor deficits (Sufian et al 2019 In Press). In this study we significantly expanded this work by blocking the eCB receptors either individually or in combination, for the first 24 hours, or the second 24 hours of development and by examining the effects on the development and morphology of primary and secondary motor neurons.

First, we determined dose-dependent effects of the antagonists by applying a range of commonly used concentrations (0.05 μM - 5μM for AM251 and 0.2 μM - 10μM for AM630) (Akhtar et al., 2013; Esain et al., 2015; Fraher et al., 2015; Tran et al., 2016) when testing survival, hatching, heart rates and morphological deficits. Based upon these results, we used a single concentration for AM251 and AM630 that was 50-70% effective for the remainder of the study. The results indicate that blocking CB_1Rs with AM251 has limited effect on gross morphology in either the first or second 24 hours of development. However, blocking the CB_2Rs in the first 24 hours resulted in significant morphological defects (Fig 1 C, D), whereas blocking CB_2Rs from 24 to 48 hpf had no obvious effects on morphology (Fig 1C, D). To examine the effects of blocking both CB_1Rs and CB_2Rs simultaneously, we incubated fertilized eggs in concentrations of the blockers that were approximately 50-60% effective. The results of these combined blockers were intriguing because they showed little effect when used from 0 to 24 hpf, but had a greater effect when used from 24-48 hpf (Fig 1C, D). Blocking the CB_2R from 0-24 hpf significantly reduced body length (Fig 1E; $p<0.05$). In contrast, inhibition of CB_1R from 24 to 48 hpf had a greater effect on body length compared to block of CB_2R (Fig 1F). Finally, treatment with both inhibitors simultaneously had greater effects when applied from 24 hpf to 48 hpf, but not when applied earlier (Fig 1E, F; $p<0.05$). Together these results suggest that the CB_2R plays a greater role in gross morphological development of zebrafish in the first 24 hours after egg fertilization while the CB_1R may play a comparatively greater role in the second 24 hours of development.

An examination of gross morphological deficits indicate that blocking CB_1R and CB_2Rs in the first 24 hours had a greater effect on pericardial edema (PE), yolk sac edema (SE), and tail and body malformations (BM) (Fig 2) compared with blocking the receptors from 24 to 48 hpf. For instance, incubation in AM630 in the first 24 hours resulted in rates of edema of 40 ± 4% (n=62) compared with 14 ± 1% (n=70) when incubated from 24 to 48 hpf (Fig 2B,

C). Similarly, blocking CB_2Rs from 0-24 hpf resulted in 43 ± 2% (n=62) rate of tail and body malformations compared with 14 ± 1% (n=70) when treated from 24 to 48 hpf (Fig 2D, E). In the case of both these treatments, combining the CB_1R and CB_2R antagonists generally did not lead to a greater effect (Fig 2).

Blocking CB_2R in the first 24 hours of development had the greatest effect on cardiac activity and resulted in heart rates of 70 ± 2 (n= 36) beats/min compared with 99 ± 1 (n=35) beats/min in control animals (Fig 2F). Blocking CB_1Rs in either the first day or the second day of development reduced heart rate from 91 beats per minute in controls to around 85 beats/min (Fig 2F, G). Blocking CB_2Rs from 24 to 48 hpf had a smaller effect on heart rate and resulted in rates of 86 ± 1 (n=35) beats min^{-1} compared with 98 ± 1 beats min^{-1} of vehicle controls (Fig 2G). Exposure to the combined antagonists from 0 to 24 hpf during the second day decreased heart rate to an intermediate level compared with each individual blocker (Fig 2F), whereas exposure to both blockers from 24 to 48 hpf had a significantly larger effect (Fig 2G). Taken together these findings implicate a more significant role for CB_2Rs in early morphological development comparted with CB_1Rs.

Morphology of Motor Neurons

Because our research focus is directed towards understanding neurodevelopment associated with locomotion, we examined whether the endocannabinoid system might be involved in the development of motor neurons in zebrafish embryos. To do this we performed immunohistochemistry to image the morphology of primary motor neurons, specifically focusing on their branching patterns. Blocking CB_1Rs in the first day of development had minimal effect on the primary branches emanating from the main axon (Fig 3B, E) but it significantly increased the number of secondary and tertiary axonal branches from control values of 13 ± 1 (n=7) to 23 ± 2 (n=7) branches per ventral motor axon ($p<0.001$, Fig 3F). In

comparison, blocking CB_2Rs gave a very interesting result and had the opposite effect of significantly decreasing the number of secondary and tertiary branches to only 5 ± 1 branches per ventral motor axon (n=6; $p<0.01$). Combining the CB_1R and CB_2R antagonists resulted in an intermediate level of branching which was not significantly different from controls (n=7; $p<0.92$) (Fig 3F).

Blocking CB_1Rs in the second day of development resulted in a significant increase in the number of secondary and tertiary branches from 11 ± 1 (n=7) in the controls to 19 ± 1 in the treated group (Fig 3L). However, application of AM630 from 24-48 hpf had no significant effect on the number of secondary and tertiary branches (Fig 3L; n=7; $p<0.92$). Application of both blockers simultaneously resulted in branch numbers that were intermediate between the effect of CB_1R and CB_2R individually (Fig 3L). These data provide some of our most interesting results and suggest that CB_1R and CB_2Rs play opposing roles with respect to the extent of motor neuron branching.

An examination of secondary motor neurons showed that exposure of the embryos to either the CB_1R or the CB_2R antagonist in the first 24 hours resulted in disruption of the lateral branches to the extent that some branches were completely absent while others were truncated or misshapen (Fig 4A-D, E). Interestingly, we found no alterations or deficits in the ventral branches of secondary motor neurons (Fig 4A-D, F). Similar results were obtained when exposures occurred over the 24-48 hpf time frame. In those experiments, we found that blocking the CB_1R or CB_2Rs altered the number and shape of the lateral branches without affecting the ventral branches (Fig 4G-L).

Locomotor Assays

Because we identified alterations in the branching patterns of both primary and secondary motor neurons, we asked whether these deficits translated into functional changes

in locomotion and movement. To address this, we allowed the fish to develop until they were 5 dpf when they become more active. However, we noted a significant number of morphological deficits in the treated groups such as pericardial edema and trunk malformations that might impact swimming. Quantification of these deficits showed significantly high levels of pericardial and yolk sac edema (Fig 5B, $p<0.05$) and tail malformation (Fig 5D; $p<0.05$) in fish treated with either antagonist. The proportion of animals exhibiting malformations was greater in animals treated in the first 24 hours compared with the second 24 hours (Fig 5B-E).

To examine larval locomotion we transferred individual animals to single wells of a 96-well plate and allowed them to acclimate to their new environment for 60 minutes before filming their activity. We found that embryos treated with AM251 in the first 24 hours exhibited approximately one half to one third the level of activity, swim velocity, number of swim bouts and cumulative duration of swim bouts compared with controls (Fig 6B-E; $p<0.052$; n=19-28). This was also evident when embryos were treated with AM251 from 24 to 48 hpf (Fig 6F-I; $p<0.048$; n=16-38). Exposure to the CB_2R antagonist, AM630 resulted in trends towards fewer and smaller swim bouts, but without significance (Fig 6B-I).

We noticed that animals exposed to AM251 tended to lie on the bottom of their holding dishes more often than animals exposed to AM630 or vehicle treated controls. Therefore, we examined their swim bladders to determine if they had developed properly (Fig 7). We found that treatment with AM251 resulted in a smaller percentage of animals with fully inflated swim bladders (Fig 7 B, E). For instance, only 36 ± 5% of animals treated with AM251 in the first 24 hours had fully inflated swim bladders, whereas 65 ± 3% of animals treated with AM630 had fully inflated swim bladders compared with controls of around 90 ± 1% (Fig 7A, B; n=58-59; $p<0.005$). Likewise, in the groups treated with AM251 from 24-48 hpf, 53 ± 2% had fully inflated swim bladders compared with 74 ± 4% in the AM630 treated group and 92 ± 1.56% in the controls (Fig 7A, E; n=54-60; $p< 0.005$). Concurrently there were greater proportions

of animals with partially inflated and non-inflated swim bladders in the AM251 treated groups compared with the AM630 treated animals (Fig 7C-D, F-G) (n=54-60). Thus, the deficits in swim bladder inflation could account for some or all of the deficits in locomotion. Together, our findings show that activity, locomotion and swim bladder development are largely influenced by activation of CB_1Rs and CB_2Rs.

Discussion

In this manuscript we show that preferentially blocking the cannabinoid receptors CB_1R or CB_2R in zebrafish embryos during either the first 24 hours of development or the second 24 hours of development resulted in differential developmental effects. Our long-term goal is to study the role of the eCB system in early synaptic development by perturbing the system at select points. Our findings show that blocking cannabinoid receptors leads to alterations in hatching, survival, heart rate and locomotion and that this occurred in a dose-dependent manner.

In previous studies we examined the effects of either over-activating the cannabinoid receptors by exposing embryos to THC and cannabidiol (CBD)(Ahmed et al., 2018), or of blocking the receptors for extended lengths of time (Sufian et al 2018 In Press). Our results were consistent with other studies and suggested that CB receptors play multiple roles during early development in events such as hatching, survival, heart rate, motor neuron development, responses to mechanical and sound stimuli, and ability to locomote (Fine and Rosenfeld, 2013; Fride, 2008; Migliarini and Carnevali, 2009). In the present study, we focus on motor neurons and locomotion, and show that inhibition of the CB_1R or CB_2R resulted in different effects depending on the time of exposure. Both antagonists had stronger effects on hatching, survival, edema, and body

malformations when used at 0-24 hpf compared with the 24-48 hpf exposure. However, blocking the CB_2Rs with AM630 was significantly more effective when applied in the first 24 hours. This was evident when comparing the morphology at 2 dpf. In fact, the morphological deficits persisted throughout development and by 5 dpf, animals exposed to the CB receptor blockers showed significantly more abnormalities than at 2 dpf.

Previous studies examined the expression of CB_1 and CB_2 receptors in developing zebrafish and found that CB_1Rs are expressed at a very low level in the first day of development, while CB_2Rs are more highly expressed (Oltrabella et al., 2017). Both receptors are expressed from as early as 1 hr following egg fertilization (Oltrabella et al., 2017) until adulthood. Zebrafish express a single form of the CB_1R (Lam et al., 2006) but 2 forms of the CB_2R due to gene duplication (Rodriguez-Martin et al., 2007). Thus, the expression of CB_1R and CB_2R mRNA were such that CB2 levels are greater in the early stages but CB1 levels rise dramatically by the time of hatching, implying a greater role for CB_2Rs in early development, and CB_1Rs in later development when the nervous system is rapidly maturing.

CB_1 receptor activity has been linked to motor neuron development through a number of factors. For instance, transgenic studies using CB_1R knockout mice ($CB1^{-/-}$ mice) show that CB_1R tune the balance between deep- and upper-layer cortical projection neurons (Diaz-Alonso et al., 2012a). The CB1 receptors are also coupled to the regulation of the Ctip2-Satb2 regulatory code by altering transcription, and are linked to the development of corticospinal tracts (Diaz-Alonso et al., 2012a). In embryonic organisms CB_1 agonists and antagonists are capable of altering axonal growth (Williams et al., 2003), and signaling through the eCB system has been shown to play chemo-attractive and chemo-repulsive roles in developing cortex (Berghuis et al., 2005; Berghuis et al., 2007). A number of other studies provide solid evidence for an interaction between the

endocannabinoid system and several different growth factors during early development. For example, neurite outgrowth of cerebellar neurons is impacted by CB_1R activation coupled to fibroblast growth factor (FGF) receptor activity (Berghuis et al., 2005). Moreover, CB_1R interaction with tyrosine kinase B (TrKB) receptors in cortical interneurons is necessary for interneuron migration and specification (Berghuis et al., 2005).

Finally, activation of CB_1R by agonists such as methanandamide increased self-renewal of neuronal-like cells in the subventricular zone via a Notch-related pathway (Xapelli et al., 2013). Importantly, activation of CB_1R also led to an increase in neurite growth and extension. Thus, the endocannabinoid system has the ability to control neuronal migration and differentiation by regulating growth factor activity.

Our finding that locomotion is altered following inhibition of cannabinoid receptors is in line with a role in neuromuscular development. Blocking CB_1Rs in either the first 24 hpf or the second 24 hpf results in an increase in the number of secondary and tertiary branches emanating from the main axons of primary motor neurons. This result was consistent and robust, but differed dramatically from the effects of blocking the CB_2Rs, which resulted in a decrease in the number of branches. Our results suggest that there may be an interplay between the actions of these receptors on neuronal growth. With regard to the secondary motor neurons we found that treatment with the CB_1R blocker altered lateral branching in approximately 50% of the cases. Surprisingly, the ventral branches were completely unaffected by any of the treatments. Our findings compare well with other studies where the eCB has been shown to impact neuronal growth, axonal branching and pathfinding (Alpar et al., 2014).

CB_1 and CB_2 receptors are differentially expressed during development. In embryonic rat the CB_1R is expressed throughout the nervous system, eyes, digestive tract, endocrine organs and lungs before gestational stage E8 to the end of gestation around E22 (Buckley et al., 1998), whereas CB_2Rs were only found in the liver from embryonic day 13 (E13), but continued to be present after birth (Buckley et al., 1998). Zebrafish express a single form of the cb1 gene and two forms of the cb2 receptor gene (Elphick and Egertova, 2001) with mRNA transcripts appearing as early as 1 hpf (Oltrabella et al., 2017). CB_2 receptor expression is present from very early in development, around the 1 hpf time point at relatively higher levels than CB_1 (Oltrabella et al., 2017). The time course for the expression of CB_1R and CB_2R show opposite patterns. Throughout the first stages of development until the end of gastrulation, CB2 mRNA levels are much greater than CB1 mRNA, implying a greater functional role for CB_2Rs in the early stages of development. This is consistent with our data which generally shows a greater effect of CB_2R than CB_1R in the first 24 hours of development. However, by day 5 CB_2R expression drops considerably and CB1 mRNA levels increase (Oltrabella et al., 2017).

In all of our experiments we used a combination of AM251 and AM630 to effectively inhibit both CB_1R and CB_2R simultaneously during the first or second 24 hours of development. Only in a few instances did we find that co-application of both antagonists resulted in an augmented effect, and often co-application resulted in an effect that was intermediate compared with application of either antagonist alone. Overall these findings suggest that CB_1 and CB_2Rs may play opposite roles during development. It is important to recognise that while we performed complete dose responses for the effects of both antagonists on hatching, survival, morphology and cardiac activity, we chose a single dose to use throughout the remainder of the study for logistical purposes. Knockout or knockdown of the CB receptors may help to determine their roles. Future studies using

morpholinos to knockdown CB_1R and CB_2R separately, and full knockouts using CRISPR-Cas9 will be critical to fully ascertain their roles during development. In fact, morpholino knockdown of the CB_1 receptor has already been performed and shows that morphants exhibit abnormal patterns in the growth of hindbrain reticulospinal neurons that are known to express cannabinoid receptors (Watson et al., 2008). Interestingly, the axonal growth of neurons that do not express CB_1Rs was unaffected (Watson et al., 2008). Our findings are consistent with this earlier study. Moreover, our findings that inhibition of CB_1R or CB_2R suppressed locomotor activity is also consistent with aberrant motor neuron branching in our treated animals, and with a previous finding in which researchers exposed 1 dpf animals to AM251 for time frames ranging from 1 hour to 96 hours (Akhtar et al., 2013). There, the researchers found that exposure to high concentrations of AM251 reduced locomotion.

In an earlier study our findings showed that zebrafish embryos exposed to cannabinoids experienced aberrant development of spinal cord motor neurons (Ahmed et al., 2018). There were additional effects on morphology, hatching, survival and cardiac activity. Furthermore, the electrical communication (mEPCs) between spinal motor neurons and muscle cells was also altered (Ahmed et al., 2018). In that earlier study the time frame of exposure to THC was only 5 hours during the developmental stage of gastrulation, whereas in the present work we attempted to block the CB_1R and CB_2Rs for the first 48 hours of development. Taken together, our results suggest that alterations in the endocannabinoid system, either by an upregulation or a reduction in activity of the cannabinoid receptors, impacts growth and development. These findings highlight the homeostatic role of the eCB system in the early stages of life.

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References

Ahmed, K. T., Amin, M. R., Shah, P. and Ali, D. W. (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, M. T., Ali, S., Rashidi, H., van der Kooy, F., Verpoorte, R. and Richardson, M. K. (2013). Developmental effects of cannabinoids on zebrafish larvae. *Zebrafish* **10**, 283-93.

Akhtar, M. T., Mushtaq, M. Y., Verpoorte, R., Richardson, M. K. and Choi, Y. H. (2016). Zebrafish as a Model for Systems Medicine R&D: Rethinking the Metabolic Effects of Carrier Solvents and Culture Buffers Determined by (1)H NMR Metabolomics. *OMICS* **20**, 42-52.

Alger, B. E. (2012). Endocannabinoids at the synapse a decade after the dies mirabilis (29 March 2001): what we still do not know. *J Physiol* **590**, 2203-12.

Alpar, A., Tortoriello, G., Calvigioni, D., Niphakis, M. J., Milenkovic, I., Bakker, J., Cameron, G. A., Hanics, J., Morris, C. V., Fuzik, J. et al. (2014). Endocannabinoids modulate cortical development by configuring Slit2/Robo1 signalling. *Nat Commun* **5**, 4421.

Baraban, S. C., Taylor, M. R., Castro, P. A. and Baier, H. (2005). Pentylenetetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. *Neuroscience* **131**, 759-68.

Berghuis, P., Dobszay, M. B., Wang, X., Spano, S., Ledda, F., Sousa, K. M., Schulte, G., Ernfors, P., Mackie, K., Paratcha, G. et al. (2005). Endocannabinoids regulate interneuron migration and morphogenesis by transactivating the TrkB receptor. *Proc Natl Acad Sci U S A* **102**, 19115-20.

Berghuis, P., Rajnicek, A. M., Morozov, Y. M., Ross, R. A., Mulder, J., Urban, G. M., Monory, K., Marsicano, G., Matteoli, M., Canty, A. et al. (2007). Hardwiring the brain: endocannabinoids shape neuronal connectivity. *Science* **316**, 1212-6.

Buckley, N. E., Hansson, S., Harta, G. and Mezey, E. (1998). Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat. *Neuroscience* **82**, 1131-49.

Carty, D. R., Thornton, C., Gledhill, J. and Willett, K. L. (2017). Developmental effects of cannabidiol and Delta9-tetrahydrocannabinol in zebrafish. *Toxicol Sci*.

de Oliveira, R. W., Oliveira, C. L., Guimaraes, F. S. and Campos, A. C. (2018). Cannabinoid signalling in embryonic and adult neurogenesis: possible implications for psychiatric and neurological disorders. *Acta Neuropsychiatr*, 1-16.

Diaz-Alonso, J., Aguado, T., Wu, C. S., Palazuelos, J., Hofmann, C., Garcez, P., Guillemot, F., Lu, H. C., Lutz, B., Guzman, M. et al. (2012a). The CB(1) cannabinoid receptor drives corticospinal motor neuron differentiation through the Ctip2/Satb2 transcriptional regulation axis. *J Neurosci* **32**, 16651-65.

Diaz-Alonso, J., Guzman, M. and Galve-Roperh, I. (2012b). Endocannabinoids via CB(1) receptors act as neurogenic niche cues during cortical development. *Philos Trans R Soc Lond B Biol Sci* **367**, 3229-41.

Elphick, M. R. and Egertova, M. (2001). The neurobiology and evolution of cannabinoid signalling. *Philos Trans R Soc Lond B Biol Sci* **356**, 381-408.

Esain, V., Kwan, W., Carroll, K. J., Cortes, M., Liu, S. Y., Frechette, G. M., Sheward, L. M., Nissim, S., Goessling, W. and North, T. E. (2015). Cannabinoid Receptor-2 Regulates Embryonic Hematopoietic Stem Cell Development via Prostaglandin E2 and P-Selectin Activity. *Stem Cells* **33**, 2596-612.

Fashena, D. and Westerfield, M. (1999). Secondary motoneuron axons localize DM-GRASP on their fasciculated segments. *J Comp Neurol* **406**, 415-24.

Fine, P. G. and Rosenfeld, M. J. (2013). The endocannabinoid system, cannabinoids, and pain. *Rambam Maimonides Med J* **4**, e0022.

Fox, M. A. and Sanes, J. R. (2007). Synaptotagmin I and II are present in distinct subsets of central synapses. *J Comp Neurol* **503**, 280-96.

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

Fride, E. (2008). Multiple roles for the endocannabinoid system during the earliest stages of life: pre- and postnatal development. *J Neuroendocrinol* **20 Suppl 1**, 75-81.

Harkany, T., Guzman, M., Galve-Roperh, I., Berghuis, P., Devi, L. A. and Mackie, K. (2007). The emerging functions of endocannabinoid signaling during CNS development. *Trends Pharmacol Sci* **28**, 83-92.

Herkenham, M., Groen, B. G., Lynn, A. B., De Costa, B. R. and Richfield, E. K. (1991). Neuronal localization of cannabinoid receptors and second messengers in mutant mouse cerebellum. *Brain Res* **552**, 301-10.

Herkenham, M., Lynn, A. B., Little, M. D., Johnson, M. R., Melvin, L. S., de Costa, B. R. and Rice, K. C. (1990). Cannabinoid receptor localization in brain. *Proc Natl Acad Sci U S A* **87**, 1932-6.

Howlett, A. C. and Abood, M. E. (2017). CB1 and CB2 Receptor Pharmacology. *Adv Pharmacol* **80**, 169-206.

Jordan, C. J. and Xi, Z. X. (2019). Progress in brain cannabinoid CB2 receptor research: From genes to behavior. *Neurosci Biobehav Rev* **98**, 208-220.

Lam, C. S., Rastegar, S. and Strahle, U. (2006). Distribution of cannabinoid receptor 1 in the CNS of zebrafish. *Neuroscience* **138**, 83-95.

Leighton, P. L. A., Kanyo, R., Neil, G. J., Pollock, N. M. and Allison, W. T. (2018). Prion gene paralogs are dispensable for early zebrafish development and have nonadditive roles in seizure susceptibility. *The Journal of biological chemistry* **293**, 12576-12592.

Liu, L. Y., Alexa, K., Cortes, M., Schatzman-Bone, S., Kim, A. J., Mukhopadhyay, B., Cinar, R., Kunos, G., North, T. E. and Goessling, W. (2016). Cannabinoid receptor signaling regulates liver development and metabolism. *Development* **143**, 609-22.

Lopez-Cardona, A. P., Perez-Cerezales, S., Fernandez-Gonzalez, R., Laguna-Barraza, R., Pericuesta, E., Agirregoitia, N., Gutierrez-Adan, A. and Agirregoitia, E. (2017). CB1 cannabinoid receptor drives oocyte maturation and embryo development via PI3K/Akt and MAPK pathways. *FASEB J* **31**, 3372-3382.

Martella, A., Sepe, R. M., Silvestri, C., Zang, J., Fasano, G., Carnevali, O., De Girolamo, P., Neuhauss, S. C., Sordino, P. and Di Marzo, V. (2016). Important role of endocannabinoid signaling in the development of functional vision and locomotion in zebrafish. *FASEB J* **30**, 4275-4288.

Meyer, H. C., Lee, F. S. and Gee, D. G. (2018). The Role of the Endocannabinoid System and Genetic Variation in Adolescent Brain Development. *Neuropsychopharmacology* **43**, 21-33.

Migliarini, B. and Carnevali, O. (2009). A novel role for the endocannabinoid system during zebrafish development. *Mol Cell Endocrinol* **299**, 172-7.

Mouslech, Z. and Valla, V. (2009). Endocannabinoid system: An overview of its potential in current medical practice. *Neuro Endocrinol Lett* **30**, 153-79.

Mulder, J., Aguado, T., Keimpema, E., Barabas, K., Ballester Rosado, C. J., Nguyen, L., Monory, K., Marsicano, G., Di Marzo, V., Hurd, Y. L. et al. (2008). Endocannabinoid signaling controls pyramidal cell specification and long-range axon patterning. *Proc Natl Acad Sci U S A* **105**, 8760-5.

Oltrabella, F., Melgoza, A., Nguyen, B. and Guo, S. (2017). Role of the endocannabinoid system in vertebrates: Emphasis on the zebrafish model. *Development, growth & differentiation* **59**, 194-210.

Onaivi, E. S., Ishiguro, H., Gu, S. and Liu, Q. R. (2012). CNS effects of CB2 cannabinoid receptors: beyond neuro-immuno-cannabinoid activity. *J Psychopharmacol* **26**, 92-103.

Pacher, P., Steffens, S., Hasko, G., Schindler, T. H. and Kunos, G. (2018). Cardiovascular effects of marijuana and synthetic cannabinoids: the good, the bad, and the ugly. *Nat Rev Cardiol* **15**, 151-166.

Palazuelos, J., Ortega, Z., Diaz-Alonso, J., Guzman, M. and Galve-Roperh, I. (2012). CB2 cannabinoid receptors promote neural progenitor cell proliferation via mTORC1 signaling. *The Journal of biological chemistry* **287**, 1198-209.

Psychoyos, D., Vinod, K. Y., Cao, J., Xie, S., Hyson, R. L., Wlodarczyk, B., He, W., Cooper, T. B., Hungund, B. L. and Finnell, R. H. (2012). Cannabinoid receptor 1 signaling in embryo neurodevelopment. *Birth Defects Res B Dev Reprod Toxicol* **95**, 137-50.

Rodriguez-Martin, I., Herrero-Turrion, M. J., Marron Fdez de Velasco, E., Gonzalez-Sarmiento, R. and Rodriguez, R. E. (2007). Characterization of two duplicate zebrafish Cb2-like cannabinoid receptors. *Gene* **389**, 36-44.

Ruginsk, S. G., Vechiato, F. M., Uchoa, E. T., Elias, L. L. and Antunes-Rodrigues, J. (2015). Type 1 cannabinoid receptor modulates water deprivation-induced homeostatic responses. *Am J Physiol Regul Integr Comp Physiol* **309**, R1358-68.

Sugiura, T., Kishimoto, S., Oka, S. and Gokoh, M. (2006). Biochemistry, pharmacology and physiology of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand. *Prog Lipid Res* **45**, 405-46.

Sylvain, N. J., Brewster, D. L. and Ali, D. W. (2010). Zebrafish embryos exposed to alcohol undergo abnormal development of motor neurons and muscle fibers. *Neurotoxicol Teratol* **32**, 472-80.

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

Trevarrow, B., Marks, D. L. and Kimmel, C. B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron* **4**, 669-79.

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

Williams, E. J., Walsh, F. S. and Doherty, P. (2003). The FGF receptor uses the endocannabinoid signaling system to couple to an axonal growth response. *The Journal of cell biology* **160**, 481-6.

Xapelli, S., Agasse, F., Sarda-Arroyo, L., Bernardino, L., Santos, T., Ribeiro, F. F., Valero, J., Braganca, J., Schitine, C., de Melo Reis, R. A. et al. (2013). Activation of type 1 cannabinoid receptor (CB1R) promotes neurogenesis in murine subventricular zone cell cultures. *PLoS One* **8**, e63529.

Figures

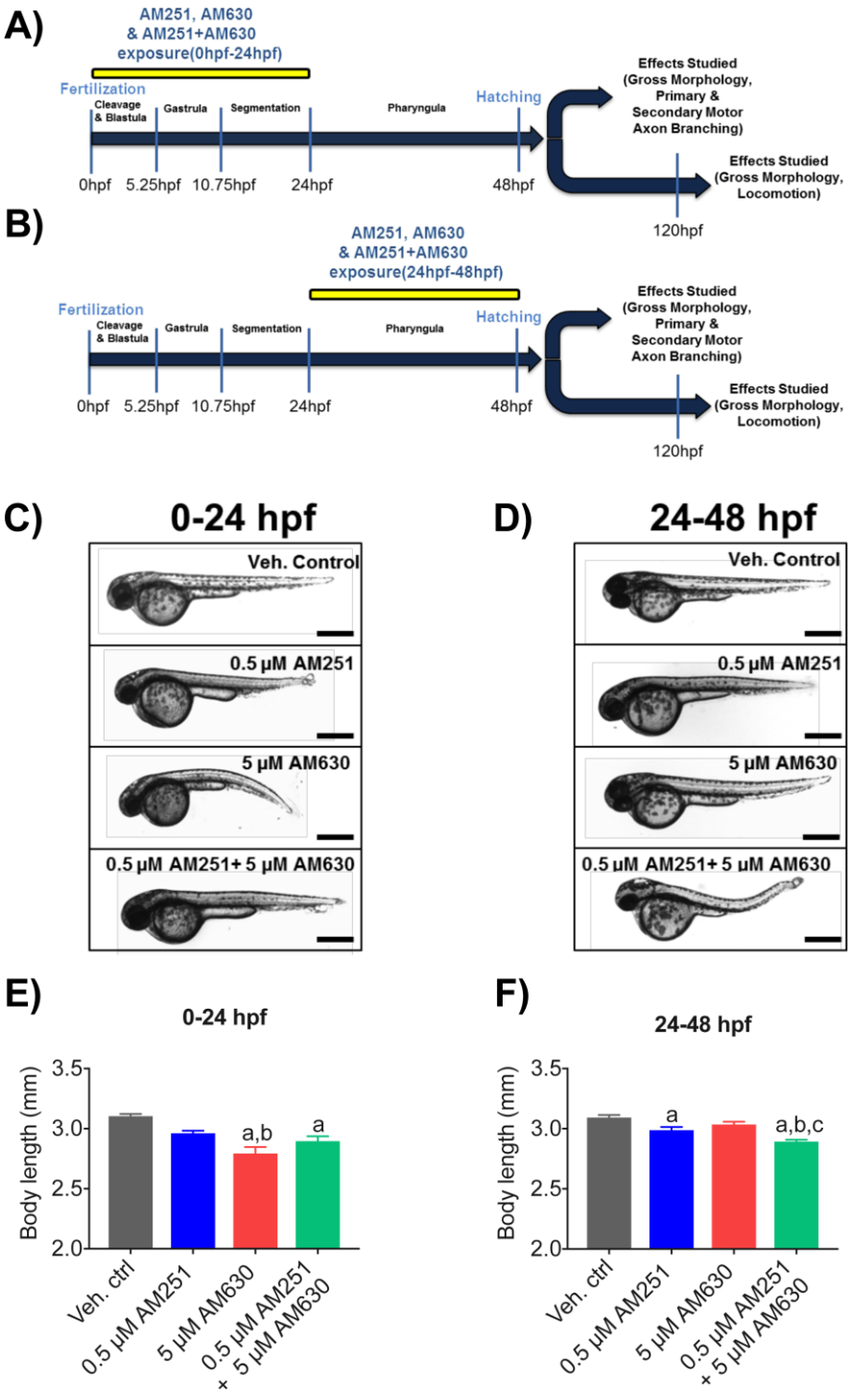

Figure 1. Effect of the endocannabinboid receptor antagonists, AM251 and AM630 on zebrafish embryo morphology. (A-B) Schematic showing the timeline for drug exposures and when experimental measurements were made. **(A)** Drug exposure during the first 24 hours

of development (0-24 hpf), and **(B)** drug exposures during the second 24 hours of development (24-48 hpf) are highlighted by the yellow bar. Embryos were allowed to develop in normal egg water after each treatment. Primary or secondary motor neuron axonal branching was investigated between 48 and 52 hpf, while locomotion was recorded at 120 hpf (5 days post fertilization). Gross morphological observations occurred at 2 dpf and 5 dpf **(A-B)**. The treatments include vehicle control (0.1% DMSO), AM251 0.5 µM, AM630 5 µM or AM251 0.5 µM + AM630 5 µM, either from 0-24 hpf or 24-48 hpf. **(C-D)** Representative images of treated embryos were taken at 48-52 hpf; Scale bar (black) = 0.5 mm. **(E-F)** Bar graphs showing body length (in mm) at 2 dpf development (n=33, n=31, n=40, n=35 for vehicle control, 0.5 µM AM251, 5 µM AM630 and 0.5 µM AM251 + 5 µM AM630 treatments from 0-24 hpf and n=29, n=27, n=37, n=34 for vehicle control, 0.5 µM AM251, 5 µM AM630 and 0.5 µM AM251 + 5 µM AM630 treatments from 24-48 hpf). Data are presented as means ± s.e.m. [a] Significantly different from vehicle control, $p<0.05$; [b] significantly different from 0.5 µM AM251, $p<0.05$; [c] significantly different from 5 µM AM630, $p<0.05$ (One-way ANOVA followed by a Tukey post-hoc multiple comparisons test).

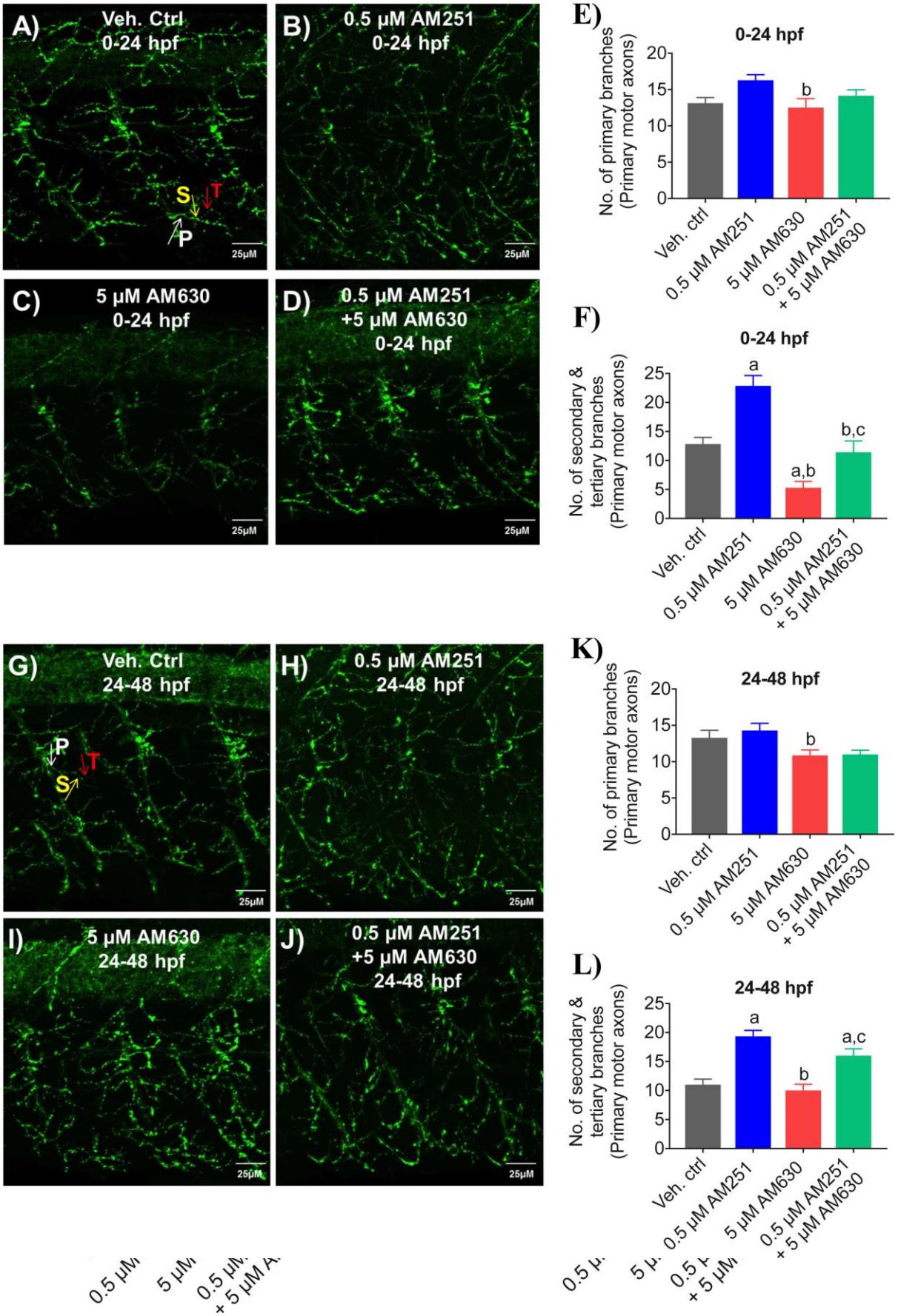

Figure 2. Effect of the endocannabinboid receptor antagonists, AM251 and AM630 on zebrafish morphological development and heart rate. (A) Incidence of pericardial edema and yolk sac edema in embryos treated with AM251 and AM630 treatments exhibit early morphological deformities such as, pericardial edema (PE), yolk sac edema (SE), tail malformation (TM) and body malformation (BM) in zebrafish embryos at 2 dpf. **(B,D)** Bar graphs show the rates of pericardial & yolk sac edema and tail & body malformation in embryos treated with AM251 and AM630 in the first 24 hours of development. (n=74, n=56, n=62, n=64 for vehicle control, AM251 0.5 µM, AM630 5 µM and AM251 0.5 µM + AM630 5 µM) **(C,E)** Bar graphs show the rates of pericardial & yolk sac edema and tail & body malformation in embryos treated with AM251 and AM630 in the second 24 hours of development. Data were obtained at 2 dpf. (n=68, n=62, n=70, n=58 for vehicle control, 0.5 µM AM251, 5 µM AM630 and 0.5 µM AM251 + 5 µM AM630) (**F-G)** Bar graphs showing the heart rate of embryos treated with AM251 and AM630 in the first 24 hours of development and the second 24 hours of development (n=35, n=35, n=36, n=40 for vehicle control, 0.5 µM AM251, 5 µM AM630 and 0.5 µM AM251 + 5 µM AM630 treatments from 0-24 hpf and n=35, n=34, n=35, n=49 for vehicle control, 0.5 µM AM251, 5 µM AM630 and 0.5 µM AM251 + 5 µM AM630 treatments from 24-48 hpf). Data were obtained at 2 dpf. Data are presented as means ± s.e.m. [a] Significantly different from vehicle control, $p<0.05$; [b] significantly different from 0.5 µM AM251, $p<0.05$; [c] significantly different from 5 µM AM630, $p<0.05$ (One-way ANOVA followed by a Tukey post-hoc multiple comparisons test)

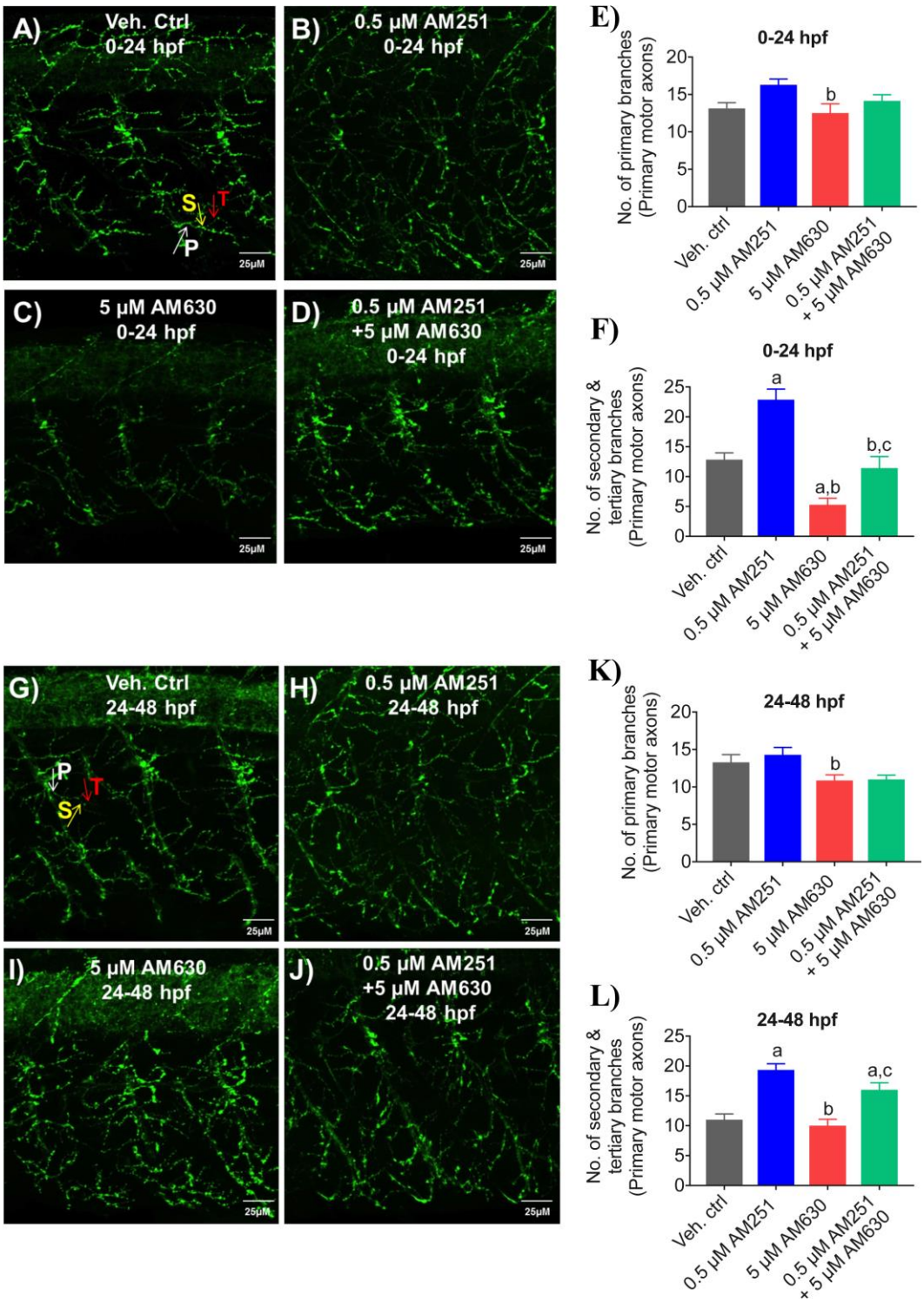

Figure 3. Effect of the endocannabinboid receptor antagonists, AM251 and AM630 on the branching patterns of primary motor axons in zebrafish embryos. (A-D) Immunohistochemical staining of primary motor neurons using the Znp-1 antibody (green) in vehicle control preparations (n=7), embryos treated with AM251 (n=7), AM630 (n=6) or

AM251 + AM630 (n=7) in the first 24 hours of development. Primary, secondary and tertiary branches in a primary motor axon are indicated with white (P), yellow (S) and red (T) arrows respectively. **(E-F)** Bar graphs show the number of primary branches, and the number of secondary and tertiary branches emanating from the main axon when treated in the first 24 hours. **(G-J)** Immunohistochemical staining of primary motor neurons using the Znp-1 antibody (green) in vehicle control preparations (n=7), embryos treated with AM251 (n=7), AM630 (n=6) or AM251 + AM630 (n=7) in the second 24 hours of development. Primary, secondary and tertiary branches in a primary motor axon are indicated with white (P), yellow (S) and red (T) arrows respectively. **(K-L)** Bar graphs show the number of primary branches, and the number of secondary and tertiary branches emanating from the main axon when treated in the second 24 hours. Scale bar (White) = 25. µm. Data are presented as means ± s.e.m. [a] Significantly different from vehicle control, $p<0.05$; [b] 0.5 µM AM251, $p<0.05$; [c] 5 µM AM630, $p<0.05$ (One-way ANOVA followed by a Tukey post-hoc multiple comparisons test).

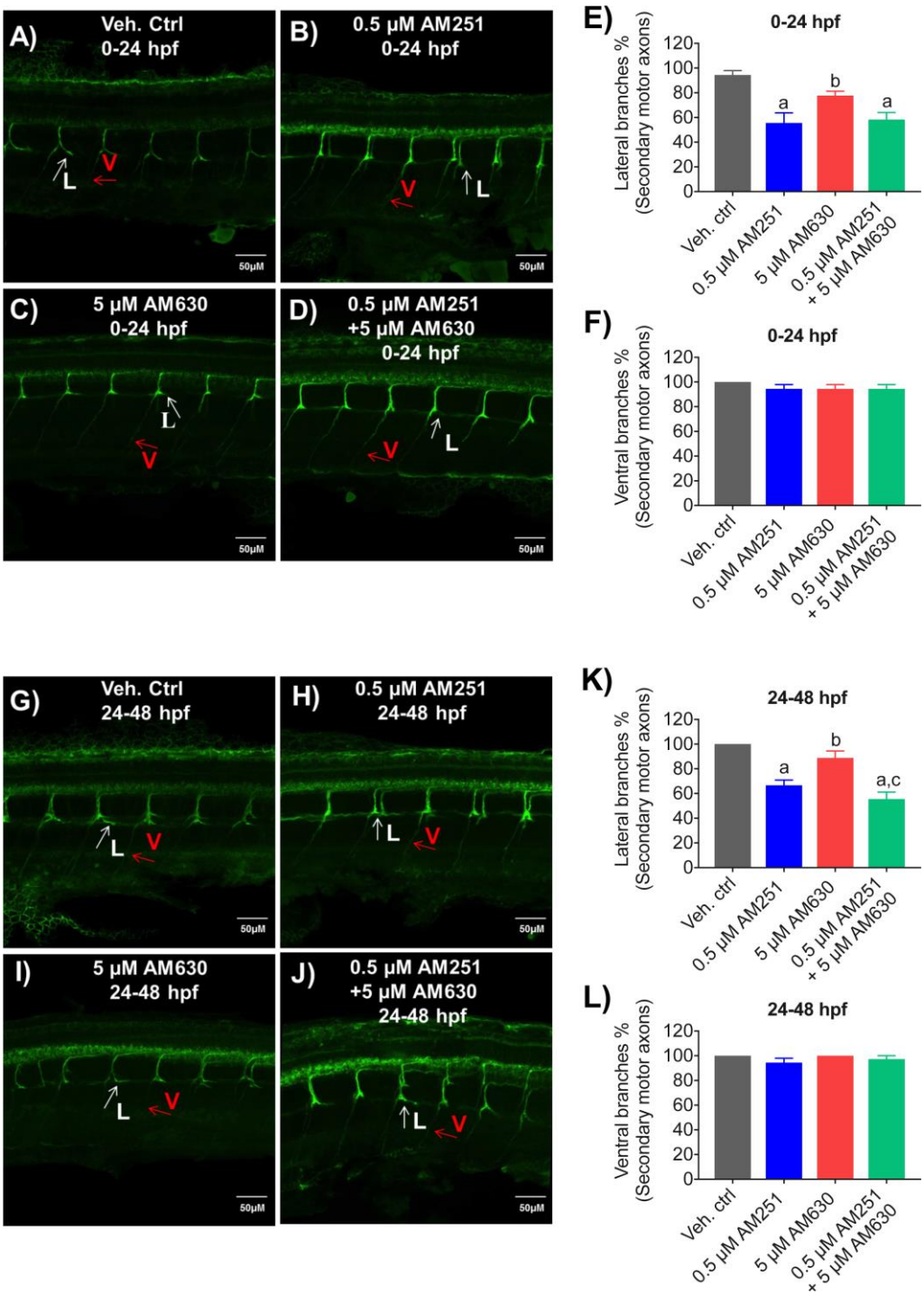

Figure 4. Effect of the endocannabinoid receptor antagonists, AM251 and AM630 on the branching patterns of secondary motor axons in zebrafish embryos. (A-D) Immunohistochemical staining of secondary motor neurons using the Zn8 antibody (green) in vehicle control preparations (n=7), embryos treated with AM251 (n=7), AM630 (n=6) or AM251 + AM630 (n=7) in the first 24 hours of development. Primary, secondary and tertiary

branches in a primary motor axon are indicated with white (P), yellow (S) and red (T) arrows respectively. **(E-F)** Bar graphs show the number of primary branches, and the number of secondary and tertiary branches emanating from the main axon when treated in the first 24 hours. **(G-J)** Immunohistochemical staining of secondary motor neurons using the Zn8 antibody (green) in vehicle control preparations (n=7), embryos treated with AM251 (n=7), AM630 (n=6) or AM251 + AM630 (n=7) in the second 24 hours of development. Primary, secondary and tertiary branches in a secondary motor axon are indicated with white (P), yellow (S) and red (T) arrows respectively. **(K-L)** Bar graphs show the number of lateral and ventral branches emanating from the secondary motor axon when treated in the first 24 hours. Scale bar (White) = 25 µm. Data are presented as means ± s.e.m. [a] Significantly different from vehicle control, $p<0.05$; [b] 0.5 µM AM251, $p<0.05$; [c] 5 µM AM630, $p<0.05$ (One-way ANOVA followed by a Tukey post-hoc multiple comparisons test).

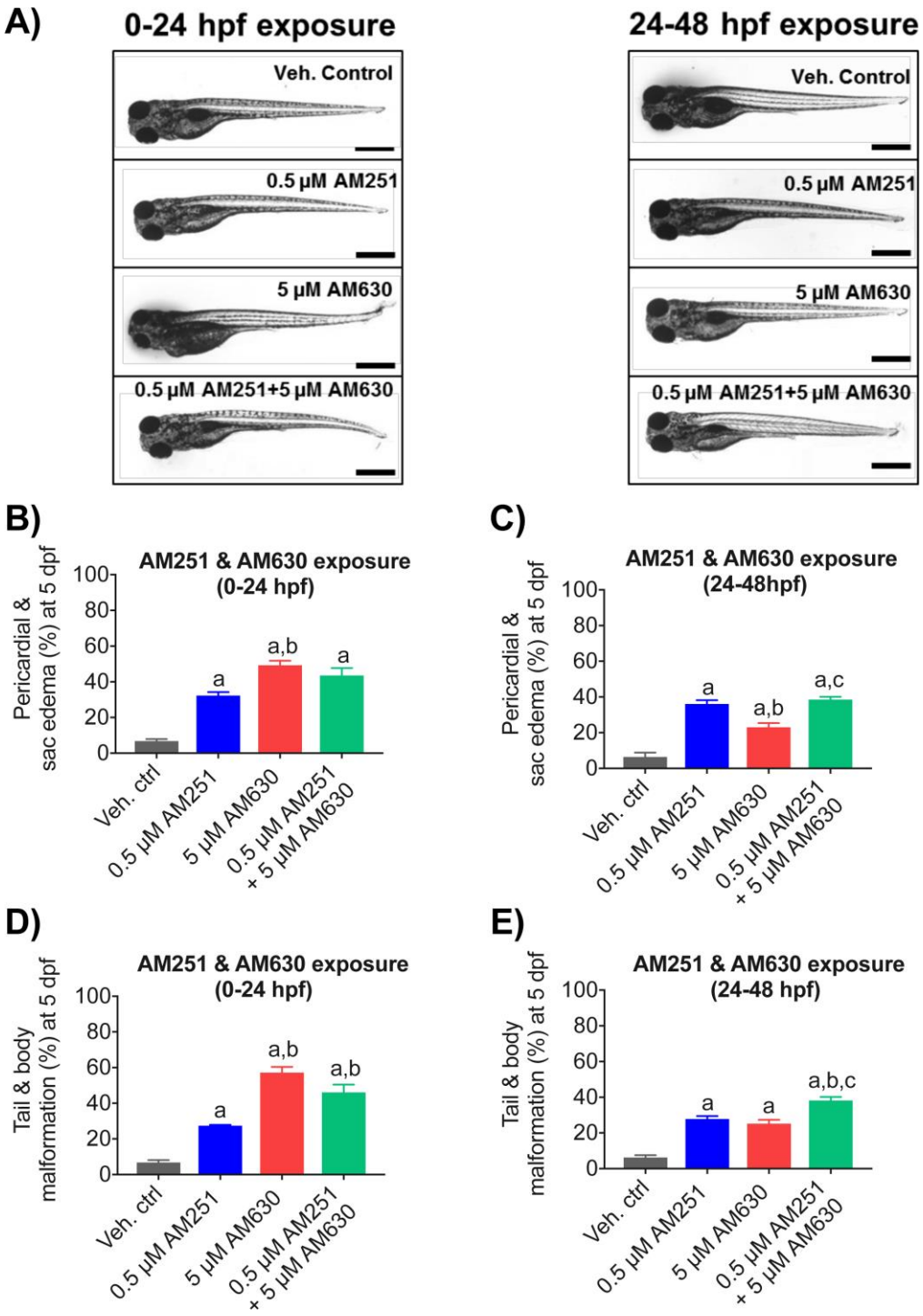

Figure 5. Effect of the endocannabinoid receptor antagonists, AM251 and AM630 on morphological development in 5 dpf zebrafish larva. (A) Incidence of pericardial edema and yolk sac edema in 5 dpf larvae when treated with AM251 and AM630 in the first 24 hours of development and in the second 24 hour of development. **(B,D)** Bar graphs showing the rates of pericardial & yolk sac edema and tail & body malformation in 5 dpf larvae treated with

AM251 and AM630 in the first 24 hours of development (n=62, n=56, n=51, n=58 for vehicle control, 0.5 µM AM251, 5 µM AM630 and 0.5 µM AM251 + 5 µM AM630). **(C,E)** Bar graphs showing the rates of pericardial & yolk sac edema and tail & body malformation in 5 dpf larvae treated with AM251 and AM630 in the second 24 hours of development (24-48 hpf) (n=74, n=56, n=62, n=64 for vehicle control, 0.5 µM AM251, 5 µM AM630 and 0.5 µM AM251 + 5 µM AM630). Scale bar (black) = 0.7 mm. Data are presented as means ± s.e.m. [a] Significantly different from vehicle control, $p<0.05$; [b] significantly different from 0.5 µM AM251, $p<0.05$; [c] significantly different from 5 µM AM630, $p<0.05$ (One-way ANOVA followed by a Tukey post-hoc multiple comparisons test).

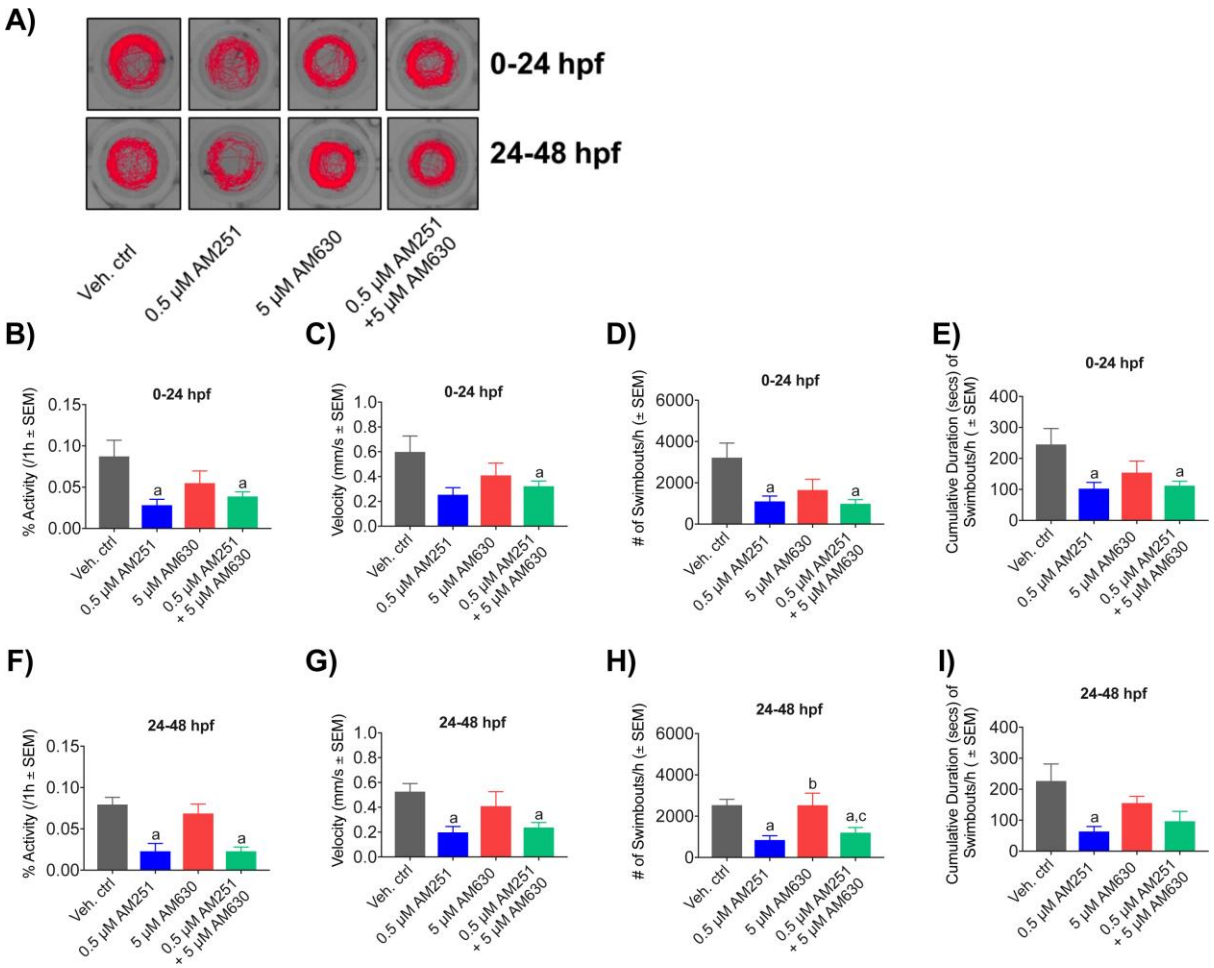

Figure 6. Effect of the endocannabinboid receptor antagonists, AM251 and AM630 on locomotor activity in 5 dpf zebrafish larva. Representative image of a portion of a 96-well plate, where each well contains an individual larva. Eight larvae per row are displayed, each representing a replicate at the dose indicated. Red lines represent movement of the fish during 60 minutes. Tracing was recorded at 5 dpf after 48 hrs treatment with AM251 and AM630 **(A)**. Bar graphs display changes in embryos mean activity (% rate for one hour), mean velocity (in mm. s^{-1} for one hour), frequency of swim bouts within one hour and cumulative duration of swim bouts (in seconds) for one hour. **(B-E)** 0-24 hpf exposures were vehicle control (n=24), 0.5 µM AM251 (n=20), 5 µM AM630 (n=19), 0.5 µM AM251 + 5 µM AM630 (n=28) **(B-E)** and 24-48 hpf exposures were vehicle control (n=38), 0.5 µM AM251 (n=16), 5 µM AM630 (n=16), 0.5. µM AM251 + 5 µM AM630 (n=24) **(F-I).** Data are presented as means ± s.e.m. [a]

Significantly different from vehicle control, $p<0.05$; [b] 0.5 µM AM251, $p<0.05$; [c] 5 µM AM630, $p<0.05$ (One-way ANOVA followed by a Tukey post-hoc multiple comparisons test).

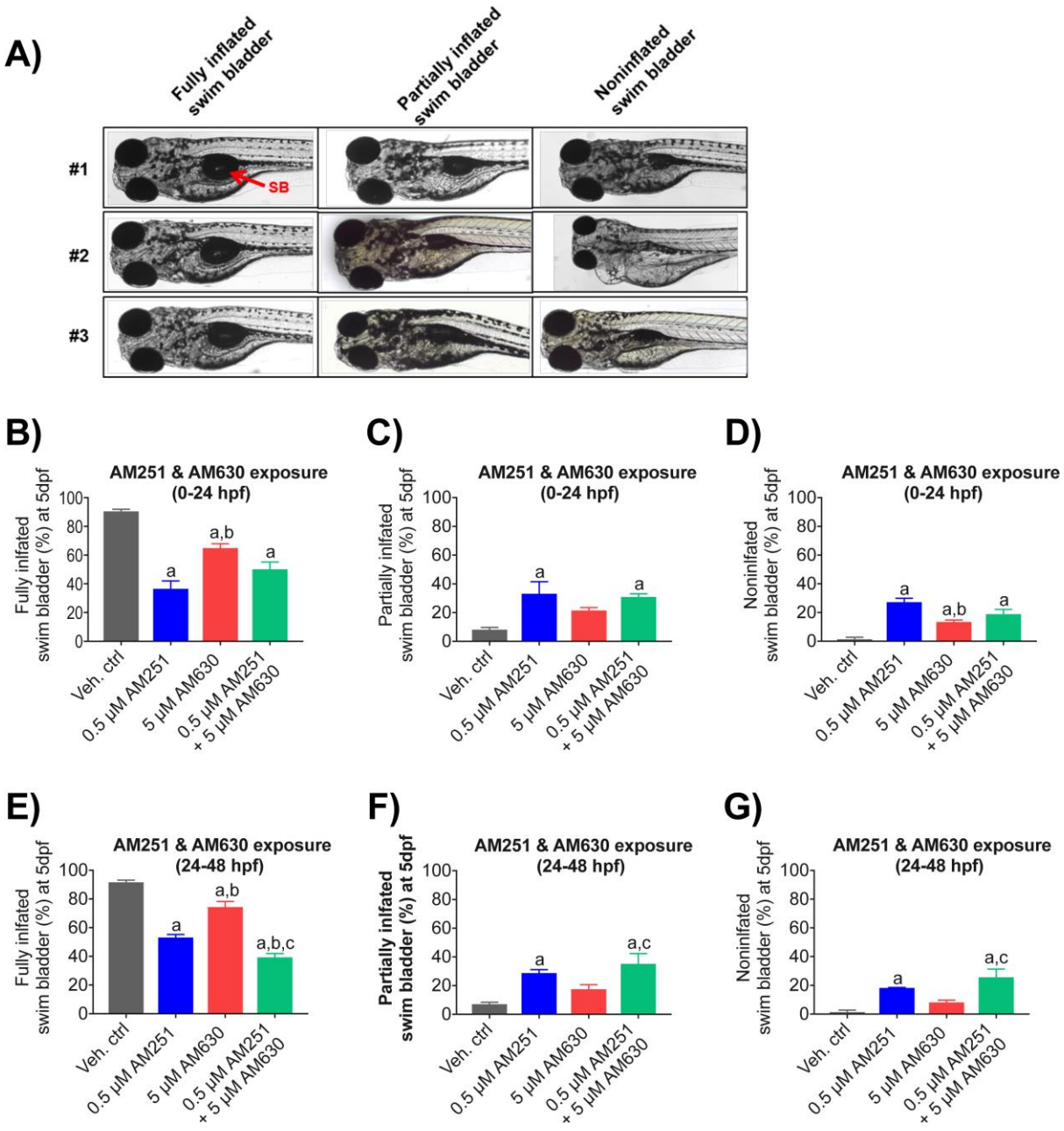

Figure 7. Effect of the endocannabinboid receptor antagonists, AM251 and AM630 on development and inflation of 5 dpf larval zebrafish swim bladders. AM251 and AM630 treatments at the early stage either from 0-24 hpf or 24-48 hpf showed fully inflated to partial or noninflated swim bladder **(SB; red arrow)** development in zebrafish larvae observed at 5 dpf. Representative images showed fully inflated, partially inflated or noninflated swim bladder in larvae **(A)**. Bar graphs show the rates of swim bladder inflation at 0-24 hpf (**Fig B-D**: n=73,n=59,n=57 and n=58 for vehicle control, 0.5 µM AM251, 5 µM AM630 and 0.5 µM

AM251 + 5 µM AM630 treatments from 0-24 hpf) and at 24-48 hpf **fig E-G**: n=78, n=58, n=61 and n=60 for vehicle control, 0.5 µM AM251, 5 µM AM630 and 0.5 µM AM251 + 5 µM AM630 treatments from 24-48 hpf). Data are presented as means ± s.e.m. [a] Significantly different from vehicle control, $p<0.05$; [b] 0.5 µM AM251, $p<0.05$; [c] 5 µM AM630, $p<0.05$ (One-way ANOVA followed by a Tukey post-hoc multiple comparisons test).