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Embryonic exposure to cannabidiol disrupts active neural circuits, an effect increased by Δ□-tetrahydrocannabinol and involving CB1R and CB2R in zebrafish

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Abstract

Considering the wide spread use of cannabis as a recreational and medicinal drug, a knowledge gap exists regarding biological mechanisms and health implication. In the light of legislative changes, delayed effects of cannabis upon brief exposure during embryonic development are of high interest as early pregnancies often go undetected. We hypothesized that brief early cannabinoid exposure impacts neural activity by affecting embryonic brain development through cannabinoid-1 (CB1R) and -2 (CB2R). Zebrafish larvae were exposed to cannabidiol (CBD) and Δ□-tetrahydrocannabinol (THC) until the end of gastrulation (1-10 hours post-fertilization) and analyzed later in development (4-5 days post-fertilization). In order to measure neural activity, we implemented the fluorescence based calcium detector CaMPARI (Calcium-Modulated Photoactivatable Ratiometric Integrator) and optimized the protocol such that a high number of samples can be economically used in a 96-well format complemented by locomotor analysis. Our results revealed that neural activity was decreased by CBD more than THC. At higher doses both cannabinoids could reduce neural activity close to a level comparable to anesthetized samples and locomotor activity was abolished. Interestingly, the decrease was more pronounced when CBD and THC were combined. At the receptor level, CBD-mediated reduction of locomotor activity was partially prevented using CB1R or CB2R inhibitors. Overall, we provided a mechanistic link to perturbed brain development *in vivo*, which seems to involve CBD and receptors, CB1R and CB2R. Future studies are warranted to reveal other cannabinoids and receptors involved in this pathway to understand the full health implications of cannabis consumption on fetal development.

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Introduction

Publicly, cannabis is used as a recreational drug [1] that is often portrayed as harmless, yet the health implications are not fully understood. Interestingly, extracts of the *Cannabis sativa* plant have been used for medical purposes for almost 5000 years [2]. However, scientific knowledge is limited and despite controversy, some countries continue to legalize cannabis for recreational use. Cannabis has been reported to be one of the most used drugs during pregnancy and key compounds, Δ□-tetrahydrocannabinol (THC) and cannabidiol (CBD), can readily cross the placenta [3-6]. We were especially interested in CBD due to the reported health benefits and availability in many natural products including oils and food [7]. In the past, the negative impacts of cannabis in connection to embryonic development have been principally associated with the psychoactive THC [8]. In humans, epidemiological and clinical studies associate maternal cannabis exposure to behavioural disturbances in the offspring linked to increased risk for neuropsychiatric disorders [9]. In rats, maternal exposure of THC changed a series of behaviours in the offspring, including water-induced grooming, increased light sensitivity and altered exploratory behaviour [10]. Recently, the negative impacts of cannabis have expanded and also include the non-psychotropic CBD, which disrupts motor-neuron development in zebrafish [11]. This study is in contrast to reports that suggest positive health benefits of CBD, by treating nausea during pregnancy [12, 13].

The mechanistic pathway(s) by which THC and CBD are toxic are elusive. Recently, these two cannabinoids were linked to the sonic hedgehog signaling pathway in mice and zebrafish, and appears to involve cannabinoid type 1 receptor (CB1R) [14, 15]. It is well established that THC binds and activates as a partial agonist two distinct classes of G-coupled protein receptors, CB1R and CB2R [16]. Both receptors are highly expressed in neuronal tissue

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where CB1R is localized mostly in the CNS [17-19] and CB2R in the peripheral nervous system [20], although CB2R has been also linked to the CNS [21-23]. CBD has been reported to interact with a lower affinity to CB1R and CB2R relative to THC, and rather antagonizes cannabinoid-induced effects indirectly through other receptors [24-28]. Additional data to illuminate signaling pathways of THC and CBD *in vivo* would be of great value.

Zebrafish embryos have several advantages that complement mammalian models. Experiments are economical because many embryos are available, and exposure studies are simple. Translucent larvae provide opportunities to implement cutting-edge fluorescing calcium sensors and measure neural activity. Further, past studies suggest that the endocannabinoid system plays a role in zebrafish development [29-31]. Both cannabinoid receptors investigated here have similar expression profiles in the CNS compared to mammals, with CB1R sharing a 70% protein sequence identity with the human homolog [18, 31]. Considering the anticipated effects of THC and CBD, high-throughput quantification of neural activity following early exposures would be of great interest. In this study, the calcium modulated photoactivatable ratiometric integrator (CaMPARI) [32], was implemented. Green fluorescing CaMPARI undergoes photoconversion (PC) to a red fluorescing protein only in the presence of intense 405 nm light and high Ca^{2+} levels. This conversion is irreversible, thus creating a temporal snapshot in a form of a ratiometric red/green output. CaMPARI is expressed exclusively in neuronal tissue due to a pan-neural promoter (*elavl3*, a.k.a. HuC) and is a direct read-out of relative neural activity. Initially, the photoconversion was not efficient, but our optimized protocol allows for high-throughput well-plate-formats. Locomotion was also quantified from the same larvae as an independent proxy. Here, we investigated the effects of the two most abundant cannabinoids

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found in the plant, THC and CBD, on neural activity and characterized CB1R and CB2R *in vivo* on the CBD effects using zebrafish.

METHODS

Animal Ethics and zebrafish husbandry

Zebrafish maintenance was approved by the Animal Care & Use Committee: Biosciences at the University of Alberta and operated under the guidelines of the Canadian Council of Animal Care. The fish were maintained in the University of Alberta fish facility at 28°C under a 14/10 light/dark cycle as previously described [33].

Engineering CaMPARI transgenic zebrafish for integrative calcium imaging.

Zebrafish expressing CaMPARI, i.e. the Tg[*elavl3*:CaMPARI (W391F+V398L)]^{ua3144} line were generated as described previously [34, 35]. We re-derived CaMPARI fish in response to a federal moratorium limiting zebrafish import into Canada [36].

CaMPARI photoconversion

For photoconversion, bright green Tg[*elavl3*:CaMPARI (W391F+V398L)]^{ua3144} larvae at 4 dpf were placed in a 96-well plate containing 150 µl egg water (made as previously decribed [33]) and acclimatized for 2 h. The central 48 wells of the plate were exposed to 405 nm LED array (Loctite) for 300 sec at a distance of 10 cm. LED array illuminated the plates entirely and evenly. The plates were floating in water at room temperature to ensure that the larvae did not

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overheat. For imaging, the larvae were anesthetized in 0.24 mg/mL tricaine methanesulfonate (MS-222, Sigma) following photoconversion. MS-222 was washed out and zebrafish were rested for a day prior locomotor activity assay.

CaMPARI analysis

The ratio of fluorescent emissions, taken as red photoconverted CaMPARI in ratio to green CAMPARI, was interpreted as relative neural activity, as previously defined [32]. Images were obtained with an InCell 2000 microscope (GE Healthcare, US). 96-well plates were run using FITC (1 second exposure) and dsRed (2 second exposure) channels. ImageJ was used to quantify the fluorescence mean of the red (dsRed) and green (FITC) channels. The area of the optic and hind brain area were quantified as those regions were most consistent in the view. A mask was applied to exclude noise outside of the CNS. Statistics used one-way ANOVA and Dunnett's multiple comparison test with GraphPad Prism Software (Version 7, GraphPad, San Diego, CA).

Quantifying Locomotor activity

The same zebrafish larvae being placed in the 96-well plate the day prior for CaMPARI analysis were quantified for locomotion using Basler GenICaM (Basler acA 1300-60) scanning camera (75-mm f2.8 C-mount lens) and EthoVision® XT-11.5 software by Noldus (Wageningen, Netherlands) as described previously [37, 38]. This set-up allowed to see whether there is a correlation between level of neural activity and the the mean activity % within a swimbout (swimbout size). Swimbout size was shown to increase in response to the convulsant PTZ

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previously [39] and here it can be a useful marker for neural activity. Transparent *Casper* strain zebrafish [40] were unable to be tracked as ususal with Noldus. Therefore, quantification of swimbouts provided an additional advantage. Clear movement was detected above the noise treshold due to movement of the pigmented eyes. Although the % swimbout is small, reliable measurements were obtained. Only clear swimbouts were quantified, which were set above a treshold of 0.1 % of activity. Figure 1F shows that measuring the mean activity of a swimbout is a useful tool in obtaining output that is clearly diminished in presence of MS-222. In addition, the output also shows sensitivity in freely swimming larvae. Statistics used here were as described above.

RMO44 staining

The zebrafish used in this experiment were of the Tuebingen Longfin strain. Set-up was similar as described above. Following the experiment, larvae were stained with anti-RMO44 (Thermo Fisher Scientific, Waltham, MA, USA) and imaged using a Zeiss LSM 710 confocal microscope (Oberkochen, Germany) as described elsewhere [41].

Results

High-throughput assessment of CaMPARI is a reliable metric of neural activity.

In this study the objective was to gain insight in the effects of THC and CBD on neural activity during early embryonic development. We measured neural activity with CaMPARI. CaMPARI photoconverts irreversibly from a green to a red fluorescing protein only if user-applied 405 nm light application coincides with high calcium levels (Fig. 1A) [32]. Red and

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green fluorescing CaMPARI are quantified as ratio (referred here as “CaMPARI activity"). We expressed CaMPARI through a pan-neural promoter, *elavl3* (a.k.a HuC) exclusively in the CNS and thus as a read-out of neural activity (Fig. 1B). Here, we optimized the use of CaMPARI zebrafish for an automated high-content INCell2000 plate-reader (Fig.1C). CaMPARI transgene expression was most suitable for this platform at 4 dpf, which is a suitable balance of fluorescing intensity and a sufficiently developed brain.

CaMPARI activity was quantified and displayed as a heatmap consisting of the red/green ratio in fluorescing intensities. At 4 dpf, optic tectum and hindbrain regions of larvae showed a clear measureable output, whereas without photoconversion CaMPARI activity was nearly zero (Fig. 1D and E). To establish a baseline of near-zero neural activity, larvae were anesthetized with MS-222, and a significant reduction in red/green ratio was obtained compared to freely swimming larvae (Fig. 1D and E). In contrast, drug-induced neural activity, with the established convulsants pentylenetetrazole (PTZ) or 4-aminopyridine (4-AP), showed a consistent increase in CaMPARI activity, even at concentrations considered to be minimal when inducing seizure (Fig. 1D, E)[34,35].

We benchmarked our CaMPARI outputs against locomotor activity of zebrafish larvae, which is an established metric of neural activity [43]. We chose 5 dpf as an optimal developmental timepoint for analyzing locomotor activity, because younger larvae are largely inactive. Larvae were left in the same 96-well plate for assessing CaMPARI and locomotion. Exposing larvae to photoconversion with LED light the day before had no impact on the mean locomotor activity (Fig. 1F). Larvae anesthetized with MS-222 did not display any detectable swimbouts (Fig. 1F). The level of neural activity correlated with locomotor activity and will be

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presented later. Overall, these results show that CaMPARI, deployed in zebrafish larvae is a reliable high-throughput tool for measuring neural activity.

CBD and THC reduce subsequent neural activity.

We sought to assess how embryonic exposure to cannabinoids impacts upon subsequent neural activity later in development. Here, the doses of CBD and THC aligned with our previous work [11], reflecting high cannabis consumption in humans. Comparisons of our dosage to humans requires various considerations: (i) blood plasma concentrations can peak up to 0.25 mg/l while smoking a single cigarette [44]; (ii) the content of THC has increased in the past 20 years; and (iii) doses of intraperitoneally administered medical CBD can vary greatly, from 5-100mg/kg, and daily maximum of 1500mg/kg [45,46]. The current study uses up 6 µg/mL of THC and 3 µg/mL CBD with an estimated 0.1-10% of this passing through the chorion to reach the embryo.

We wanted to gain insight into the effects of CBD and THC separately (Fig. 2A), and therefore each was applied in a dose-response format. This validation was necessary as CaMPARI has not been used in studying cannabinoids. Compounds were added to the bath early in animal development at (0.5 hpf) and then washed out towards the end of gastrulation (10 hpf). CaMPARI was imaged at 4 dpf and locomotor activity was assessed at 5 dpf (Fig. 2B). Animals that were exposed to CBD at concentrations of 2 µg/ml and 3 µg/ml exhibited a dose-dependent reduction in CaMPARI activity (Fig. 2C, F). These reductions in CaMPARI output are substantial when compared against the ~50% reduction we observed in anesthetized larvae, where little neural activity is expected. Coordinate with this, locomotion was also reduced

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starting at 1.5 µg/ml and was significant at 3 µg/ml (Fig. 2F). THC had a similar effect than CBD and also reduced neural activity at higher doses, 4 µg/ml and 6 µg/ml, (Fig. 2F and G). Locomotor activity exhibited a more extensive reduction at 6 µg/ml. We compared neural activity and locomotion in the same individuals exposed to effective doses of cannabinoids and found significant correlations (Fig. S2; CBD r=0.52 and THC r=0.71). Indeed, most larvae, that displayed reduced neural activity also showed reduced locomotion. Together, our findings show that both CBD and THC reduced neural activity when exposed early in development.

Antagonistic effects of CBD on neural activity is enhanced when combined with sub-effective doses of THC. Cannabis consumption during pregnancy exposes the fetus to THC and CBD in concert. In order to investigate whether CBD and THC have a combined effect that is different from either compound on its own, neural activity was measured when larvae were exposed to both cannabinoids, using the same timeline as the previous experiment (Fig. 2B). First, various sub-effective doses of CBD were applied from 0.5 to 1.5 µg/ml in the presence of 2 µg/ml THC (identified as sub-effective in Fig. 2). Preliminary dosage attempts revealed small, but significant differences on neural activity when combining 0.5 or 1.5 µg/ml CBD with 2.0 µg/ml THC (Fig. S3A and B). Locomotor activity also trended towards a dose-dependent reduction during concerted application of CBD and THC, but was statistically non-significant (Fig. S3C). Next, CBD and THC were added in a 1:1 ratio mixture at 1.0 and 2.0 µg/ml each. An enhanced antagonistic effect was revealed on neural activity when both CBD and THC were added at 2.0 µg/ml. CBD by itself at 2.0 µg/ml significantly reduced neural activity (Fig.3D) consistent with results in Fig.2D, but CBD and THC together, further reduced neural activity compared to CBD or THC alone (Fig. 3D and E). Locomotor activity was mainly affected by

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CBD with no additional reduction when compared to CBD and THC combined (Fig. 3F). No significant effect on neural activity or locomotor activity was obtained when 1.0 µg/ml of CBD and THC was applied (Fig. 3A-C). Due to the effect on locomotion, we also wanted to assess the integrity of neurons in connection to motor axons. Reticulospinal neurons in the hindbrain were stained with RMO44 antibody targeting NEFM, an established marker for neuronal damage [47, 48]. Indeed, RMO44 immunostaining was further reduced when CBD and THC were combined at 2.0 µg/ml suggesting that both CBD and THC affect neuronal health (Fig. 4S). Together, these results suggest that CBD and THC are more potent in reducing neural activity when applied in combination.

CB1R and CB2R are both required for CBD-induced reduction of locomotor activity. Some reports suggest that CBD mediates its actions through CB1- and CB2- receptors [24]. To test whether these two receptors are involved in the neuronal toxicity mediated by CBD *in vivo*, we deployed corresponding receptor inverse agonist AM251 and antagonist AM630. As described previously, the experimental timeline is as depicted in figure 2B with CBD being added immediately after AM251 and AM630. When analyzing CaMPARI activity, our results revealed that the CBD-mediated reduction in neural activity could not be completely prevented with concentrations from 0.1 to 10 nM AM251 or AM630 in the presence of 3 µg/ml of CBD (Fig. 4A-C, D). In contrast, CBD-mediated reduction of locomotor activity was prevented with a concentration of AM251 as low as 1 nM (Fig. 4D) or with 10 nM of AM630. In the absence of CBD, when using the maximum dose (10 nM) of AM251 or AM630, neither antagonist had an effect. This finding excludes the possibility that the effect of AM251 or AM630 on locomotion were independent of CBD (Fig. 4G and H). Overall, this suggests a role for both CB1R and

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CB2R receptors in CBD-mediated effects on locomotor activity. In sum, inhibiting CB1R and CB2R can ameliorate the negative impacts of early CBD on late CNS function, at least with respect to locomotion.

Discussion

In some jurisdictions, including Canada, cannabis has been legalized despite the lack of mechanistic understanding and health implications. Cannabis consumption has increased in the past decade [49, 50] and reports emerged associating prenatal cannabis consumption with stillbirths[51] and autism spectrum disorder, a neurodevelopmental syndrome [52, 53]. Yet, cannabis and CBD oil have been used by consumers to treat labor pain [54]. Cannabis has been shown to have detrimental effects on developing embryos in rats and chicken [10, 55]. More recently, new evidence revealed that exposure to not only the more widely studied THC, but also CBD, during gastrulation impacts the overall development in zebrafish [11]. Interestingly, the effect of the two key cannabinoids, CBD and THC, were delayed, and observed after the exposure had ended.

In vitro, CBD and THC can function through CB1R and CB2R, which are expressed in neuronal tissue [16-22, 24]. Our goal was specifically to investigate the effects of CBD, and how it might interact with the more widely studied THC, during early development and characterize the impact on neural activity *in vivo* using our CaMPARI assay. We found that neural activity was reduced later in zebrafish development upon brief exposure of not only THC, but also CBD early in development. Surprisingly, CBD had similar impacts on neural activity at lower concentrations compared to THC. Notably, the impact of CBD was increased by sub-effective

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doses of THC. Our data quantifying locomotion, suggested that the CBD-mediated effect involves CB1R and CB2Rs.

CaMPARI Assay

We optimized CaMPARI such that this innovation can be used for large number of samples to screen compounds in future studies. Zebrafish larvae can be deployed in a 96-well set-up quantifying locomotion [39, 42, 56-61]. Nevertheless, locomotor activity is not a direct measurement of neural activity. Calcium sensors, such as the GCaMP series [62, 63], or more recently GECO [64, 65], are more accurate, but CAMPARI became more practical once we optimized the assay. CaMPARI provides a temporal-snapshot of neural activity in the freely swimming animal, which can be anesthetized and imaged after the experiment [32]. Furthermore, the ratiometric red/green signals are imaged together and mitigate inter-individual differences in expression levels or focal plane. We optimized conditions for a well-plate set-up analyzing neural activity in the optic tectum and hindbrain. Unfortunately, less defined areas or specific neurons could not be quantified. Furthermore, when interpreting our data as neural activity, it is important to consider that when anesthetized a clear red to green CaMPARI ratio can be still measured. This is unlikely due to autofluoresence because larvae that were not photoconverted yielded zero CaMPARI activity. We cannot exclude the possibility that due to longer photoconversion some CaMPARI has converted into the red fluorescence version even when anesthetized. It is well established that when anesthetized with MS-222, neurons are barely active [69, 70]. Indeed, our behavioural assay shows no locomotion in the presence of MS-222. We believe this method is a suitable technique to measure substantial changes in neural activity

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in the larger parts of the zebrafish CNS. Considering the advantages, this method outweighs its limitation and could serve for future studies, especially in connection with epilepsy.

Delayed implications after early embryonic exposure of CBD and THC

Both CBD and THC have been implicated in embryonic development including abnormal changes to neurons. Delayed abnormalities after early exposure of key cannabinoids have only recently been investigated [11]. Here, early exposure of CBD and THC during the first 10 hours of embryonic development reduced neural activity when measured days later, in a dose-dependent manner. These later effects may be due to abnormal neurodevelopment and/or the slow release of the cannabinoids from the tissue due to their lipophilic nature. Consistent with this finding, zebrafish larvae with a similar exposure (5-10 hpf) to cannabinoids have shown effects that could be due abnormal development (at 5 dpf). This was not exclusive to neurons, but included other changes such as morphological abnormalities, decreased survival rate, decreased mEPCs activity (may relate to muscle development), decreased heart rate and delayed hatching [11]. Considering the specific time-window of exposure (first 10 hours in development), an abrupt effect on the developmental program including neurons seems plausible. Interestingly, CBD seemed to impact neural activity at a lower concentration than THC, which also aligns with our previous study [11]. It is important to acknowledge that CBD consumption has been documented to help with seizures and cancer by reducing pain [71, 72]. Nevertheless, our study suggests caution during pregnancies.

Considering that cannabis contains large proportions of both CBD and THC, we looked at the effects of these two compounds in combination and found a more potent effect in

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decreasing neural activity. Reticulospinal neurons in the hindbrain of the zebrafish control motor neurons during swimming [47, 73]. In line with a reduction in locomotion, we observed reduced staining when exposed to both cannabinoids, suggesting that the reticulospinal neuronal integrity was indeed compromised. However, this finding did not explain why locomotor activity was mainly influenced by CBD. This could be due in part that CBD has a greater effect at lower concentration than THC. The effect of CBD may be amplified by effecting motor neurons and potentially muscle development. Overall, these findings were surprising, because CBD was found to negate the effects of THC on CB1R [26]. One possibility is that at higher concentrations both, CBD and THC, affect the developmental program. In contrast, at lower concentrations and in fully developed brains the impact of CBD and THC is different. This could result in overriding the negating effects of CBD. Indeed, several receptors have been shown to have different affinities to THC and CBD [24]. A second possibility, therefore, is that at high concentration CBD and THC affect receptors other than CB1Rs and CB2Rs. This could explain why inhibiting CB1R and CB2R did not fully prevent CBD-mediated reduction of neural activity, as further discussed below.

Potential mechanism involved in the delayed effect of CBD and THC

At this stage, it is unclear what mechanism(s) account for the reduction in neural activity later in development when embryos are exposed briefly to CBD and THC in the first hours after fertilization. In this study, we were especially interested in the less widely studied CBD. Using HEK cells several receptor candidates have been identified showing to interact with CBD [24]. A recent study supports a functional role of CB1R and CB2R role in zebrafish [33]. Here, by

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inhibiting CB1R, and CB2R, we found that the reduction in locomotor activity mediated by CBD was partially prevented at nanomolar concentrations of the inhibitors suggesting that, mechanistically, CBD acts through both receptors. Previous studies have implied that CBD may bind to both CB1R and CB2R *in vitro* [24] and our study is in support that these interactions are important *in vivo*. In line with this, CBD can lead to abnormal craniofacial and brain development in mice and zebrafish, which is mediated through CB1R and the hedgehog signaling pathway [15]. Whether CB2R can mediate its effects through the same pathway remains to be determined. An explanation to why inhibiting CB1R and CB2R did prevent the maximum effect of CBD, which is delayed, could relate to the fact that the CB1R and CB2R receptors are expressed very early in development and found on stem cell progenitor cells [74]. As mentioned above an early effect may impact the developmental program such that changes persist until later even if cannabinoids are no longer present in the circulation. Changes may not be specific to the CNS as CB receptors are not only expressed in neuronal progenitor cells, but in stem cells forming other types of tissues [74]. This could explain our previous study CBD affected heart-rate and overall morphology after 5 dpf when exposed only briefly during gastrulation [11]. The CBD-mediated reduction in neural activity was not prevented when either CB1 or CB2Rs were inhibited. While the reason for this is unclear, it could relate that the effects of CBD are larger outside the optic tectum and the hindbrain and more visible when quantifying locomotion. It is possible that other receptors such as TRPV1 are involved [27, 75, 76], but this needs further investigation.

Conclusion

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Overall, both CBD and THC exposure during embryonic stages had a negative impact on later neural activity, which was additive when combined. Together, this could mean that the impact of cannabis on early development is higher than using isolated compounds and profound. Our findings also support an *in vivo* mechanism of CBD functioning through CB1R and CB2R. We believe this study opens up a new path for investigating detailed mechanisms, including other receptors, in specific neurons that are affected by cannabinoid toxicity early in development and persist. The accumulating evidence for prenatal cannabis consumption having negative consequences on neurodevelopmental disorders in the offspring [3] has to be considered. As the recreational use is becoming legalized and socially acceptable, more studies are warranted to not only fully understand the impact on human development, but also the mechanisms of the diseases.

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DISCLOSURE

None of the authors have any conflicts of interest to disclose.

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AUTHOR'S CONTRIBUTIONS

RK, MRA, WTA, DWA contributed to the experimental design. RK, MRA, LFL, DDB, AMO, performed the experiments and analyzed the data. RK, MRA and DWA interpreted the results. RK, WTA and DWA wrote the manuscript. All authors read and approved the manuscript.

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Figure 1: High-throughput quantification of neural activity in freely-swimming zebrafish larvae. A, Calcium-modulated photoactivatable ratiometric integrator (CaMPARI) photoconverts from green fluorescent emission to red emission in presence of both high calcium concentrations and a bright 405 nm light source. This photoconversion is irreversible and thus creates a temporal snapshot, integrated over the time window of light application, of increased calcium levels (neural activity) as a red-to-green ratio of fluorescing CaMPARI. **B**, Lateral view of green fluorescing CaMPARI merged with brightfield image shows expression of this neural activity biosensor in the CNS due to a pan-neural promoter (*elavl3* aka HuC) allowing the red-to-green ratio of CaMPARI to be interpreted as a direct output of relative neural activity of freely-swimming larvae. **C**, Larvae were transferred to 48 wells in the centre of a 96-well plate to ensure that the 405 nm LED Flood Array covers all larvae entirely. To minimize overheating, the plate was floating in a waterbath at 10 cm distant to the LED while CaMPARI was photoconverted (PC) by the LED Flood Array. Variations in fluorescence intensity of a sample due to variable larvae positioning in different wells are normalized due to the ratiometric feature of the CaMPARI output. **D**, Lateral view of zebrafish akin to panel B with Exemplar "CaMPARI Activity" heat maps that were acquired in the 96 well plate using an automated INCell 2000 high-content microscope and reveal a consistent 2-fold reduction in neural activity when the larvae were anesthetized with MS-222 and an expected increase when neural activity was stimulated with convulsants, pentylenetetrazole (PTZ) and 4-aminopyridine (4-AP). Top, Enlarged lateral view from freely swimming larvae illustrating the optic tectum and hindbrain areas (dotted line) from which the R/G ratio was obtained. Heat maps show ratio of red/green (R/G) fluorescent output as indicated by the calibration bar and can be interpreted as relative calcium levels or neural activity. CNS regions with higher levels of neural activity translate to

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higher R/G values and are reported here as "hotter" pixels in image. **E**, Neural activity, inferred from the mean R/G ratio in optic tectum and hindbrain, increases and decreases following application of stimulants or anesthetics, respectively. CaMAPRI is reduced to baseline in MS222-anaesthetized fish and photoconversion is undetectable when CaMPARI photoconverting light is omitted. Dashed green line represents mean for MS-222 anesthetized samples, which show a clear reduction in signal compared to freely swimming larvae and provides a baseline "near-zero" activity level for reference in subsequent experiments. Drivers of neural activity, such as the convulsants PTZ and 4-AP induce a significant increase in CaMPARI signal **F**, Locomotor activity was measured using behavioural tracking software. PC light from the day prior at 4 days post-fertilization did not affect locomotion at 5 dpf. Anesthetic (MS-222) treatment abolished any swimbouts. Biological replicates are individual larvae = n. ** Significantly different from freely swimming samples, $p<0.01$.

Figure 2: Early application of Cannabidiol (CBD) and Δ□-tetrahydrocannabinol (THC) reduce subsequent neural activity and locomotor activity. A, The chemical structures of key cannabinoids used in this study: CBD and THC. **B**, Timeline of experimental set up where zebrafish embryos were treated with drugs between 0.5 and 1 hour post-fertilization (hpf) and washed out at 10 hpf towards the end of gastrulation. CaMPARI imaging was obtained at 4 days post-fertilization (dpf) and locomotor activity was tracked at 5 dpf. **C** and **F**, Representative CaMPARI activity maps obtained from treated larvae and corresponding quantification and statistics shown in **D** and **G**, respectively. **E** and **H**, Locomotor activity of the same larvae. **C**, CBD reduces neural activity and locomotor activity as shown in **D** and **E**. **F**, THC requires a higher does than CBD to reduce neural activity and locomotor activity as shown in **G** and **H**.

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CaMPARI heat maps show ratio of R/G channels as indicated by the calibration bar., with higher ratios (hotter colours) representing greater neural activity. Green-dashed lines depict mean baseline (zero) value for MS-222 anesthetized samples (From Fig 1). Biological replicates are n=8-19. * is p<0.05; ** p<0.01 compared to the vehicle control (0.3 % MeOH in experiments with CBD and 0.6 % MeOH for THC).

Figure 3: Antagonistic effect of CBD on neural activity is enhanced when combined with sub-effective doses of THC. Zebrafish larvae were exposed to a series of CBD and THC concentrations by themselves or in combination, which are mostly sub-effective, or in the case of CBD, were minimally effective. **A** and **D**, Exemplar CaMPARI activity heat maps show an additive effect (minimizing neural activity) when exposed to 2 µg/ml (**D**) of each CBD and THC compared to CBD or THC by themselves (as plotted in **E)**. When 1 µg/ml of 1:1 CBD and THC was applied, ratios shows no additive effect as illustrated by quantifications and statistics in **B**. **C** and **F**, Locomotor activity from the same well at 5 dpf shows a clear reduction when CBD and THC is combined at 2 µg/ml each, but reduction mediated by CBD alone is almost as low as when combined with THC suggesting that CBD is the main component affecting locomotor activity. R/G is indicated by the calibration bar. Green-dashed lines depict mean values for MS-222 anesthetized samples (from Figure 1). Biological replicates are n=15-25. * compared to vehicle control (equal amount of MeOH in all experiments); # compared to CBD plus THC. One symbol is p<0.05; Two symbols is p<0.01.

Figure 4: Cannabinoid receptor 1 (CB1R) and CB2R are both involved in CBD-mediated reduction of locomotor activity. Zebrafish larvae were exposed to CBD (3 µg/ml) with CB1R-

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and CB2R inhibitors, AM251 and AM630, respectively. **A** and **B**, Exemplar CaMPARI heat maps showing R/G ratios obtained at 4 dpf. **C** and **D**, Corresponding quantifications showing CBD mediated-reduction of neural activity is not fully rescued when CB1R is inhibited with AM251 or CB2R with AM630 in the nanomolar range. **E** and **F**, Locomotor activity at 5 dpf shows a clear rescue when inhibiting either CB1R or CB2R with 0.1 nM AM251, or 10 nM AM630, respectively. **G** and **H**, shows that applying AM251 or AM630 without CBD did not affect CaMPARI activity or locomotion. All samples contained the same amount of vehicle DMSO (0.1 %) and MeOH (0.3 %) including the vehicle control (Veh.). Green-dashed lines depict mean values for MS-222 anesthetized samples. Biological replicates are n=13-30. * is $p<0.05$; ** $p<0.01$ compared to CBD.

Figure S1: Supplementary figure accompanying Fig.2. Re-plotting data to illustrate that CBD shows an effect at lower concentration than THC. Quantifications of CaMPARI activity (left) or locomotor activity (right) showing a statistical difference between CBD and THC at 4 or 5 days post-fertilization when adding 3.0 µg/ml of either cannabinoids. Green-dashed lines depict mean values for MS-222 anesthetized samples. Biological replicates are n=14-28. Statistics using unpaired t-test shows ** $p<0.01$ compared to CBD.

Figure S2: Supplementary figure accompanying Fig.2. Re-plotting data from groups that were untreated, treated with vehicle, CBD (3 µg/ml) or THC (6 µg/ml) to assess CaMPARI activity vs. Locomotor activity within the same individual larvae. There is a significant positive linear correlation between neural activity and locomotor activity when corresponding vehicle controls

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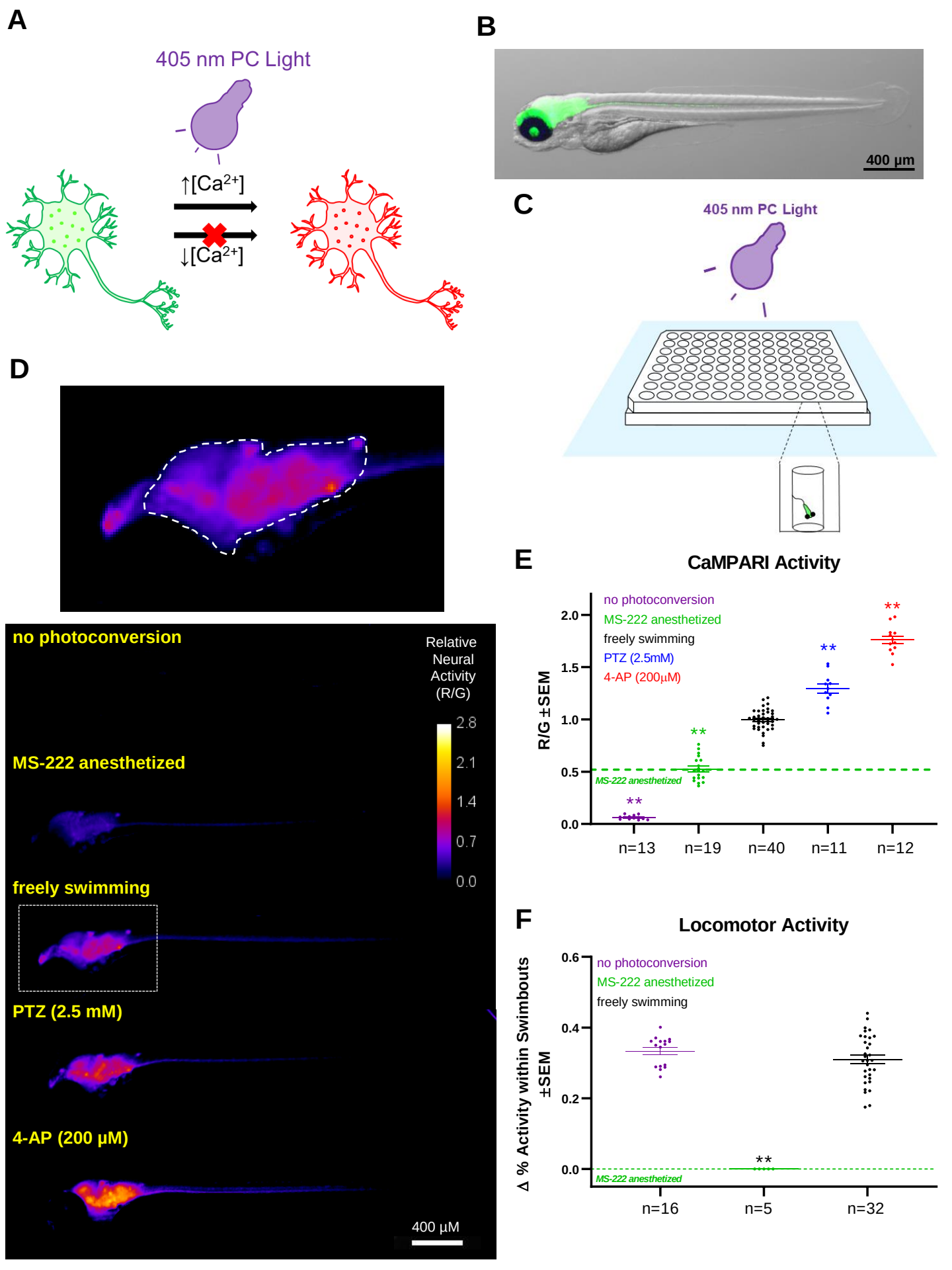
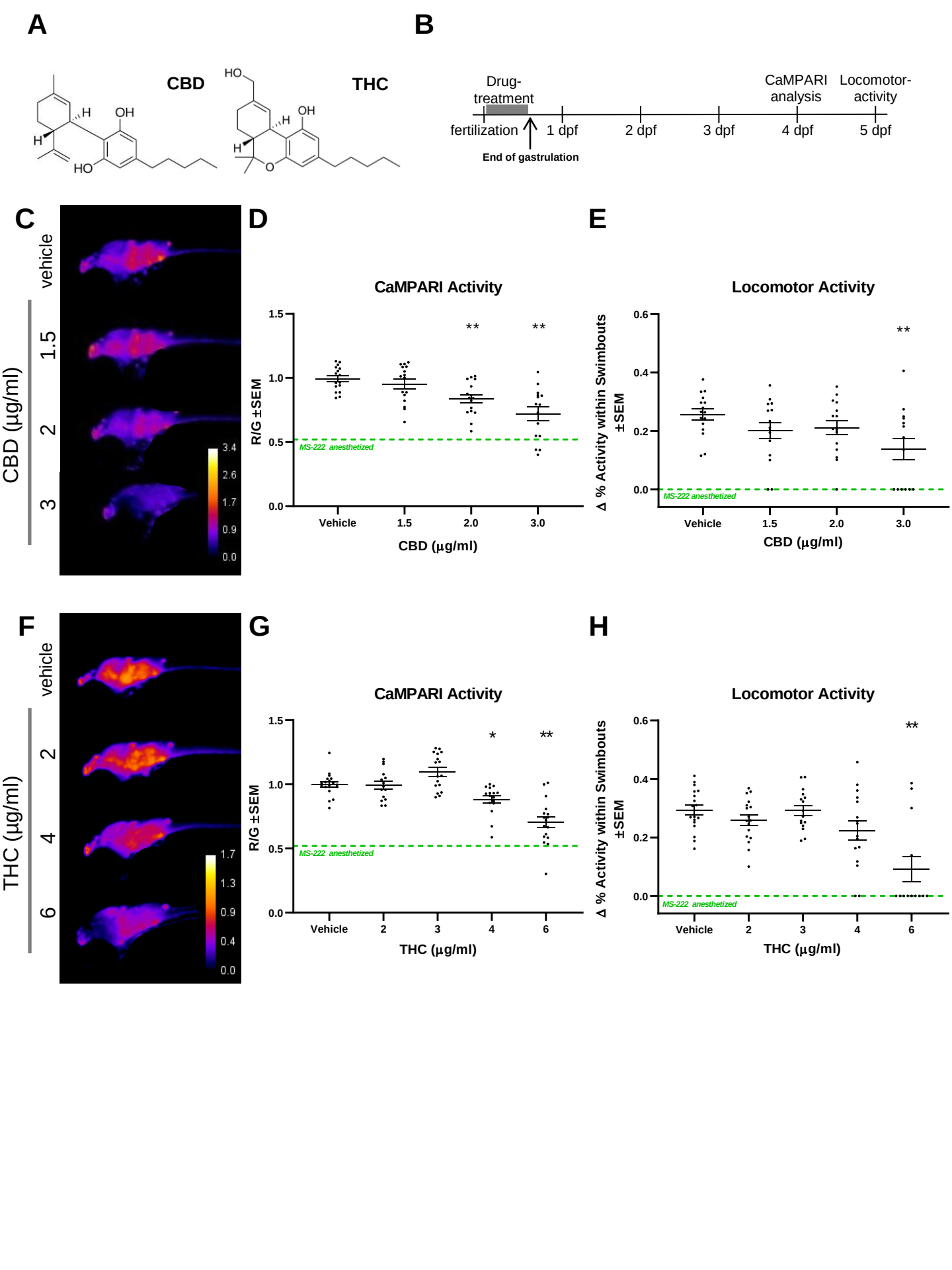
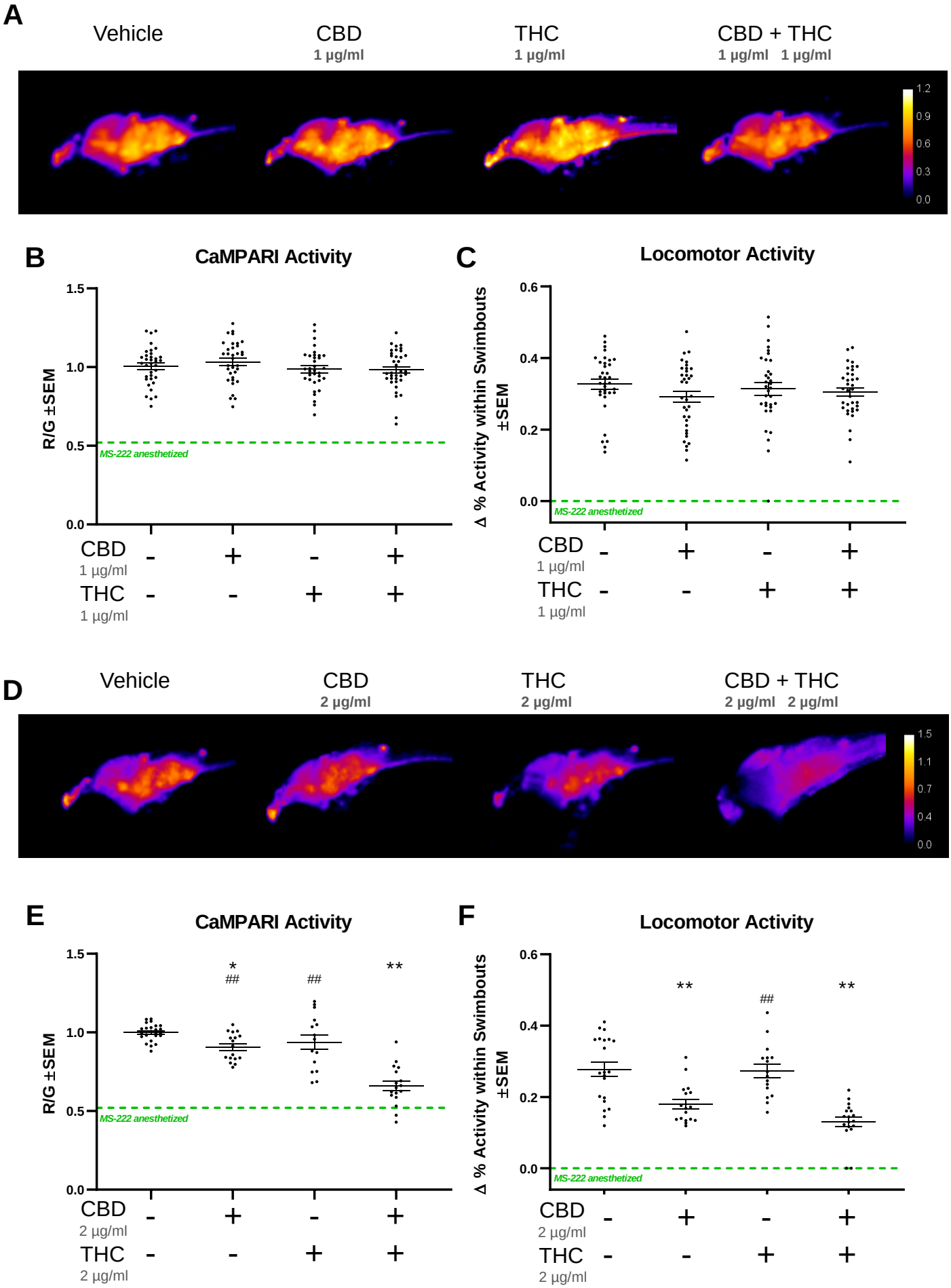
(black circles) were plotted with either CBD (A, red circles) or THC (B, blue circles). Green-dashed lines depict mean values for MS-222 anesthetized samples.

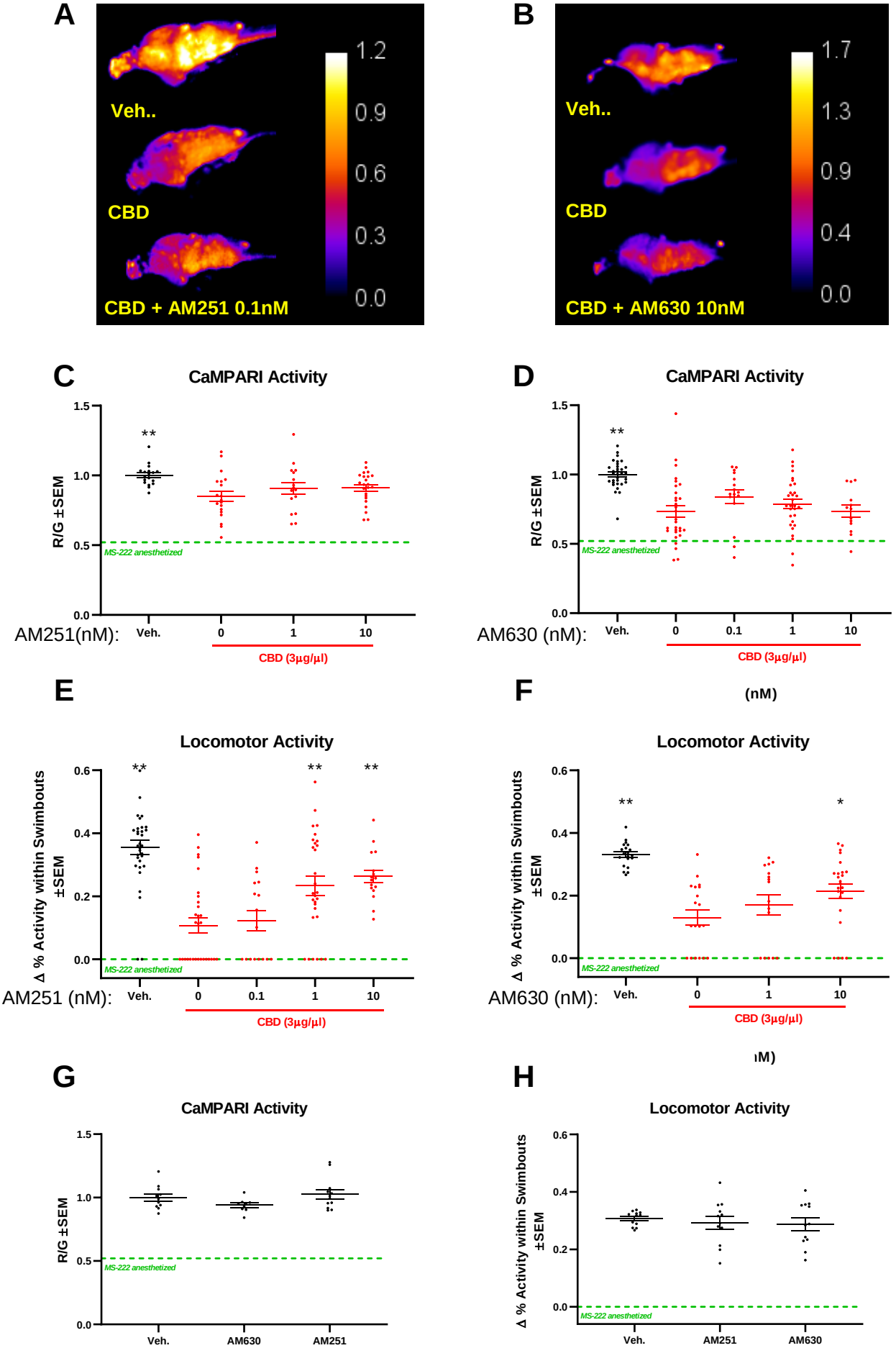
Figure S3: Supplementary figure accompanying Fig.3. Additive effects in reducing neural activity are also obtained at lower CBD concentrations while THC concentration is kept constant. **A**, Exemplar CaMPARI activity heat maps obtained at 4 days post-fertilization show an effect at 0.5 and 1.5 µg/ml of CBD when combined with 2 µg/ml THC compared to CBD as illustrated by quantifications in **B**. **C**, Locomotor activity from the same well at 5 dpf. R/G as indicated by the calibration bar. Green-dashed lines depict mean values for MS-222 anesthetized samples. Biological replicates are n=14-28. * is $p<0.05$; ** $p<0.01$ compared to CBD.

Figure S4: Supplementary figure accompanying Fig.3. RMO44 staining of reticulospinal neurons in the hindbrain shows a decrease in fluorescence intensities when combining CBD and THC at 2 µg/ml at 5 dpf compared to when CBD or THC was added alone. Images show exemplar biological replicates, left to right, from corresponding treatment group, top to bottom.


A
405 nm PC Light
↑[Ca2+]
↓[Ca2+]
B
400 µm
C
405 nm PC Light
D
no photoconversion
MS-222 anesthetized
freely swimming
PTZ (2.5 mM)
4-AP (200 µM)
Relative Neural Activity (R/G)
2.8
2.1
1.4
0.7
0.0
400 µM
E
CaMPARI Activity
no photoconversion
MS-222 anesthetized
freely swimming
PTZ (2.5mM)
4-AP (200µM)
R/G ±SEM
2.0
1.5
1.0
0.5
0.0
MS-222 anesthetized
**
n=13
n=19
n=40
n=11
n=12
F
Locomotor Activity
no photoconversion
MS-222 anesthetized
freely swimming
Δ % Activity within Swimbouts ±SEM
0.6
0.4
0.2
0.0
MS-222 anesthetized
**
n=16
n=5
n=32


A
CBD
THC
HO
OH
H
B
Drug-treatment
fertilization
1 dpf
2 dpf
3 dpf
4 dpf
5 dpf
CaMPARI analysis
Locomotor-activity
End of gastrulation
C
vehicle
1.5
2
3
CBD (µg/ml)
3.4
2.6
1.7
0.9
0.0
D
CaMPARI Activity
R/G ±SEM
MS-222 anesthetized
Vehicle
1.5
2.0
3.0
CBD (µg/ml)
E
Locomotor Activity
Δ % Activity within Swimbouts ±SEM
MS-222 anesthetized
F
vehicle
2
4
6
THC (µg/ml)
1.7
1.3
0.9
0.4
0.0
G
CaMPARI Activity
R/G ±SEM
MS-222 anesthetized
THC (µg/ml)
H
Locomotor Activity
Δ % Activity within Swimbouts ±SEM
MS-222 anesthetized
THC (µg/ml)


A
Vehicle
CBD
1 µg/ml
THC
1 µg/ml
CBD + THC
1 µg/ml 1 µg/ml
1.2
0.9
0.6
0.3
0.0
B
CaMPARI Activity
R/G ±SEM
1.5
1.0
0.5
0.0
MS-222 anesthetized
CBD
1 µg/ml
THC
1 µg/ml
C
Locomotor Activity
Δ % Activity within Swimbouts ±SEM
0.6
0.4
0.2
0.0
MS-222 anesthetized
CBD
1 µg/ml
THC
1 µg/ml
D
Vehicle
CBD
2 µg/ml
THC
2 µg/ml
CBD + THC
2 µg/ml 2 µg/ml
1.5
1.1
0.7
0.4
0.0
E
CaMPARI Activity
R/G ±SEM
1.5
1.0
0.5
0.0
*
##
##
**
MS-222 anesthetized
CBD
2 µg/ml
THC
2 µg/ml
F
Locomotor Activity
Δ % Activity within Swimbouts ±SEM
0.6
0.4
0.2
0.0
**
##
**
MS-222 anesthetized
CBD
2 µg/ml
THC
2 µg/ml


A
Veh..
CBD
CBD + AM251 0.1nM
1.2
0.9
0.6
0.3
0.0
B
Veh..
CBD
CBD + AM630 10nM
1.7
1.3
0.9
0.4
0.0
C
CaMPARI Activity
R/G ±SEM
MS-222 anesthetized
AM251(nM):
Veh.
0
1
10
CBD (3µg/µl)
D
CaMPARI Activity
R/G ±SEM
MS-222 anesthetized
AM630 (nM):
Veh.
0
0.1
1
10
CBD (3µg/µl)
E
Locomotor Activity
Δ % Activity within Swimbouts ±SEM
MS-222 anesthetized
AM251 (nM):
Veh.
0
0.1
1
10
CBD (3µg/µl)
F
(nM)
Locomotor Activity
Δ % Activity within Swimbouts ±SEM
MS-222 anesthetized
AM630 (nM):
Veh.
0
1
10
CBD (3µg/µl)
ıM)
G
CaMPARI Activity
R/G ±SEM
MS-222 anesthetized
Veh.
AM630
AM251
H
Locomotor Activity
Δ % Activity within Swimbouts ±SEM
MS-222 anesthetized
Veh.
AM251
AM630