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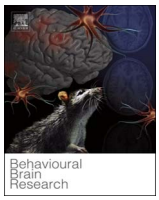

Research report

Comparison of cannabinoids with known analgesics using a novel high throughput zebrafish larval model of nociception

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ABSTRACT

It has been established that both adult and larval zebrafish are capable of showing nociceptive responses to noxious stimuli; however, the use of larvae to test novel analgesics has not been fully explored. Zebrafish larvae represent a low-cost, high-throughput alternative to traditional mammalian models for the assessment of product efficacy during the initial stages of drug development. In the current study, a novel model of nociception using zebrafish larvae is described. During the recovery from an acute exposure to low levels of acetic acid, larvae display innate changes in behaviour that may be indicative of nociception. To assess the usefulness of this model for testing potential analgesics, three known synthetic pain medications were assessed (ibuprofen, acetaminophen and tramadol) along with three naturally occurring products (honokiol, tetrahydrocannabinol and cannabidiol). When the effect of each compound on both the acetic acid recovery and control activity was compared there appeared to be both similarities and differences between the compounds. One of the most interesting effects was found for cannabidiol which appeared to oppose the activity change during the recovery period of AA exposed larvae while having a nominal effect on control activity. This would appear to be in line with current research that has demonstrated the nociceptive properties of cannabidiol. Here we have provided a novel model that will complement existing zebrafish models and will expand on the potential use of zebrafish larvae for studying both nociception and new analgesics.

1. Introduction

The development and testing of analgesics is arduous given that pain is a complex physiological phenomenon with multiple etiologies. Numerous targets exist for potential analgesics and different analgesic classes can treat different pain modalities from relatively mild (non-steroidal anti-inflammatory drugs-NSAIDs, paracematol) to more severe and chronic pain (opioids) [1]. Often a single analgesic is only efficacious in a subset of patients or can only reduce the level of pain by a certain degree but cannot eliminate it [2]. Along with the difficulty in targeting the causes of pain, choosing the right analgesic is often hampered by the side effect profile. This is especially true in the case of opioids where their potential for abuse has become an epidemic [3,4].

In an effort to avoid strong side effects many people look towards naturally-derived compounds for pain management [5]. One such product that has steadily gained interest in the field of pain management is cannabis since it has been shown that the endocannabinoid system plays a distinct role in the modulation of pain [6,7]. While the use of medical cannabis is gaining mainstream support, challenges still exist with its use, such as dosing, side effect profiles (psycho-activity, toxicity) and an overall lack of knowledge of its mechanism of action, which often makes clinicians hesitant to prescribe it [8]. Some of these challenges stem from the fact that in addition to the most characterized cannabinoids, tetrahydrocannabinol (THC) and cannabidiol (CBD), cannabis contains numerous other cannabinoids as well as terpenes and flavonoids that all may have biological activity but are poorly characterized [9]. Thus, a model that can test the bio-activity and provide a toxicity profile for both plant-derived material and extracted chemicals would aid in the further development of cannabis and cannabinoids as therapeutics.

Drug testing models using zebrafish larvae have gained popularity as a starting point for the testing of potential therapeutics as they offer a low-cost, high-throughput alternative to more costly mammalian tests [10]. It has been shown that zebrafish possess a similar endocannabinoid system to that of mammals and may be useful for identifying the therapeutic potential of cannabinoids [11]. Zebrafish models may also provide information on both the target specificity of the compounds being tested along with potential off target effects that

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may be detrimental or lead to toxicity [12]. Additionally, a large number of disease models have been developed using zebrafish that can be used to test the efficacy of potential therapeutics [13,14]. With respect to pain, it is now widely accepted that fish can detect painful stimuli (nociception) and have similar somatosensory neural networks to higher vertebrates [15,16]. Zebrafish behavioural models of nociception have been developed using both adults and larvae [15,17–27]. Adult pain models have attempted to mirror those developed for other fish species involving the injection of acetic acid (AA) into either the lips [17,23] or subcutaneously along the trunk [21]. While these studies produced changes in behaviour that were indicative of nociception, one of the lures of using zebrafish as a drug testing model comes from the high throughput and low cost of larval studies[28–30]. A recent study has highlighted the potential usefulness of a larval nociception model as replacement for adult studies [27]. However, there currently are only a limited number of zebrafish models of nociception using larvae [18,19,22,27]. It has been shown that chemical and thermal stimuli can produce changes in larval behaviour that are indicative of nociception such as changes in activity [22,27] and temperature aversion [18,19]. In addition to behavioural responses thermal stimuli can produce gene expression changes that may be related to the activation of nociceptive pathways [20].

In the current study we have developed a novel model of pain assessment in which 5 day old larvae are briefly exposed to acetic acid followed by an analysis of their subsequent patterns of behaviour during their recovery. We have shown that exposure to acetic acid causes localized tissue damage along the tail of the larvae and leads to a unique, multifaceted, change in behaviour that is indicative of a nociceptive response. In order to evaluate the efficacy of the model for testing the therapeutic potential of cannabinoids, known synthetic and naturally-derived health products were tested for their effects on the larval model of nociception and compared with the effects of THC and CBD.

2. Materials and methods

2.1. Animals

Zebrafish (*Danio rerio)* were maintained according to standard animal care protocols (Westerfield 1995) and in accordance with the Canadian Council on Animal Care guidelines. AB/Tubingen adults, embryos and larvae were maintained on a re-circulating Tecniplast aquatic system at 28oC +/− 1oC and between pH 7.0–7.5 on a 14/10 h light/dark (L/D) cycle. Embryos were collected from multiple AB/Tubingen breeding pairs and pooled. Following 4–6 h in an incubator in E3 media (5 mM NaCl, 0.17 mM KCL, 0.33 mM CaCl2·2H2O, 0.33 mM MgSO4) unfertilized embryos were removed. Larvae were placed in Aquatic Habitats mesh-bottom baby baskets on the recirculation system until use.

2.2. Propidium iodide staining

Five day old zebrafish larvae were placed 2 larvae per well in 12 well plates fitted with 15 mm Corning Netwell mesh bottom baskets. Larvae were exposed to acetic acid (AA) solutions (0.15, 0.25, 0.5%) made from a 5% AA stock solution in E3 media for 60 s. Larvae were washed in HEPEs buffered E3 media (HE3, E3 + HEPEs 10 mM, pH 7.2), and transferred to 2 ml of tricaine HE3 (50ul of 4 mg/ml tricaine per 2 ml HE3). Propidium Iodide was added to the well (0.75ul of 1.5 mM PI) and swirled briefly for a final concentration of 0.5 ug/ml. Larvae were removed from the well and placed on a depression slide (3 per slide). The media was removed and replaced with ~100ul of 1.5% methyl cellulose w/tricaine (50ul per 2 ml). Larvae were imaged using a Nikon AZ100 fluorescent microscope at 4 × 2 zoom, dark: 400 ms/2.8X gain, light: 100 ms, 1X gain. All images were processed using NIS Elements AR 3.2 software.

2.3. Behavioural testing

Five day old zebrafish larvae were placed 2 larvae per well in 12 well plates fitted with 15 mm Corning Netwell mesh bottom baskets. Larvae were incubated in HEPEs buffered E3 media (HE3, E3 + HEPEs 10 mM, pH 7.2) or HE3 media with the therapeutic being tested for 2 h in a light incubator. Acetic acid (AA) solutions were prepared using E3 media as the HE3 buffer contained enough HEPEs to reduce the acidity of the AA solutions reducing their effect on the larvae. An initial 5% stock solution in E3 was prepared from glacial AA. The stock solution was used to make the appropriate concentration of AA to be tested. Larvae were transferred from the pre-exposure solution to the AA or E3 control plate with the netwells. Following the required exposure time the netwells were washed 2 times with HE3 in 12 well plates then the larvae were transferred to a 24 well plate containing the appropriate recovery solution, 1 larvae per well, using a transfer pipette. The plates were placed immediately into the Zebrabox plate holder comprised of a recirculating water bath used to heat the plates, maintaining the plate temperature at 28 °C. Larval activity was immediately tracked using the Viewpoint video tracking system and software (Viewpoint Life Sciences Inc., Montreal, Quebec, Canada). Images were scanned at a rate of 40fps and initially binned into 60-s windows. The behavioural testing protocol was begun immediately and consisted of a 2.5 h exposure to light followed by a 5 min dark-light cycle for 30 min. Following the behavioural protocol larvae were scored for any mortality, tissue damage or other abnormalities that may affect their behaviour. The initial concentrations tested of for each compound are as follows: Ibuprofen 25–100 μM; Acetaminophen 1–10 mM; Tramadol 1–20 μM; Honokiol 1–5 μM; THC 0.1–1 μM; CBD 0.1–2.5 μM.

2.4. Data analysis

The entire distance travelled was used as the behavioural metric and data was initially pooled into 60 s bins. Each 24 well plate was split into two treatment groups for each run (n = 12/group). The data from multiple experiments conducted on separate days was pooled for further analysis (n = 36–60 for drug treatment groups, n = 188 for E3 controls, n = 208 for AA controls). Following the initial analysis the activity was binned into the following time frames: 1–5, 10–30, 80–90 and 140–150 min. In addition the total activity for the second light cycle was subtracted from the total activity during the second dark cycle. Comparisons were made between experimental groups by testing significant differences using a standard 2-tailed *t*-test with a two sample unequal variance that compared data for each bin. Multivariate statistical analysis and PCA was performed on the software Unscrambler X (v10.3) from CAMO. The dataset was built by averaging the observations recorded for 12 larvae and each sample was described by 150 variables obtained from the mobility measured within 60 s bins over the 2.5 h of light exposure. PCA results were obtained after mean centering the data matrix for each variable and discussed by interpreting both score and loadings plots.

3. Results

Previous zebrafish models have shown that continuous exposure of larvae to low levels of acetic acid (AA - 0.0025–0.025%) leads to a general and often sustained increase in activity [24] while exposure to higher levels of AA (0.1–0.25%) produces a decrease in activity [27]. In order to design an alternative behavioural model of nociception using zebrafish larvae that would complement the existing studies and may more closely resemble both adult and rodent injection studies we have developed a model where larvae were exposed to higher levels of AA for short durations followed by recovery in a buffered solution. The goal was to produce some peripheral tissue damage that would be akin to a localized injection without causing overt morphological abnormalities or lethality. We initially exposed larvae to AA between 0.1 & 0.5% for