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Impact of analgesic drugs on the behavioural responses of larval zebrafish to potentially noxious temperatures

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ABSTRACT

Recent studies have demonstrated that fish exhibit behavioural responses to noxious stimuli, including mechanical, chemical or thermal stimulation. In many cases, these responses are characterised by a reduction in the locomotor activity, which in turn can be ameliorated by using appropriate analgesia. However, studies with larval forms are scarce. This study explores the behavioural responses of larval zebrafish after stimulation with hot and cold temperatures and screens a range of analgesics to determine whether these can ameliorate the potential noxious effect of thermal stimulation. Therefore, we aimed to validate the use of these young forms in experimental pain studies as replacement for adults. Five-days post fertilisation zebrafish were exposed to hot (35 and 40 °C) and cold (7, 10 and 15 °C) temperatures for one minute. Pre- and post-stimulation behaviour (velocity and time spent active) was recorded using a novel tracking software in 25 fish at once. Fish exposed to 7, 10 and 40 °C showed significant reductions in both the velocity of swimming and overall activity whereas exposure to 15 and 35 °C had a lesser effect. The efficacy of aspirin, lidocaine, morphine and flunixin as analgesics via immersion after exposure to water at 40 °C was tested. To understand whether this efficacy was similar at lower temperatures, morphine was tested in fish exposure to 10 °C. Administration of 5 mg/l of lidocaine and 48 mg/l of morphine ameliorated the reduction of the activity when larvae were exposed to 40 °C but the group exposed to morphine showed intermediate values between controls and larvae exposed to 10 °C. These results suggest that larval zebrafish responded to heat and cold stimulation and that the behavioural changes induced by hot temperatures were a response to noxious stimulation. In contrast, responses to cold were probably a consequence of both nociception and some kind of stress and/or anaesthetic effect. This novel approach to study nociception and to test analgesic efficacy justifies the replacement of adult zebrafish with non-protected larval forms.

1. Introduction

For some species (rainbow trout, Atlantic cod, goldfish and Atlantic salmon), evidence from empirical studies suggests that fish may have the capacity to experience painful stimuli and the associated discomfort (Dunlop and Laming, 2005; Eckroth et al., 2014; Sneddon, 2003a). Nociception, the detection of potentially harmful stimuli, is the basic mechanism for the sensation of pain, i.e., interpreting the nociceptive stimulus. Recent investigations (Sneddon, 2002) have demonstrated that teleost fish have nociceptors, receptors to detect potentially painful stimuli, which are very similar to those found in mammals (Ashley et al., 2007; Roques et al., 2012). The use of zebrafish in pain and nociception studies has dramatically risen in the last few years (Curtright et al., 2015; Gomes Lima et al., 2012 Gonzalez-Nunez and Rodríguez, 2009) because of the similarities that both the peripheral and central nociceptive processing systems of this species have with other vertebrates and mammals (Curtright et al., 2015 Gonzalez-Nunez and Rodríguez, 2009). However, the high number of adults used in these kind of studies should be drastically reduced if we are to adopt an ethical 3Rs approach to research (Russell and Burch, 1959). In addition, larval stages of zebrafish also possess a similar organization of molecular nociceptive circuits compared with mammals (Caron et al., 2008; Gau et al., 2013) and display comparable behavioural responses to adults after a potentially painful procedure (Curtright et al., 2015; Steenbergen and Bardine, 2014; Lopez Luna et al. MS submitted). Classical studies on nociception use mechanical, electrical and chemical stimulation systems to

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explore the behavioural response in both adult and larval zebrafish (Currie, 2014; Steenbergen and Bardine, 2014), but thermal nociception has not been studied yet in this species.

The acute application of a high-intensity thermal stimulus to the skin is one of the most commonly used models to assess nociceptive processing as an assay to screen for the analgesic activity of a drug or physiological manipulation. This manipulation activates sensory fibres which eventually evoke a behavioural response in animals (Allen and Yaksh, 2004; Minett et al., 2014). In fish, the presence of polymodal and mechanothermal nociceptors has been demonstrated (Mettam et al., 2012; Sneddon, 2003a). Indeed, these nociceptors are known to respond to heat when temperature above 30 °C is applied in rainbow trout (Ashley et al., 2007). Previous evidence show that fish have a thermal threshold, i.e. the temperature at which the subject shows an escape response from a thermal stimulus, or latency to withdraw (Nordgreen et al., 2009). However, this response has not been studied in larval zebrafish. These young forms, when exposed to high temperatures, present the characteristics of mammalian animal model suffering of tissue burns and pain (Malafoglia et al., 2014). Although the critical lethal maximum temperature in zebrafish is between 39 and 41 °C approximately (Cortemeglia and Beitinger, 2005), larvae show behavioural responses when exposed to temperatures above 32 °C (Prober et al., 2008).

Similarly, cold nociceptors have been described in birds and mammals (Necker, 2000; Simone and Kajander, 1997), with mechanothermal and polymodal nociceptors known to respond to cold stimuli (Cain et al., 2001). However, cold nociceptors could not be found on the head of trout (Ashley et al., 2007), suggesting that it may not be undergoing a nociceptive or painful situation when exposed to cold temperatures. The critical lethal minimum temperature for zebrafish has been established in approximately 10 °C for animals acclimated to 30 °C (Cortemeglia and Beitinger, 2005). However, changes in behaviour can be observed with higher temperatures. In a study by Pritchard et al. with adult zebrafish (Pritchard et al., 2001), it was found that the activity was reduced when fish were exposed to low temperatures (15 and 20 °C) compared to higher temperatures (25 °C). To date, only one study has investigated the effects of potentially noxious temperatures on the locomotor activity in larval fish (Colchen et al., 2016). However, no studies have been conducted in larval zebrafish.

Noxious heat stimulation has been successfully used to investigate the efficacy of analgesics in birds (Caplen et al., 2013; Hughes, 1990), mammals (Nagakura et al., 2003) or fish (Nordgreen et al., 2009) but the combination of temperature and analgesia has been never been explored in zebrafish. Appropriate analgesia should be applied to minimize the impact on animals undergoing experimental procedures that cause tissue damage (Sneddon, 2015), however, 5 days post fertilisation (dpf) zebrafish larvae are not considered sufficiently developed enough to experience suffering. In addition, behavioural alterations elicited by any noxious stimulation should be reduced by the use of analgesics or painkillers (Sneddon et al., 2014).

Current legislation protects fish until the moment when they become capable of independent feeding, i.e. 5 dpf (Animals (Scientific Procedures) Act 1986; Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes). The objective of our study was to provide a scientific basis for the application of the principle of replacement in zebrafish. i.e., if similar behavioural responses to potentially noxious stimulation are observed in protected and non-protected animals, then the replacement is justified. To achieve this, we explored the use of 5 dpf zebrafish as a valid replacement for adult fish through quantifiable behavioural measurements and to test potential analgesics to inform the development of analgesic protocols. Drugs with analgesic properties dissolved in the tank water should ameliorate the potential noxious responses elicited by hot and cold temperatures, which would present an important refinement in juvenile and adult zebrafish experimentation.

2. Materials and methods

2.1. Experimental animals

All experiments were conducted according to the guidelines of research ethics as approved by the Ethics Committee at the University of Liverpool (40/3534). Five days post-fertilisation (dpf) zebrafish larvae of AB wild type were used for the purposes of this experiment. Eggs were provided by the in-house breeding programme. Briefly, adult zebrafish were held in breeding pairs and eggs collected the morning after. Eggs were then kept in 3 L plastic tanks (length 33.3 cm, width 11.4 cm, height 15.2 cm; Pentair Aquatic Habitat, Apopka, USA) in a closed aerated recirculation system supplied with filtered, aerated freshwater at a temperature of 28.0 ± 0.5 °C and on a 12 h: 12 h light:dark cycle until 5 dpf at which point fish were selected at random for experiments. Water quality parameters were kept ideal for this species (pH 7.2; Nitrite = 0 mg/l; Nitrate <20 mg/l; Ammonia = 0 mg/l). Any animals not used in the present study were either held as stock for other experiments or were humanely killed before reaching 6 dpf by being placed in an Eppendorf on dry ice for use in another study investigating genomics.

2.2. Apparatus

Larvae movements were analysed by placing them individually in a custom built plastic plate of 25 square wells (length: 16.5 mm; width: 16.5 mm; depth: 8 mm) mounted to the side of a 3 L plastic tank (Pentair Aquatic Habitat, Apopka, USA) and secured with clear silicon (AquaMate, Everbuild, Leeds, UK). The plastic plate had a 53 μm mesh bottom (Zebrafish Management Ltd., UK) which allows water to be rapidly flushed in and out. The plate was positioned atop an infrared light stage to maximize contrast and facilitate tracking of dark targets on a light background. The experimental tank was supplied from a glass sump tank (45 × 35 × 40 cm) with filtered and maintained at a constant temperature of 28.5 ± 0.5 °C and with aeration provided via an air stone (12 cm) and air line from a compressed air supply.

2.3. Video acquisition

Video of spontaneous free-swimming was recorded at 25 frames/s using a digital monochrome infrared-sensitive camera (IDS UI-1240LE-NIR-GL; STEMMER IMAGING, Surrey, UK) with an attached lens (SPACE-COM JHF25M-5MP; SPACE inc., Tokyo, Japan) placed above the 25 well plate. The camera was mounted to a tripod at a height of 1.8 m and videos were acquired and saved without compression (.avi), via IDS software (uEye Cockpit; IDS Imaging Development Systems GmbH). The camera was connected to a laptop computer (HP, DSC HM87, Palo Alto, CA, USA). For video analysis, a novel tracking software based on an object automated detection, tracking and monitoring algorithm was developed at the University of Liverpool for this project. Briefly, the algorithm can be divided into four different stages, namely pre-processing, object detection, post-processing and monitoring of the object physical activity. In order to monitor the individual behaviour of the larvae, a user-friendly Graphical User Interface (GUI) has been designed and developed using open-source MATLAB functions (MathWorks, Inc.; Natick, MA, USA). Data files generated by the tracking software were then processed with the bespoke algorithm in MATLAB, which can detect various behavioural larvae patterns larvae based

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