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Research report

Development of an opioid self-administration assay to study drug seeking in zebrafish

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ABSTRACT

The zebrafish (*Danio rerio*) has become an excellent tool to study mental health disorders, due to its physiological and genetic similarity to humans, ease of genetic manipulation, and feasibility of small molecule screening. Zebrafish have been shown to exhibit characteristics of addiction to drugs of abuse in non-contingent assays, including conditioned place preference, but contingent assays have been limited to a single assay for alcohol consumption. Using inexpensive electronic, mechanical, and optical components, we developed an automated opioid self-administration assay for zebrafish, enabling us to measure drug seeking and gain insight into the underlying biological pathways. Zebrafish trained in the assay for five days exhibited robust self-administration, which was dependent on the function of the μ-opioid receptor. In addition, a progressive ratio protocol was used to test conditioned animals for motivation. Furthermore, conditioned fish continued to seek the drug despite an adverse consequence and showed signs of stress and anxiety upon withdrawal of the drug. Finally, we validated our assay by confirming that self-administration in zebrafish is dependent on several of the same molecular pathways as in other animal models. Given the ease and throughput of this assay, it will enable identification of important biological pathways regulating drug seeking and could lead to the development of new therapeutic molecules to treat addiction.

1. Introduction

In recent years, opioids have become the second most commonly initiated drug of abuse. A study by the *National Survey of Drug Use and Health* revealed that 12.5 million Americans reported opioid abuse. As a consequence, the incidence of overdose is reaching alarming rates across North America [1].

A major limiting factor in the fight against opioid abuse is the limited number of therapies currently available. Despite the current situation, there is no effective medical treatment for this type of addiction. Substitution therapies are the only options currently available, consisting of a slow acting opioid such as methadone [2] or in some cases a combination of partial agonists (buprenorphine) and antagonists (naltrexone) [1]. Despite some success in controlling drug intake, these therapies do not treat drug seeking directly and are often unsuccessful [1], leading to a high rate of relapse. Therefore, increasing efforts are being put toward the development of drug addiction models to study the biological mechanism of substance abuse and to find new treatment options. So far, rodents and non-human primates have been used almost exclusively [3].

Over the years, two main categories of assays have been developed to study addiction in animal models. The first consists of the non-contingent assays, including locomotor sensitization and the conditioned place preference paradigms. The second category consists of the contingent models, including different types of self-administration assays requiring a self-operant response in order to receive a dose [4]. Contingent assays are considered more significant and have been shown to be an efficient way to identify compounds affecting addiction [5]. One of the main differences between these two categories resides in the fact that conditioned place preference is a form of passive administration, as opposed to self-administration, which is an active administration. This distinction is important because studies have demonstrated that active administration leads to different molecular and structural changes in the addicted brain [6,7].

An interesting alternative to rodents or non-human primates in fundamental research is *Danio rerio* (zebrafish), which is becoming an important model for dissecting complex neurological disorders [8,9]. Previous studies demonstrated that zebrafish are sensitive to a wide variety of drugs of abuse [10–15], including opioids [11,16]. In fact, several important neuronal networks implicated in addiction in humans are conserved in zebrafish [17,18]. As with other models, fish show signs of addiction as well as withdrawal symptoms [19–22]. In

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addition, given its ease of genetic manipulation and the ability to perform *in vivo* small molecule screening, zebrafish have potential to be a powerful addition to the addiction research community.

Thus far, most substance abuse research in zebrafish has been based on the conditioned place preference paradigm [11,13,14,23]. In these assays, a drug is delivered in a specific area of a tank combined with a specific visual cue, and trained fish developed a preference for the area presenting the cue. Alternatively, a choice assay has been described in which fish larvae are given the option between an opioid solution in one end of the tank and a drug-free environment at the other end. Zebrafish larvae preferentially swim toward the side with morphine, and this preference is dependent on the dopamine pathway [16]. So far, a very limited number of active administration assays have been reported with zebrafish. Recently, an active alcohol administration protocol was designed in which fish are trained to voluntary consume gelatin containing various percentages of ethanol. Using this technique, it was shown that fish consume a larger amount of a 10% EtOH gelatin compared to gelatin without EtOH [24]. Although this assay is an active form of administration, there was no increase in administration over time and no evidence of addiction development. Therefore, the use of zebrafish as an addiction model remains limited by the absence of a *bona fide* self-administration model.

In order to overcome such limitations, we developed an opioid self-administration assay using young adult zebrafish, modeled after the self-administrations used in mammals. In this assay, fish are trained to trigger the delivery of a hydrocodone solution by swimming across a specific underwater platform within a test arena. Fish trained to self-administer opioids demonstrate an escalation in the number of doses received, and self-administration is dependent on the μ-opioid receptor as well as two key pathways for drug addiction: the dopamine and glutamate pathways. The development of this assay will enable improved understanding of the biological mechanism driving drug seeking.

2. Methods

2.1. Animal housing

Ekkwill strain zebrafish (*Danio rerio*) (EkkWill Waterlife Resources) were maintained and embryos were obtained according to standard fish husbandry protocols and with the approval of the Massachusetts General Hospital and University of Utah Institutional Animal Care and Use Committees.

2.2. Experimental apparatus

2.2.1. Design of the testing arena

The conditioning arena consists of a plastic tray (4-3/4 Gallon Shallow Tray – 17-1/2"L x 15-1/2"W x 5"H, USPlastic, USA) with delimited submersible square platforms and connected to a larger water reservoir (15 gallons plastic bin, USPlastic, USA) equipped with a pump (Supreme Aqua-Mag, Thatfishplace, USA) to generate a continuous recirculating flow of water (Fig. 1A). The arena is illuminated with a warm white light source (2700 K, 40 W, CFL bulb, McMasterCarr, USA) providing just enough light to allow the fish to identify the different platforms without affecting behavior (Fig. 1A).

Infrared cameras (PiNoir camera, Adafruit, USA) are installed over each platform and are each connected to a mini-computer Raspberry Pi 2 (Adafruit, USA) to monitor movement above each platform. To generate optimal light conditions for the camera, an LED strip light (850 nm, Environmental Lights, USA) was installed above the arena.

One of the platforms is called the “active platform” and is yellow while the “inactive platform” is identical but is white. The color yellow was chosen because fish do not seem to have a natural preference for this color [25,26]. The computer connected to the camera above the active platform also controls a peristaltic 12 V pump (Adafruit, USA) and a small green LED (Adafruit, USA). A small silicone tube is fixed to one side of the active platform to allow direct delivery of the drug at the platform.

2.2.2. Development of the coding script

The Raspberry Pi2 used in our assay runs on the latest *Raspbian* operating system and a homemade Python script was written to control the assay. The script was designed to detect the movement generated by a fish swimming across the platform by comparing the pixel difference between the image of the current frame with the average image of the previous frames. Movement above a platform was defined as a triggering event when the pixel difference was higher than a manually set threshold. The threshold was set to the minimum value for which the circulating water was not triggering the pump and was set for each experiment. The code was also designed to record the elapsed time, save an image of the frame in which the motion is detected and record the total number of triggering events detected. Finally, when a triggering event was detected above the active platform, the pump and the LED light were activated for 0.3 s to deliver a dose of drug and to provide a secondary reinforcing cue.

2.3. Animal conditioning

2.3.1. Animal pre-conditioning

Two to three-month-old fish were used as they are large enough to provide good movement detection yet small enough to maintain in large groups.

The main goal of this assay was to condition fish to associate the action of swimming over the active platform with receiving a dose of drug. Because the arena was a novel environment which could be a source of stress [27] and the fact that opioids can be aversive for naïve animals [28], we decided to perform pre-conditioning sessions. The pre-conditioning protocol was divided in two steps, the first one consisted of performing one habituation session of 50 min daily for 5 days in the arena, allowing the fish to swim freely in the arena. During those sessions, fish food (Larval, AP100, Zeigler, USA) was administered when motion was detected above the active platform, thus providing a reward for this action. These sessions allowed the animals to acclimatize to the arena and forged a positive association with the action of swimming across the active platform. At the opposite end, swimming above the inactive platform did not trigger anything, thus no positive reinforcement was developed toward this platform.

The second step of the pre-conditioning was to expose the animals to hydrocodone (1.5 mg/L) for 60 min in a separate tank following the session in the arena. Such pre-exposure to an opioid has been shown to improve opioid self-administration training in rodents [28,29].

Additionally, to further reduce the stress associated with our assay, we decided to condition the animals in groups of 15 fish, as it has been shown that social isolation is a stressful condition for zebrafish [30]. The number of animals was selected based on early preliminary tests in the arena (data not shown). To avoid pre-selection bias and to get a more uniform training across the different groups, fish were re-grouped each night in large tanks containing 50–70 animals.

The combination of pre-training in the arena and pre-exposure to opioids served to acclimatize fish to the assay, forge a positive association with the active platform and sensitize the animals to opioids.

2.3.2. Opioid self-administration conditioning

The opioid self-administration conditioning followed an approach similar to the pre-conditioning. Small groups of 15 animals were trained for 50 min daily for 5 consecutive days. As with the pre-conditioning, to avoid any bias toward gender or potential genetic predisposition between the different groups, as well as to generate uniform trainings, animals were kept in large groups between sessions and conditioned in randomly selected subgroups of 15.

As with the pre-conditioning, fish were allowed to swim freely in the