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

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Highlights:

- Zebrafish can be conditioned to self-administer opioids
- Conditioned fish developed several hallmarks of addiction
- Self-administration in zebrafish relies on the same molecular pathways utilized in other animal models

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 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.

Keywords: addiction, opioid, zebrafish, self-administration, animal model

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 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 (Figure 1A). The arena is illuminated with a warm white light source (2,700K, 40W, CFL bulb, McMasterCarr, USA) providing just enough light to allow the fish to identify the different platforms without affecting behavior (Figure1A).

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 (850nm, 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 12V 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 seconds 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 minutes 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 minutes 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 minutes daily for 5 consecutive days. As with the pre-conditioning, to avoid any bias toward gender or potential genetic pre-disposition 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 arena but with the added factor of triggering the release of a dose of 1.5 µg of hydrocodone from a solution of 6mg/L diluted in water from the main system by swimming across the active platform. Each triggering event resulted in drug delivery, in accordance with a fixed ratio (FR) training previously established in other models[3,28]. The continuous flow of water prevented the saturation of the system and forced fish to re-activate the pump in order to receive another dose of drug.

2.4 Animal behavior

2.4.1 Novel tank assay

The novel tank assay was performed in a 1.5L tank (15.2 cm height x 7.1 cm width x 27.9 cm long from Aquatic Habitats, Apopkam, USA) filled with water from the main system. The assay was performed between 12pm and 4pm and recorded for 5 minutes. We tested 3 fish at the same time as it has been suggested that small groups could yield more consistent result than individual fish[31]. The animals were transferred to the middle of the novel tank. The *ActualTrack* software (ActualAnalysis, Scotland) was used to analyze the videos. To analyze the exploration rate, two zones were created in the software, the center and the rest of

the tank. The software recorded the position of the fish in the tank and generated the data for the latency to enter the different zones, time spent, number of visits in the zones. The software gave the position (x, y) of the body center for each fish, the latency to enter the other area, the total time spent in the different areas and the number of visits. The coordinates of the body center of each fish were used to calculate the distance between each pair of animals with the equation: $\sqrt{(x2-x1)^2+(y2-y1)^2}$.

2.5 Chemical treatment

The following chemicals were use in this paper; Hydrocodone hydrochloride (Sigma-Aldrich, USA), Naloxone hydrochloride dihydrate (Sigma-Aldrich, USA), SCH23390 hydrochloride (Tocris, UK), MK801 maleate (Tocris, UK), Ketamine hydrochloride (Sigma-Aldrich, USA) and CNQX (Tocris, UK).

3. Results

3.1 Opioid conditioning

To test our conditioning protocol, we used a wild-type strain of zebrafish (Ekkwill) and analyzed the number of triggering events produced in every training session for the active and the inactive platforms. The analysis of the number of triggering events revealed that on the first day of training, there was no significant difference detected between the active platform (1249± 338 events) compared to the inactive platform (956± 380 events) (Figure1B).

As the week progressed, the number of triggering events increased for the active platform and decreased for the inactive platform such that the difference between the two platforms became significant (Figure1B). By day 5, there were 1771±369 triggering events detected above the active platform, representing a 41 % increase compared to day 1, whereas, we observed a total of 139±51 triggering event at the inactive platform representing an 85% decrease in triggering events for the inactive platform between the first and last day of training. Thus, the increase in events detected is specific for the active platform. As mentioned earlier, in this assay, each triggering event detected for the active platform resulted in one pump activation and thus the delivery of one dose of hydrocodone.

In order to confirm that this increase in motion events was caused by the presence of hydrocodone in the solution and not just a preference for the color yellow or a consequence of the pre-conditioning with food, we repeated the training with pre-conditioned animals but the hydrocodone solution was substituted with only water from the main system. Without opioid delivery, training failed to produce any increase in the number of triggering events for the active platform and caused the total activation for both platforms to decrease by more than 50% from day 1

(Figure1C). We also observed a similar number of triggering events detected through the week between the active and inactive platforms.

The activity of opioids is mainly dependent upon their binding to the μ-opioid receptor[32]. Therefore, to confirm that the self-administration of hydrocodone in our assay was dependent on this receptor, we treated fish with the μ-opioid receptor antagonist, naloxone. It has been shown that naloxone can block CPP and opioid self-administration in different animal models[33]. We treated fish with naloxone for 60 minutes before each pre-conditioning session as well as before each training session. As expected, blocking the μ-opioid receptor prevented development of self-administration behavior. As observed with the water treated groups, the total number of motion events decreased by more than 75% for both platforms as the week progressed and no difference was observed between the platforms (Figure1D).

These results suggest that the specific increase in motion events observed for the active platform is dependent on the administration of the opioid and implicate the μ-opioid receptor[11,16].

3.2 Hallmarks of addiction

3.2.1 Self-administration despite adverse consequences

One of the main characteristics of addiction is the repeat administration of a drug despite adverse consequences such as physical or mental pain. For example, addicted animals will exhibit sustained administration while faced with a physical punishment such as electric shock[18,34-37]. We therefore sought to determine if zebrafish would continue to exhibit self-administration in the face of conditions normally aversive to them.

It has been previously shown that zebrafish prefer deep water, and that shallow water can trigger anxiety[38,39]. This particular behavior allowed us to create an aversive condition in our assay by raising the height of the active platform. As a consequence, the water level above the active platform was reduced by ≈25%. Instinctively, fish should avoid swimming above the elevated platform unless the reward associated with it is powerful enough.

Before testing hydrocodone-conditioned fish, we confirmed that non-conditioned fish would avoid this elevated platform by testing a group of fish conditioned to self-administer food but not pre-exposed to hydrocodone. For the testing session, apart for the change in the platform height, the assay was performed with the same parameters as previously described. With these animals, we observed a reduction in the total number of triggering events at the elevated platform when compared with an assay performed with the regular platform (Figure2A). Interestingly, there was no difference in the number of triggering events measured at the inactive platform (Figure2A).

When hydrocodone-conditioned fish were tested with the elevated active platform, there was no significant difference in the total number of pump activations observed with the elevated platform versus the regular platform (Figure2B). This suggests that conditioned fish will actively cross the active platform despite an aversive condition. Even when the water depth above the active platform was further reduced to ≈50% of the initial level (triple platform), fish still visited the active platform a significantly higher number of times than the inactive platform thus confirming that fish are willing to overcome an aversive condition to receive a dose of the drug.

3.2.2 Self-administration following a progressive ratio

As mentioned previously, in our assay, the animals were conditioned with a fixed ratio, but to further establish that our assay is a *bona fide* self-administration assay, we decided to test a different conditioning protocol: the progressive ratio[28,40-43]. In this assay, animals are required to perform an increasing number of actions in order to receive a dose of the drug as opposed to the fixed ratio where each action triggers release of a dose. For example, a mouse would be required to press a lever five times in order to activate the delivery of the drug and this number is increased until the addicted animal gives up and loses interest. This value is called the break point for this particular animal. In most cases, the progressive ratio is tested with animals previously trained with a fixed ratio[44-46].

We thus tested progressive ratio training by modifying our python script so the pump would only be activated after a variable number of triggering events. In other words, fish would have to swim across the active platform multiple times to receive a single dose. Using this method is informative because if the fish simply developed a place preference for the location of the active platform in the arena, the number of motion events should not change and the fish should lose interest if no drug is delivered. We decided to test the progressive ratio paradigm in our assay by setting the number of triggering events required to activate the pump to 5, 10, 15 and 20. The number of triggering events required to activate the pump was increased on successive days and not during the same session.

We observed that conditioned fish increased their total number of visits each time the number of triggering events required for a dose was increased. This trend was observed with 5 events/dose and became significant with 10 and 15 events/dose, but at 20 events/dose, the difference was no longer significant when compared to the fixed ratio. As a result, there were about 4 times more triggering events detected at the active platform for the 15 events/dose setting as compared with the fixed ratio (Figure2C). Interestingly, the increase in triggering events observed with the setting at 15 events/dose was such that the actual dose received by the animals was similar to the dose received with 5 events/dose (Figure2D). Furthermore, the fact that the number of visits doesn't increase at 20 events/dose suggests that this value could be considered the break point for the group of fish tested.

The increase in total triggering events detected with the progressive ratio demonstrates that hydrocodone-conditioned fish are willing to perform the required action multiple times to get a dose. This result combined with the administration despite an adverse consequence suggests that the activation of the pump is not simply a conditioned place preference but rather an active action to self-administer the drug.

3.3 Evidence of withdrawal symptoms

Among the major hallmarks of drug addiction are the intense physical and psychological manifestations triggered by withdrawal from the drug. These include an increase in anxiety and stress levels in both human and animal models, which are thought to promote continued drug seeking in addicted patients[47]. In zebrafish, previous studies have demonstrated that withdrawal can cause profound physical manifestation such as reduced exploration behavior, increased erratic movements, changes in freezing behavior and changes in locomotion [19,48]. We therefore investigated the behavior of conditioned fish 48 hours after their last self-administered dose of hydrocodone in the Novel Tank assay[19]. In this assay, fish are introduced into a new tank and the behavior of the fish is recorded. The inter-fish distance and the patterns of swimming can be used as measures of anxiety and stress level in zebrafish [27,49-51].

Stress and anxiety have been shown to increase social cohesion in zebrafish[31,52]. We therefore analyzed the effect of withdrawal on shoaling by measuring the distance between each fish in a group of three fish for a period of five minutes. Hydrocodone-conditioned fish had an average distance of 4.32 cm as compared to 5.92 cm for naïve animals. Thus, as hypothesized, fish undergoing withdrawal from hydrocodone form a tighter group than naïve animals (Figure3).

We also measured the exploration behavior of the fish by analyzing the time spent swimming in the center of the tank versus the outer area. Stressed animals have been shown to exhibit reduced exploration of a novel environment[19]. As expected, zebrafish undergoing withdrawal displayed reduced exploration behavior as evidenced by a longer latency to explore the outer area of the tank as it took an extra 103 seconds for the conditioned group to enter the outer area (Figure4B). In accordance with this result, hydrocodone-conditioned fish had a lower number of visits in the outer area resulting in a shorter total visit time (Figure4C-D).

The results suggest that conditioned fish in withdrawal display signs of stress and anxiety as demonstrated by tighter shoals and reduced exploration activity. This increase in stress during withdrawal further supports the idea that conditioned zebrafish develop an addiction to hydrocodone.

3.4 Molecular characterization of the assay

All drugs of abuse share some similar modes of action, and one of the reasons for their addictive potential is the fact that they target the natural brain reward system. This system relies heavily on two major neurotransmitters: dopamine and glutamate [53,54]. Dopamine is released in the brain upon activation of the reward system and is responsible for the sensation of pleasure [55]. Many drugs of abuse directly influence the release or recycling of dopamine, thus enhancing the association between the drug and a positive experience[56,57]. It has been shown that blocking the dopamine pathway can reduce drug seeking in other animal models[11,58].

On the other hand, glutamate is an excitatory neurotransmitter that signals through two majors receptors, the *α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid* (AMPA) *receptor* and the *N-methyl-D-aspartate* (NMDA) *receptor* [59]. As with dopamine, it has been shown that the glutamate pathway is required for drug seeking[53].

Because of their crucial roles in addiction processes, we decided to investigate the involvement of dopamine and glutamate pathways in the self-administration behaviors we observed, to confirm that similar pathways are implicated in zebrafish self-administration. Fish conditioned for hydrocodone self administration for at least seven days were treated with the dopamine receptor-1 antagonist SCH23390 [11,53] to block dopamine signaling. Fish were treated for 60 minutes prior to the self-administration assay and then introduced in the arena for 30 minutes. Treatment with SCH23390 reduced the number of triggering events for the active platform and no difference was observed between the numbers of triggering events at the active and inactive platforms (Figure5).

Similarly, to test the importance of glutamate signaling in our assay, we selected the NMDA receptor antagonists MK-801 and ketamine and AMPA receptor antagonist CNQX. Each of these compounds has been shown to reduce drug seeking in other animal models[11,58]. As with SCH23390, conditioned fish were treated for 60 minutes and introduced in the arena for a 30-minute self-administration assay. The treatments with each compound reduced the number of triggering events registered for the active platform when compared to control hydrocodone-conditioned fish. In addition, the preference for the active platform was also abolished, as the fish no longer triggered the active platform at a higher rate than the inactive one (Figure5).

Taken together, these results suggest that drug seeking in zebrafish relies on molecular pathways similar to those required in other animal models and supports the validity of the assay.

4. Discussion

4.1 Opioid conditioning assay

In order to better understand the molecular and biological pathways controlling drug seeking, we developed an opioid self-administration assay using young adult zebrafish. The design of this assay was inspired by self-administration assays used in other models. In these assays, animals are required to press a lever or a button to receive a dose of drug. Since this kind of action would be hard to reproduce with fish, we decided to use a motion detection approach. Instead of pressing a lever, fish were required to swim across a submersible platform to receive a dose of drug. As with other self-administration assays, there was also an inactive platform for which no dose delivery was triggered. With this assay, we conditioned zebrafish to self-administer the opioid hydrocodone in as few as five days of training. The presence of the inactive platform allowed us to discriminate a simple increase in locomotion induced by opioids[60-62], as opposed to a specific increase in triggering actions at the active platform. The number of pump activations can be used as a read-out of drug seeking in zebrafish.

Interestingly, we observed an increase in triggering events detected at the active platform from day 3 to day 5 with an average of 20.69 events per minute at day 3 and 35.42 events per minute on the fifth day. Furthermore, when fish were trained for several additional days, the number of events rose to 60.5 events per minute, suggesting that we were able to reproduce the escalation in self-administration observed in addicted subjects/models.

Although there is an increase in triggering events at the end of the five days of training, the total of events on the initial day is already quite high. We believe that this high number is caused by the exploration of the arena as the number of triggering event for the inactive platform is also high on day 1. This hypothesis is reinforced by the fact that both the control fish and the naloxone treated fish also have a high number of triggering events for both platforms on the first day. We could also imagine that because we pre-conditioned these animals to self-administer food in the arena, some of the triggering event detected could be caused by food seeking as these fish might be exploring the arena to find food.

It is also interesting to note that the number of triggering event detected for the inactive platform for all training conditions, as well as the active platform for the control and the naloxone groups, both decreased as the week progressed and the number of triggering events for both platforms are quite similar throughout the week. This could reflect a loss of interest in either platform if no reward is provided.

One of the main advantages of zebrafish as a research model is the ability to perform small molecule screening *in vivo*[63], which gives us the possibility to develop a small scale screen to identify compounds that block drug seeking. In addition, we can train a large number of fish in a single week opening up the possibility of screening small chemical libraries. Such a screen could uncover unexpected

biological mechanisms important for seeking and could lead to the identification of potential treatment candidates. The opportunity of performing this kind of screen is a much-needed addition to the field of addiction research.

In addition to performing a small molecule screen, this assay also gives us the possibility to study the opioid sensitivity of different mutants. The ease and efficiency of genetic manipulation techniques such as CRISPR-Cas9 in zebrafish could allow us to rapidly generate mutant lines for genes of interest and test their involvement in drug seeking.

Finally, in the present paper, we limited ourselves to opioids, but the assay could easily be applied to train zebrafish to self-administer other drugs of abuse. We could, thus, determine if an interesting hit from the screen is opioid specific or could affect a global pathway regulating drug seeking. Since we can also perform food self-administration conditioning, we could test the effect of a potential compound on non-drug seeking, thus further determining if the effect is specific to a drug or affects reward seeking in general. The ultimate compound would be specific for a drug of abuse without impacting the tendency to seek natural rewards such as food.

4.2 Evidence of self-administration and hallmarks of addiction

Our initial results demonstrated that fish learned to swim across the active platform to receive a dose of drug. However, since our assay is based on motion detection, the establishment of a place preference for the active platform could have explained this behavior.

One of the main differences between conditioned place preference and self-administration assays is the conditioning phase. Typically, a place preference assay involves a passive delivery of the drug to the animal paired with an environment. Previous studies in zebrafish have used this paradigm by restricting the animals to a specific chamber during the conditioning phase[14,64,65]. On the other hand, self-administration assays require animals to perform an action to receive a dose of drug while they are moving freely in the testing chamber. This type of assay also suggests that the animal has to make the decision to receive a dose. Since in our assay, fish still need to perform the action of swimming across the platform to receive a dose and are free to swim in the whole arena, we believe that these parameters are in line with self-administration assays develop with other animal models.

In addition to the conditioning parameters, the fact that conditioned fish displayed other hallmarks of addiction also suggests that these animals were indeed consciously self-administering the drug.

The progressive ratio training gives an indication of the level of effort a conditioned subject/model is willing to expend to receive a dose of drug. The break point is a key value that has been used to estimate and compare the maximum effort different subjects are willing to expend to receive a dose[66]. The progressive ratio suggests

that the animal associates the action with release of the drug. The increase in triggering actions to compensate for the reduced dose ratio suggests that conditioned animals perceive the correlation of swimming across the platform and the delivery of a dose of drug. Thus, despite the fact that there might be some overlap between self-administration and place preference, based on our conditioning parameters and the fact that conditioned animals can successfully perform the progressive ratio, we believe that the number of triggering event is indeed a measure of self-administration.

The fact that hydrocodone-conditioned fish can also be tested with the progressive ratio is very interesting since it suggests a potential assay that would determine the sensitivity of different mutants to addiction. It is possible to imagine that a mutant might display no difference during the conditioning phase but could show differences in the progressive ratio assay. The breaking point could potentially be used to test the effect of diverse chemical treatments or genetic lesions on the rewarding effects of opioids [40].

Self-administration despite adverse consequences, as observed in this model, is noteworthy because it is one of the main hallmarks of drug addiction. Addicted subjects are often willing to go to great lengths to receive a drug of abuse and continue to seek the drug despite severe consequences on their health or quality of life. The fact that conditioned fish are willing to overcome their natural aversion for shallow water may suggest a similar phenomenon in fish.

In addition, the fact that we see a large reduction in the number of triggering events for both the active and inactive platforms when conditioning is performed with water or when the fish are treated with naloxone, also suggest that unless a reward is provided fish will avoid swimming across those platforms. The platforms measure ≈1.6cm high and seem to represent an obstacle for the animals. Thus, even the simple singular platform could be slightly aversive for fish, reinforcing the notion that fish require a positive reinforcement in order to swim over one of the platforms.

4.3 Withdrawal syndrome

The novel tank assay has been show to be an effective way to measure anxiety and stress levels in zebrafish. One of the parameters that can be measured with such an assay is the exploration rate of the animals. The latency to enter the top zone is the value most often used to determine the willingness of animals to explore their new environment. Upon performing the assay, we noticed that the conditioned animals were reluctant not only to enter the top zone but also to explore the rest of the tank. We also noticed that hydrocodone-conditioned fish formed a tighter group than the naïve animals. Both the reduction in the exploration rate and the formation of a tighter group are indications of stressed animals and suggest that conditioned fish are suffering from withdrawal symptoms when they are not given access to hydrocodone.

4.4 Molecular characterization of the assay

As a final step toward assay validation, we decided to begin the molecular characterization of drug seeking in zebrafish. It was already postulated that zebrafish replicate findings from other animal models of addiction at the behavioral level, but we wanted to test whether it was also the case at the molecular level. So our first step was to test the involvement of the two major neurotransmitters, dopamine and glutamate, on drug craving in zebrafish. Our study shows that blocking the dopamine and glutamate pathways also reduces drug seeking in zebrafish. This is an important result demonstrating that key pathways that are important for drug seeking in other models are also required in zebrafish[18,67], thus suggesting that drug seeking in zebrafish relies on similar pathways as other animals models. This also support the use of small molecule screening to enhance our understanding of the biological pathways controlling drug seeking, since potential hits from such a screen could be relevant for controlling addiction beyond zebrafish.

5. Conclusion

This novel self-administration assay provides an opportunity to study addiction and drug seeking in a model organism amenable to scalable drug screening and facile genomic manipulation. The self-administration behavior observed relies on pathways conserved in other animals models and humans, supporting the ability to use this assay to perform small molecule screening or genetic studies of pathways important for drug seeking, potentially leading to new knowledge and novel addiction therapeutics.

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Supplementary videoS1: Example of conditioned animals

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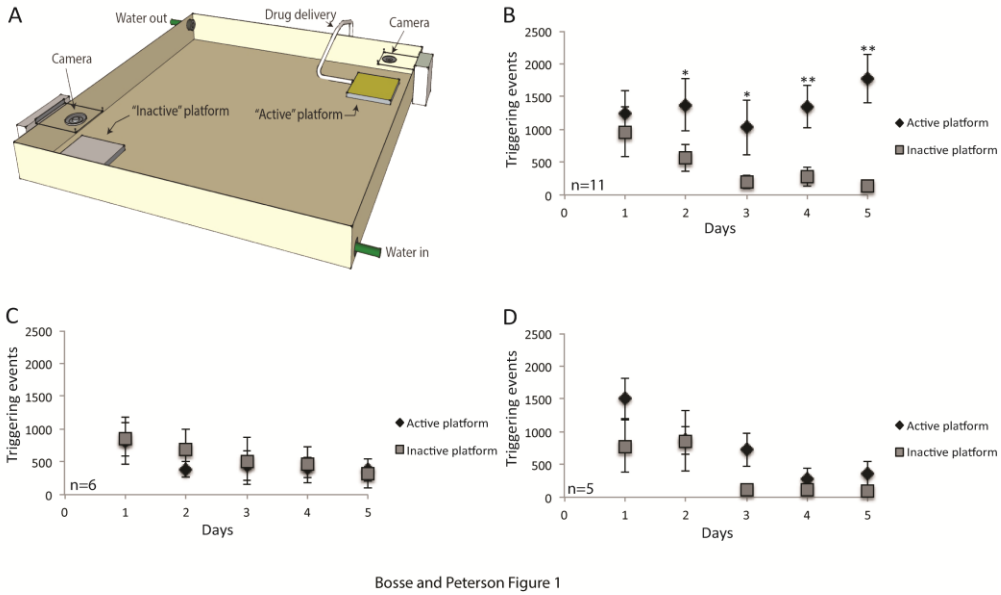
8. Figure legends

Figure 1. Opioid self-administration conditioning. A: Layout of the conditioning arena. B: Fish generated a specific increase in triggering events at the active platform (n=11) C: Fish trained with only water from the main system did not increase the number of triggering events (n=6). Treatment with naloxone (2.7μM) [11], for 60 minutes before each sessions abolished the increase in triggering events generated at the active platform (n=5). Error bars represent a 95% confidence coefficient. *p-value <0.01, ** p-value <4e-5.



Bosse and Peterson Figure 1

Figure 2. Hydrocodone-conditioned fish displayed evidence of self-administration. A: Non-conditioned animals generated fewer triggering events at the active platform with the double stacked platforms (n=8). B: There was no difference detected between the regular and the double stack platform with the conditioned fish (n>6). C: Hydrocodone-conditioned fish can be conditioned with a progressive ratio; animals significantly increased the number of triggering events at the active platforms with 10-15 events required to activate the pump, but not at 5 and 20 events. D: The doses of drug received by the animals under the different progressive

ratio conditions are lower than with a fixed ratio but similar across the different settings. Error bar represents confidence coefficient of 95%. *p-value< 1.99 E-19, ** p-value <1.7e-7, *** p-value <0.05, # p-value <0.01.

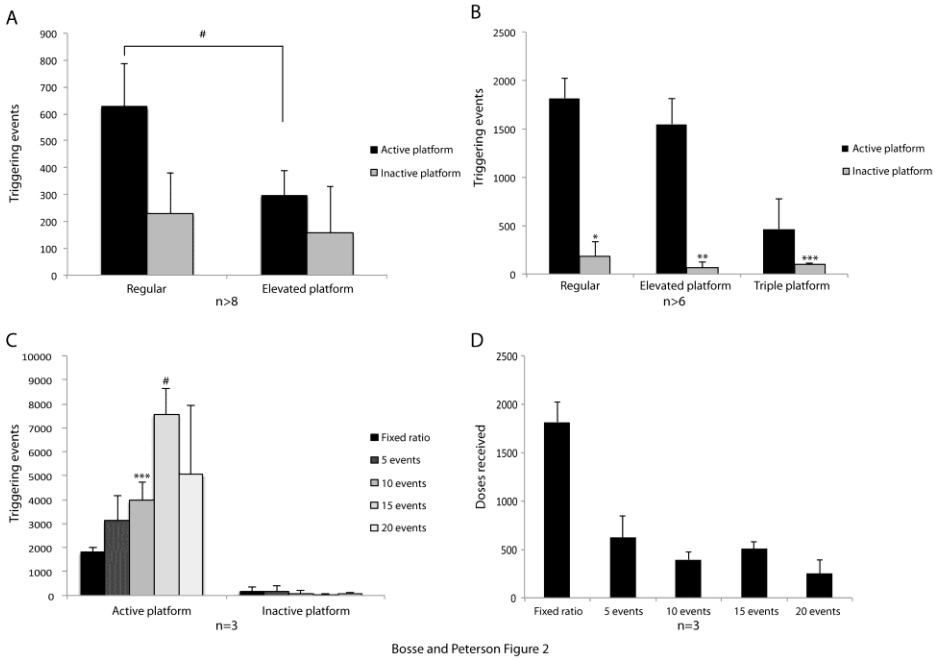


Figure 3. Hydrocodone-conditioned animals in withdrawal form a tighter group. The average distance between pairs of conditioned animals (cm) is shorter than naïve animals. Naïve group n=15, conditioned animals n=10. Error bar represents a 95% confidence coefficient. p-value<0.05.

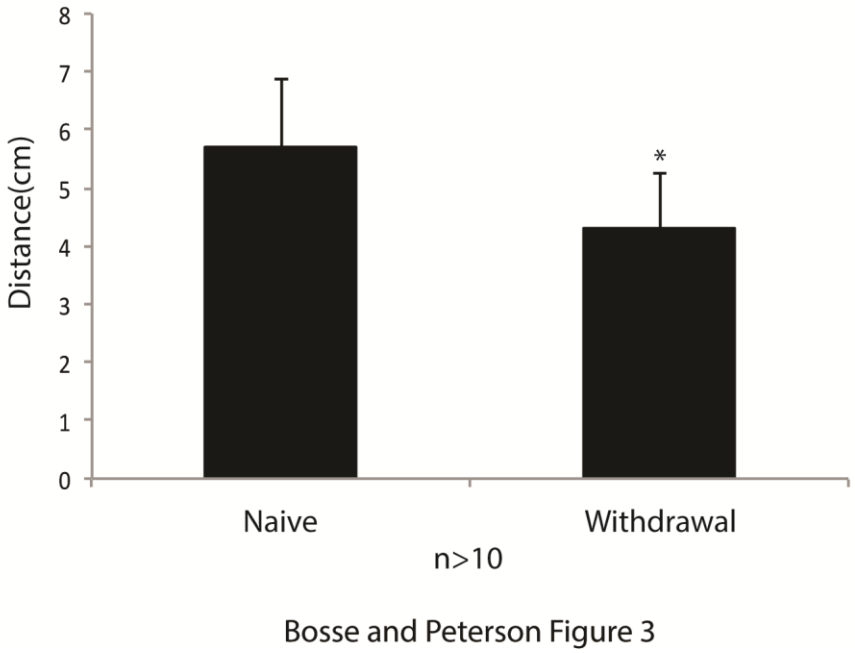
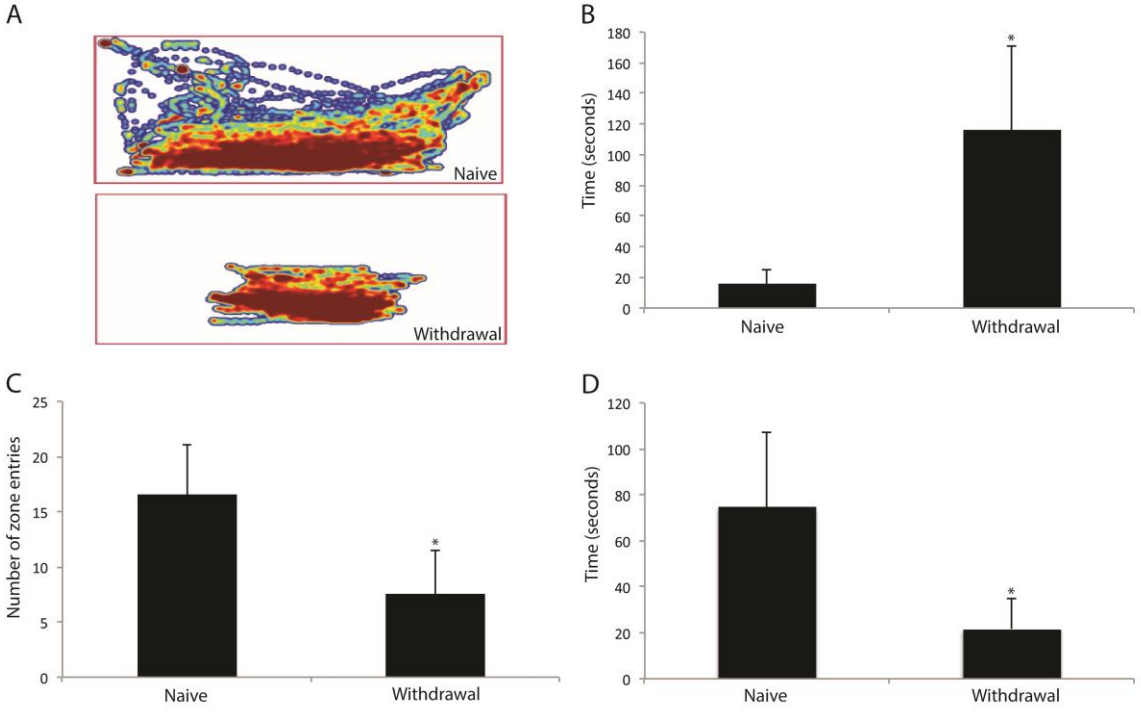
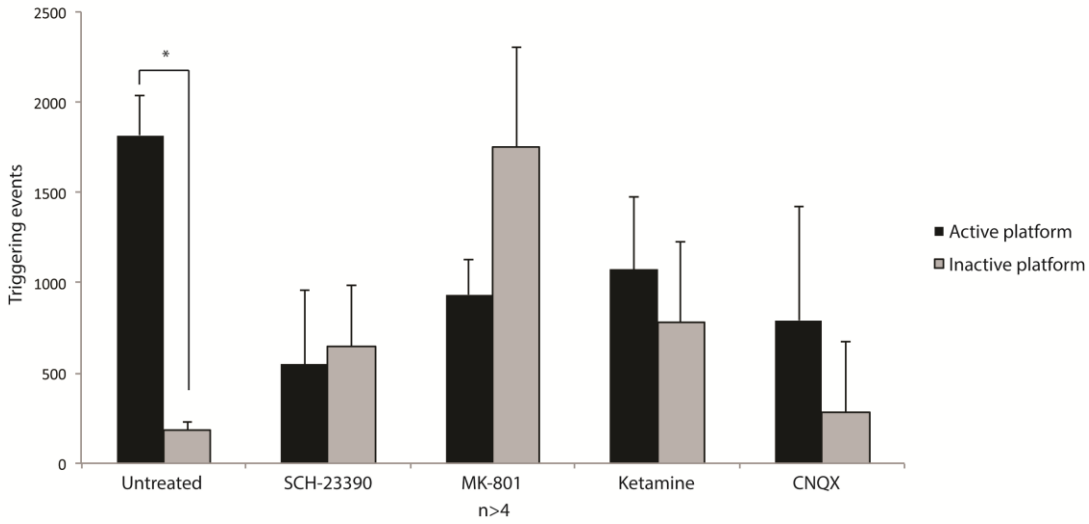


Figure 4. Hydrocodone-conditioned animals in withdrawal had a reduced exploration rate in the novel tank assay. A: Representative heat map of the location of a fish in the tank. B: Conditioned animals had a longer latency (seconds) to enter the outer area of the tank. C: The total number of visit in the outer area was smaller for these animals. D: Animals in withdrawal also spend less time (seconds) in the outer area of the tank than naïve animals. Naïve groups n=15, conditioned groups n=10. Error bar represents a 95 % confidence coefficient. *p-value<0.05.



Bosse and Peterson Figure 4

Figure 5. The dopamine and glutamate pathways are required for drug seeking in zebrafish. Treatment with the dopamine receptor antagonist SCH-23390 (1mM) [16], the NMDA receptor antagonist, MK-801 (20 μM) [68-70] and Ketamine (20mg/L) [71] or the AMPA receptor antagonist CNQX (10 μM) [72], abolished the preference for the active platform. Untreated n=41, SCH-23390 n=5, MK-801 n=4, ketamine=5, CNQX n=4. Error bar represent a 95% confidence coefficient. *p-value < 1.99E-19.



Bosse and Peterson Figure 5