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Conditioned place preference behavior in zebrafish

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Abstract

This protocol describes conditioned place preference (CPP) in zebrafish following a single exposure to a substance. In the CPP paradigm, animals show a preference for an environment that has previously been associated with a substance (drug), thus indicating the positive-reinforcing qualities of that substance. The test tank consists of two visually distinct compartments separated by a central alley. The protocol involves three steps: the determination of initial preference, one conditioning session and the determination of final preference. This procedure is carried out in ~2 d; other reported CPP protocols take up to 2 weeks. An increase in preference for the drug-associated compartment is observed after a single exposure. Establishment of this high-throughput protocol in zebrafish makes it possible to investigate the molecular and cellular basis of choice behavior, reward and associative learning. The protocol is also a tool for testing psychoactive compounds in the context of a vertebrate brain.

INTRODUCTION

Conditioned place preference (CPP), which likely engages mechanisms similar to those of classical conditioning, has been widely used to determine the reinforcing effect of naturally rewarding (e.g., food) or addictive substances (e.g., cocaine and amphetamine) in rodents¹. In the place conditioning paradigm, the primary motivational property of a natural reward or an addictive substance serves as the unconditioned stimulus. When it is paired with a previously neutral set of environmental stimuli (the conditioned stimulus), an approach behavior for the paired environment can be elicited. Despite the broad utilization of CPP in behavioral studies, its underlying molecular and cellular basis is not well understood.

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The zebrafish (*Danio rerio*), a prominent vertebrate model organism for developmental studies², is increasingly used for molecular genetic dissection of neural circuits and behavior^{3,4}. Zebrafish are small and can be maintained in large numbers (10 + fish per 2-liter tank). They produce many rapidly developing progeny (hundreds of embryos per pair mating) on a weekly basis. By 5 d after fertilization, transparent larval zebrafish are free living and show simple patterns of behavior, including feeding, swimming and escape response. Although many visually guided behaviors are readily observable in young larvae, olfactory- or learning-related behaviors only appear at later stages approaching adulthood. The recent development of the adult transparent *casper* line⁵ has made the adult brain potentially accessible to *in vivo* imaging or optogenetic manipulations. These facts, together with advancing molecular genetic technologies and a central nervous system similar to but less complex than that of mammals, make the zebrafish a promising animal model for elucidating neural circuits and behavior.

In 1998, Mattioli *et al.*⁶ presented a CPP paradigm in goldfish, which assessed the reinforcing effect of the H1 histamine antagonist chlorpheniramine. In that study, they used an aquarium that consisted of a black and a white chamber, with a half-white and half-black start area. Once the doors were removed, the start area became part of the two main chambers. As fish have an innately biased preference for black versus white environments^{4,7-9}, the authors used a biased experimental design (referring to a design in which animals show an innate initial preference for the conditioning environment). In 2001, Darland and Dowling¹⁰ reported the first CPP behavior in adult zebrafish elicited by the addictive substance cocaine. The testing apparatus they used was a 2-liter rectangular tank divided into two halves containing distinct visual cues (white versus dotted pattern). The tank had a perforated wall that allowed complete, although somewhat impeded, movement. After an initial assessment of baseline preferences, the fish were restricted to the least-preferred side for cocaine exposure. The authors used a non-balanced design (referring to a design in which animals are conditioned to one compartment with the substance but are not conditioned to the other compartment in the absence of the substance). CPP paradigms have since been used by other laboratories to determine the reinforcing effects of amphetamines, salvinorin A, alcohol and nicotine in zebrafish¹¹⁻¹⁴. In our own lab, we have obtained robust preferences for food¹⁵, the opiate drug morphine¹⁵ and ethanol¹⁶ using the unbiased and balanced single-exposure protocol described here. An overview of the design of published CPP procedures in zebrafish is provided in Table 1.

Strengths and limitations of the CPP assay in zebrafish

In zebrafish, simple visual cues are sufficient to elicit CPP in association with diverse types of rewarding stimuli. Often, only a single pairing session is needed to obtain significant place preference. Reinforcing agents can be easily delivered to the central nervous system through direct dissolution in the tank water. As the rewarding effect of the drug is measured in animals that are 'drug-free' during final preference testing and because of the short-term (2 d) nature of the experiment, a large number of compounds can be efficiently tested using this protocol; thus it is the ideal choice for initial screening of rewarding drugs. Automated tracking in the current protocol eliminates observer bias and makes it amenable to high-throughput applications.

Despite all these advantages, there are a few potential disadvantages related to the use of a CPP procedure. Finding the right dose can be time-consuming, as there might be no effect over a range of lower doses and a sudden maximal effect over a higher dose range¹⁷. The exact concentration of the reinforcing agents available to the central nervous system can be influenced by multiple factors, including metabolism and permeability across the blood-brain barrier. Therefore, techniques such as high-performance liquid chromatography–tandem mass spectrometry (LC/MS-MS) need to be used in order to precisely determine the substance level in the brain.

Considerable time must be spent in designing and testing the CPP tank to ensure that animals are not biased toward any compartment. Adequate control animals and control experiments are needed to rule out effects on locomotor activity, vision, learning and memory, and environmental factors including noise, room temperature and feeding times also have to be carefully controlled. Strategies to address these issues are included below.

Because the experimenter controls the animal's access to the drug, place conditioning studies are sometimes criticized as being less relevant as compared with procedures in which animals self-administer the drug¹⁸. However, the important contribution of Pavlovian learning processes to drug addiction is well recognized in contemporary approaches^{19,20}.

Potential uses

Although CPP in zebrafish has been described before, the protocols used so far involve lengthy and repeated conditioning sessions or manual tracking that introduces observer bias; these protocols are not amenable to a high-throughput experimental design^{12,13,21}. Although in many cases it may be necessary to use lengthy protocols to study drug withdrawal, the present protocol is ideal for conducting a rapid screen to identify mutants with altered drug response or to carry out drug screening to identify compounds with reinforcing or aversive properties.

Experimental design

An image of the CPP tank used in our laboratory is shown in Figure 1 and a flow chart depicting the CPP protocol is shown in Figure 2.

Bias and balance.—As learned preferences and aversions are superimposed on unlearned preferences in a biased design, which can be confounding because of potential anxiolytic effects of certain substances²², we use an unbiased apparatus, in which time spent by naive animals in each compartment approaches a normal distribution, to increase our chances of detecting conditioned preference or aversion. Apparatus bias is tested separately for each subject population (wild type versus mutant, different strains, etc.) by introducing individual animals into the apparatus and tracking the time each animal spends in each compartment. As individual animals may show preferences for one compartment over another, we discard animals that show excessive bias (>70% preference) and pair the drug with the initially non-preferred compartment. We counterbalance each group such that both the drug trial and the vehicle trial are received, and the order in which each type of trial is received (e.g., drug trial

first versus vehicle trial first) is randomized. Control animals are handled in exactly the same way as drug-treated animals, except that they receive the vehicle only.

Temporal parameters.—Place conditioning procedures involve several important temporal parameters²³. In our experiments, animals are exposed to the drug while being restricted to one compartment of the CPP tank; accordingly, the blood and brain concentrations of the drug are typically rising during the beginning of the trial. Depending on the pharmacokinetics of the drug being tested, it may be necessary to have a short delay between exposure to the drug and exposure to conditioned stimulus to allow time for drug absorption²⁴. Duration of exposure to the conditioned stimulus can affect the strength of CPP or conditioned place aversion²⁵. In the present protocol, we exposed animals to the conditioned stimulus in the presence of drug for 20 min and found that this was effective in inducing CPP. In our experiments, we usually insert a delay of ~16 h between the conditioning session and the session of determining the final preference. During this time, we do not provide food to the animals, as this leads to variable responses. Test duration for both initial and final preference is 5 min.

Cohort size.—A cohort size of eight is used because this is the maximal number of animals that can be simultaneously tracked using our four-camera setup. Each cohort includes both control and drug-treated animals; multiple cohorts can be examined in a given experiment to reach a desirable number of observations.

Zebrafish husbandry and handling.—Generally, 4- to 12-month-old zebrafish are used for behavioral experiments. The sex, age and body weight of each tested animal are recorded, so that the potential effect of these variables on behavior can be discerned. Handling stress is a major confounding factor in behavioral testing. The following precautions should be taken to minimize handling-evoked stress: (i) Care should be taken to move fish gently between home tanks and test tanks, as well as between home tanks and conditioning tanks. (ii) All fish should be handled the same way. (iii) The dividers used to restrict the fish to the start area should be such that they can be removed very smoothly from the top of the tank with minimal disturbance to the fish. (iv) The water level inside the tank should be about one-third of the total tank/divider height, so that there is minimal disturbance to the fish when the experimenter removes the dividers; additionally, the tank should be inside a brown box to prevent the test animals from seeing the experimenter.

Control behavioral tests.—Behavioral tests to assess locomotor activity¹⁵ and visual sensory acuity^{15,26} are important for interpreting the results of altered CPP responses on genetic or pharmacological perturbations. The procedural details for these behavioral tests are not provided in this protocol, but can be found in the references cited above.

Drug bioavailability assays.—The bioavailability of drugs to the central nervous system can be assayed, e.g., using colorimetric enzymatic assays (for ethanol)^{16,27} or LC/MS-MS (for morphine)^{15,28}, when it becomes necessary to determine whether a lack of CPP or altered CPP response is due to a lack of or altered bioavailability of the drug, respectively.

MATERIALS

REAGENTS

- Zebrafish; strain choice (i.e., the use of a specific wild-type or mutant strain) will depend on the design of the experiment. Zebrafish should be age- and size matched (see REAGENT SETUP) ! **CAUTION** All animal experiments require the approval of the Institutional Animal Care and Use Committee (IACUC) at the researcher's institution.
- Reverse osmosis water
- Live brine shrimp (Platinum-Grade Argemina Brine Shrimp, Argent Chemical Laboratories)
- Flake food (Tropical Flakes, Aquatic Eco-Systems)
- Instant ocean salts to maintain system water salinity (Aquatic Systems, cat. no. SS15–10)
- Sodium bicarbonate to maintain system water pH (Aquatic Systems, cat. no. SC12)
- Ethanol (Rossville Gold Shield Ethyl Alcohol, Gold Shield Chemical, cat. no. 94545). Prepare fresh 0.5%, 1% and 1.5% (vol/vol) ethanol solutions in system water and avoid evaporation ! **CAUTION** It is a flammable liquid and vapor. It may cause central nervous system depression, liver, kidney and heart damage. Use gloves and a lab coat, use in well-ventilated area and wash thoroughly after handling.
- • Morphine sulfate (a controlled substance, available from the National Institute on Drug Abuse or Sigma-Aldrich, cat. no. M8777). Prepare fresh morphine sulfate solution in system water to desired concentration (1.5–15 μ M) ! **CAUTION** It may be fatal if swallowed, harmful if inhaled or absorbed through skin, an allergen or a narcotic. Use respirator and local exhaust ventilation when handling the powder. Use gloves, lab coat and safety glasses and develop techniques that limit exposure. Store in tightly closed container in a cool, dry and ventilated area, and protect from direct sunlight. Storage location must comply with all Drug Enforcement Agency (DEA) regulations.
- Tricaine (Sigma-Aldrich, cat. no. MS222)

EQUIPMENT

- Housing tanks (AHAB system, Aquatic Habitats, 1.5-liter tank (see EQUIPMENT SETUP))
- Fishnets (nylon, Aquatic Habitats)
- CPP tank (Custom-made glass tank, dimensions are 52 cm (length) $\times$ 16.6 cm (width) $\times$ 22.9 cm (height). The tank is divided into two halves (white and dotted) by a 7.6-cm-wide gray central alley or start area. The dotted half consists

of 16 blue dots of 2-cm diameter in a fixed pattern (see EQUIPMENT SETUP)

▲ **CRITICAL** All parts of the CPP tank, including edges and dots, should provide enough contrast so that the animal can be tracked. Interior must be waterproof so that nothing comes off and hinders the movement of the animal or interferes with tracking. Water should not be so deep that the animal goes out of focus and cannot be tracked.

- Opaque dividers for each CPP tank
- Brown box for each CPP tank
- Video camera (Sony High-definition Handycam Camcorder, Sony)
- Video recorders (Noldus)
- Automated tracking software. (We have used the Noldus Ethovision XT Version 5 (Noldus); other similar tracking software can also be used.)
- Computer (4 GHz processor, 2 GB internal memory, Hard disk with 1 GB free space, 64 MB video memory, 1024 × 768 graphics card, >24-bit TFT monitor)

REAGENT SETUP

Zebrafish husbandry—Adult zebrafish are maintained and bred following standard procedures²⁹. Zebrafish aged 3 months and older are considered as adults. They are fed twice a day with a mixture of live brine shrimp (Platinum-Grade Artemia Brine Shrimp, Argent Chemical Laboratories) and flake food (Tropical Flakes, Aquatic Eco-Systems). They are maintained on a 14:10-h light/dark cycle at 28 °C. Illumination provided by standard ceiling lights is similar to that in the fish housing room and is sufficient for the animal to distinguish the pattern and to be tracked. All experiments are carried out in accordance with National Institutes of Health (NIH) guidelines regarding the care and use of animals for experimental procedures.

System water—Dissolve 5 g of instant ocean salts and 3 g sodium bicarbonate in 20 liters of reverse-osmosis water. A closed system that recirculates the water after purification is usually used. Water out of the facility's water system is dripped into clean tanks for the experiments.

EQUIPMENT SETUP

Behavior room setup—The behavior room is an isolated soundproof room, with 14:10-h day/night cycle and 28 °C temperature. Illumination provided by ceiling lights is similar to that in the fish housing room and is sufficient for the animal to distinguish the pattern and to be tracked. Additional lighting closer to the CPP tank is avoided, as it may stress the animal and produce reflections in the water, which will interfere with tracking. White noise is provided by a fan that remains on for the duration of the experiment. Experimental tables are covered with foam boards to absorb vibrations. Cameras are mounted on overhead racks above the test tanks and video recorders (Noldus) are used to record behavior simultaneously from four cameras.

Transport and housing equipment—Zebrafish are housed in an AHAB system (Aquatic Habitats) and separated into individual tanks a few days before the experiment. Zebrafish are transported to and from the behavior room in their 2-liter housing tanks.

CPP tank—The CPP tank is positioned below the recording camera such that the animal can be tracked at all points within the tank. It is filled with system water and kept in a brown box so that the animal cannot see the experimenter. Opaque dividers are positioned and individual animals are gently netted into the central alley or start area. Dividers are withdrawn 2 min after the introduction of animals into the central alley; this is followed by 5 min of recording. Separate CPP tanks and fishnets are used for preference testing and for conditioning to ensure no cross-contamination. Tanks, dividers and fishnets are rinsed with deionized water before and after each use.

Data collection setup—Videos recorded by the camera are analyzed with Ethovision software. Tracks generated by the software allow visualization of the movements of the animal. Moreover, the software calculates multiple parameters, including the distance moved and swimming speed of the animal, as well as the number of times the animal enters or leaves a particular compartment.

Animal disposal after the experiments—Animals are used for one round of experiments and are subsequently euthanized by an overdose of anesthetic (0.05 mg ml⁻¹ tricaine).

PROCEDURE

▲ **CRITICAL** Animals must be housed in individual tanks and assigned numbers 1–2 d before the experiment.

Setup (Day 1)

1. Mount four cameras on the shelf above the behavioral testing table and connect them to the power and video recorder in a switch-on mode.
2. Place a CPP test tank below each camera inside a brown box and check that all parts of the tank are viewable on the camera screen.

▲ **CRITICAL STEP** Lighting should be sufficient to allow animals to visually discriminate between the two compartments. Intense illumination can affect tracking and produce aversion behavior. Verify that animals show similar preferences for both compartments before starting experiments. Expose several non-experimental animals to the tank before the real experiment; if they show preference/aversion, then re-evaluate the lighting, tank features and behavior room setup.

? TROUBLESHOOTING

3. Ensure that the output of all four cameras can be seen on the computer screen through the video recorder.

? TROUBLESHOOTING

4. Fill each tank with 6.6 liters of system water.
5. Place opaque dividers on either side of the central alley in all four tanks.
6. Transport individual tanks of animals to be tested from the housing room to the behavior room.
7. Leave tanks undisturbed in the behavior room for 30 min to habituate.

Assessment of initial preference (Day 1)

1. Gently, but quickly, net individual animals from the home tank into the central alley or start area of the CPP test tank.

▲ **CRITICAL STEP** Ensure that all animals are handled in a consistent manner and that each animal is placed in the central compartment in the same manner. Avoid prolonged handling, as this can lead to hypoxia and stress³⁰.
2. Allow each individual animal to explore the area for 2 min.
3. Remove both dividers slowly and simultaneously, taking care not to startle the animal.
4. Record the animal's behavior for 5 min and return to its individual home tank.

▲ **CRITICAL STEP** Be prepared to begin recording as soon as the dividers are removed.

? TROUBLESHOOTING

5. Determine the initial preference for white or the dotted environment of the CPP tank with Ethovision software, according to the software manual. An overview is provided in Box 1.

? TROUBLESHOOTING

Conditioning (Day 1)

1. Of the animals that show an initial preference for the white compartment in Step 12, assign half to the vehicle-treated group and half to the drug-treated group. Proceed similarly for the animals that prefer the dotted compartment.
2. Outline the sequence in which animals will be conditioned so that an equal number of drug-treated animals receive drug first versus vehicle first. In our experience, CPP is stronger in animals that receive the drug after receiving the vehicle. All drug-treated animals receive the drug when they are placed in their non-preferred compartment and vehicle in their preferred compartment.
3. Set up four CPP tanks with dividers so that two contain system water alone and two contain system water containing appropriate concentration of the drug (i.e., unconditioned stimulus).

! CAUTION Use appropriate personal protective equipment, ventilation and handling precautions, as listed in the material safety data sheet (MSDS) of the drug being tested.

4. Net the animal into the least preferred compartment containing the drug and let the animal swim freely for 20 min.

▲ CRITICAL STEP Animals should be kept in their home tank, not too far away from the conditioning tank, to minimize the time needed for transfer from the home tank to the conditioning tank. This is important to minimize the effect of the stress of hypoxia due to prolonged netting. Experimenters must avoid unnecessary movements and noise; physical barriers must be used to block views outside the tank and white noise must be provided to mask sounds.

? TROUBLESHOOTING

5. At the end of 20 min, put the animal in a tank containing system water for 1 min to ensure that the drug is washed off externally.
6. Net the animal into the preferred compartment containing only system water for 20 min.

Note: Randomize the order of Steps 16 and 18. Expose vehicle-treated animals to system water in both preferred and non-preferred compartments. It is possible to condition two animals per tank and a maximum of eight animals simultaneously.

? TROUBLESHOOTING

7. Gently net the animals out, pass them through three tanks containing system water, put them back to individual housing tanks and return them to the fish system.

! CAUTION Dispose of tank water containing drugs in accordance with DEA guidelines.

8. Clean tanks, nets and dividers thoroughly, and then let dry.

Assessment of preference following conditioning (Day 2)

1. After 16 h, repeat Steps 1–12 to determine the final preference for white or the dotted environment of the CPP tank. As the final preference has to be assessed when the drug is cleared from the nervous system, 16 h is the minimal intervening period that we have used before determining final preference.

? TROUBLESHOOTING

2. Clean tanks, nets and dividers thoroughly, and then let dry.

Data analysis

1. Quantify the following parameters using a tracking software such as Ethovision: time spent in each zone, velocity in each zone and number of entries made into each zone. The percentage of total time spent on white and dotted sides for each

animal before and after conditioning is calculated using the total duration of recording (5 min or 300 s).

2. For each animal, note the side in which it received the drug and calculate the change in preference for the conditioned compartment.

? TROUBLESHOOTING

3. Express the result as change in percentage preference (percent of time in the drug-paired compartment after conditioning – percent of time in the same compartment before conditioning).

? TROUBLESHOOTING

4. Use ANOVA followed by Dunett's *post hoc* test or Student's *t*-test (available in software packages such as the GraphPad Prism) to compare vehicle- and drug-treated groups.
5. If necessary, carry out additional control experiments (locomotor activity assay, visual acuity assay and so on)¹⁵ to rule out any effect of the drug on locomotor activity or vision that can affect CPP results.
6. If necessary, use appropriate assays (colorimetric, HPLC or mass spectrometry)^{15,16,27,31} to estimate the amount of drug in the brain, as it is difficult to predict the bioavailability of the drug after administration in the tank water.
7. Euthanize animals by overdose with tricaine, in accordance with IACUC regulations.
8. Steps 1–29 are described for a cohort of eight animals. Repeat Steps 1–28 for additional cohorts as necessary, e.g., to determine the effects of drugs at different concentrations.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

● TIMING

Steps 1–7, Setup: for a cohort ($n = 8$) of experimental animals: 75 min

Steps 8–11, Initial preference recording: 16 min per cohort

Step 12, Video analysis to determine initial preference: 80 min per cohort

Steps 13–20, Conditioning: 80 min per cohort

Steps 21 and 22, Final preference recording and clean up: 60 min per cohort

Steps 23–30, Video analysis to determine final preference: 80 min per cohort

ANTICIPATED RESULTS

An increase in the time spent in the drug-paired compartment after conditioning suggests that the substance has a positive reinforcing effect (drug-induced place preference). Shown in Figure 3 are data we obtained for zebrafish using two reinforcing drugs, morphine and ethanol. In both cases, on the test day, animals conditioned with drug show a significant ($P < 0.005$ for morphine sulfate; $P < 0.05$ for ethanol) increase in change of preference for the drug-associated compartment, as compared with animals previously treated with vehicle only. Average swim velocity during final preference testing was similar in vehicle- and drug-treated groups. Thus, interpretation of the preference data is not affected by variation in locomotor activity.

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Box 1 |**DETERMINING INITIAL PREFERENCE WITH ETHOVISION SOFTWARE**

1. Specify the entire CPP tank as one arena.
2. Divide the arena into three zones (white, dotted and middle alley).
3. Calibrate the arena and specify detection settings (maximum and minimum pixels and contrast settings) to track a dark object (fish) on a light background.
4. In the analysis profile, specify duration in each zone and velocity as output parameters.
5. Analyze initial and final preference and conditioning recordings in the same fashion. Velocity measurements from the final minutes of conditioning will give information about the possible effect of the drug on locomotor activity.

▲CRITICAL STEP An animal is considered to be in a compartment when two-thirds of the body length of the animal has crossed the boundary.

Figure 1 |. CPP apparatus. **(a,b)** Image showing the CPP tank **(a)** and a graphical representation of the tank **(b)** with the position of the dividers and dimensions indicated, adapted from a previously published paper¹⁵.

Figure 2 |.

Flow diagram showing the CPP procedure and time required for each step.

Figure 3 |.

Conditioned place preference behavior in adult zebrafish.

(a) Place preference behavior of naive adult zebrafish treated with morphine sulfate (MS) at different concentrations. MS was dissolved in system water and appropriate volumes of this stock solution were added to the tank water before conditioning. Mean percent preferences $\pm$ s.e.m. values are shown. <p> C = control ($n = 25$), MS 1.5 μ M ($n = 9$), MS 3 μ M ($n = 23$), MS 7.5 μ M ($n = 13$) and MS 15 μ M ($n = 13$). Dunett's T3 test, ** $P < 0.005$.

(b) Change in preference for the conditioned compartment of control ($n = 8$) and drug-treated animals treated with 0.5% ($n = 11$), 1% ($n = 11$) and 1.5% ($n = 8$) (vol/vol) ethanol. Change in preference = (percent time in the ethanol-paired compartment after conditioning) – (percent time in the same compartment before conditioning). Error bars are means $\pm$ s.e.m. Student's t -test, $P = 0.0409$ (*) for 1.5% (vol/vol) ethanol compared with 0% ethanol. Panel a is adapted from a previously published paper¹⁵.

CPP behavior in zebrafish.

TABLE 1 |

Paper and aim of study	Apparatus	Animals and the design of test	Route of substance administration	Number of conditioning trials	Secondary behavioral assays	Outcome
Darland and Dowling ¹⁰ Assay for cocaine's reinforcing effect and screen for zebrafish mutants	Two chambers with half-white and half-dotted patterns, separated by a perforated wall	Adults (8 to 12 months old), unspecified strain Unbiased ^a Non-balanced ^b	In the tank water	One	Visual threshold measurement; T-maze test	Increase of preference for the cocaine-paired compartment by ~15% on single pairing with 10 mg per liter cocaine in the water
Ninkovic <i>et al.</i> ¹¹ The reinforcing effect of amphetamine and the role of the acetylcholinesterase (<i>AChE</i>) gene	Two chambers with a dark half colored brown and a light half colored white, with two black circles at the bottom of the tank	Female adults (3 to 6 months old) of AB strain Unbiased, balanced	Intraperitoneal injection	Three	T-maze test	Increase of preference for the amphetamine- paired compartment by ~27% on three pairings with 40 µg g ⁻¹ of D-amphetamine
Lau and Guo ¹⁵ The reinforcing effect of food, morphine and the role of the <i>tol/fez12</i> gene	Three chambers: a middle alley, a white and a dotted compartment	Adult zebrafish of AB/EK background, unspecified age Unbiased, balanced	In the tank water	One	Locomotor activity test; visual acuity test	Increase of preference for the morphine- paired compartment by ~30% on a single pairing with 3 µM morphine sulfate in the water
Braida <i>et al.</i> ¹² The reinforcing effect of the hallucinogen salvinorin A	Similar to ref. ¹⁰	Adult zebrafish of unspecified genetic background or age Unbiased, balanced	Intramuscular injection into the caudal musculature	One	Swimming activity test	Increase of preference for the salvinorin A- paired compartment by ~33% on a single pairing with 0.5 µg kg ⁻¹ salvinorin A
Kily <i>et al.</i> ¹³ The reinforcing effects of nicotine and ethanol and underlying gene expression changes	Two chambers with black spots on one side and black and white stripes on the other	Approximately 4-month-old adults of Tuebingen strain Unbiased, balanced Manual tracking	In the tank water	Single acute exposure 1, 3 or 4 weeks of daily conditioning	None	Increase of preference for the drug-paired compartment by ~58%. However, water-pairing also increased preference

^aUnbiased design refers to a design of the conditioning apparatus such that naive animals do not show a significant preference for one of the conditioning compartments on initial exposure.

^b A balanced design refers to a design such that animals are conditioned to one conditioning compartment with the substance and the other compartment without the substance.

TABLE 2 |

Troubleshooting table.

step	problem	possible reason	solution
2	One or more CPP tanks or part of tank not visible on camera	Camera is not connected to power supply; cap is still on lens; camera is out of focus or not properly oriented	Check the power supply; check that cap is off; reorient tank and camera
3	One or more CPP tanks not visible on computer screen	Camera is not connected to video recorder or computer cannot detect camera	Check connections from camera to video recorder. Restart computer
11, 16, 18	Animal jumps out of the aquarium or freezes in the aquarium	Background noise; excessive handling; hypersensitivity to stress in certain animals (usually low percentage of ani-mals, < ~1% if handled appropriately)	Use white noise (either from a fan or played by a radio); use physical barriers (e.g., a large box) to contain the behavioral testing tank; discard the few animals that do freeze or jump
11	Animal shows a strong initial preference for either the white or the dotted compartment	Behavioral room setup causing biased preference; intrinsic preference of the animal (low percentage, < ~1%)	Keep the behavioral room setup identical from session to session; discard animals that show strong intrinsic preference (>70% preference)
11, 16, 18	Animal shows excessive thigmotaxis	Stress; reflective nature of the tank	Minimize extrinsic stress-inducing factors such as background noise, excessive and rough handling; use non-reflective material to construct the tank
12, 21	Objects in addition to the animal are tracked	Debris in the tank; lighting conditions and contrast setup are not optimized; dots in the pattern are tracked	Keep a dropper handy to remove debris from the tank; provide optimum lighting in the behavioral room; specify maximum and minimum pixel size to exclude dots
24, 25	No CPP obtained	Inconsistent procedures for assessing the initial and final preference; crosscontamination; excessive stress shown by the animals; the nature of the drug and concentration (e.g., the drug may affect sensory, motor or learning capabilities) Genetic background; differences in size of animals	Carry out initial and final preference testing at the same time of day in all animals; use separate tanks and nets for vehicle- and drug-treated groups; carry out additional conditioning trials; test a wide concentration range; control for potential confounding effects of the drug using additional behavioral tests to assess sensory, motor and learning capabilities; use age- and size-matched animals