Chapter 14

Intraperitoneal Injection as a Method of Psychotropic Drug Delivery in Adult Zebrafish

Adam Stewart, Jonathan M. Cachat, Christopher Suciu, Peter C. Hart, Siddharth Gaikwad, Eli Utterback, John DiLeo, and Allan V. Kalueff

Abstract

Zebrafish behavioral phenotypes are often evaluated in response to pharmacological modulation by various psychotropic drugs. An important step in this process is the method of drug administration. While the most popular drug administration technique in zebrafish research is by immersion, systemic intraperitoneal injection is another effective alternative. This method is useful for drugs that are difficult to dissolve in water, or which require a better control over the amount of drug delivered to an individual animal. Here we outline a simple protocol for the intraperitoneal injection of drugs in adult zebrafish.

Key words: Zebrafish, intraperitoneal injection, drug exposure, drug administration method, anxiety.

1. Introduction

Zebrafish exhibit robust behavioral phenotypes, which can be examined in simple and reliable assays for drug screening (1–4). Our group has made extensive use of these paradigms, often in conjunction with video-aided analysis, to correlate the behavioral and endocrine indices of anxiety-like behavior evoked by psychotropic drug exposure (1, 5–7) (**Fig. 14.1**).

One of the most important steps in using pharmacological agents to study animal behavior is the method of drug exposure (8–10). Indeed, a proper uniform administration of the chosen drug is crucial to the outcome of the study. In zebrafish, drug exposure is usually performed via immersion in a drug-containing

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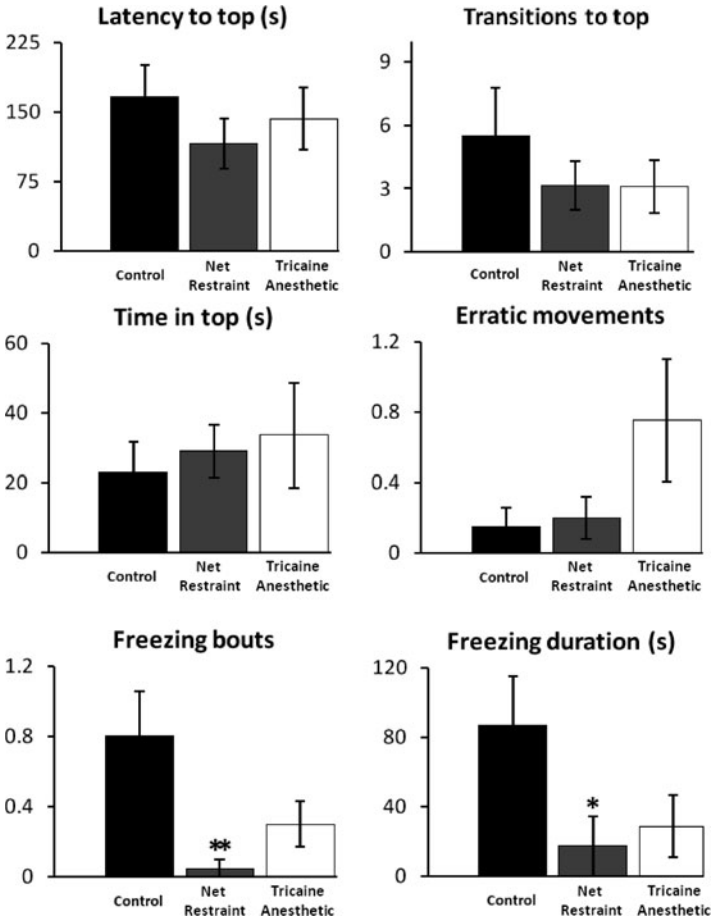

Fig. 14.1. Behavioral data comparing controls (water immersion only) and intraperitoneal (i.p.) injection (via net immobilization) with i.p. injection using Tricaine anesthesia ($n = 13$–14). *$p<0.05$, **$p<0.01$, vs. control.

solution for a specified duration (6, 7, 11, 12). However, certain drugs do not readily dissolve in water and can therefore be arduous to administer (13).

An alternative approach is to administer the drug via systemic intraperitoneal (i.p.) injection. This is a procedure that, when performed correctly, can be a viable and effective technique for drug delivery. Intraperitoneal administration has been long used for big fish species, such as rainbow trout, Atlantic halibut, tilapia, and crucian carp (14–17), as well as in smaller fish, such as goldfish (18–22), minnows (23–25), and guppies (26–28). Systemic i.p. injections have also been used in several published zebrafish studies (29–33). For example, zebrafish have been used to model the effects of environmental toxins implicated in the pathogenesis of Parkinson's disease, with various doses being administered intraperitoneally (29). Furthermore, i.p. injection has also been

used as an effective administration route to study the reinforcing properties of drugs of abuse in zebrafish (33).

Somewhat more stressful for animals (than immersion), systemic i.p. injections are usually needed when the immersion method of drug delivery is infeasible. While some agents, such as diazepam or 1,3,5-Trinitroperhydro-1,3,5-triazine (RDX), are insoluble in water, but can be dissolved in an acceptable alternative solvent. However, the solvent must be conducive to the health of the fish, as well as have no known reactivity with zebrafish behavior. Again, an additional "solvent" control group must be added to the experimental design. In cases where these two criteria are not met, i.p. injection becomes a viable alternative (34, 35). Whereas i.p. administration is a more precise method than immersion, it is also often the preferred method of administering expensive or rare drugs (36), as well as drugs affecting sideline receptors (*see* (37) for details about the role of administration precision and receptor interaction). Likewise, administration of small volumes of oily substances (e.g., some steroid hormones or similar hydrophobic agents) via i.p. injection may be the preferred method of drug delivery. Other situations where i.p. injection may be preferred involve drugs that can irritate gills (38) or agents that are highly unstable in water (39). Here, we outline a protocol utilizing i.p. injection for drug delivery in *adult* zebrafish for their subsequent testing of a variety of behavioral assays.

2. Potential Limitations

There are also several limitations of using i.p. injection. For instance, it is often necessary to use anesthetics when carrying out the procedure, which may have undesirable effects on the examined behavior and physiology (40). Likewise, age, sex, strain, previous drug exposure, and even time of day of exposure can have important impacts on anesthetic drug responses in various animals, including rodents and fish (41). Another limitation of this method is that it involves considerably more skill (relative to immersion), as care and precision are needed to avoid puncturing the animal's organs, as well as to minimize behavioral anomalies induced by pain (40, 42) (*see* Note 1). Likewise, the procedure requires more time than the immersion method. Furthermore, the i.p. injections can only be performed using small volumes of the drugs (e.g., 5 or, less preferably, 10 μl), and hence, this method may not be appropriate for applying high doses of certain drugs (which would require higher injection volumes). Finally, while the immersion method can be used for chronic drug

administration, *repeated* i.p. injections cannot be performed in small animals such as zebrafish.

3. Methods and Materials

3.1. Animals and Housing

Adult zebrafish (e.g., 6–8 month-old; ≈50:50 male:female ratio) can be obtained from a local commercial distributor, and housed in groups of approximately 20–30 fish per 40-L tank. Tanks should be filled with filtered water, with room and water temperatures maintained at ≈25°C and water pH at 7.0–8.0. Illumination can be provided by ceiling-mounted fluorescent light tubes on a 12–12 or 10–14 h cycle, consistent with the zebrafish standard of care (43).

3.2. Equipment

1. Small 5–10 μL Hamilton syringe (e.g., Hamilton Company, Reno, NV, USA).
2. Net (for immobilizing zebrafish) (e.g., Fisher Scientific, Pittsburgh, PA, USA).
3. Treatment beaker for Tricaine solution.

4. Procedure

4.1. Acclimation and Pre-treatment for Intraperitoneal Injection

1. Transport the animals from their holding room to the experimental room for acclimation 1 h prior to testing. After acclimation, the fish will be individually treated with the chosen drug via i.p. injection. Importantly, this must be organized in intervals of ~10 min to correspond to the time allotted per each 6-min trial, with a ~4 min left-over for preparation for the next one.
2. To administer the drug, anesthetize the fish by immersion in Tricaine (100–120 mg/L; Sigma-Aldrich, St. Louis, MO) for ~30–60 s, until only the gills are moving. Slightly tap on the beaker to see if the fish is still capable of movement to ensure that it is fully anesthetized. Do not leave the fish in the Tricaine longer than necessary, as this is a time-sensitive procedure, and death can result if exposure is prolonged by as little as an additional ~20–30 s.
3. Remove the fish from the Tricaine and lay it down on a sterile surface, turning the animal so its ventral side is facing upwards.

4. Quickly inject 5–10 μL of the drug solution into the peritoneal cavity using a small Hamilton syringe. Note that control fish must be treated by injecting an equal amount of vehicle (e.g., saline or water) solution. The site of injection is in the midline cranial to the base of the pelvic fin. For a general reference, the place of injection should lie about 1 "fin-length" ahead of the pelvic fin base. For details on troubleshooting, refer to Note 1.
5. Move the fish to a 3–4 L holding beaker filled with ~3 L water for the desired pre-treatment time (which, like the dosage, should be determined by a prior literature search or calculation from previous human or rodent studies).

4.2. Behavioral Testing

Fill the apparatus with the specified amount of room-temperature filtered water. After the necessary pre-treatment time has elapsed, begin video recording, and proceed to carefully move the fish to the apparatus. For details on troubleshooting, refer to Notes 2–3.

4.3. Endocrine Analysis

Once all of the behavioral data has been collected and analyzed, a comparison of the cortisol levels between the control and experimental groups can be performed (*see* Chapter 11, this volume). This will allow for the behavioral phenotypes to be paralleled with their respective physiological measurements of anxiety.

4.4. Data Analysis

If a control and single experimental groups were used, utilize the Mann-Whitney *U*-test for comparing these two groups (Student's *t*-test may be used for normally distributed data). If more than one drug dosage was applied, use an Analysis of Variance (ANOVA), followed by an appropriate post-hoc test, such as Tukey, Dunn, Newman-Keuls, or Dunnet tests.

5. Notes

1. *Death results from the procedure.* While general care is needed throughout the procedure, it is most vital to avoid piercing the animal's vital organs during injection. The needles should be only long enough to penetrate the abdominal wall, otherwise one can easily inject into the abdominal organs causing injury. However, an alternate factor to consider is the duration spent in the Tricaine during anaesthetization. Leaving the fish in the solution for too long can be fatal. During the exposure, check for subtle gill movement to rule this out.
2. *Observed anxiety levels are unusually high.* Careful handling of the fish during injection is crucial. If the anesthesia is not

administered properly, or the injection is done roughly, pain and a heightened state of anxiety can result. This may last well into the trial, thereby affecting the observed behavior as well as cortisol levels. Drug and humor leakage in injected fish is not uncommon, and may strongly alter results due to unpredictable dose levels (44).

3. *Abnormally low levels of locomotion*. If the injection is performed too roughly, lasting pain can result and continue into the trial. This can have confounding effects on the data, especially since one of the notable phenotypes of pain in zebrafish is lethargy (exhibited by freezing and decreased locomotion). Exclude the fish from subsequent trials and discard the data, allow fish 7–10 days to heal further.

6. Anticipated Results

Our group has obtained good results with the method of i.p. injection described here. When performed correctly, behavioral and endocrine results are generally similar to those obtained using the immersion method (**Figs. 14.1 and 14.2**). We have recently conducted a pilot study to determine if adding a

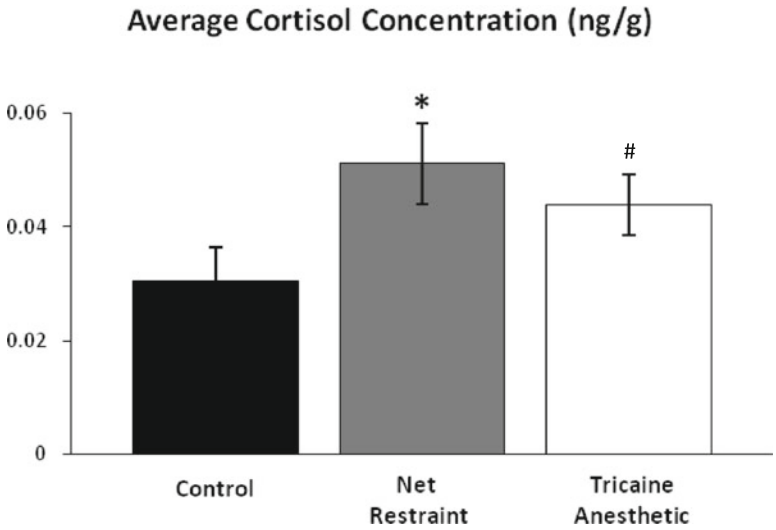

Fig. 14.2. Cortisol levels among controls (water immersion only) vs. intraperitoneal (i.p.) injection via net immobilization or using Tricaine anesthesia ($n = 13$–14). Fish receiving i.p. injection after net immobilization expressed elevated levels of cortisol vs. controls ($p<0.05$). Fish receiving i.p. injection after Tricaine anesthesia did not exhibit significant elevations in cortisol levels, compared to control fish (*$p<0.05$, #$p=0.05$–0.1 (trend) vs. control).

substance via i.p. injection affects behavior or cortisol levels in zebrafish. Zebrafish immobilization was achieved using two different methods. One group was trapped via net during injection, while another group was anesthetized by Tricaine for drug administration. A third (control) group remained immersed in water and did not receive i.p. injection. Overall, fish receiving i.p. injection while anesthetized by Tricaine did not show significant alterations in behavior in the 6-min novel tank test (**Fig. 14.1**), also displaying unaltered cortisol levels relative to controls (**Fig. 14.2**). However, fish immobilized via net for i.p. injection did demonstrate significant increases in cortisol vs. controls. Thus, Tricaine immobilization may be a better option for i.p. injections to avoid the confounding influences of net stress. In line with this, we have utilized this method in experiments investigating the effects of neuromodulating drugs, such as lysergic acid diethylamide (LSD). As can be seen in **Fig. 14.3**, the i.p. injection produces the results similar to those observed with the immersion method (6).

While we used i.p. injections for drug administration, other groups utilize this technique for other purposes in zebrafish, such as the injection of infectious agents to study innate immunity and bacterial pathogenesis (45, 46). As the use of biomarkers is becoming increasingly prevalent in zebrafish research, various labeling compounds can also be injected intraperitoneally, useful for the tracking of small animals and for revealing internal morphology (47–49).

While i.p. injection is not the only method of injection-based systemic drug delivery, some methods routinely used in other animals can be problematic in fish. For instance, intravenous (i.v.) injection can be difficult due to the small vessel diameter of zebrafish (50). More practical methods include intramuscular (i.m.) injections, which already were used in zebrafish studies for compounds such as salvinorin A (51), methionine enkephalin (52), the neurotoxin MPTP (53), the prostaglandin PGE2 (54), and the fluorescent tracer rhodamine dextran (55). However, this method is often not ideal as the skin seals poorly over the injection site, and large amounts of the injected substance can easily leak out (56). Likewise, intracerebral (i.c.b.) drug administration has also been applied to fish for a variety of compounds (57), but its application may be less feasible due to the animal's small size and the need for specific equipment. Subcutaneous (s.c.) injection, representing another standard practice in research involving fish, is more commonly used as an identifying marker of the animal (58, 59), but can also be applied to deliver psychotropic drug in zebrafish. Nevertheless, for most zebrafish research purposes involving treatment with pharmacological agents, i.p. injection appears to be a viable alternative to immersion.

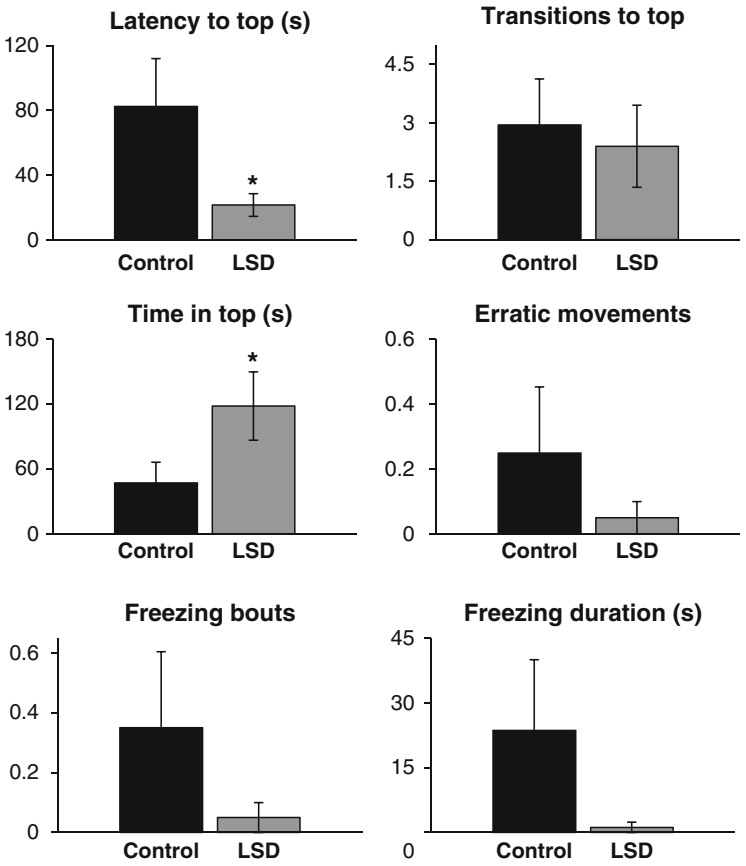

Fig. 14.3. Behavioral effects of lysergic acid diethylamide (LSD), administered to zebrafish via intraperitoneal (i.p.) injection. Control group was injected with 10 μL/fish saline solution ($n = 10$), while LSD-injected fish were injected with 10 μL of a 250 μg/L stock concentration of LSD ($n = 10$). Fish then spent 20 min in a 1 L holding beaker prior to the 6-min novel tank test. Similar to our data (6) obtained from the immersion method, LSD-injected fish had significantly lower latency to the top, more time spent in top, and tended to spend less time frozen (* $p<0.05$ vs. control).

7. Summary

Intraperitoneal injection represents a valuable technique in psychopharmacological research in zebrafish (also see (60) for a detailed review). Importantly, as new methods of behavioral quantification emerge, various effective routes of drug administration must also be available to suit the experimental design of a particular study. From this viewpoint, i.p. injection offers an easy and efficacious route of drug administration, and can complement the immersion method of drug delivery in zebrafish-based behavioral pharmacological research.

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