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Research report

Unique and potent effects of acute ibogaine on zebrafish: The developing utility of novel aquatic models for hallucinogenic drug research

Jonathan Cachat[a,b], Evan J. Kyzar[a,b], Christopher Collins[a], Siddharth Gaikwad[a], Jeremy Green[a], Andrew Roth[a], Mohamed El-Ounsi[a], Ari Davis[a], Mimi Pham[a], Samuel Landsman[a], Adam Michael Stewart[a,b], Allan V. Kalueff[a,b,c,*]

[a] Department of Pharmacology and Neuroscience Program, Tulane University Medical School, 1430 Tulane Avenue, New Orleans, LA 70112, USA
[b] Zebrafish Neuroscience Research Consortium (ZNRC), Tulane University Medical School, 1430 Tulane Avenue, New Orleans, LA 70112, USA
[c] ZENEREI Institute, 309 Palmer Court, Slidell, LA 70458, USA

HIGHLIGHTS

- Ibogaine is a potent hallucinogenic drug with multiple psychoactive effects.
- Ibogaine exerted robust anxiolytic-like effects on zebrafish behavior.
- Ibogane altered shoaling and coloration, but not cortisol or *c-fos* expression.
- Our results support the utility of zebrafish for hallucinogenic drug research.

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ABSTRACT

An indole alkaloid, ibogaine is the principal psychoactive component of the iboga plant, used by indigenous peoples in West Africa for centuries. Modulating multiple neurotransmitter systems, the drug is a potent hallucinogen in humans, although its psychotropic effects remain poorly understood. Expanding the range of model species is an important strategy for translational neuroscience research. Here we exposed adult zebrafish (*Danio rerio*) to 10 and 20 mg/L of ibogaine, testing them in the novel tank, light–dark box, open field, mirror stimulation, social preference and shoaling tests. In the novel tank test, the zebrafish natural diving response (geotaxis) was reversed by ibogaine, inducing initial top swimming followed by bottom dwelling. Ibogaine also attenuated the innate preference for dark environments (scototaxis) in the light–dark box test. While it did not exert overt locomotor or thigmotaxic responses in the open field test, the drug altered spatiotemporal exploration of novel environment, inducing clear preference of some areas over others. Ibogaine also promoted 'mirror' exploration in the mirror stimulation test, disrupted group cohesion in the shoaling test, and evoked strong coloration responses due to melanophore aggregation, but did not alter brain *c-fos* expression or whole-body cortisol levels. Overall, our results support the complex pharmacological profile of ibogaine and its high sensitivity in zebrafish models, dose-dependently affecting multiple behavioral domains. While future investigations in zebrafish may help elucidate the mechanisms underlying these unique behavioral effects, our study strongly supports the developing utility of aquatic models in hallucinogenic drug research. High sensitivity of three-dimensional phenotyping approaches applied here to behavioral effects of ibogaine in zebrafish provides further evidence of how 3D reconstructions of zebrafish swimming paths may be useful for high-throughput pharmacological screening.

1. Introduction

Ibogaine is an indole alkaloid derivative with psychoactive properties, which can be isolated from the African shrub *Tabernanthe iboga* [1,2]. At different doses, it has been used by native Western Africans as a stimulant, appetite suppressant and aid in religious ceremonies [3]. In addition to potent hallucinogenic effects [4], ibogaine is effective in the treatment of addiction to

* Corresponding author at: Department of Pharmacology, Room SL-83, Tulane University Medical School, 1430 Tulane Ave., New Orleans, LA 70112, USA.
Tel.: +1 504 988 3354; fax: +1 504 988 3354.
E-mail address: avkalueff@gmail.com (A.V. Kalueff).

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opiates, methamphetamine, cocaine, and some other drugs [3,5]. Despite these potential therapeutic applications, the mechanisms of ibogaine action remain poorly understood. The pharmacological profile of ibogaine is very complex and involves multiple neuromediator systems. Structurally resembling serotonin (5-HT), ibogaine inhibits serotonin and dopamine transporters [1] and activates serotonin- (e.g., 5-HT_{2a}, 5-HT_{2c}) [6,7], opioid- (mu and kappa) [8,9] and sigma- (1 and 2) receptors [4,10,11]. The drug also acts as an antagonist of glutamate NMDA receptors [11–13], and a weak inhibitor of cholinergic muscarinic and nicotinic receptors [14]; see [15] for details of ibogaine receptorome.

While ibogaine is a controlled substance in various countries, including the United States (Schedule I), the drug does not appear to be commonly abused and is administered in medical settings in South Africa and Mexico [2]. In humans, ibogaine produces intense dream-like hallucinations which subjectively differ from those caused by classic serotonergic psychedelics [2,16] and include a vivid 'visual' phase followed by a longer 'introspective' phase [2,16]. Ibogaine can occasionally cause acute psychoses [17], whereas its anti-addictive properties have also been reported in the literature [5,18], including lasting anti-craving effects after a single ibogaine dose [19,20]. Further supporting the complex nature of ibogaine action are the prolonged effects of ibogaine in attenuating addiction and depressive symptoms [3].

Although ibogaine has been previously tested in rodent models, its effects on various animal phenotypes remain poorly understood. For example, it enhances nociception and other opioidergic effects in rodents [21] and reduces locomotion and central activity in novel environments [22]. In the plus-maze test, acute ibogaine decreases aversion to the open arms, interpreted as anxiolysis [23], while other studies reported anxiogenic-like responses [24]. Rats trained to discriminate ibogaine from vehicle did not respond to other serotonergic hallucinogens, such as lysergic acid diethylamide (LSD) and 3,4-methylenedioxymethamphetamine (MDMA) [25]. In addition to direct effects on multiple receptors, ibogaine modulates several molecular pathways, up-regulating the expression of glial cell line-derived neurotropic factor [26] and early proto-oncogenes *egr-1* and *c-fos* in the brain [27]. The development of novel high-throughput models and expanding the range of model species are important strategic directions in biological psychiatry [28], particularly useful to tackle complex effects of psychotropic drugs. Recently, there has been a remarkable resurgence of interest in hallucinogenic drugs, focusing on the mechanisms of their action in various species, as well as side effects and potential clinical applications [16,28–34]. Zebrafish (*Danio rerio*) possess high physiological similarity to humans [35–41], robust behavioral responses and a fully characterized genome [42,43], and are emerging as a sensitive and promising model for the investigation of hallucinogen-evoked states. Recent studies have reported the effects of LSD [44], MDMA [45], mescaline, phencyclidine (PCP) [46], ketamine [47,48] and salvinorin A [49,50] in adult zebrafish, emphasizing the role of specific receptor systems in the observed hallucinogenic-like phenotypes.

The pharmacological profile of ibogaine includes receptor targets that are shared with serotonergic psychedelic hallucinogens (e.g., LSD, mescaline, psilocybin), dissociative glutamatergic hallucinogens (e.g., ketamine) and hallucinogenic drugs acting via opioidergic systems (e.g., salvinorin A) [4,10,13]. The unique aspect of ibogaine action is that it affects all these targets simultaneously, most likely resulting in a complex profile that may theoretically include the actions of LSD, mescaline, psilocybin, MDMA, ketamine, PCP and salvinorin A combined. The sensitivity of zebrafish to all these drugs (see above) renders them a potentially useful experimental model to further elucidate the profile of acute ibogaine exposure. The present study aimed to evaluate the potential effects of ibogaine in several behavioral paradigms in adult zebrafish. In addition to behavioral markers, selected physiological biomarkers, validated in previous zebrafish studies, including *c-fos* gene expression (as a measure of neuronal activation [51]), and cortisol levels (as a measure of the neuroendocrine axis activation [52–54]), were examined following ibogaine treatment.

2. Methods

2.1. Animals and housing

A total of 500 adult (5–8-month-old) "wild type" short fin zebrafish (~50:50 male:female ratio) were obtained from a commercial distributor (50 Fathoms, Metairie, LA). All fish were given at least 14 days to acclimate to the laboratory environment and housed in groups of 20–30 fish per 40-L tank. Tanks were filled with filtered system water and maintained at 25–27 °C. Illumination (1000–1100 lx) was provided by ceiling-mounted fluorescent lights on a 12-h cycle (on: 6.00 h, off: 18.00 h) according to the standards of zebrafish care [55]. All fish used in this study were experimentally naïve and fed Tetramin Tropical Flakes (Tetra USA, Blacksburg, VA) twice a day. Following behavioral testing, the animals were euthanized in 500 mg/L Tricaine (Sigma–Aldrich, St. Louis, MO) and dissected on ice for further analysis. Animal experimentation in this study fully adhered to national and institutional guidelines and regulations.

2.2. Behavioral testing

Behavioral testing was performed between 11:00 and 15:00 h using tanks with water adjusted to the holding room temperature. The present study used several different behavioral tests, including the novel tank, open field (OFT), social preference, shoaling and mirror stimulation tests, as described in [44,56]. To avoid the test battery effect, each test was performed on a separate cohort of naïve fish. Prior to testing, fish were pre-exposed in a 1-L plastic beaker for 20 min to either drug-treated or drug-free vehicle solution (0.1% dimethyl sulfoxide DMSO, commonly used in zebrafish behavioral assays [46]). During testing, zebrafish behavior was recorded by 2–3 trained observers blind to the treatments, who manually scored different behavioral endpoints (inter- and intra-rater reliability in all experiments >0.85) with subsequent automated analysis of generated traces by Ethovision XT7 software (Noldus IT, Wageningen, Netherlands).

The novel tank test, used to assess zebrafish anxiety and locomotion [45,57–59], was a 1.5-L trapezoidal tank (15 cm height × 28 cm top × 23 cm bottom × 7 cm width; Aquatic Habitats, Apopka, FL) maximally filled with water and divided into two equal virtual horizontal portions by a line marking the outside walls (Fig. 1). In Experiment 1, fish were individually pre-exposed to ibogaine (10 or 20 mg/L) for 20 min (see details further), and tested in the standard 6-min novel tank test. Zebrafish behavior was recorded by trained observers, scoring the latency to reach the top half of the tank (s), time spent in top (s), number of transitions to top, as well as the number and duration (s) of freezing bouts. Freezing was defined as a total absence of movement, except for the gills and eyes, for >2 s. Trials were also recorded to a computer using a USB webcam (2.0-megapixel, Gigaware, UK) and subsequently analyzed by Ethovision XT7, assessing distance traveled (m), velocity (m/s), and meandering [44]. Ethograms in this test were also constructed by manually scoring episodes of bottom swimming, top swimming, bottom freezing and erratic movements, in order to visualize the occurrence of behaviors and the transitions between them, with the diameter of each circle reflecting the frequency of the behavioral activity, and the width and direction of each arrow representing the frequency of transitions between behaviors [44].

The light–dark test (Experiment 2), based on the natural preference of zebrafish for dark environments [60,61], was a rectangular tank (15 cm height × 30 cm length × 16 cm width) filled with water to a height of 12 cm, and divided into two equal vertical portions, demarcated by black and white coloration (Fig. 2A) [61]. Fish (n = 13 in each group) were individually introduced into the black half (facing the wall), for 5 min, and manually scored for the latency to enter(s), time spent (s), average entry duration (s), and the number of entries to the white half (due to the dark background, zebrafish behavior in the black compartment was not assessed here).

The OFT (Experiment 3) consisted of a white plastic cylinder (21 cm diameter, 24 cm height) filled with water to a height of 12 cm (Fig. 2B). Following drug pretreatment, the animals (n = 12–13 in each group) were individually placed in the center of the tank, and video-recorded from the top view for 6 min, using Ethovision XT7 to calculate the distance traveled (m), average velocity (m/s) and meandering (°/m), as defined in [62]. Since zebrafish establish robust preferred loci (homebases) in the OFT [63], the homebase behavior was examined in this study in detail by comparing zebrafish activity in their preferred homebase quadrant with averaged activity in non-homebase quadrants (see details in Fig. 2B). Homebase quadrant was defined for each fish as described in [63,64], calculating the average time spent, frequency of visit and distance traveled in homebase vs. non-homebase quadrants. In addition, thigmotaxis behavior (preference for walls vs. center) was assessed in this study by virtually dividing the OFT arena into two zones – periphery (area within 2.5 cm from the walls) and the central arena. Using Ethovision XT7, the time spent, distance traveled, average velocity and frequency of visits were calculated for each zone in this test. Given previous rodent data and zebrafish data on glutamatergically

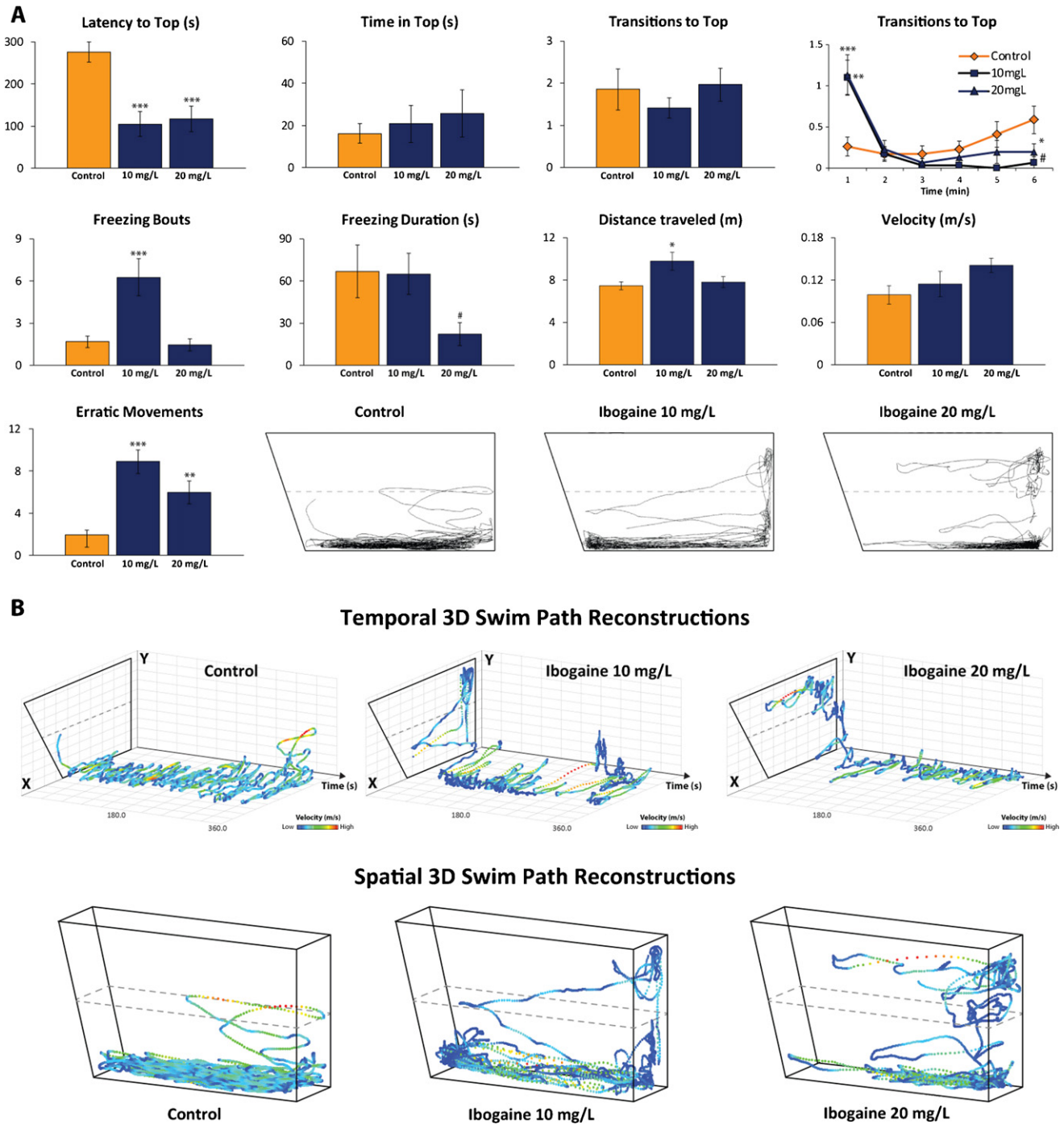

Fig. 1. Behavioral effects of acute 20-min exposure to ibogaine (10 and 20 mg/L) in the novel tank test. Panel A shows behavioral endpoints recorded in standard 6-min novel tank test ($n = 29$–34 per group), as well as two-dimensional (2D) representative traces generated in XY coordinates (side view) by Noldus EthoVision XT7. Panel B shows temporal and spatial three-dimensional (3D) reconstructions of zebrafish swim paths. Temporal 3D graphs plotted *XY*-coordinates (generated in EthoVision XT7) on respective *XY*-axes, with experimental time plotted across the *Z*-axis [66,67]. Spatial 3D graphs were generated in a similar fashion, with spatial coordinates from a coordinated, top-view recording plotted on the *Z*-axis representing zebrafish trajectories in *XYZ* coordinates. Track color reflects changes in velocity (m/s) (blue to green = lower velocity, yellow and red = higher velocity). In all experiments, representative traces selected after rating the activity of each subject/cohort from 1 to *n* based on activity level, and reaching consensus by three highly-trained investigators. Data are reported as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; post hoc Tukey test for significant ANOVA data. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

mediated circling/rotation behavior (rev. in [48]), we also analyzed zebrafish circling phenotypes using 2D video-track data generated by Ethovision XT7 replayed in slow motion. According to [48], rotation was defined as a full 360° circle of ~5 cm (~2 fish body lengths) in diameter. The data was then analyzed for total rotations, and the number of fish demonstrating 'high rotation' behavior (defined as 5 or more full rotations per a 6-min trial) in this study.

The social preference test (Experiment 4) examined zebrafish social behavior and locomotor activity, as described in [44] (Fig. 3A). The target *conspecific* fish was introduced to an exposure compartment (conspecific box), separated by transparent sliding doors from the rest of the apparatus. To avoid lateral bias in zebrafish cohorts, the left/right location of target fish alternated between the trials. After a 20-min pre-treatment, control and ibogaine exposed zebrafish ($n = 15$ in each group) were introduced individually to the central zone, temporarily separated by sliding doors from the two arms of the corridor. Following a 30-s interval (to reduce transfer/handling stress), the two sliding doors were gently lifted, and the zebrafish was released to explore the apparatus for 6 min. Fish behavior was scored manually by

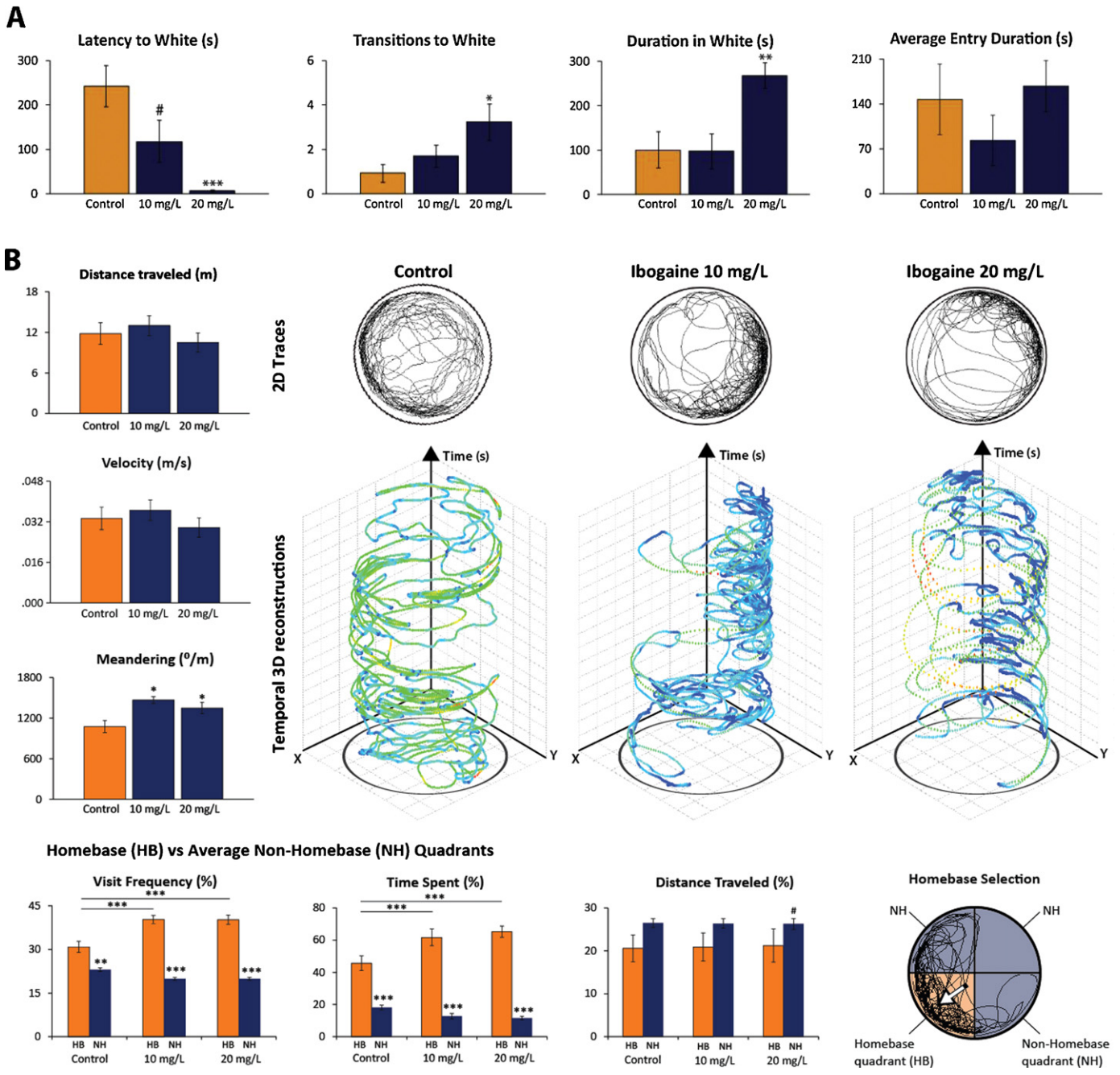

Fig. 2. Behavioral effects of acute 20-min exposure to ibogaine (10 and 20 mg/L) in the light–dark box and open field tests. Panel A shows behavioral effects of ibogaine on zebrafish in the light–dark box ($n = 13$ per group). Panel B shows the behavioral effects of ibogaine on zebrafish in the open field test ($n = 12$–13 per group). Representative 2D traces were generated by Noldus Ethovision XT7 software using the top-view video-recording. In all experiments, the traces were examined for each experimental cohort, rated from 1 to n (based on similarity to each other), and the middle trace was selected as representative by consensus of three highly-trained investigators, to illustrate the patterns of zebrafish locomotion observed in the open field test. Homebase behavior was examined in this study by comparing zebrafish behavior in their preferred homebase location (quadrant) with average results in non-homebase quadrants. Data are reported as mean ± SEM, $^{\#}p = 0.05$–0.08 (trend), $^{*}p < 0.05$, $^{**}p < 0.01$, vs. control; post hoc Tukey test for significant ANOVA data.

trained observers, assessing the number of center entries, time spent in center (s), the number of "conspecific" arm entries, the number of "non-conspecific" (empty) arm entries, total arm entries, as well as time spent (s) in the respective zones of the apparatus. The ratios of conspecific:empty and conspecific:total entry and time spent were calculated based on this data [44].

The shoaling test (Experiment 5) was performed to examine the effects of ibogaine on social behavior of zebrafish shoals (Fig. 3B). Groups of 8 zebrafish were pre-exposed to either ibogaine or drug-free vehicle for 20 min, and group-tested in the novel tank. Zebrafish shoaling behavior was video-recorded for 6 min, and analyzed using 8 screenshots made every 20 s during the last half of the observation period. A total of 16 screenshots from ibogaine-treated or control cohorts were used for analyses in this study. Each screenshot was properly calibrated and analyzed by trained observers, manually measuring the distances (cm) between each fish in the group, and then averaging this data to obtain an average inter-fish distance per screenshot (final shoaling data for control and experimental cohorts represented averaged results for 16 screenshots per group).

The mirror stimulation test (Experiment 6) represented another modification of the novel tank test, also relevant to social behavior (Fig. 4). The mirror image stimulation is a well-established fish paradigm, traditionally used for studying their exploratory and social/aggressive behavior [56]. Similar to other fishes, zebrafish display boldness by approaching, butting or biting the mirror when placed in the novel tank with it. The present study used the same novel tank apparatus in Experiment 1, with the addition of the mirror to a vertical wall, as described in [56]. In this 6-min test, zebrafish ($n = 15$ per group) were introduced to a novel tank with

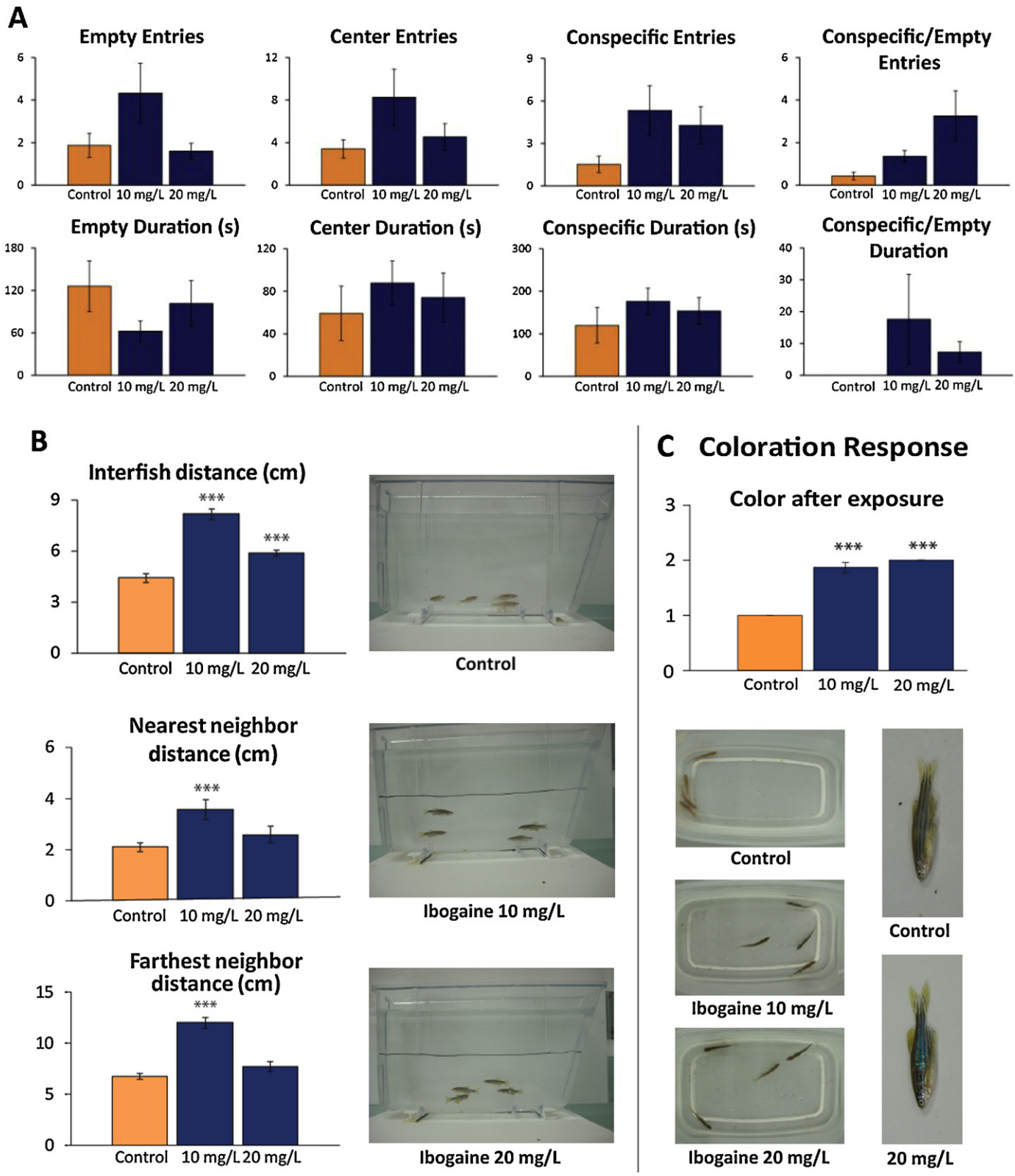

Fig. 3. Behavioral effects of acute 20-min exposure to ibogaine (10 and 20 mg/L) in the social preference, shoaling and body coloration tests. Panel A shows behavioral endpoints obtained from the standard 6-min social preference test ($n = 15$ per group). Bar graphs in panel B show behavioral endpoints of zebrafish shoaling behavior in control ($n = 96$) and ibogaine (10 and 20 mg/L, $n = 64$ per group). Panel C illustrates a significant increase in the color index for both ibogaine-treatment groups, which was found across all experimental designs. The body color was examined for each fish using the colorations scale (1 = pale, 2 = dark) by three highly-trained investigators, and representative photographs were selected on a consensus basis, to illustrate the patterns of zebrafish coloration (also see Section 3 for a similar profile detected using pixel-based analyses by ImageJ software). Data are reported as mean ± SEM, $^{***}p < 0.001$ vs. control; post hoc Tukey test for significant ANOVA for behavioral data (B); or $p < 0.005$ vs. control; post hoc *U*-test with Bonferroni correction for significant Chi-square body coloration data (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

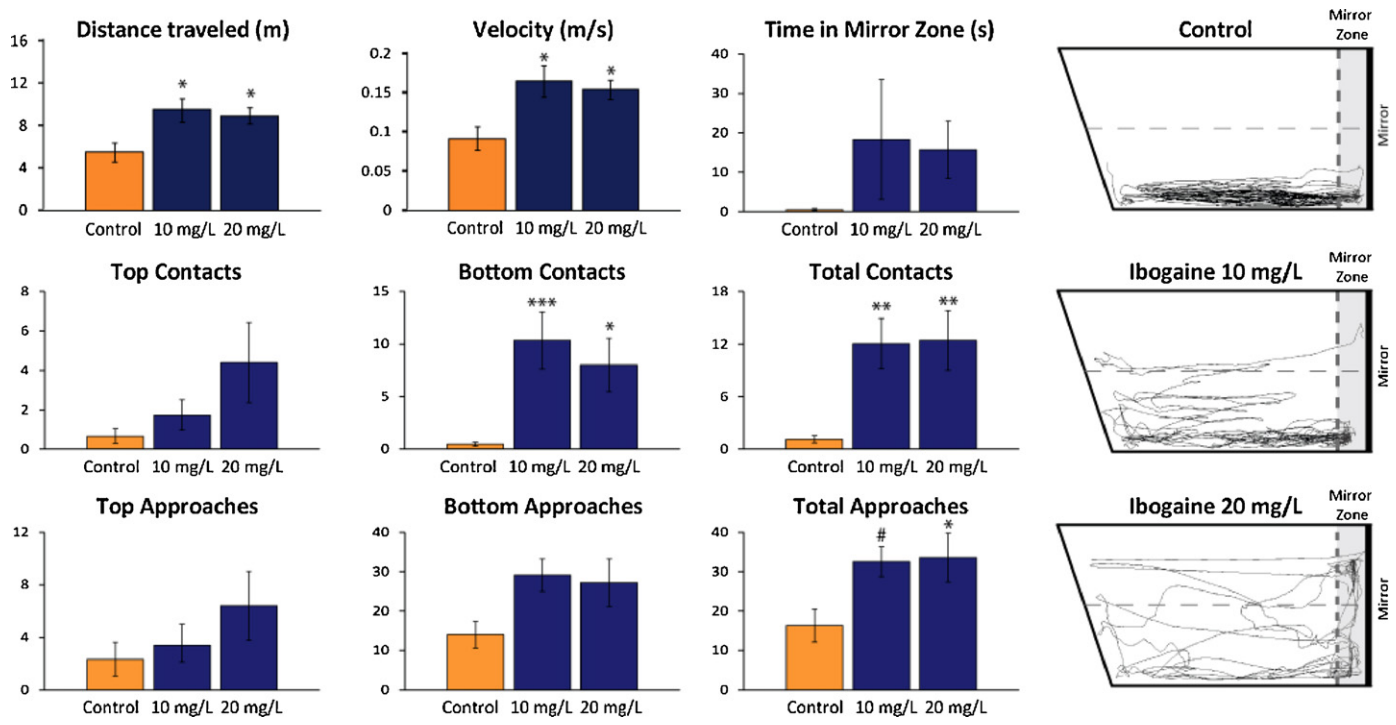

Fig. 4. Behavioral effects of acute 20-min exposure to ibogaine (10 and 20 mg/L) in the mirror stimulation test. In this 6-min test, zebrafish ($n = 15$ per group) were introduced to a novel tank containing a mirror on the vertical side wall. The mirror zone was defined as the area within 3 cm from mirror, which included the mirror contact zone (the immediate area within 0.5 cm of the mirror) and the mirror approach zone (the area within 1 body length (2.5 cm) from the mirror contact zone). Two-dimensional (2D) representative traces were generated using side-view video analysis by Noldus EthoVision XT 7. Data are reported as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; post hoc Tukey test for significant ANOVA data.

a mirror fixed to the vertical side wall. The mirror zone was defined as the area within 3 cm from mirror, and included the mirror contact zone (the immediate area within 0.5 cm of the mirror) and the mirror approach zone (the area within 1 body length (2.5 cm) from the mirror contact zone). In addition to traditional novel tank endpoints, Ethovision XT7 examined the number of entries and time spent in direct contact with mirror (mirror contact zone) and approaches to mirror (mirror approach zone). Ethograms in this test were also constructed (similar to Experiment 1 for the novel tank test) by 2–3 highly trained observers manually scoring episodes of bottom swimming, top swimming, bottom freezing, erratic movements and mirror biting (contact with the mirror), and the transitions between them.

2.3. Coloration response analyses

During the course of this study, we consistently observed that ibogaine darkened the color of exposed zebrafish (especially on the dorsal ridge of their bodies) for both doses tested and across all tests used (Fig. 3C). This phenotype was unusual and particularly prominent, based on our experience with screening in zebrafish over 20 different psychotrophic drugs (2009–2012, own systematic observations). In order to more fully quantify zebrafish coloration response to ibogaine, we first employed a standardized color rating scale (1 = pale body color; 2 = dark body color) assessed visually by 2–3 highly-trained observers (blinded to the treatments) immediately after the 20-min exposure to 10 and 20 mg/L ibogaine. The resulting responses were analyzed based on consensus between all observers. To confirm reliability of manual coloration data collected in this study, we also performed Spearman correlation between the observers scoring fish coloration in a separate experiment using 10 control and 10 experimental fish exposed to 20 mg/L ibogaine for 20 min. This analysis yielded strong and highly significant ($R = 0.82$, $p < 0.01$) inter-rater reliability of manual coloration observations. Photographs showing representative fish from control, and 20 mg/L ibogaine cohorts (Fig. 3C) further illustrate the robust coloration reponse evoked in zebrafish by this drug. Similarly, the same phenotype can also be observed on photographs of zebrafish shoals (made from the top; Fig. 3B), where all drug-treated fish are visibly darker compared to controls.

To further objectively quantify zebrafish coloration responses, we complemented manual data with pixel-based analysis, applying an open-access ImageJ software (NIH, Bethesda, MD) [65] to a separate cohort of control and ibogaine-exposed fish (20 mg/L) mentioned above. Briefly, following a 20-min treatment, fish ($n = 10$ per group) were euthanized and immediately photographed (two images per fish, left and right sides) against a white, laminated sheet of paper that served as the background. Photographs were taken from a 15-cm distance with a 5.0-megapixel iSight camera from an iPhone 4S (Apple, Cupertino, CA) mounted on a ring stand to reduce potentially confounding variables (i.e., lighting, distance, subject size). Body color quantification was performed in ImageJ [65]. To standardize the images prior to quantification, each photo was processed in Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA), using the 'Magic Wand' tool to select/delete the background and remove transparent pixels. Images were converted to 8-bit grayscale TIF files, cropped (560 × 200 pixels) to isolate zebrafish body and rotated to a uniform horizontal orientation (head facing left). A region of interest (300 × 60 pixel rectangle, representing a substantial part of zebrafish body) was positioned over each fish image in a standardized manner, starting at the gill and incorporating the lateral body surface. The mean gray value, ranging from 0 (black) to 255 (white), was then measured for this selected region by ImageJ software, averaged between left and right-side images for each individual fish, and presented as the average for the group.

2.4. Video-tracking

During manual observation, videos were recorded in MPEG1 format with the maximum sample rate 30 fps for each trial by auto-focusing 2.0 MP USB webcams, placed 50 cm in front of or on top of the tanks, and attached to laptop computers. Recorded videos were analyzed with Ethovision XT7 software, as described previously [44,46,66]. All environments were calibrated for each arena, and the calibration axes were placed to designate the origin (0,0) at the center of each tank. The track data for each fish was exported as raw data into separate spreadsheets. The exported traces were independently rated on a consensus basis from 1 to n (based on similarity to each other) by three trained observers blinded to the treatments. The median trace was selected as representative of the group to illustrate the spatial pattern of zebrafish swimming [44,46,66].

For each experiment, raw track data was exported into Excel spreadsheets, pre-processed and formatted to generate 3D swim path reconstructions, as previously described in detail [66,67]. Briefly, each track was interpolated to replace missing values within the Track Editor of Ethovision XT7. This step replaced missing spatial coordinates by a linear interpolation of the nearest neighbor detection points, or the previous and most recent valid detection coordinates. Raw track files were formatted so that column headers containing independent variables were in the first row of the spreadsheet. A "find and replace" procedure was performed to replace null values ("-") with blank cells. After removing Trial Identification information, track files were renamed to provide this information (i.e., "Control1side.xlsx"). Each track file was then saved as a comma separated value (CSV) file and imported into RapidMiner 5.0 software. Each column (Independent Variable) was designated as either a real or integer value-type based on its contents and no special attributes were assigned. Temporal 3D reconstructions were created in a Scatter 3D Color plot, in which

X-center, time, and *Y*-center were attributed to the *X*,*Y*- and *Z*-axes, respectively. Dependent variables were actively cycled across the path using the color attribute, and tracks were explored using rotation and zooming features. For comparison, axis ranges were standardized, and reconstructions were saved as image files. Representative reconstructions for each experimental manipulation were selected by comparing the complete set of 2D and 3D swim path images, rating from 1 to *n* based on their similarity to each other (by three observers on a consensus basis) and choosing the middle track as representative [50].

2.5. Brain c-fos and whole-body cortisol assays

QT-PCR was performed for zebrafish *c-fos* mRNA from samples obtained in Experiments 1 and 2. The brains were quickly dissected on ice, and 2–4 brains were pooled to obtain 6 samples per group, RNA was extracted from these samples, and cDNA was synthesized using random primers and iScript Select cDNA Synthesis Kit (Bio Rad, Hercules, CA). For QTPCR, cDNA was amplified with c-fos primers for zebrafish and compared against the reference gene (elongation factor 1 alpha), expressing *c-fos* data as the relative fold change vs. control (taken as 100%) [48]. Whole-body samples were taken from subjects used in the same experiments, cortisol was extracted with diethyl ether (Fisher Scientific, Pittsburgh, PA) and its concentrations were assessed using ELISA human salivary cortisol assay kit (Salimetrics LLC, State College, PA), and presented as relative concentrations per gram of body weight for each fish, according to [53].

2.6. Pharmacological manipulations

Ibogaine for this study was obtained through the NIDA Drug Supply Program (NIH/NIDA, Bethesda, MD). Its doses (10 and 20 mg/L) were chosen based on our pilot studies with the drug with a wider dose range (5–25 mg/L). A standard 20-min pretreatment time was chosen here based on our experience with other hallucinogenic drugs in zebrafish, including LSD [44], MDMA [45], mescaline [46], ketamine [47,48], PCP [46] and salvinorin A [50]. Based on our experience, this exposure time was also sufficient for provoking the physiological (e.g., cortisol and *c-fos*) responses of zebrafish. Drug exposure in this study was performed by submerging individual zebrafish (or a shoal of zebrafish in Experiment 5) in a 1-L plastic beaker for 20 min prior to the testing, as described in [48]. The solution was regularly changed after each exposure to ensure that each fish is exposed to a consistent concentration of ibogaine; control fish were exposed to drug-free vehicle for the same treatment time, as described above.

2.7. Statistical analyses

The behavioral and cortisol data was analyzed using one-way ANOVA(factor: dose) followed by post hoc Tukey testing for significant ANOVA data. Intra-session habituation in Experiment 1 was analyzed using repeated measures ANOVA (factor: dose) for per-minute data, and the paired Wilcoxon-Mann–Whitney *U*-test to compare minute 1 vs. minute 6 data. Two-way ANOVA (factors: dose and location) was used to analyze homebase vs. non-homebase behavioral endpoints for the homebase analysis of the OFT. *C-fos* expression for each dose was individually compared with the respective control group, and analyzed using non-paired *U*-test. Inter- and intra-rater reliability for the observers was determined by Spearman correlation. Since zebrafish body coloration data generated manually were based on a 2-point scale, this categorical data was analyzed using Chi-square test to test the hypothesis of no association between ibogaine treatment and coloration response (for coloration analyses using three groups, Chi-square test was followed by a pairwise post hoc comparisons with Bonferroni correction). Automated coloration data generated by ImageJ software was analyzed using *U*-test to compare control with 20 mg/L ibogaine treatments. Manual and software-generated coloration data were also analyzed using Spearman correlation. Data were expressed as mean ± SEM, and significance was set at $p < 0.05$ in all experiments of this study.

3. Results

In the novel tank test (Experiment 1), ibogaine induced robust behavioral responses, significantly affecting the latency to top, erratic movements and freezing bouts ($F_{2,90} = 12.1$, 14.8 and 10.8, $p < 0.001$, respectively). The drug effect on freezing duration approached statistical significance ($F_{2,90} = 2.8$, $p = 0.07$), with ibogaine at 20 mg/L reducing freezing duration and at 10 mg/L increasing frequency of freezing (Fig. 1A). Overall, while control fish froze for much longer, ibogaine 10 mg/L evoked multiple freezing bouts of shorter duration per bout, at 20 mg/L increasing time swimming in the novel tank. Both doses significantly reduced the latency to enter the top, compared to control cohorts (Fig. 1A). Significant effects were also found for total distance traveled ($F_{2,90} = 3.3$, $p < 0.05$), but not velocity, time spent in top or transitions to the top ($F_{2,90} = 0.3$–0.5, NS). Examining the per-minute distribution of transitions to top shows that control fish gradually increased the number of transitions, displaying a typical habituation response to a novel environment, described in detail in zebrafish [68]. In contrast,both ibogaine doses completely reversed this reponse, with drug-treated fish showing initial top swimming followed by gradual descending to the bottom (Fig. 1A and B). Repeated measures ANOVA (group × time) revealed significant time effect ($F_{5,540} = 15.4$, $p < 0.001$) and time × dose effects ($F_{10,540} = 5.2$, $p < 0.001$), but not dose effect ($F_{2,540} = 0.9$, NS), with ibogaine-treated zebrafish showing more transitions to top during minute 1, and significantly less transitions in the minute 6 of the novel tank test. This drug-evoked phenotype was further illustrated by temporal 3D reconstructions of zebrafish swimming paths (Fig. 1B), where control fish showed initial bottom dwelling behavior, followed by incrementally larger horizontal sweeps along the bottom with brief vertical excursions into the upper region of the novel tank. As can be seen in Fig. 2B, ibogaine 10 mg/L evoked a short-lived initial top dwelling, followed by a slow descent made to the bottom of the tank. Once at the bottom, ibogaine induced another interesting behavioral pattern, as fish seemed to prefer the bottom corners (likely investigating their reflection) but, within seconds, quickly swam to the opposite corner, where it remained still facing the wall, then slowly turned and returned back. These phenotypes represented the most frequent movement patterns of ibogaine-treated cohorts, with the 'intial top, slow to bottom' profile observed in 58 ± 6.6% and 'bottom side-to-side' profile in 72 ± 6.0% vs. 2.3 ± 2.3% and 9.5 ± 4.5% in controls, respectively ($p < 0.0001$, *U*-test). Spatial 3D reconstructions of the zebrafish locomotion in XYZ coordinates provided further confirmation of the observed locomotor patterns evoked by acute ibogaine exposure in zebrafish in the novel tank test (Fig. 1B).

In the light–dark box test (Experiment 2), ibogaine-treated zebrafish showed a marked attenuation of scototaxic behaviors, spending more time in the white than the black, with a significant effect for latency to enter the white chamber ($F_{2,36} = 9.6$, $p < 0.001$), where the drug dose-dependently decreased the latency (Fig. 2A). Additionally, sigificant effects were found in transitions to white ($F_{2,36} = 3.9$, $p < 0.05$) and duration in white ($F_{2,36} = 6.9$, $p < 0.005$), where ibogaine at 20 mg/L induced more transitions and time spent in the white chamber (Fig. 2A).

In the OFT (Experiment 3), there were no overt effects of ibogaine on circling-like behavior (Fig. 2B), distance traveled and velocity ($F_{2,35} = 0.7$–1.0, NS), with a significant effect on meandering ($F_{2,35} = 4.5$, $p < 0.05$) which was increased by ibogaine (Fig. 2B). Ibogaine treatment significantly altered the overall spatial exploration strategy of zebrafish in this experimental paradigm. Examination of zebrafish spatial dynamics in the OFT illustrates the latter profile particularly well. Recent studies have shown that zebrafish establish clear preferred loci (homebases) in novel environments [63,69]. Although all fish established clear homebases in this test, two-way ANOVA (factors: dose and location) established significant dose ($F_{6,138} = 2.8$, $p < 0.05$), location ($F_{3,68} = 114.7$, $p < 0.001$) and dose × location ($F_{6,138} = 4.6$, $p < 0.001$) effects on *normalized* (expressed as percentage of total) homebase activity, including time spent, distance traveled and transitions to homebase area (Fig. 2B). Subsequent ANOVA analysis of homebase vs. non-homebase activity within each group provided additional insight into this behavior. Overall, there were significant effects for normalized visit freqency in control, ibogaine 10 mg/L and 20 mg/L cohorts ($F_{1,24} = 15.4$, 193.2 and 142.7, $p < 0.001$, respectively), as well as for for the normalized time spent in zone ($F_{1,24} = 52.8$, 81.9 and 152.2, $p < 0.001$, respecively), but not the distance traveled in zone (Fig. 2B). The effects of ibogaine treatment on OFT exploration and homebase behavior were further illustrated in the representative swim paths. 2D traces (generated by the top-view video-recording) show that ibogaine-treated fish established a homebase and swam

within this preferred quandrant more, compared to other areas of the OFT (Fig. 2B). While control fish did establish a homebase quandrant, they showed higher exploration throughout the entire arena with lesser homebase-centric behavior. These differences in behavioral patterning are further emphasized by examining the temporal 3D swim path reconstructions, showing that control zebrafish spent more time exploring the arena prior to establishing a preferred homebase quadrant (lower half of 3D reconstructions), then venturing from the homebase more often exploring non-homebase quandrants (Fig. 2B). In contrast, ibogaine treatment markedly decreases this initial exploration phase and latency to homebase formation in a dose-dependent manner. For example, the representative ibogaine 20 mg/L reconstruction demonstrates that homebase formation occured very quickly during the test, and the subsequent fish swimming was generally restricted to this preferred quadrant, except for short-lasting, high-velocity exploration excursions to non-homebase quadrants (Fig. 2B).

In the social preference test (Experiment 4), we assessed the ability of zebrafish to interact with conspecifics (vs. empty compartment). The ibogaine-treated zebrafish in this test did not display an overt preference for any zone (empty arm, center, or conspecific arm) or exhibit an altered ratio of conspecific:empty arm entries (Fig. 3A). In the shoaling test (Experiment 5), ibogaine significantly disrupted shoal formation (Fig. 3B), revealing significant effects on average interfish distance, nearest neighbor distance and farthest neighbor distance ($F_{2,221}$ = 39.3, 7.4 and 44.5, $p < 0.001$, respectively). In general, ibogaine treatment increased each index, reflecting less cohesive shoaling compared to control fish.

As mentioned earlier, we also consistently observed in all experiments that ibogaine exposure at both doses markedly darkened the color of zebrafish, especially on the dorsal ridge of their bodies. To objectively quantify this coloration response, we first used a color rating scale that revealed a significant treatment effect (χ^2 (2) = 37.6, $p < 0.005$) on the body color after a 20-min exposure to ibogaine (Fig. 3C). A similar result was obtained in 10 control and 10 experimental fish (treated with 20 mg/L of ibogaine) used in pixel-based analyses of fish coloration (χ^2 (1) = 9.9, $p < 0.005$, see further). Photographs showing representative control and ibogaine-treated fish further illustrate the robust coloration reponse evoked by this drug. Similarly, the same phenotype can also be observed on photographs of zebrafish shoals, made from the top, where all drug-treated fish are visibly darker compared to untreated controls (Fig. 3C). These ibogaine-evoked coloration responses were retained by zebrafish throughout their testing in each of the behavioral paradigms used here, and were also present following euthanasia using Tricaine (data not shown). While only representative images are shown in Fig. 3C, the coloration resposes evoked by ibogaine were invariably observed in every experiment of this study (data not shown), and reliably scored by several highly trained observers. Automated pixel-based analysis of zebrafish coloration using ImageJ software further reconfirmed our findings obtained using manual observation. First, a significant difference was observed between 'mean gray value' data in control group (50.4 ± 3.6) vs. fish treated with 20 mg/L ibogaine (35.8 ± 2.3; $p < 0.005$, U-test, $n = 10$ per group), indicating that ibogaine-treated fish were significantly darker, as assessed by ImageJ software. Second, a significant negative correlation (Spearman $R = -0.44$, $p < 0.05$) was found between manual and pixel-based analyses performed in control and ibogaine-treated fish. Specifically, considering ibogaine-treated fish as 'dark', human observers assigned to this group higher scores on a manual 2-point scale (1–pale, 2–dark), while ImageJ software detected lower 'mean gray value', based on the scale from 0 (black) 255 (white). The fact that both methods showed significant correlation in assessing fish color responses confirms that both approaches applied here can be used to characterize body coloration phenotypes in zebrafish.

In the mirror stimulation test (Experiment 6), which represented a modification of the novel tank test, ibogaine treatment evoked several prominent behavioral responses, including significant effects on total distance traveled ($F_{2,42}$ = 4.9, $p < 0.05$), average velocity ($F_{2,42}$ = 5.1, $p < 0.01$), bottom contacts ($F_{2,42}$ = 5.8, $p < 0.01$), total approaches ($F_{2,42}$ = 4.0, $p < 0.05$) and total contacts with the mirror ($F_{2,42}$ = 6.2, $p < 0.005$), but not bottom approaches ($F_{2,42}$ = 3.2, NS). Representative 2D swim traces further illustrate these behavioral changes, as controls swam along the bottom of the tank, and displayed less overall exploratory behavior (compared to the two ibogaine cohorts that showed wide-spread tank exploration with a notable tendency to investigate the mirror; Fig. 4).

Ethogram-based analyses used in this study provided another intuitive way to assess the overall behavioral organization of zebrafish activity. As shown in Fig. 5, in both the novel tank and the mirror stimulation tests (Experiments 1 and 6), ibogaine-treated cohorts displayed significant alternations in overall behavioral patterning of zebrafish responses. In the novel tank test, significant effects were found for erratic movements, total transitions and for various transitions, including from bottom to top swimming, from bottom swimming to erratic movement, from top to bottom swimming, from erratic movement to bottom swimming, and from erratic movement to top swimming ($F_{2,85}$ = 3.3–18.6, $p < 0.001$–0.05). In the mirror stimulation test, while there were no significant effects for bottom swimming, top swimming or freezing ($F_{2,42}$ = 2.6–3.0, NS), we observed significantly altered transitions from bottom swimming to erratic movements, from top swimming to erratic movements, from top swimming to mirror biting, from erratic movement to bottom swimming, and from mirror biting to freezing ($F_{2,42}$ = 3.3–8.5, $p < 0.001$–0.05). Finally, in a striking contrast to the robust behavioral effects of ibogaine in multiple tests, the drug at either dose tested failed to affect physiological indices, including whole-brain *c-fos* expression and whole-body cortisol levels (data not shown).

4. Discussion

This study is the first report of ibogaine effects in zebrafish, showing high sensitivity of this model organism to multiple acute behavioral effects of this hallucinogenic drug. The use of fish to study the effects of hallucinogenic drugs complements traditional rodent-based models, enabling rapid testing of novel psychoactive compounds. The history of zebrafish as an animal model in genetics and developmental biology has resulted in several genetic techniques applicable to zebrafish more easily than in mammals, raising the possibility of using zebrafish models to study pharmacogenetics of drug abuse. The behavioral effects of ibogaine in various animal models remain poorly understood, and are based on complex interactions of several neurotransmitter systems. However, as will be discussed further, the accumulating body of evidence from screening psyhoactive drugs with different mechanisms of action in zebrafish provides important insights into the complex profiles of hallucinogenic drugs, such as ibogaine.

In general, the unique action of ibogaine across all tests was the reversal of several normal zebrafish behaviors. In the novel tank test, ibogaine reduced geotaxis, evoking atypical intial top exploration followed by gradual swimming toward the bottom. Upon reaching the bottom, fish exhibited an unusual side-to-side movement pattern, rapidly swimming to a corner, staying there for several seconds facing the wall and then rapidly reaching the

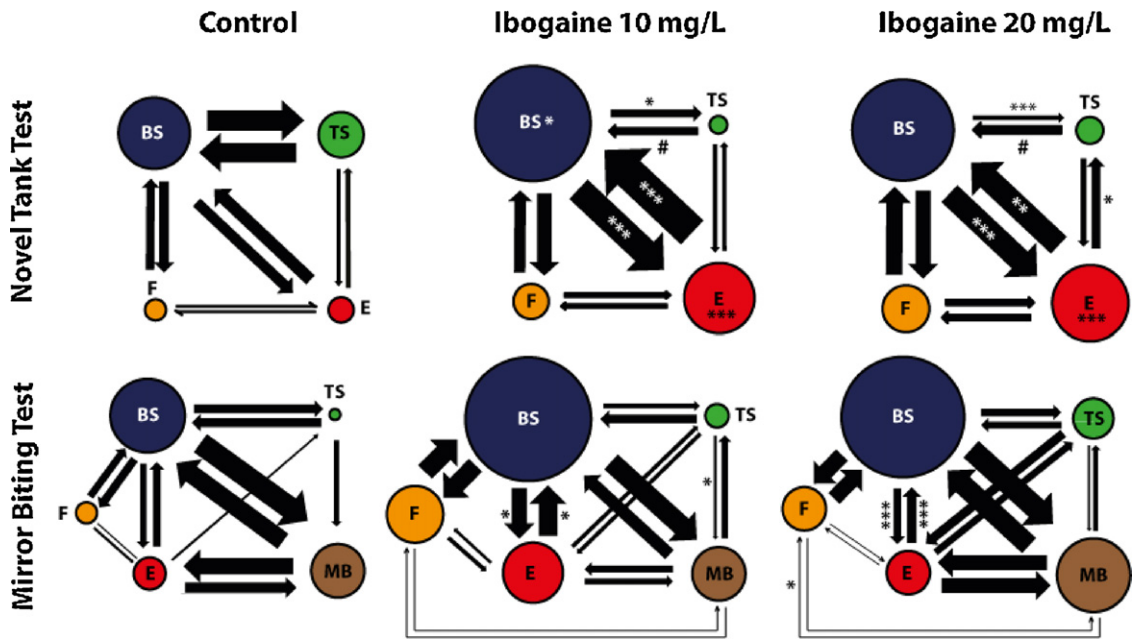

Fig. 5. Ethogram-based analysis of the effects of acute 20-min exposure to ibogaine (10 and 20 mg/L) on zebrafish behavioral patterning in the novel tank (Fig. 1) and mirror stimulation (Fig. 4) tests. Ethograms were generated based on frequencies and transitions between each individual behavioral activity, as described in [44,46]. The diameter of each circle corresponds to the frequency of each individual behavioral activity (BS – bottom swimming, E – erratic movements, F – freezing, MB – mirror biting, TS – top swimming episodes). The arrow width and direction reflect the frequency of transitions between these behaviors (asterisks within circles indicate significant differences in types of behavioral activity, asterisks placed next to arrows denote significantly different transitions). Data are reported as mean ± SEM, $^{\#}p = 0.05–0.08$ (trend), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; post hoc Tukey test for significant ANOVA data.

opposite bottom corner, remaining there briefly before repeating this pattern (Fig. 1B). In the light–dark box text, the drug attenuated zebrafish scototaxis, acting similarly to LSD [44], but with more pronouced effects (Fig. 2A). While elevated light behavior in the light–dark test can be due to higher locomotion [44], this phenotype can also be explained by distorted perception of light/visual cues, commonly described clinically during ibogaine experience [70]. Overall, the similarity of observed behavioral effects of LSD and ibogaine suggests the important role of serotonergic mechanisms in in these tests, which may be further dissected using specific genetic or pharmacological manipulations.

Although ibogaine did not alter most of OFT behaviors, it increased meandering (suggesting more changes in swimming direction compared to controls; Fig. 2B). While there were no differences in center vs. peripheral swimming activity between the groups (data not shown), ibogaine strongly affected homebase formation. For example, control fish generally investigated the entire arena before choosing a preferred homebase area, whereas ibogaine reduced this intial investigation phase. In line with earlier theoretical predictions [63], our study provided the first experimental evidence of the utility of homebase analyses for pharmacological screening using zebrafish. Similar to serotonergic drugs LSD and MDMA, ibogaine affected shoaling, reducing this innate group behavior in zebrafish (Fig. 3B). Ethogram-based analyses of zebrafish novel tank and mirror stimulation test revealed disrupted patterning of fish behavior (Fig. 5).

Finally, the drug evoked robust changes in zebrafish coloration (Fig. 3C), confirming the utility of skin pharmacology [71] for high-throughput screening of psychotropic agents in drug discovery and development [72]. In zebrafish, body coloration is emerging as an important phenotype sensitive to various experimental manipulations, including social [73] or physiological stress and pharmacological modulation [74,75]. Serotonergic modulation of melanophores has been reported in various vertebrates, including fish [76,77], and may underlie the coloration effects observed here. However, glutamatergic mechanisms may also be implicated, since PCP and kynurenic acid, two other glutamatergic antagonists, both induce strong coloration responses in zebrafish (own unpublished data), similar to ibogaine. Collectively, our findings (Fig. 3C) support the potential of screens for small molecules with selected psychoactive properties based on zebrafish coloration. Our results also show that automated image-analyzing software, such as ImageJ program used here, can be particularly promising for objective high-throughput quantification of zebrafish coloration phenotypes.

Given the robust behavioral effects of ibogaine in multiple tests here, we expected that physiological biomarkers, including brain *c-fos* expression and whole-body cortisol levels, may also be affected in this study. In line with this notion, rodent data implicate ibogaine in elevated *c-fos* expression [27,78] and corticosterone levels [79,80]. Our results, however, did not show ibogaine effects on these indices, raising the possibility that the drug may be inactive on physiological responses at doses where it affects zebrafish behavior. It may also exert opposite influences from different modulated neurotransmitters. For example, a combination of serotonergic LSD-like (elevating zebrafish cortisol [44]) and glutamarergic ketamine-like (reducing zebrafish cortisol [48]) effects of ibogaine may result in unaltered cortisol responses consistently observed here. The lack of effects on *c-fos* expression may also have similar explanation, given the relation of *c-fos* to stress and anxiety, and the lack of anxiogenic-like behavioral responses of ibogaine induced here. Additionally, we examined whole-brain *c-fos* expression, which may be a potential limitation of this study, since rodent data showed region-specific changes in *c-fos* and *egr-1* expression following ibogaine administration [27,81]. The alternative possibility is that ibogaine exerts some effects in species-specific manner, resulting in fish phenotypes that can differ from those of rodents and humans. Indeed, given the complex pharmacological profile of ibogaine (targeting several modulatory neurotransmitter systems), it has been difficult to evaluate induced psychological and behavioral states in traditional tests. For example, in several clinical studies examining ceremonial and therapeutic use of ibogaine,

the drug's effects seem to heavily depend on the subject's 'set and setting', with both anxiogenic [70,82] and positive, mood-elevating [3,83] effects being reported. Additionally, in rodent studies differences in the behavioral test and ibogaine doses have resulted in both anxiogenic-like [24,84] and anxiolytic-like profiles [23]. Our novel tank and light–dark box results (Figs. 1A and 2B) suggest that a reduction in anxiety (as interpreted by reduced geotaxis and scototaxis) may be part of the drug's complex and potent profile in zebrafish. However, the profound reversal of innate responses in zebrafish, and consideration of variable clinical and rodent reports, suggest that the psychopharmacology of this potent drug merit further in-depth translational investigation in various behavioral tests and across different model species.

There were several other notable limitations of this study. First, we analyzed behavior in wild-type zebrafish, in approximately ~50:50 male to female ratio (similar to other studies using adult zebrafish [85–87]). Given sex and strain differences in zebrafish responses to various drugs of abuse (e.g., [88,89]), the analyses of these differences in sensitivity to ibogaine merit further scruitiny. Likewise, while the present study examined acute ibogaine effects at 10–20 mg/L, several rodent reports indicate potential differences in the behavioral effects of subacute, acute and chronic ibogaine at different doses [23,90], which may be examined further using zebrafish models. Finally, we did not focus here on the effects of ibogaine on addiction-like phenotypes in zebrafish, which again provides opportunities for future studies with high translational relevance.

Nevertheless, the ability to use zebrafish to dissect potential roles of different mediators in ibogaine-induced responses seems promising, offering an additional, evolutionarily relevant 'reference' point for cross-species analyses. For example, glutamatergic NMDA antagonists (e.g., ketamine, PCP and MK-801) evoke robust circling behavior, also reported in clinical, rodent and zebrafish studies (see [46,48] for discussion). Since ibogaine is an NMDA antagonist, we could expect increased circling behavior in our study. While circling was not commonly assessed in rodent studies with ibogaine, an early report did show increased circling following its administration in mice [91]. In the present study, however, zebrafish circling behavior was not affected by ibogaine, dissimilar to ketamine, MK-801 and PCP that all evoke tight circling [46,48]. Therefore, the lack of overt circling here may be interpreted as the relatively lesser glutamatergic contribution to this phenotype, as compared to other mediator systems modulated by ibogaine. At the same time, despite the initial top preference, the lack of overt sustained surfacing behavior (typical in zebrafish for serotonergic drugs such as LSD [44] and MDMA [45]) suggests that serotonergic-like profile may only be a part, rather than the predominant action, of complex ibogaine profile observed here.

A potential difference in the outcomes of some tests, such as the social preference (Experiment 4) and mirror stimulation (Experiment 6), also merits discussion. For example, in the social preference test, all cues were present since the target and conspecific fish shared the same water, and while ibogaine may affect visual perception, the fish had other sources of information (e.g., vibration or chemosensory cues) to draw from the conspecific. In contrast, during the mirror stimulation test, there was only a reflection in the mirror, which, combined with altered visual perception, could evoke higher responses in ibogaine-treated fish. Clearly, further studies are needed to dissect the complexity of the effects of ibogaine on different social behaviors. Furthermore, this study highlights another important aspect of zebrafish neurophenotyping, which only recently became recognized. The ability of zebrafish to swim in 3D space represents a unique advantage of this aquatic species over other models, such as rodent paradigms, which generally include 2D locomotion in horizontal plane [66]. High sensitivity of 3D phenotyping approaches applied here to the effects of ibogaine in zebrafish (Figs. 1B and 2B) provides further evidence of how 3D reconstructions of fish swimming paths may be useful for high-throughput pharmacological screening [66].

Finally, our analyses of psychotropic activity of ibogaine reveal interesting parallels between the relative efficacy of this compound relative to other psychotopic hallucinogenic-like drugs tested in zebrafish previously. In the present study, behavioral effects of ibogaine were observed acutely following both 10 and 20 mg/L treatments. In zebrafish literature, the effective doses of other related compounds were established to be 0.1–0.25 mg/L LSD, 80–160 mg/L MDMA, 20 mg/L mescaline and 20–40 mg/L ketamine (see [46] for review). Thus, ibogaine in zebrafish was approximately 100–200 times less potent than LSD, 8 times more potent than MDMA, and equally potent to mescaline and ketamine. In humans, acute psychoactive experiences can be observed with 500–700 mg ibogaine [19], as well as with <1 mg LSD, 200 mg mescaline and MDMA, and 125 mg ketamine (see [46] for discusson). Therefore, ibogaine clinically appears to be 500–1000 times less potent than LSD, twice less potent than mescaline or MDMA, and 4 times less potent than ketamine. While species differences in pharmacokinetics and pharmacodynamics may contribute to some differences in its relative efficacy, these analyses show that the effects of ibogaine and other common hallucinogens in fish generally parallel those of humans. Together with a rich spectrum of robust behavioral phenotypes identified for ibogaine in this study (Figs. 1–5), this strongly supports the growing translational value of zebrafish models for hallucinogenic and drug abuse research. Future studies will be able to utilize zebrafish models to increase our understanding of the mechanisms of behavioral effects of ibogaine in various model organisms.

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