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A Novel Behavioral Test Battery to Assess Global Drug Effects Using the Zebrafish

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The zebrafish (*Danio rerio*) has been at the forefront of neurobiological research and is steadily gaining favor as a model for behavioral applications. The ease of handling, high yield of progeny, and efficient mode of drug delivery make this species a particularly useful model for behavior. Here, we append to the growing body of literature on zebrafish behavior by introducing a novel behavioral battery of tests aimed at identifying drug induced alterations in social and motoric behaviors. In a series of experiments, zebrafish were exposed to MK-801 (0, 2 µM, 20 µM), SFK 38393 (0, 10 µM, 100 µM), and ethanol (0, 0.5%, 1.0%) for one hour and overt locomotor behaviors were scored. Following a one-hour treatment exposure, circling behavior (a thigmotaxic display typical of dysregulated glutamate function) was scored from videotape at specific time points over a 37-minute session. In a separate experiment the zebrafish's natural tendency to shoal (social display) was analyzed using a novel open-field paradigm that examined fish distribution over quadrants. Most notably, MK-801 (20 µM) significantly increased circling behavior compared to controls. However, shoaling displays were disrupted when zebrafish were exposed to both MK-801 and SKF 38393 (20 µM and 100 µM respectively). Our results, in part, complement existing knowledge about zebrafish behavior following acute drug exposure. Additionally, our novel approach to assessing shoaling behavior, reported here, introduces an alternative view of social/group behavior in the zebrafish that is sensitive to both NMDA and dopaminergic manipulation.

Viable animal models (i.e. rodent and primate) have enabled researchers to infer about the fundamental features of human behavior and physiology. Since the zebrafish's introduction as a model for neural development by Streisinger in the 1960's (Grunwald & Eisen, 2002), its promise as a genetic tool for biological research has nearly been realized (Beis & Stainier, 2006; Driever et al., 1996; Guo, 2004; Haffter et al., 1996). Recent years, however, have seen a steady increase in the use of this species in behavioral applications (Miklósi & Andrew, 2006; Spence, Gerlach, Lawrence, & Smith, 2008). Many features of the zebrafish make it a particularly attractive candidate for inferring higher-level vertebrate behavior. The anatomical similarities to other vertebrates and nervous system structure, albeit in a less complex form, of this particular species of fish have allowed researchers to draw conclusions regarding the function of the human nervous system. Zebrafish central nervous system development closely resembles that of other vertebrates and has been the focus of most research thus far (for a review see Blader & Strähle, 2000). Recently, commercial resources (e.g. Zebrafish Information Resource Center, ZIRC) and the availability of selective genetic progeny (Sprague, Doerry, Douglas, & Westerfield, 2001) make research on the zebrafish an efficient and inexpensive addition to behavioral inquiries.

There have been an increasing number of research investigations highlighting the behavioral spectrum of the zebrafish and drug challenges. Because the zebrafish model affords an alternative and efficient mode of drug delivery via the gills, submersion has been the primary method used (Gerlai, Lee, & Blaser, 2006; Levin, Bencan, & Cerutti, 2007; Lockwood, Bjerke,

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Kobayashi, & Guo, 2004). Exogenous compounds, such as ethanol, have been shown to rapidly enter systemic circulation following introduction into the tank environment (Dlugos & Rabin, 2003). Acute ethanol exposure elicited a variety of behavioral effects in adult zebrafish on locomotor activity and shoal cohesion (Gerlai, Lahav, Guo, & Rosenthal, 2000). The reported effects on motoric function follow a characteristic inverted U-shaped function with intermediate doses of ethanol increasing locomotor activity and higher doses suppressing it. Interestingly, the effects of ethanol exposure on shoaling tendency are less clear. Fish treated with lower doses of acute ethanol and tested individually spent significantly more time in the vicinity of their reflection indicating possible shoaling tendencies, according to the authors (Gerlai, 2003). However, subsequent studies of group behavior revealed reduced shoal cohesion and increased scattering throughout the testing environment (Gerlai et al., 2000). The former may be due to aggressive tendencies elicited by acute ethanol exposure, as the author states. Decreased predator avoidance has been demonstrated following ethanol exposure leading researchers to draw conclusions regarding anxiolysis in the zebrafish previously seen in the rodent model for anxiety (Gerlai et al., 2006). The N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 increased circling behavior when measured in 13-cm diameter round glass dishes (Swain, Sigstad, & Scalzo, 2004). Exposure to MK-801 also induced behavioral changes on measurements such as locomotor activity and swim location.

The zebrafish shares more similar features to humans than other genetically homologous models, such as the *Drosophila* (Guo, 2004). Likewise, it is also postulated that the anatomical similarities of neurotransmitter pathways, like dopamine, may indicate comparable neural functionality. Dopaminergic modulation has been shown to produce overt changes in swim behavior that included disorientation and marked decreases in movement in the zebrafish (Anichtchik, Kaslin, Peitsaro, Scheinin, & Panula, 2004). Dopamine agonists (e.g. SKF 38393) elicited a variety of behavioral responses in the rodent model including hyperactivity and increased locomotion (Sobrian, Jones, Varghese, & Holson, 2003). Taken with the above evidence, it is highly plausible that zebrafish would exhibit a similar behavioral repertoire to pharmacological exposure. To date, little focus has been given to a comparative approach aimed at comparing and contrasting the behavioral changes that result from pharmacological intervention.

The current sets of experiments were designed to extend on the existing research regarding zebrafish behavioral assessment. We sought to investigate the effects of three established pharmacological agents (ethanol, MK-801, and SKF 38393) on previously reported behaviors seen in the *Danio* model (Engeszer, Da Barbiano, Ryan, & Parichy, 2007; Gerlai et al., 2000; Gerlai et al., 2006; Speedie & Gerlai, 2008). The drugs chosen were based on neurotransmitter systems effected and their potential roles in locomotion and shoaling displays. Furthermore, MK-801 (Chartoff, Heusner, & Palmiter, 2005), SKF 38393 (Rosengarten, Bartoszyk, Quartermain, & Lin, 2006), and ethanol (Colombo et al., 1998) have been shown to effect locomotor activity and produce stereotypic behavior in several animal models on a variety of behavioral paradigms. First, we examined the effect of each drug on individual behaviors previously shown to be sensitive to water-soluble compounds. The effect of drug exposure on shoaling displays was then explored in a separate

series of experiments using a novel open-field paradigm that assessed fish distribution over time. The results of the current study append to the growing body of literature on zebrafish behavior and introduce a novel approach to studying drug induced changes in overt locomotive behavior and shoaling tendency.

Method

Subjects

Adult zebrafish (*Danio rerio*) were housed in a community tank system at a temperature of approximately 27°C. The community system consisted of two 10-gallon tanks (holding approx. 30 fish per tank) and four 5-gallon tanks (holding approx. 10 fish each) connected to a main reservoir. Additionally, a separate (autonomous) 10-gallon tank was used to hold approximately 30 fish. Fish were kept on a 14 h on and 10 h off light cycle and were fed flake food twice daily (TetraMin, Blacksburg, VA). Tank system contained aeration and filtration units with de-chlorinated H_2O. Drug exposure and testing took place during the light cycle between 8:00 a.m. and 5:00 p.m. Both male and female fish were used in the experiments.

General procedure

In these series of reported investigations, subjects were videotaped and later observed to assess the effects of ethanlol, MK-801, and SKF 38393 on behaviors that reflect typical motor and schooling output as well as the assessment of side effects of drug exposure in zebrafish. As part of this three-tiered test battery, fish were individually exposed to treatment and tested both during exposure (overall mobility in a 250ml beaker) and for 37 minutes after exposure (circling behavior). In the final phase (open-field/schooling) we quantified the shoaling displays of zebrafish during drug exposure and tested subjects by using a group setting design that allowed for the display of social behaviors.

In the first study, subjects (n=30) were acutely exposed to the NMDA antagonist MK-801 at concentrations of 0.0 µM, 2 µM, and 20 µM (based on the dose range used by Swain et al., 2004). In the second study, a separate group of subjects (n=30) were immersed in micromolar concentrations (0.0 µM, 10 µM, 100 µM) of the dopamine D_1 subtype agonist SKF 38393. Doses of SKF 38393 were chosen to reflect minimum and maximal (but non-lethal) ends of a proposed dose range. The last study exposed a final group of fish (n=30) to three concentrations of ethanol: 0.0%, 0.5%, and 1.0% (v/v) to evaluate the aforementioned behavioral effects, where the dose range was acquired from numerous investigations of ethanol and zebrafish behavior (Dlugos & Rabin, 2003; Gerlai et al., 2006; Lockwood et al., 2004). Ethanol (95%, 190 proof) was diluted and prepared prior to fish exposure. All drugs were mixed with dechlorinated tank water at a temperature of approximately 27ºC. In all three studies subjects were randomly placed into one treatment group for each of the drugs in the investigation (fish were only tested once). Drug doses were calculated using the weights of the salts with the exception of ethanol, which was calculated as a percentage concentration (volume ethanol/volume pre-conditioned tank water).

Drug exposure behavior

Solutions were mixed in a 250 mL beaker containing 200 mL of de-chlorinated tank water. Each subject was randomly assigned to a treatment group and immersed in a solution containing the drug for one-hour. During this time, all behavior was videotaped using a Sony 8 mm camcorder positioned perpendicular to the 250 mL beakers for later assessment and scored by observers blind to treatments conditions. Scoring was done at set time intervals for thirty seconds at specific time points (5, 10, 20, 30, 45, and 55 min.) based on increments outlined by Swain and colleagues (2004). Observers scored for three behaviors when subsequently viewing videotape: *immobility time, erratic swimming,* and *top time*. With the exception of fin movement, immobility time was defined as the time a subject spent without movement in any direction. Erratic swimming was observed as the amount of time a subject spent swimming in an irregular and jostling fashion and included darting motions and rapid looping movements around the beaker. Finally, top time was the amount of time spent occupying the top half of the exposure

beaker. To minimize the potential influence of nearby subjects, opaque barriers were placed between the testing beakers to obscure the view of neighboring fish.

Circling behavior

The tendency for zebrafish to engage in repetitive circling behavior around the testing environment following acute exposure to the NMDA antagonist MK-801 has been reported previously (Swain et al., 2004). We sought to replicate, in part, these findings and to compare the known effects with two of the other agents under investigation in the current study (ethanol and SKF 38393). In this study, circling behavior was analyzed following a one-hour exposure to a treatment condition; this is in contrast to Swain and colleagues (2004), they measured drug-induced circling behavior in zebrafish during exposure time. Zebrafish were placed in a 6.5 x 13 cm Pyrex dish filled with 550 mL de-chlorinated tank water after being exposed for one hour to one of the following treatment conditions (in a separate 250 mL beaker): ethanol (0, 0.5%, 1.0%), SKF 38393 (0.0 µM, 10 µM, 100 µM), or MK-801 (0.0 µM, 2 µM, 20 µM). A camcorder was placed aerially above the dishes to record circling behavior exhibited by the fish. Following one-hour exposure at one of the aforementioned treatment conditions, subjects were placed in the dish of de-chlorinated tank water for 37 minutes while their behavior was recorded and subsequently scored by raters blind to treatment conditions. Observers would later score these videos and record behavior based on the number of laps (complete 360 degrees around test dish) swam in 30 seconds at five different time points (5, 10, 15, 20, 25 min.) as outlined by Swain et al. (2004).

Open-field – schooling/shoaling displays

Zebrafish are naturally a schooling fish (Gleason, Weber, & Weber, 1977). We tested their tendency to shoal in a separate series of experiments investigating the effects of the highest doses of the treatment regimens used, as defined by the results of the first two series of experiments. We used a novel open-field paradigm to investigate the distribution of fish in a set of quadrants over a given time frame. Two methods were used to examine the shoaling display of zebrafish when exposed to the highest dose of ethanol (1.0%), SKF 38393 (100µM), MK-801 (20µM), or control (de-chlorinated tank water). Nonparametric analyses were first applied to evaluate if the distribution of fish at a particular time point was significantly different from a known probability distribution. In the first method, the thirty-minute session time was broken into six five-minute time blocks (0-5, 5-10, 10-15, 15-20, 20-25, 25-30 minutes) to demonstrate change in schooling habits during drug exposure as a function of time. For each time block, the average frequency of fish (tabulated every 10 seconds) was calculated per quadrant (Cartesian system). The frequencies were then analyzed with the exact multinomial test to compare to a known frequency distribution. Secondly, multinomial tests were run at every ten-second interval for each five-minute time block to account for variations in independent measurements. An average p-value (cumulative probability) was then computed and graphed to illustrate significant shoaling on the part of the treatment and control groups. This was also done to complement quadrant statistical analysis. Groups consisted of either 5000 mL of tank water (control), 1% ethanol solution in 5000 of mL of tank water, SKF38393 (100µM in 5000 mL of tank water), or MK-801 (20µM in 5000 mL of tank water). Fish (n=10 for each group) were placed in a plastic tub (13 cm x 18 cm x 29 cm) filled with 5000 mL of regular de-chlorinated or drug-treated de-chlorinated tank water for 30 minutes. Each tub was divided into four equal quadrants with markings on the back wall of the tank so that the observers' views would not be hindered by the quadrant marking and fish would remain visible at all times. Schooling behavior was recorded via video and later scored in ten second intervals throughout the 30-minute session. At each interval, the number of fish in each quadrant was tabulated and recorded. In the case where a fish was isolated against a quadrant marking, the head of the fish was counted as the midpoint of the body axis and the corresponding quadrant was recorded (this procedure was used to represent swim path on the part of the fish). Quadrants were labeled using the Cartesian coordinate system and labeled in a counterclockwise fashion.

Statistical analyses: drug exposure, circling behavior, shoaling activity

Individual behaviors (immobility, erratic swimming, top time, and circling behavior) were analyzed using a mixed model ANOVA to assess significance for both within (time) and between (treatment) subjects effects. Post hoc analysis (Fisher's LSD) was used for multiple

comparisons of significant effects where found. Open-field/shoaling data were analyzed using the exact multinomial test to assess the frequency distribution of fish over the quadrants. Each 30-minute session was broken into six five-minute time blocks to evaluate schooling behavior change over time. The frequency of fish was averaged across quadrants for each time block and analyzed using the multinomial test. Frequencies were truncated to the .10 decimal place and rounded to the nearest integer before multinomial analysis. Independence of measures was accounted for by calculating cumulative probabilities at each ten-second interval using the multinomial test. Exact probabilities (p-values) were then averaged for each time block for subsequent illustration.

Results

Drug exposure behavior

The effects of ethanol, SKF 38393, and MK-801 were evaluated during a one-hour exposure time in an effort to characterize the behavioral effects of three drugs previously reported to be biologically active in zebrafish (Gerlai et al., 2000; Gerlai, Lee, & Blaser, 2006; Miller & Gerlai, 2007; Speedie & Gerlai, 2008). The most compelling effect was seen with the NMDA antagonist MK-801. Exposure to this drug resulted in increased time spent by each subject closer to the surface of the water. There was a significant effect of time [$F(2.52,62.93) = 3.21$, $p<.05$] and a significant interaction [$F(5.03,62.93) = 5.09$, $p<.01$] in the highest dose group (20 µM) for top time behavior. Figure 1 illustrates the effects of MK-801 on top time during an acute one-hour exposure. Post hoc testing (Fisher's LSD) revealed that at 45 and 55 minutes into the exposure session, fish in the highest dose group (20 µM) spent significantly ($p<.05$) more time in the top half of the exposure beaker than fish in the other treatment groups. No significant effects of the dopamine agonist SKF 38393 or ethanol were seen on any of the behaviors measured during acute drug exposure.

Circling behavior

Prior exposure to MK-801 (for 1 hour) and testing immediately afterward in "clean" tank water revealed a significant increase in circling behavior when compared to control [$F(2,21) = 3.77$, $p<.05$]. Post hoc analysis (Fisher's LSD) revealed that the 20.0 µM dose significantly increased circling activity over the two other treatment groups of 2.0 µM and 0 µM ($p<.05$; Fig. 2) for time measurements at five and ten minutes. SKF 38393 and Ethanol did not elicit any significant effects on circling behavior at the doses used in the current study.

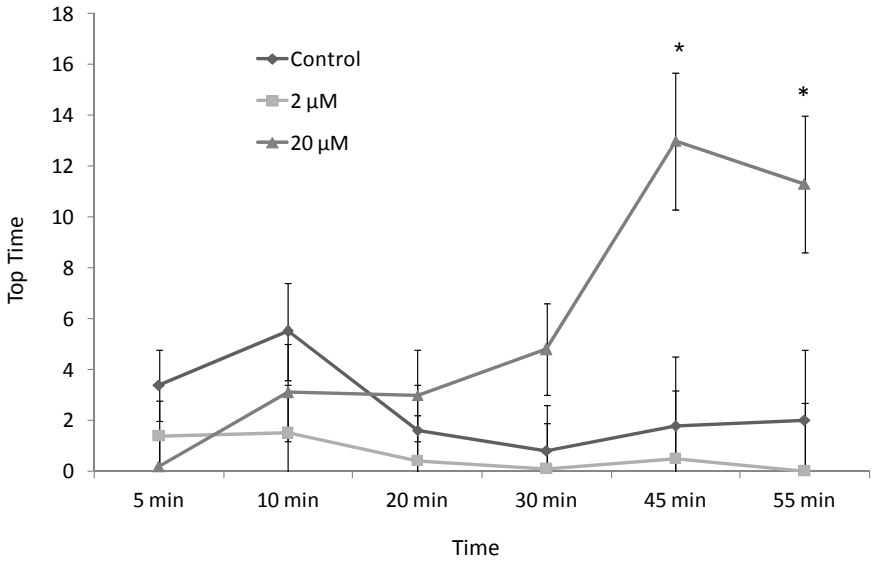


Figure 1. Effects of MK-801 on top time in adult zebrafish. There was a significant effect of session time and a significant interaction effect during acute exposure to the NMDA antagonist. As the session progressed, zebrafish increased the amount of time spent in the top half of the exposure beaker at the highest dose (20 µM; $p<.05$). Mean differences were analyzed using a mixed model ANOVA and Fisher's LSD post-hoc tests were used to indicate direction of effects where significant. Mean (±SEM) time (seconds) for each condition is shown, *$p<.05$.

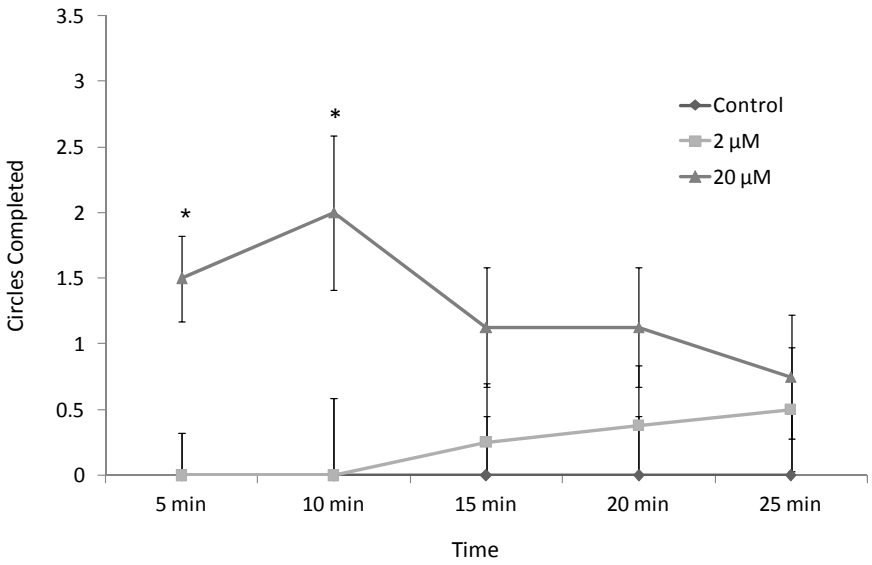
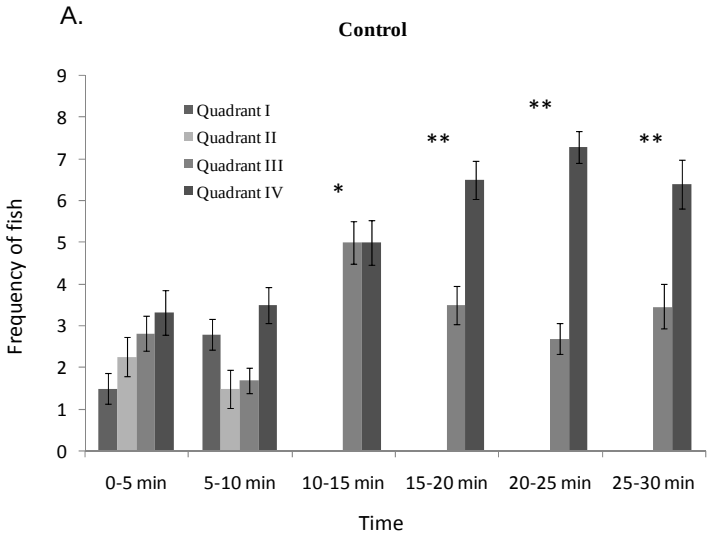


Figure 2. Effects of circling behavior following acute treatment with MK-801. The effects of MK-801 on circling behavior show the largest dose (20µM) significantly increased circling behavior over the 2 µM dose and controls ($p<.05$; n=24). Mean differences were analyzed using a mixed model ANOVA and Fisher's LSD post-hoc tests were used to indicate direction of effects where significant. Mean (± SEM) circles completed during 30 sec for each time point for each dose are shown.

Shoaling and schooling behavior

Shoaling behavior was notably affected by two of the three drugs under investigation in the current study. During these experiments subjects were tested in tanks containing either drug infused water or "clean" tank water (control). Results of the multinomial analysis for MK-801 (20µM in 5000 mL of tank water) and shoaling displays can be seen in Figures 3a,b and 4. Analysis of the control group revealed an initial disorganization for the first ten minutes of testing evidenced by non-significant multinomial probabilities for the 0-5 and 5-10 minute time blocks (*p*'s>.05). The distribution of control fish of each time block for the last twenty minutes was found to be statistically significant (*p*'s<.05) indicating an acclimation period in which fish eventually began to shoal as the session progressed. Results of the MK-801 group indicated a markedly different distribution over time. The distribution (average frequency) of fish was, in the first time block (0-5 min), found to be briefly significantly grouped (*p*=.03). The remaining distributions for the session were not significantly different from chance (*p*'s>.05). Figure 4 illustrates the average exact probability (p-value) for each time block during MK-801 treatment and largely agrees with quadrant analyses. Although the graphical representation of p-values shows increasing and decreasing slopes, the interpretation of the data is strictly dichotomous. Average p-values found above the indicated significance level should, in theory, represent non-significant results in regards to the behavior of interest (lack of shoaling).



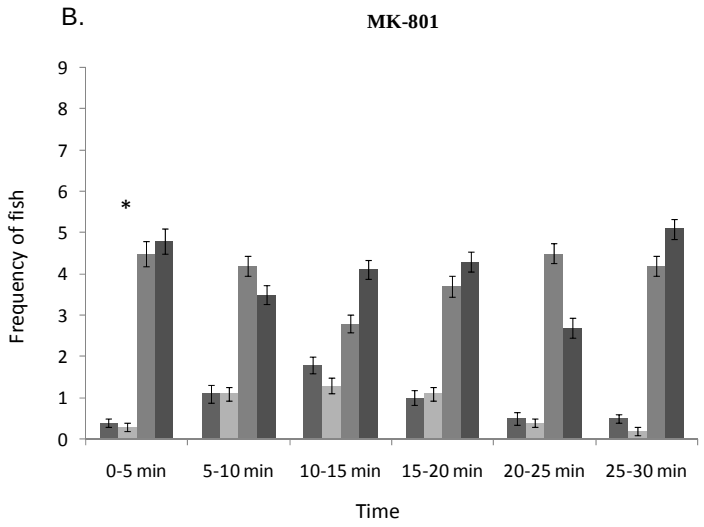


Figure 3a,b. Effects of the NMDA antagonist MK-801 (20µM) on shoaling displays. (a) The control group (n=10) initially shows a lack of shoaling behavior before becoming acclimated to their surroundings; fish began shoaling around 10 minutes into the session as shown by significant multinomial probabilities across the remaining time blocks (*p*'s<.05). (b) Fish exposed to MK-801 (n=10) exhibited greater disruption of shoaling behavior than controls. Shoaling behavior was briefly displayed (*p*=.03) but as the session progressed, fish began to display disorganization that proceeded until the end of testing. Multinomial statistical analyses were used to calculate p-values from mean frequencies of each quadrant. Mean (±SEM) frequencies are shown, *p<.05 **p<.001.

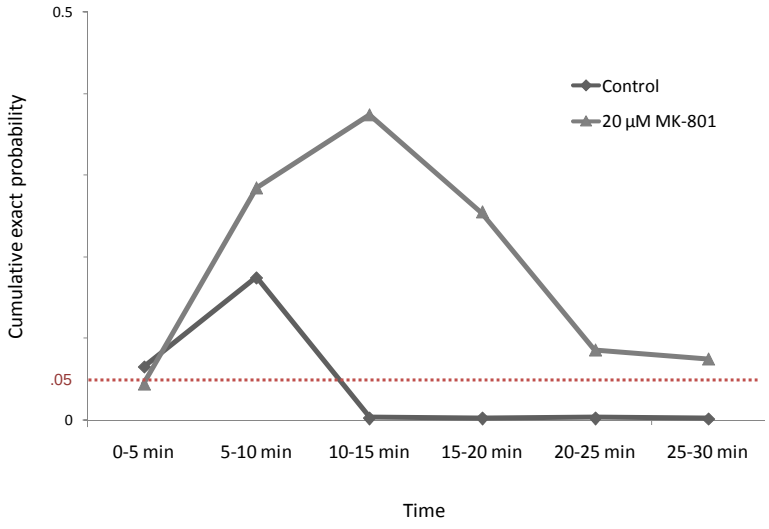
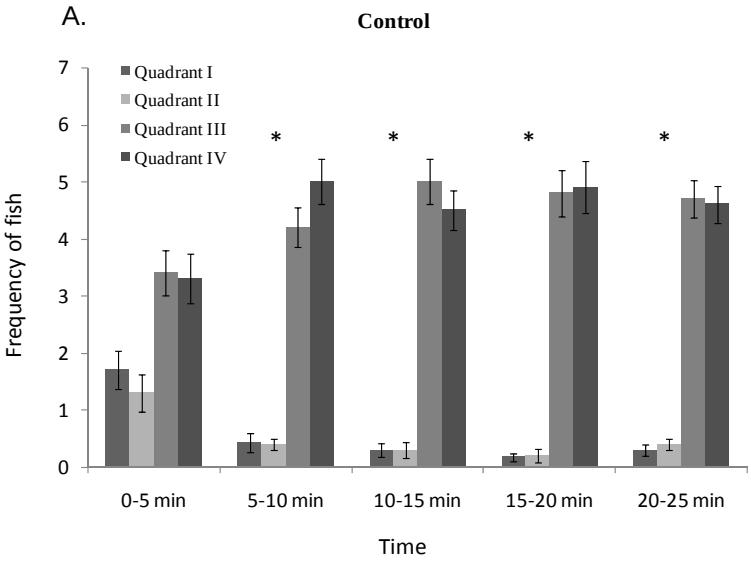


Figure 4. Distribution of cumulative probabilities reflecting the effects of MK-801 (20µM) on shoaling behavior. Data points represent average p-values of each time block for control and drug groups. Interpretation of data indicates a dichotomous relationship with respect to significance. Cumulative probabilities show similar trends to quadrant analyses. The first ten minutes reveals non-significant p-values for control fish after which they become and remain significant throughout the session. Fish exposed to MK-801 briefly shoal (*p*=.03; 0-5 min) the group then becomes disrupted and disorganized for the remainder of the session (*p*'s>.05). Multinomial tests were used to calculate exact probabilities at 10 second intervals during open-field testing. Significance is indicated by the dashed line labeled at .05.

The results of exposure to SKF 38393(100μM in 5000 mL of tank water) also indicated a deviation in shoaling distribution over time as compared to controls. Figure 5a,b illustrates the results of multinomial analysis of quadrant frequency during SKF 38393 exposure. Similar to control fish during MK-801 testing, subjects not exposed to the dopamine agonist showed an initial (0-5 min. time block) acclimation period for five minutes followed by shoaling display for the remainder of the session. Multinomial tests were significant for each quadrant for the remainder of the session (p's<.05). Exposure to SKF 38393 initially mirrored that of controls with a non-significant p-value during the first five minutes (p=0.86) followed by brief shoaling in the 5-10 minute time block (p<.05). Results of statistical analyses revealed non-significant p-values for the remaining time blocks in the session; cessation of shoaling (p's>.10). A graphical representation of the cumulative probabilities for SKF 38393 can be seen in Figure 6. Again, fish exposed to the dopamine agonist initially follow the same results as control fish until the 10-15 minute time block where the average multinomial probability becomes non-significant and shoaling behavior is disrupted.



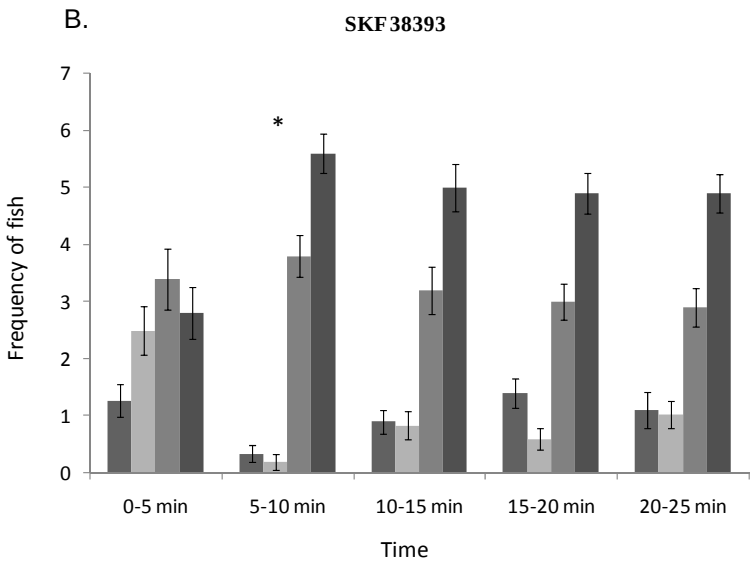


Figure 5a,b. Effects of the dopamine D_1 subtype agonist SKF 38393 (100µM) on shoaling displays. (a) The control group (n=10) after 5 minutes of acclimation display shoaling for the remainder of the session as shown by significant multinomial probabilities across the remaining time blocks (*p*'s<.05). (b) Fish in the SKF 38393 group followed the same trend as the control group in which probabilities were not significant for the first 5 minutes (*p*=.86) and significant during the 5-10 minute time block (*p*=.03). The remainder of the session witnessed a disruption in shoaling behavior with non-significant multinomial probabilities (*p*'s>.05). Multinomial statistical analyses were used to calculate p-values from mean frequencies of each quadrant. Mean (±SEM) frequencies are shown, *p<.05.

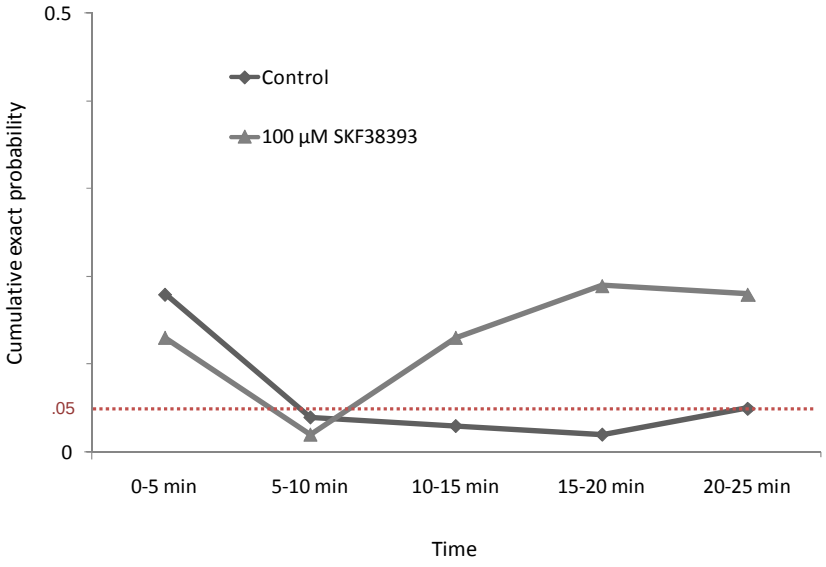


Figure 6. Distribution of cumulative probabilities reflecting the effects of the dopamine agonist SKF 38393 (100µM) on shoaling behavior. Data points represent average p-values of each time block for control and drug groups. Multinomial tests were used to calculate exact probabilities at 10 second intervals during open-field testing. Significance is indicated by the dashed line labeled at .05.

The non- effect of ethanol (1% ethanol solution in 5000 of mL of tank water) on shoaling behavior can be seen in Figure 7a,b. Multinomial probabilities throughout the entire session were found to be statistically significant for each time block (*p*'s<.01). A distribution of the cumulative probabilities calculated from each ten-second interval can be seen in Figure 8. Like quadrant analyses, multinomial probabilities were found to be significant for both control fish and fish exposed to 1% ethanol. Therefore, 1% ethanol yielded no disruption of shoaling display when compared to controls.

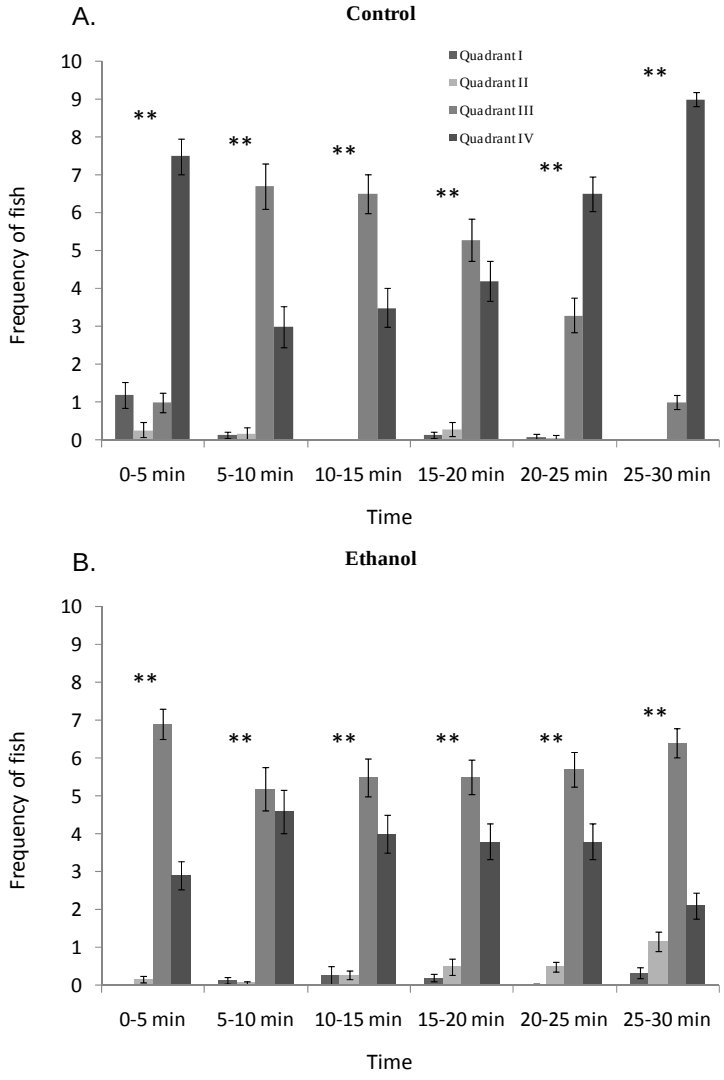


Figure 7a,b. Effects of 1% ethanol on shoaling displays. (a) Multinomial probabilities were found to be statistically significant throughout the shoaling session for the control group (*p*'s<.01). (b) Fish exposed to 1% ethanol displayed similar shoaling behavior to controls with significant probabilities for the entire exposure session (*p*'s<.01). Multinomial statistical analyses were used to calculate p-values from mean frequencies of each quadrant. Mean (±SEM) frequencies are shown, **p<.001.

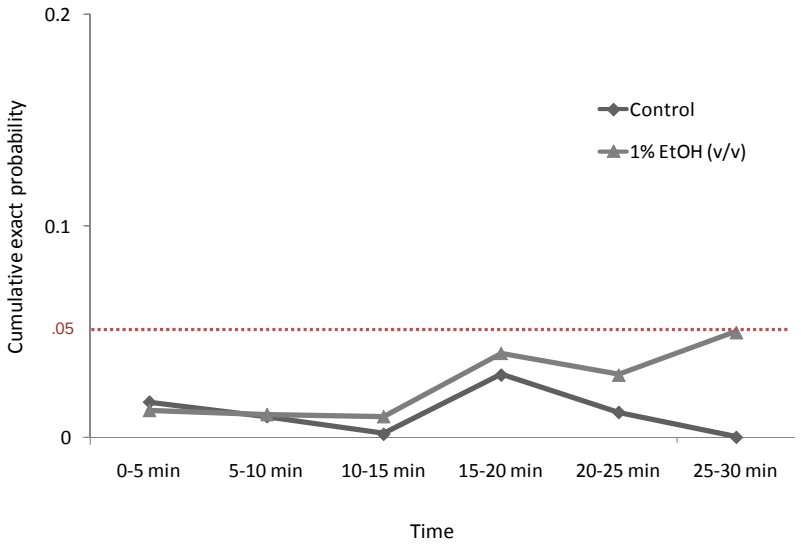


Figure 8. Distribution of cumulative probabilities reflecting the non-effect of 1% ethanol on shoaling behavior in zebrafish. Data points represent average p-values of each time block for control and drug groups. Multinomial tests were used to calculate exact probabilities at 10 second intervals during open-field testing. No difference in average cumulative probability was found at each time block for ethanol and control. Significance is indicated by the dashed line labeled at .05.

Discussion

The current studies reported here introduce a novel behavioral test battery aimed at identifying drug-induced alterations in social and motoric behaviors. The drugs used in the current study (ethanol, MK-801, and SKF 38393) were chosen for their observed effects on both rat and human performance. Previous reports of induced alterations in swim behavior following treatment with ethanol (Dlugos & Rabin, 2003; Gerlai, 2000) and MK-801 (Swain et al., 2004) also provided an impetus and rationale for the current investigations. In the studies reported here, both individual and group behaviors were significantly affected by exposure to one or more of the agents under investigation. Exposure to the NMDA antagonist MK-801 (0.0 µM, 2 µM, 20 µM) led to an increase in the amount of time spent at the water surface during drug exposure and an increase in circling behavior following a one-hour exposure, but only with the largest dose used in this study. Measures on these two tests (drug exposure and circling behavior) yielded no difference from control groups when fish were exposed to SKF 38393 (0.0 µM, 10 µM, 100 µM) or ethanol (0%, 0.5%, 1.0%). Most notably, when employing the use of a novel open-field paradigm designed to measure shoaling displays, subsequent analyses revealed distinctive differences between drug and control groups for MK-801 (20µM) and SKF 38393 (100µM). Both of these drug treatment groups exhibited a period where shoaling activity was displayed briefly, during early stages of the observation session. Eventually, fish began to disperse, and shoaling ceased as the sessions progressed (approximately 5 and 10 minutes for MK-801 and SKF 38393, respectively). This behavior can be plausibly

correlated to the duration of time needed for drug absorption via the gills, and time needed for the drug to systemically circulate and arrive at the receptor. Exposure to ethanol (1%) had no significant effect on shoaling behavior as compared to control fish. Control groups for all three investigations (MK-801, SKF 38393, and ethanol) exhibited stable and reliable baselines of the species-specific behavior of interest, shoaling.

Our results make an interesting complement to previous behavioral assessments of MK-801 (e.g., Swain et al., 2004). The current study observed circling behavior *following* a one-hour exposure to MK-801, in contrast to the aforementioned study that assessed behavior *during* drug exposure. This deviation from previously reported methods enabled the illustration of a time course for circling behavior and likely, the drug's effect at the receptor site. Our results showed a distinct decrease in circles as a function of time for the group exposed to the 20µM dose. One possible explanation could be that the outward behavioral exhibition of circling is a function of MK-801's effects at the receptor level. The fact that circling behavior decreased across all subjects as the session progressed further supports this idea. Using the post-exposure method in the current study might also explain the discrepancies found between the current results and those reported by Swain et al. (2004) regarding the significance of the lower dose (2 µM) and mean number of circles completed during a session. Our findings on top time behavior *during* MK-801 exposure are consistent with the zebrafish literature. Previous reports of increased surface time have been hypothesized to be the result of a possible disruption in swim bladder functioning (Swain et al., 2004), which is often coupled with a fish's inability to remain upright. However we did not observe this behavior. An alternative, and potentially more plausible, explanation might be the increasing need for an oxygen-rich environment due to an overall global impairment resulting from exposure to the highest dose, 20 µM (Fig. 1). This behavior has been previously demonstrated in the zebrafish exposed to environmental toxins and was coupled with increased respiration and darkened pigmentation (Bretaud, Lee, & Guo, 2004).

The novel open-field paradigm used in these studies can reliably gauge shoaling behavior in the zebrafish and this behavior appears to be sensitive to both NMDA and dopamine receptor manipulation. This is particularly evident for the drugs MK-801 and SKF 38393, by which the overall distribution of fish increased as time progressed resulting in a decrease in shoaling behavior. Changes in shoaling activity during MK-801 exposure (20 µM) may be associated with increased disorientation, as seen in other animal models, or a change in overall activity level. Decreased shoaling behavior, in this case, is probably not due to a decrease in motor activity. 20 µM MK-801 as reported produced an increase in circling behavior, which is indicative of hyperactivity. In rodents, thigmotaxic displays have previously been reported as an increase in behavioral responding, likely do to anxiety (Lukoyanov & Paula-Barbosa, 2000; Prut & Belzung, 2003). The supposition that increased locomotor activity may account for decreased shoaling behavior is further supported by findings that high doses (20 µM and 50 µM) of MK-801 have been shown to increase swim speed and turning angle in the zebrafish (Panula et al., 2006).

The lack of effect for ethanol on this three-tiered test battery (exposure, circling behavior, shoaling displays) reported here might seem at first glance, to be inconsistent with previous reports of acute exposure in the zebrafish. The

most notable of these discrepancies is the purported disruption of shoaling behavior resulting from ethanol exposure using concentrations of 0.25%, 0.50%, and 1.0% reported elsewhere (Dlugos & Rabin, 2003; Gerlai, 2003; Gerlai et al., 2000). Consider that our shoaling paradigm is a novel design that has the essential element of assessing the frequency distribution of group behavior over a set of quadrants. This is in contrast to previous shoaling analyses where inter-individual distances were the dependent measure (Dlugos & Rabin, 2003) and another study which assessed a zebrafish's reaction to its own reflection (Gerlai, 2003). Results obtained by Gerlai found that conspecific preference (time spent in proximity to stimulus fish) was reduced in the presence of ethanol and the effect was dose dependent. This novel open field paradigm assesses the overall behavior of a group, instead of looking at individual differences. Future directions dictate that a wider dose range for ethanol be tested, as shoaling display is sensitive to changes induced by NMDA and dopaminergic manipulation (evidenced by exposure to MK-801 and SKF 38393).

The results presented here append to the growing body of literature utilizing the zebrafish as a behavioral model of investigation. We present for the first time, a novel paradigm that measures shoaling displays based on a modified open field design. This task in conjunction with the drug exposure and circling behavior tasks form a comprehensive test battery that can profile drug-induced alterations in social and motoric behaviors. Although only our first report this novel task is sensitive to NMDA and dopamine receptor manipulation. While both manipulations disrupted shoaling displays only the NMDA manipulation also yielded higher than normal circular swimming displays and increased surface swimming across the entire test battery. That is, MK-801 (20 μM) disrupted group-shoaling behavior when tested on the novel task. This same dose when administered to fish individually (during two different experiments) caused exposed fish to exhibit circular swimming patterns more frequently than controls and during drug exposure caused individual fish to spend significantly more time in the top half of the testing apparatus. This pattern of impairment suggests that MK-801 (20 μM) increases motoric output and may also cause the zebrafish to require a more oxygen-rich environment due to an overall global impairment. Compare this to the application of SKF 38393 (100 μM), where dopaminergic manipulation resulted in no behavior change during individual drug exposure or assessment on the circular swimming task. However, group-shoaling displays were significantly impaired when fish were exposed to SKF 38393 (100 μM).

The use of nonparametric analyses to date has not been utilized when assessing shoaling displays in the zebrafish. Our findings suggest that the design is appropriate for analysis of fish distribution as it relates to shoaling behavior. The fact that quadrant analysis and average probability trends compliment and agree with one another further supports the reliability of nonparametric analysis use when assessing these behaviors. Furthermore, consistent significant grouping behavior for control fish indicates uniformity among independent variables. Taken together, these tasks form a comprehensive battery of tests aimed at unambiguously identifying drug-induced alterations in social and motoric behaviors in the zebrafish. Lastly, we have presented additional evidence that supports the utility of this species in behavioral applications.

References

Anichtchik, O. V., Kaslin, J., Peitsaro, N., Scheinin, M., & Panula, P. (2004). Neurochemical and behavioural changes in zebrafish *Danio rerio* after systemis administration of 6 hydroxydopamine and 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine. *Journal of Neurochemistry,* **88**, 443-453.

Beis, D. & Stainier, D. Y. R. (2006). *In vivo* cell biology: Following the zebrafish trend. *TRENDS in Cell Biology,* **16**, 105-112.

Blader, P. & Strähle, U. (2000). Zebrafish developmental genetics and central nervous system development. *Human Molecular Genetics,* **9**, 945-951.

Bretaud, S., Lee, S., & Guo, S. (2004). Sensitivity of zebrafish to environmental toxins implicated in Parkinson's disease. *Neurotoxicology and Teratology,* **26**, 857-864.

Chartoff, E. H., Heusner, C. L., & Palmiter, R. D. (2005). Dopamine is not required for the hyperlocomotor response to NMDA receptor antagonists. *Neuropsychopharmacology,* **30**, 1324-1333.

Colombo, G., Agabio, R., Lobina, C., Reali, R., Vacca, G., & Gessa, G. L. (1998). Stimulation of locomotor activity by voluntarily consumed ethanol in Sardinian alcohol-preferring rats. *European Journal of Pharmacology*, **357**, 109-113.

Dlugos, C. A. & Rabin, R. A. (2003). Ethanol effects on three strains of zebrafish: Model system for genetic investigations. *Pharmacology, Biochemistry and Behavior,* **74**, 471-480.

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C. F., Malicki, J., Stemple, D. L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J., & Boggs, C. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development,* **123**, 37-46.

Engeszer, R. E., Da Barbiano, L. A., Ryan, M. J., & Parichy, D. M. (2007). Timing and plasticity of shoaling behaviour in the zebrafish, *Danio rerio*. *Animal Behavior*, **74**, 1269-1275.

Gerlai, R. (2003). Zebra Fish: An uncharted behavior genetic model. *Behavioral Genetics,* **33**, 461-468.

Gerlai, R., Lahav, M., Guo, S., & Rosenthal, A. (2000). Drinks like a fish: Zebrafish (*Danio rerio)* as a behavioral genetic model to study alcohol effects. *Pharmacology, Biochemistry and Behavior,* **67**, 773-782.

Gerlai, R., Lee, V., & Blaser, R. (2006). Effects of acute and chronic exposure on the behavior of adult zebrafish (*Danio rerio*). *Pharmacology, Biochemistry and Behavior,* **85**, 752-761.

Gleason, P.E., Weber, P.G., & Weber, S.P. (1977). Effect of group size on avoidance learning in zebrafish, *Brachydanio rerio*. *Animal Learning and Behavior*, **5**, 213-216.

Grunwald, D. J. & Eisen, J. S. (2002). Headwaters of the zebrafish: Emergence of a new model vertebrate. *Nature Reviews Genetics,* **3**, 717-724.

Guo, S. (2004). Linking genes to brain, behavior, and neurological diseases: What can we learn from zebrafish? *Genes, Brain, and Behavior,* **3**, 63-74.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A.,Odenthal, J., van Eeden, F. J. M., Jiang, Y. J., Heisenberg, C. P., Kelsh, R. N., Furutani Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., & Nüsslein-Volhard, C. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development*, **123**, 1-36.

Levin, E. D., Bencan, Z., & Cerutti, D. T. (2007). Anxiolytic effects of nicotine in zebrafish. *Physiology and Behavior,* **90**, 54-58.

Lockwood, B., Bjerke, S., Kobayashi, K., & Guo, S. (2004). Acute effects of alcohol on larval zebrafish: A genetic system for large-scale screening. *Pharmacology, Biochemistry and Behavior*, **77**, 647-654.

Lukoyanov, N. V., & Paula-Barbosa, M. M. (2000). A single high dose of dizocilpine produces long-lasting impairment of the water maze performance in adult rats. *Neuroscience Letters*, **285**, 139-142.

Miller, N. & Gerlai, R. (2007). Quantification of shoaling behavior in zebrafish (*Danio rerio*). *Behavioural Brain Research*, **184**, 157-166.

Miklósi, A. & Andrew, R. J. (2006). The zebrafish as a model for behavioral studies. *Zebrafish*, **3**, 227-234.

Panula, P., Sallinen, V., Sundvik, M., Kolehmainen, J., Torkko, V., Tiittula, A., Moshnyakov, M., & Podlasz, P. (2006). Modulatory neurotransmitter systems and behavior: Towards zebrafish models of neurodegenerative diseases. *Zebrafish*, **3**, 235-247.

Prut, L. & Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs onanxiety-like behaviors: A review. *European Journal of Pharmacology*, **463**, 3-33.

Rosengarten, H., Bartoszyk, G. D., Quartermain, D., & Lin, Y. (2006). The effect of chronic administration of sarizotan, 5-HT1A agonist/D3/D4 ligand, on haloperidol-induced repetitive jaw movements in rat model of tardive dyskinesia. *Progress in NeuroPsychopharmacology & Biological Psychiatry*, **30**, 273-279.

Sobrian, S. K., Jones, B. L., Varghese, S., & Holson, R. R. (2003). Behavioral response profiles following drug challenge with dopamine receptor agonists and antagonists in developing rat. *Neurotoxicology and Teratology*, **25**, 311-328.

Speedie, N. & Gerlai, R. (2008). Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). *Behavioural Brain Research*, **188**, 168-177.

Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). The behaviour and ecology of the zebrafish, *Danio rerio*. *Biol. Rev.*, **83**, 13-34.

Sprague, J., Doerry, E., Douglas, S., & Westerfield, M. (2001). The zebrafish information network (ZFIN): A resource for genetic, genomic and developmental research. *Nucleic Acids Res.*, **29**, 87-90.

Swain, H. A., Sigstad, C., & Scalzo, F. M. (2004). Effects of dizocilpine (MK-801) on circling behavior, swimming activity, and place preference in zebrafish (*Danio rerio*). *Neurotoxicology and Teratology*, **26**, 725-729.