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Behavioral effects of bidirectional modulation of brain monoamines by reserpine and d-amphetamine in zebrafish

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Abstract

Brain monoamines play a key role in the regulation of behavior. Reserpine depletes monoamines, and causes depression and hypoactivity in humans and rodents. In contrast, d-amphetamine increases brain monoamines' levels, and evokes hyperactivity and anxiety. However, the effects of these agents on behavior and in relation to monoamine levels remain poorly understood, necessitating further experimental studies to understand their psychotropic action. Zebrafish (*Danio rerio*) are rapidly emerging as a promising model organism for drug screening and translational neuroscience research. Here, we have examined the acute and long-term effects of reserpine and d-amphetamine on zebrafish behavior in the novel tank test. Overall, d-amphetamine (5 and 10 mg/L) evokes anxiogenic-like effects in zebrafish acutely, but not 7 days later. In contrast, reserpine (20 and 40 mg/L) did not evoke overt acute behavioral effects, but markedly reduced activity 7 days later, resembling motor retardation observed in depression and/or Parkinson's disease. Three-dimensional 'temporal' (X, Y, Time) reconstructions of zebrafish locomotion further supports these findings, confirming the utility of 3D-based video-tracking analyses in zebrafish models of drug action. Our results show that zebrafish are highly sensitive to drugs bi-directionally modulating brain monoamines, generally paralleling rodent and clinical findings. Collectively, this emphasizes the potential of zebrafish tests to model complex brain disorders associated with monoamine dysregulation.

Keywords

zebrafish; reserpine; d-amphetamine; anxiety; depression; monoamines; locomotor activity; novel tank test

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1. Introduction

The monoamines dopamine, serotonin and norepinephrine play a key role in the regulation of brain functions in animals and humans (Baumeister et al. 2003). Active clinically and in experimental (animal) models, d-amphetamine and reserpine exert opposing effects on brain monoamine levels. D-amphetamine is a traditional psychostimulant drug which elevates brain monoamines via several mechanisms, including competitively inhibiting monoamine oxidase (Miller et al. 1980; Filingier and Stefano 1982), and plasma membrane monoamine transporters (Sulzer et al. 1995; Sabol and Seiden 1998), also reversing these transporters to promote the synaptic release of monoamines (Seiden et al. 1993). The clinical effects of d-amphetamine include mania, euphoria (Janowsky 2003; Hall et al. 1996), addiction, hallucinations, agitation, anxiety/panic (Kokkinidis and Anisman 1981; Hall et al. 1996), as well as elevated body temperature, blood pressure, heart rate and plasma cortisol (Hamidovic et al. 2010; Sofuoglu et al. 2008). In animals, d-amphetamine evokes anxiety in the elevated plus maze (Biala and Kruk 2007) and other paradigms (Lapin 1993; Markham et al. 2006; Rotllant et al. 2010).

Reserpine is a natural alkaloid isolated from *Rauwolfia serpentine* (Shamon and Perez 2009; Huffman and Stern 2007), which depletes monoamines by irreversibly blocking their vesicular monoamine transporter (VMAT) (Erickson et al. 1992). In humans, this agent generally induces antipsychotic, calming (tranquilizing) and pro-depressant effects (Quetsch et al. 1959; Freis 1954; Baumeister et al. 2003; Estes 1995; Bigelow 2006; Yaniv and Bachrach 2005). In rodents, reserpine causes hypoactivity (Williams and Pirch 1974), motor stereotypies (Neisewander et al. 1991), akinesia (Dolphin et al. 1976), lethargy (Sigg et al. 1965) and anhedonia (Skalisz et al. 2002), relevant to depression (Borison et al. 1978; Lee et al. 2012) and Parkinson's disease (PD) (Duty and Jenner 2011). The drug also exerts conflicting action on clinical (Shamon and Perez 2009; Sarwer-Foner and Ogle 1956; Starkweather 1959) and animal anxiety, including no effects (Angrini et al. 1998; Heslop and Curzon 1999), increased (Angrini et al. 1998; Heslop and Curzon 1999; Haggendal and Lindqvist 1963; Ahlenius and Salmi 1994; LaBuda and Fuchs 2002) or reduced anxiety (Xu et al. 1992) (also see tranquilizing effects in some fish species (Turner and Carl 1955; Cano 1959)).

However, further experimental studies to understand the psychotropic action of d-amphetamine and reserpine are need to fully understand their effects on behavior as well as their relation to monoamine levels (Baumeister et al. 2003; Haggendal and Lindqvist 1963; Duty and Jenner 2011; Labonte et al. 2012; Hu et al. 2002). Increasing the spectrum of animal model organisms has recently been recognized as the strategic direction in translational biological psychiatry research (Kalueff et al. 2007; Stewart et al. 2011a), potentially targeting 'core' disordered domains and pathways that may be evolutionarily conserved across species. Zebrafish (*Danio rerio*) are rapidly emerging as a novel species for biomedical research (He et al. 2006; Hogan et al. 2008; Champagne et al. 2010), as they exhibit substantial physiological and genetic similarity to humans, and possess all major brain structures, neurotransmitters, receptors, and hormones (Panula et al. 2006; Alsop and Vijayan 2009). Zebrafish also become a promising aquatic model for efficient drug screening, demonstrating high sensitivity to psychotropic agents of various classes (Egan et al. 2009; Berghmans et al. 2007; Chakraborty et al. 2009; Collins 2012). Based on recent advances in drug and biomarker screening in this species, the present study examined the acute and long-term effects of d-amphetamine and reserpine on zebrafish behavior and physiology. Overall, this study suggests that zebrafish may hold potential for investigating brain disorders associated with monoamine dysregulation, as well as the pathogenic mechanisms underlying the comorbidity of drug abuse and anxiety. In particular, as simpler model organisms, zebrafish may be optimal to detect the effects of monoaminergic drugs

due to their less complex, yet robust and sensitive, behavioral responses, and may therefore offer significant potential as a model organism for antidepressant, antipsychotic and/or anti-PD drug discovery.

2. Methods

2.1 Animals and housing

A total of 151 adult (5–7 month old) male and female (approximately 50:50) wild-type short-fin zebrafish were obtained from a local commercial distributor (50 Fathoms, Metairie, LA). All fish were housed in groups of 15–30 fish per 40-L tank (filled with filtered facility water maintained at 25–27°C) on a 14:10-h cycle. Fish were fed TetraMin Tropical Flakes twice daily. All fish used in this study were experimentally naïve. Following behavioral testing, the animals were euthanized in 500 mg/L Tricaine (Sigma-Aldrich, St. Louis, MO), and immediately dissected on ice for further analysis. Animal care and experimentation adhered to national and institutional regulations. Animal experiments were approved by TU and ZENEREI Institute LLC.

2.2 Behavioral testing and apparatuses

Behavioral testing was performed between 11.00 and 15.00 h with tanks filled with water adjusted to the holding room temperature. Zebrafish were acclimated to the behavioral testing room 1 h prior to experimentation. To avoid the test battery effect, each test was performed on a separate cohort of naïve fish. Zebrafish behavior was recorded by two trained observers (inter-rater reliability >0.85), manually scoring different behavioral endpoints with subsequent analysis of traces by Ethovision XT8.5 (Noldus IT, Wageningen, Netherlands).

The novel tank test, used to assess zebrafish anxiety and locomotion (Bencan et al. 2009; Levin et al. 2007; Egan et al. 2009), 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 line marking the outside walls (Egan et al. 2009). Zebrafish behavior was assessed by scoring the latency to reach the top half of the tank (s), time spent in top (s), number of entries to the top, erratic movements, as well as the number and duration (s) of freezing bouts. Erratic movements were defined as sharp or sudden changes in direction of movement or repeated darting behavior (Cachat et al. 2010d). Freezing was defined as a total absence of movement, except for the gills and eyes, for 2 s or longer (Blanchard et al. 1998).

2.3. Video-tracking

During manual observation, videos were recorded in MPEG1 format with the maximum sample rate 30 fps for each trial by autofocusing 2.0 MP USB webcams, placed 50 cm in front of or on top of the tanks, and attached to laptop computers. For each experiment, raw track data was exported into Excel spreadsheets, pre-processed and formatted to generate 3D swim path reconstructions, as described previously (Cachat et al. 2010c; Cachat et al. 2011). 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. Generated 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 (on a consensus basis) as representative of the group, to illustrate the spatial pattern of zebrafish swimming, as explained in (Cachat et al. 2011; Grossman et al. 2010; Kyzar et al. 2012b).

2.4 Pharmacological manipulations

Because prior literature is unavailable on effective doses of d-amphetamine and reserpine in zebrafish, we undertook a pilot pharmacological study. In pilot studies, 0.5 mg/L d-amphetamine (NIDA, NIH, Bethesda, MD) did not evoke robust changes in behavior, while 20 mg/L caused prominent sedation/freezing, preventing behavioral observations (data not shown). Therefore, the use of mild doses (5 and 10 mg/L) of d-amphetamine was chosen for this study. We also found that treatment with 0.5 and 5 mg/L reserpine (Sigma-Aldrich, St. Louis, MO) acutely did not significantly affect zebrafish behavior. Prior to all experiments, zebrafish were pre-exposed for 20 min (based on previous experience with similar compounds (Riehl et al. 2011b; Grossman et al. 2010; Stewart et al. 2011c; Kyzar et al. 2012c)) to either water or an experimental dose (5 or 10mg/L for d-amphetamine and 20 or 40mg/L for reserpine) in a 3-L plastic beaker. The fish ($n = 11$ – 15 in each group) were then tested in the standard, 6-min novel tank test. We also examined the long-term effects of d-amphetamine and reserpine, exposing zebrafish to a 6-min novel tank test 7 days after a single 20-min treatment.

2.5 Statistical analysis

Data obtained in the novel tank test was analyzed in SPSS for each drug separately, using one-way ANOVA (factor: dose), followed by a post-hoc Tukey test for significant results. Data were expressed as mean $\pm$ SEM. Significance was set at $p < 0.05$ in all experiments

3. Results

Overall, acute d-amphetamine (5 and 10 mg/L) evoked an anxiogenic-like effect in zebrafish, modulating several behavioral endpoints, including the latency to enter the top ($F_{(2, 35)} = 5.196$; $P < 0.05$), the time spent and the number of entries to the top of the tank ($F_{(2, 35)} = 5.532$ and 5.684 , respectively; $P < 0.01$). Interestingly, while acute d-amphetamine treatment significantly decreased the time spent and the number of entries to the top of the tank, it shortened the latency to enter the top. The shortened top latency, combined with a lack of freezing, suggests acute d-amphetamine also evokes an increase in locomotion concurrent with the anxiogenic response observed here (Fig. 1A). The 3D spatiotemporal reconstructions of zebrafish locomotion further supported this observation, revealing a mild hyperlocomotion with bouts of increased velocity for d-amphetamine treated fish (Fig. 1A). In general, we also observed the lack of overt effects in the 6-min novel tank test 7 days after a single 20-min treatment with d-amphetamine. The exception to this observation was a slight modulation of erratic movements ($F_{(2, 33)} = 3.453$; $P < 0.05$), characterized by a trending, but non-significant, elevation in erratic behavior for the 10 mg/L group (Fig. 1B).

In contrast to d-amphetamine, reserpine (20 and 40 mg/L) did not evoke overt acute behavioral effects (Fig. 2A), but markedly reduced activity 7 days later. Specifically, reserpine-exposed fish exhibited alterations in several behavioral indices on Day 7, including latency to enter the top and freezing duration ($F_{(2, 44)} = 3.452$ and 3.641 , respectively; $P < 0.05$). The long-term effects of single reserpine exposure markedly increased both the top latency and time spent frozen (Fig. 2B). Notably, zebrafish swim patterning on Day 7 resembled the motor retardation observed in depression and/or PD, further coupled with a distinct “droopy tail” phenotype (Fig. 2C). This observation was further supported by 3D swim path reconstructions, showing a hypolocomotion characterized by a decrease in velocity and distance traveled, with an often restricted range of overall swimming throughout a given trial (Fig. 2C).

4. Discussion

This study is the first report examining in-depth the effects on anxiety and locomotion produced in zebrafish by d-amphetamine and reserpine, the two compounds that differentially modulate central monoaminergic systems. Overall, d-amphetamine evoked prominent anxiogenic-like responses acutely, but did not induce long-term effects on zebrafish behavior (Fig. 1). In contrast, reserpine had no overt effects on zebrafish behavior acutely, but produced marked hypolocomotion 7 days later (Fig. 2).

While d-amphetamine was tested in some zebrafish behavioral models previously, these studies have mostly focused on reward behavior (Webb et al. 2009; Ninkovic and Bally-Cuif 2006; Ninkovic et al. 2006) and did not assess anxiety or motor domains. Our present findings of increased anxiety following d-amphetamine administration in adult zebrafish (Fig. 1) indicates that their affective behavior (like in rodents and humans (Biala and Kruk 2007; Lapin 1993; Markham et al. 2006; Rotllant et al. 2010; Janowsky 2003; Sulzer et al. 1995)) is strongly affected by this drug. Given the growing clinical importance of the comorbidity between anxiety and drug abuse (Alegria et al. 2010; Simon 2009; Brady and Verduin 2005), including amphetamine-induced anxiety (Janowsky 2003; Sulzer et al. 1995), the potential of using zebrafish models to target this aspect of pathogenesis becomes particularly promising. Furthermore, plasma membrane monoamine transporters, targeted by psychostimulants d-amphetamine and cocaine, also include plasma membrane serotonin transporter (SERT, which is selectively blocked by fluoxetine, and is present in zebrafish, along with other monoamine transporters). The anxiogenic-like action of acute d-amphetamine observed here (Fig. 1) resembles the effects exerted in zebrafish by acute cocaine in both adult (Stewart et al. 2011d) and larval fish (Irons et al. 2010), suggesting that global inhibition of monoamine transporters can induce anxiety in zebrafish. However, since this effect strikingly differs from acute fluoxetine (not anxiogenic in the zebrafish novel tank test (Stewart et al. 2011d)), alternative mechanisms (other than SERT-mediated) may underlie acute anxiety-like effects of amphetamine in zebrafish. This possibility is in line with observations that mutant mice lacking dopamine transporter (Gainetdinov et al. 1999; Spielwoy et al. 2001), but not SERT (Bengel et al. 1998), show altered activity and exploration following d-amphetamine treatment. At the same time, the lack of evident long-term effects of d-amphetamine (Fig. 2) and cocaine (own unpublished observations) on zebrafish behavior here differs from the prominent anxiolytic-like effects evoked by fluoxetine (Egan et al. 2009; Wong et al. 2010), suggesting that serotonergic/SERT-mediated mechanisms contribute relatively more (than other monoamines) to reduced anxiety following chronic monoamine transporter blockage.

As already mentioned, mounting evidence shows complex effects of reserpine in humans and animals. Interestingly, while reserpine did not affect rat anxiety, it significantly affected their immobility behavior (Angrini et al. 1998), paralleling our zebrafish findings of the lack of short- or long-term anxiety-like responses, but a pronounced hypolocomotion following long-term treatment (Fig. 1). Similar to behavioral inhibition observed in other rodent studies (Pirch and Rech 1968; Carvalho et al. 2006), this profile is generally in line with “depression-like” motor retardation and/or PD-like hypoactivity (Duty and Jenner 2011) observed in mammalian or clinical reserpine studies. The fact that zebrafish hypoactivity in the novel tank was seen globally, in both the top and bottom portions of the tank, and without affecting top:bottom ratios, supports the lack of anxiety in the present study. Evidence of depressive-like states evoked by reserpine in humans (Freis 1954; Quetsch et al. 1959) and animals (Messiha 1988) seem to support the notion that long-term treated zebrafish developed depressive-like behavior (also see similar effects in other model species (Sigg et al. 1965; Fierman 1955)) in our study, most likely representing a motor retardation-like phenotype associated with dopamine depletion.

The long-term (but no short-term) behavioral effects of a single reserpine exposure, observed in this study (Fig. 1), are in line with findings in rats (Haggendal and Dahlstrom 1971; Carvalho et al. 2006) and teleost fish (Turner and Carl 1955; Finnin and Reed 1973), and well-known pro-depressant action of an irreversible blockade of VMAT. An alternative explanation meriting further studies involves a high level of dopamine depletion, similar to PD, where hypolocomotion and specific “droopy tail” signs in fish represents part of the phenotype (Domellof et al. 2011; Lewis et al. 2005) (also see (Carvalho et al. 2006) for similar rodent data, and Fig. 2C for droopy tail phenotypes universally seen in reserpine-treated fish in this study). Finally, it is also possible that both mechanisms contribute to the observed zebrafish and rodent phenotypes in reserpine tests, representing an interesting ‘comorbidity’ (depression/PD) model, as has recently been suggested for other species (Skalisz et al. 2002; Tadaiesky et al. 2010).

Interestingly, while there were no prior behavioral studies in zebrafish treated with reserpine, larval data show the sensitivity of their morphological biomarkers to this agent (Phylonix 2012). Likewise, injection of reserpine to adult zebrafish produces robust effects on peripheral monoamines (Saroya et al. 2009), supporting the sensitivity of zebrafish models to this agent. Our present study, assessing multiple behaviors in adult zebrafish, reports robust reserpine-induced phenotypes, potentially relevant to depression and PD.

One of advantages of using zebrafish is that as simpler model organisms, they may better detect the effects of the drugs due to their less complex, yet robust and sensitive, behavioral responses (Cachat et al. 2010b; Stewart et al. 2012; Stewart et al. 2010). Another advantage of aquatic models is that they may be used for high-throughput low-cost drug screening, including utilizing long-term reserpinized fish as a model for antidepressant, antipsychotic and/or anti-PD drug discovery. For example, reserpine-treated rodents have been successfully used for antidepressant drug screening (Maj et al. 1983; Duty and Jenner 2011), and the possibility of developing conceptually similar, but very high-throughput and low-cost, zebrafish models seems indeed feasible. Moreover, our findings allow interesting insights into various effects produced in zebrafish models by psychotropic drugs with similar pharmacological targets. For example, both reserpine and 3,4-methylenedioxy-*N*-methylamphetamine (MDMA) inhibit VMAT (Erickson et al. 1992; Rudnick and Wall 1992), but unlike the lack of overt behavioral effects of acute reserpine here, MDMA increases top swimming in zebrafish (Stewart et al. 2011d), similar to a serotonergic ligand lysergic acid diethylamide (LSD) or chronic fluoxetine (Grossman et al. 2010; Egan et al. 2009), a reuptake inhibitor that selectively blocks SERT. Collectively, this suggests that while an anxiolytic-like action in zebrafish is likely mediated via serotonergic mechanisms (Maximino and Herculano 2010; Maximino et al. 2011), they do not seem to play a major role in the acute action of reserpine.

Finally, the translational value of zebrafish models is illustrated by comparative cross-species analyses of relative potencies of monoaminergic drugs (Grossman et al. 2010; Stewart et al. 2011b; Kyzar et al. 2012b; Riehl et al. 2011a; Partilla et al. 2006). For example, the effective acute doses in zebrafish were reported as 0.1–0.25 mg/L for LSD, 80–160 mg/L for MDMA and 0.5 mg/L for cocaine (Kyzar et al. 2012b; Cachat et al. 2012; Cachat et al. 2010a; Lopez-Patino et al. 2008). Therefore, active doses of d-amphetamine (5–10 mg/L) used here were approximately 50–100 times less potent than LSD, 10–15 times more potent than MDMA, and 10–20 times less potent than cocaine. In humans, acute psychoactive effects can be evoked with approximately 2.5 mg d-amphetamine (Kelly et al. 2009), >0.025 mg LSD (Hoffer 1965), 200 mg MDMA (Bedi et al. 2009) and 2 mg cocaine (Vongpatanasin et al. 1999). Thus, d-amphetamine clinically appears to be ~100 times less potent than LSD, ~10 times more potent than MDMA, and similarly potent as cocaine. While direct cross-species comparisons for efficient *long-term* treatments, such as reserpine,

are difficult to perform due to differences in pharmacokinetics and pharmacodynamics (that may affect relative efficacy), the relative potency of d-amphetamine vs. other monoaminergic psychoactive compounds in zebrafish generally parallel clinical observations.

Importantly, this study further exemplifies how zebrafish phenotyping research can significantly benefit from using visualization-based (3D) analyses of zebrafish locomotion (Cachat et al. 2011). As demonstrated here, the dissection of hyper- and hypolocomotion, including alterations in swim velocity and distance traveled, from traditional manual indices has only recently been made possible with the application of video-tracking analyses (Cachat et al. 2010c; Cachat et al. 2011; Grossman et al. 2010). In particular, the long-term motor retardation produced by reserpine exposure, was only quantifiable following 3D spatiotemporal reconstruction of zebrafish activity, revealing a marked hypolocomotion and restricted range of overall swimming (Fig. 2B).

In summary, this study demonstrates the high sensitivity of zebrafish to drugs bi-directionally modulating brain monoamines, further emphasizing the developing utility of zebrafish models for psychopharmacology research. Given the growing importance of drug and biomarker screening in biological psychiatry, investigation into monoaminergic drug targets becomes a critical task, for which (as our study suggests) zebrafish offer a promising and sensitive novel model. This study also further supports the potential of zebrafish for investigating brain disorders associated with monoamine dysregulation, as well as the pathogenic mechanisms underlying the comorbidity of drug abuse and anxiety.

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Highlights

- Zebrafish are sensitive to drugs bi-directionally modulating brain monoamines
- D-amphetamine evokes anxiogenic-like effects in zebrafish acutely
- Reserpine leads to reduced activity and motor retardation 7 days after exposure
- The effects of d-amphetamine and reserpine parallel rodent and clinical studies

Figure 1. Behavioral effects of 20-min d-amphetamine exposure in adult zebrafish tested in the novel tank

Behavioral endpoints were obtained in the standard 6-min novel tank test for 5 and 20 mg/L d-amphetamine ($n = 11-12$ per group). Fish were tested following (A) acute exposure, as well as (B) 7 days after the single 20 min treatment. (C) Temporal 3D graphs plotted X, Y coordinates (generated in Ethovision XT8.5) on respective X,Y-axes, with experimental time plotted across the Z-axis (Cachat et al. 2011; Cachat et al. 2010c) for acute d-amphetamine treated fish. Track color reflects changes in velocity (m/s) (blue to green = lower velocity, yellow and red = higher velocity). * $p < 0.05$ vs. control; post-hoc Tukey test for significant ANOVA data.

Figure 2. Behavioral effects of 20-min reserpine exposure in adult zebrafish tested in the novel tank

Behavioral endpoints were obtained in the standard 6-min novel tank test for 20 and 40 mg/L reserpine ($n = 12-15$ per group). Fish were tested following (A) acute exposure, as well as (B) 7 days after the single 20 min treatment. (C) Temporal 3D graphs plotted X, Y coordinates (generated in Ethovision XT8.5) on respective X,Y-axes, with experimental time plotted across the Z-axis (Cachat et al. 2011; Cachat et al. 2010c) for reserpine-treated fish 7 days after the single 20-min treatment. Track color reflects changes in velocity (m/s) (blue to green = lower velocity, yellow and red = higher velocity). * $p < 0.05$ vs. control; post-hoc Tukey test for significant ANOVA data.