D-Amphetamine Exposure Differentially Disrupts Signaling Across Ontogeny in the Zebrafish

Bradley J. Serpa, MS,¹ Jennifer D. Bullard, MS,¹ Victoria C. Mendiola, BS,¹ Crystal J. Smith, MS,² Brandon Stewart, BS,¹ and Lisa R. Ganser, PhD¹

Abstract

Background: Prescriptive and illicit amphetamine (AMPH) use continues to increase along with the likelihood that during an individual's lifetime, the drug deleteriously influences the growth and connectivity of behavior circuits necessary for survival. Throughout ontogeny, neural circuits underlying these behaviors grow in complexity, gradually integrating many sensory inputs that trigger higher order coordinated motor responses. In the present study, we examine how AMPH disrupts the establishment of these circuits at critical neurodevelopmental periods, as well as the communication among established survival circuits.

Materials and Methods: Zebrafish embryos (from 1 hpf) were raised in AMPH solutions, growth parameters and escape behavior were assessed at 24 and 48 hpf, and spinal cord tissues analyzed for differences in excitatory-inhibitory signaling balance among treatments. Adult fish were fed an acute dosage of AMPH over an 11-day conditioned place preference (PP) paradigm during which behaviors were recorded and brain tissues analyzed for alterations in dopaminergic signaling.

Results: AMPH negatively affects embryonic growth and slows the execution of escape behavior, suggesting an imbalance in locomotor signaling. Although local spinal circuits provide primary escape modulation, no differences in inhibitory glycinergic, and excitatory glutamatergic signaling were measured among spinal neurons. AMPH also influenced place preference in adult zebrafish and resulted in the increased expression of dopamine signaling proteins (DRD1) in brain areas governing survival behaviors.

Keywords: D-amphetamine, escape behavior, spasticity, experiential learning, dopamine, conditioned place preference

Introduction

FROM ITS EARLIEST DEVELOPMENTAL stages to its final hours, the chemical perturbations encountered by individuals during their lifetime can permanently alter neural circuits that underlie behaviors necessary for learning, memory, and survival. Although the epidemic of opioid abuse continues to grow rampantly, the population of amphetamine (AMPH) users consistently rises, including the number of individuals prescribed AMPH, as well as with illicit users who often pair other drugs of abuse with AMPH.¹ AMPHs are commonly prescribed to children as young as 3 years old through middle-aged adults who experience attention disorders, depression, and binge eating disorder.² Between 2005 and 2014, attention disorder diagnoses more than doubled from 6.8% to 14.4%, and were paired with a significant increase in AMPH prescriptions (3.57–8.51%) among children and adults.³

Successes in treating behavioral disorders with consistent, low doses of AMPH to increase concentration, sharpen focus, and awaken alertness in these individuals⁴ greatly outweigh the common side effects of dizziness, anxiety and fear, loss of appetite, insomnia, and headaches.⁵ In extreme cases, stimulant-induced psychosis has been reported.⁴ Along with an increase in physician-directed AMPH use, however, by 2002, 35.5% of surveyed American undergraduates reported illicit or incident use of prescription AMPHs to improve academic performance.⁶ Between 2001 and 2009, incident use jumped eight-fold in individuals 18–44 years of age.¹ Although studies have not correlated AMPH use with academic performance, the perception that AMPH use improves study habits and academic ability only strengthens the likelihood of illicit AMPH use among students desperate to succeed.⁷ Misuse, especially in this population of neocortex-forming and childbearing-aged individuals is not only on a

¹Department of Molecular and Cellular Biology, Kennesaw State University, Kennesaw, Georgia.

²Medical University of South Carolina, Charleston, South Carolina.

consistent upward trajectory, even incidental exposure introduces changing brain chemistries to artificially inflated concentrations of neurochemicals that alter the establishment of balanced signaling that governs behavior, mood, memory, and learning.

Prescribed AMPH use among adolescents with attention deficits yields measurable improvements in concentration and task performance. As a nervous system-wide stimulant, the AMPH-induced increases in excitatory neurotransmitter concentration also influence the development of behavioral circuits, as well as balanced signaling, depending on the time, magnitude, and duration of exposure.⁸ The adolescent brain experiences critical periods of growth during which rapid increases in volume and complexity result from the addition of experience-related circuits. This growth, especially in the dopaminergic reward circuits, lays the groundwork for an individual's ability to reason, practice self-control, persist, learn, store memory, and execute complex motor behaviors.⁶ Because these circuits are forged from childhood into an individual's 20s, chronic exposure to AMPH during these developmental critical periods, likely impact adolescent brain development that will later govern adult behaviors.

Evidence of these behavioral deficits, for instance, was common in adolescent rats that after AMPH treatment, showed impaired accuracy in task performance, took significantly longer to learn tasks, and were more often hindered in learning new tasks by previously learned tasks.⁹ Similarly, adolescent rats exposed to AMPH showed decreases in initial reversal learning challenges compared with exposed adults and controls,¹⁰ meaning that AMPH exposure resulted in more difficulty in task switching and novel learning. AMPH-treated rats were also more impulsive and took longer to habituate from initial exposure through adulthood.¹¹ These studies demonstrate how strongly AMPH influences the development of essential behavior circuits that govern learning, memory, and adaptability, and for which deficits are often hallmarks of psychiatric disorders, including depression, anxiety, and addiction. Although the precise AMPH-induced neuroplastic mechanisms and changes remain somewhat a mystery, AMPH exposure, clearly alters the function of these behavioral circuits that govern survival behaviors.¹¹

How detrimental, though, are the effects of earlier AMPH exposure on the development of behavioral circuits? Currently, AMPH prescriptions are approved by the U.S. Food and Drug Administration for children as young as three.⁴ The wide range of generally prescribed doses starts at 2.5 mg tablets daily, usually in the youngest children (3–6 years of age), 5–40 mg/day for children ages 6–12, and up to 60 mg daily for adults.² The young age that many children are prescribed AMPH stresses the pertinence for identifying and understanding both acute and long-term consequences of AMPH exposure during the earliest critical brain development periods. Likewise, aside from direct ingestion of prescription AMPH, fetal and newborn exposures will alter the most nascent neural circuits. For example, AMPHs have been detected in breast milk for up to 24 h after consumption by the breastfeeding mother, noting a marked clearance of AMPH after 48 h postingestion.¹²

As a central nervous stimulant, the desired effects of attentiveness and appetite suppression popularize the use of AMPHs among women of childbearing age, although actual prevalence remains difficult to accurately estimate. Prenatal drug testing methods vary, and the willingness for AMPH

using pregnant or nursing women to seek treatment or be tested for drugs is low. Even though historical data have not differed greatly in the time during the pregnancy when samples were obtained, consistent records of prenatal AMPH exposure do not exist from which to draw reliable measurements, and data from human and animal studies were insufficient to understand the specific mechanisms by which AMPH affects growth and development.¹³ AMPH readily crosses the placenta, though, and the type of anomaly and the severity of these exposure-related changes varies depending on fetal developmental stage.¹³

While the strongest AMPH-induced morphological effects result from the earliest fetal growth periods when developmental programs for structure and organogenesis are laid down, AMPH exposure during later stages of gestation would make subtle or latent behavioral changes affecting overall brain organization and morphogenesis, including altered growth rate, skewed behavioral circuit formation, imbalanced expression of neural signaling chemicals, and deficits in overall brain organization.¹³

At these later fetal stages, the effects of AMPH exposure manifest themselves during childhood and adolescent development. Lower birth weights and continued growth deficits through adolescence were measured in 65 children born to women who abused AMPH during pregnancy.¹⁴ Throughout a 14-year postnatal observation period, a majority of these children displayed sex-specific delayed or altered growth rates and continued behavioral deficits. Female children were significantly shorter and weighed less than their peers at 10 years of age, compared with the AMPH-exposed males who were heavier and taller than peers through age 14. A meta-analysis of gestational growth estimates and birth weights confirmed that fetal exposure to AMPH during pregnancy stifled fetal growth, increased the likelihood of preterm birth, and resulted in low birth weight.¹⁵

Aside from disrupted growth, cognitive deficits were common among children who endured AMPH exposure during fetal development, however, these deficits were unrecognizable between ages 1 and 3 years.¹⁶ By 6–7 years of age, however, children exposed to methamphetamine *in utero* performed poorly on neurocognitive tests of IQ, memory, visual motor acuity, and fine motor skills, compared with unexposed control children of the same age.¹⁵ *In utero* AMPH-exposed children also scored significantly lower in academics compared with their nonexposed peers.¹⁴ Underlying these cognitive defects, are detectably smaller subcortical volumes in neonates, specifically thalamic and caudate nuclei, which are primary integrating, and physiological motivation units involving dopaminergic signaling.¹⁶ Growth deficits and neural defects are latent consequences that likely persist due to the influence of fetal AMPH exposure during brain morphogenesis and subsequent behavioral circuit formation.

Zebrafish escape circuit model AMPH-induced neuroplasticity

The timing, magnitude, and duration of AMPH exposure determine the degree and type of developmental consequences. Although the central nervous targets of AMPH and resulting anomalies have been observed and modeled, the exact mechanisms and neuroplastic changes resulting from AMPH exposure are not widely known. In the current study,

the zebrafish serves as an ideal model for understanding developmental, behavioral, and neuroplastic changes resulting from AMPH exposure. Detailed timing of somatic development, mapping of nervous system growth, and the emergence of stereotyped escape behaviors provide a window into the deleterious influence of AMPH at different times during the life history of an organism. The zebrafish escape behavior follows a pattern of development over which the increasing complexity of the behavior parallels the addition of more circuit components and descending inputs influencing both the detection of stimulus and execution of escape.^{17,18}

During embryonic development, the escape circuitry matures through three major stages: (1) Spontaneous coiling takes place between 17 and 21 hpf, when interneurons synapsing onto motor neurons in the spinal cord mediate side-to-side coils. (2) Touch-evoked coiling is predominant between 21 and 27 hpf when touch stimuli are detected by transient, sensory Rohon-Beard cells that activate Mauthner neurons whose axons extend from the hindbrain down the length of the body and tail. Activation of Mauthner cells initiates glutamatergic interneuronal inputs onto motor neurons that mediate a C-shaped bend away from the stimulus, and simultaneous glycinergic interneuronal inputs onto opposing motor neurons for the reciprocal inhibition. (3) After 27 hpf, a touch stimulus initiates a C-bend away from the stimulus, and is followed initially by a series of counterbends that eventually mature to a coordinated, patterned swim away from the stimulus.

These behaviors are mediated by hindbrain trigeminal and Rohon-Beard activation of Mauthner cells to interneurons to motor neuron circuits that coordinate axial bending.^{17,18} At each of these developmental stages, the circuits underlying the behaviors change, such that stimulus detection and inputs onto motor neurons grow in connectivity.¹⁹ Spinal interneurons synapsing onto motor neurons activate spontaneous coiling from 17 to 21 hpf. From 21 to 27 hpf, descending hindbrain inputs from trigeminal neurons detect touch stimuli on the head to activate descending Mauthner neurons that govern touch-activated escape response.^{20,21}

Trunk and tail touch stimuli, at this developmental stage, stimulate transient sensory Rohon-Beard neuron inputs onto Mauthner neurons for quick escape.²⁰ Descending input from the hindbrain elongates, and special DAergic neurons, diencephalospinal neurons (DDN), are present at 24 hpf at the diencephalic/midbrain border, extend continuously to innervate the spinal cord by 4 days postfertilization (dpf). Before reaching the spinal cord, DDN play a role in encouraging motor neuron growth into the spinal cord.¹⁹ Figure 1 illustrates the primary circuits governing these motor behaviors throughout the embryonic period, and the zebrafish correlates of mammalian dopaminergic nuclei driving locomotor behaviors.

After 72 hpf, the zebrafish changes from trigeminal/Rohon-Beard/Mauthner touch-induced escape to lateral line/octavolateral (cranial nerve VIII, auditory/vestibular, A/V) sensation of environmental disturbance igniting escape behavior.^{22,23} A/V inputs continue to be the primary afferent signals mediating escape during adulthood.²⁴ The efferent response circuits grow in connectivity with descending cerebral inputs from dopaminergic integrating pathways.¹⁹ Under normal conditions, the reward system controls an individual's response to natural rewards, such as food, sex, and social interactions, and is therefore an important determinant

of motivation and incentive drive.²⁵ Repeated exposure and subsequent activation of this DAergic pathway commits these stimuli to memory so it can be repeated in the future.²⁶ Genes encoding dopamine (DA) synthesis and communication proteins, as well as central nervous pathways modulating physiological drive, are highly conserved, arising in taxa that preceded the chordate expansion.²⁷ In mammals, these neural circuits form a three-part path, including the nigrostriatal (NS) pathway that underlies locomotor motivation, the mesolimbic pathway motivates behaviors of self-preservation and meeting physiological need, and the mesocortical (MC) pathway that is more prominent in species with higher cognitive ability, mediating pleasure, resilience, impulse control, and understanding consequence associated with a stimulus.^{28,29} Communication among DA-synthesizing nuclei (ventral tegmental area, nucleus accumbens, substantia nigra, and prefrontal cortex [PC]) and DAergic reward circuits are modulated by GABAergic (inhibitory) and glutamatergic (excitatory) inputs.^{30,31}

The locomotor circuits in the adult zebrafish driving avoidance or escape behavior, as well as motivating seeking behavior, are activated by midbrain dopaminergic efferents to motor circuits responding to stimuli from olfactory, visual, and A/V afferents. To understand how AMPHs influence neural signaling at this late stage in ontogeny, the second part of this study explores how AMPH-induced neuroplasticity of these DAergic pathways affects the production of adult locomotor responses. Because the communication of DA among these circuits modulates behavior, the changes in DA-mediated signaling can directly influence locomotor behaviors essential for survival. The role of the DAergic reward pathway in learning and memory essentially puts into action self-preservation behaviors, including locomotor responses of avoidance and seeking.³⁰

Altered DA communication among reward pathway neurons yield numerous locomotor and psychiatric disorders, like Parkinson's disease, spasticity, and addiction. According to the National Institute on Drug Abuse (NIDA), addiction is defined as a chronic, relapsing brain disease characterized by compulsive drug seeking and use, despite harmful consequences. This implies that the addicted-state is not triggered instantaneously upon exposure to drugs of abuse. Rather, repeated exposure produces neuroplastic changes that contribute to pathological drug-related behaviors.³² These changes result from alterations in gene expression, protein regulation, neuroanatomy, and synaptic function that collectively influence the neural circuits that govern reward, motivation, and higher order cognitive control to produce maladaptive behaviors.^{33,34} Aside from assays measuring changes to higher order cognition, the locomotor behavioral responses and the changes in DA signaling circuits of the adult zebrafish are reliable indicators of neuroplastic change after AMPH exposure.

The early embryonic development and function of zebrafish escape circuits drive survival, and because these circuits grow in complexity to give way to more elaborate patterned behaviors, the zebrafish provides an ideal model for understanding how AMPHs interfere with the development of circuits modulating survival behavior. Because AMPH is known to exert plasticity on brain and behavior throughout the lifespan of an organism,³⁵ the zebrafish model illustrates a detailed picture of the AMPH-induced ontogenic alterations

FIG. 1. Locomotor circuit development. Brain and escape circuit and behavior development in the zebrafish at three major critical periods of development. Illustrations of (1) the brain morphology, (2) the neuronal circuits contributing to reflexes, and (3) generalized behaviors that result from a touch stimulus (arrows) are represented at three embryonic stages. **(A1)** Twenty-one to 27 hpf the brain expands and folds, defining tripartite regionalization: Te and Di, Me, Hb, and SC. **(A2)** When touch-induced escape behavior is detected by trigeminal neurons, CN V (dark blue) on the head and RB afferents (light blue) from the trunk. Touch stimuli are received by MN somata (red) located in the hindbrain, and signals are sent down axons (red) that decussate and extend the length of the trunk. Signals from MN stimulate PMN (orange) onto axial muscles, while triggering CoLo glycinergic interneurons (purple) that inhibit Mauthner axons extending down the length of the trunk ipsilateral to the stimulus. By 24 hpf, early developing dopaminergic dorsal DDN (green) innervate the spinal cord where dopamine encourages growth of PMN to their targets. **(A3)** While exhibiting touch-induced side-to-side coil responses, the embryo still practices spontaneous coils, and both responses are governed primarily by local spinal interneuron networks onto MN and PMN. **(B1)** Forty-eight to 72 hpf, the brain becomes more defined, especially the OT of the midbrain. **(B2)** As with 21–27 hpf circuits, touch stimuli detected by CN V and RB trigger MN or reticulospinal neurons regulating rhythmic motor behaviors in the Hb to somite-specific interneurons and PMN. This developmental period is marked by increasing integration of descending hindbrain innervation to the spinal cord and the elongation and connectivity of the dopaminergic nuclei from the ME, Di, and Te. Adapted from Sallinen et al.⁶⁷ and Schweitzer et al.,⁴⁹ the DAergic nuclei and tracts in the brain are color coded by depth, with the most dorsal in chartreuse, green for medial, and ventral most in dark green. **(B3)** Typical escape behavior advances from a touch-induced coil away from the stimulus to the initial coil followed by subsequent counterbends that mature into a coordinated swim. **(C1)** Greater than 96 hpf as the brain grows into its adult morphology, defining the OB, T, OT, Cb, MO, and SC. **(C2)** At this time point, the primary triggers of escape are detected by the octavolateralis system (CN VIII). Larvae incorporate special sensory inputs onto dopaminergic nuclei that trigger locomotor behaviors motivated by signals integrated in these dopaminergic circuits. RAC are the last DAergic cells to differentiate. **(C3)** Pectoral fins emerge, larvae are free swimming, and escape behavior, triggered by various sensory inputs, results in a quick turn away from the stimulus followed by a rapid swim. (D1) Illustrations of zebrafish dopaminergic nuclei and (D2) the mammalian homologs, (D3) anterior to posterior diencephalic nuclei (D1–D7), OB, SP, POa, PO, Pr, Cb, cerebellum; CN V, cranial nerve V (trigeminal nerve); CoLo, commissural local; DA, dopamine; DDN, diencephalospinal neurons; Di, diencephalon; Hb, hindbrain or rhombencephalon; Me, mesencephalon; MN, Mauthner neuron; MO, medulla oblongata; OB, olfactory bulbs; OT, optic tectum; PMN, primary motor neurons; PO, preoptic area; POa, anterior preoptic area; Pr, dorsal pretectum; RAC, retinal amacrine cells; RB, Rohon-Beard; SC, spinal cord; SP, subpallium; Te, telenkephalon. Adapted from Refs.^{18,19,21,24,27,48–51,54–56,73,74}

in circuitry and behavior. In the present study, we explore the essential effects of AMPHs on escape circuits and behavior at both ends of ontogeny, first, measuring how AMPH influences embryonic growth, the development of escape circuitry, and the execution of escape behaviors, and second, in adults, measuring AMPH-induced behavioral and brain changes associated with dopaminergic, striatal-like circuits that influence physiologically driven survival behaviors.

Materials and Methods

Fish care and embryo rearing

Experiments were carried out on wild-type strains TAB (Tubigen $\times$ AB) and OWT (Optimum Wild Type, a fish store strain from Atlanta, GA). Adults were housed in a flow-through, filtered aquatic system recirculating premade water consisting of deionized water with added salts, and mounted LED lights maintained a 14-h light/12-h dark cycle at 28°C. Embryos were collected from crosses of these wild-type adults following the removal of a divider simultaneously with the turning on of morning lights. Embryos were raised in Petri dishes in a 28°C incubator maintained on a 14-h light/10-h dark cycle.

Embryo experiments

AMPH treatment. Embryos (1–2 cell stage) from several clutches were collected and pooled within an hour after fertilization. Embryos were randomly distributed among four treatment groups each with 20 individuals. Repeat treatments were run simultaneously when embryo numbers allowed. Treatment concentrations and AMPH exposure protocols followed previous zebrafish studies.³⁶ D-Amphetamine (Sigma-Aldrich, St. Louis) was dissolved in 10 mL zebrafish system water for the following treatment groups: Control (0 μ g/mL), 10, 20, and 30 μ g/mL AMPH. Embryos were subject to treatment for 48 h total. At 24 hpf the embryos were dechorionated, behavior was analyzed, and treatment solutions were renewed, then after behavior was measured again at 48 hpf, embryos were euthanized for antibody staining. Water quality parameters (pH, chlorine, nitrate, nitrite, and total hardness) for each treatment, measured at 0, 24, and 48 hpf indicated that AMPH was not adding other deleterious variables by affecting water quality.

Natural chorion emergence. To determine whether AMPH exposure affected natural developmental processes, we measured the timing of natural embryo emergence from the chorion. The treatment protocol was adapted from a study that examined the developmental effects of environmental uranium concentrations.³⁷ Zebrafish embryos generally emerge from the chorion between 48 and 72 hpf.³⁸ The fish were placed into Petri dishes with respective treatments within 1 h of fertilization and stored in an incubator at 28°C. A 50% treatment change was implemented at 24 hpf to preserve the fragile chorion and maintain observation of natural emergence.

During preliminary trials, 100% treatment changes induced early hatching. At 24 hpf, half of the treatment was carefully removed by transfer pipette from the top of the embryos in the dish and the new treatment was slowly pipetted down the outer edge of the dish and gently swirled to incorporate new treatment.

Embryos were quantified at 24 hpf (for mortality), then at 48, 54, 72, 78, 96, and 102 hpf for the proportion of embryos emerged per time. Percent emergence was calculated at each time point by dividing total number of embryos hatched by the total number of surviving embryos (hatched and unhatched) for each treatment group. Complete hatching was achieved when the entire embryo (head and tail) was free of the chorion. Deceased embryos were counted and removed from dishes to prevent potential health risk to other embryos.

Body length. At 48 hpf, each embryo was placed in its arena to analyze behavior. Body length (snout to tail tip) was also measured, and subsequent body length measures were recorded for each fish at 72, 96, and 120 hpf. Embryos were photographed under the AxioScope using a Zeiss camera, recorded, and analyzed using the Tracker function within the Zeiss Zen Blue software package. Embryos were briefly chilled at 4°C for 5 min to slow movement and reduce handling stress for measurement. Each photograph included a 2 μ m slide micrometer as a reference point. Tracker's calibration tape/stick function was used to obtain accurate lengths of individual fish with minimal manipulation. Morphological abnormalities for each embryo were also recorded and photographed at these time points.

Statistical analyses of growth parameters. A one-way analysis of variance (ANOVA) ($\alpha=0.05$) was used to identify variance in average body length within and among treatments. Pairwise comparisons between each treatment group were analyzed with Tukey's tests, using a Bonferroni correction to avoid type 1 error ($\alpha=0.0125$). The effect of AMPH on survivorship was analyzed using *t*-tests ($\alpha=0.05$), and the differences among AMPH treatments in the timing of natural chorion emergence was measured using Chi-square test with a Bonferroni correction applied to avoid the identification of a false positive through multiple comparisons of the same data sets ($\alpha=0.0125$).

Behavioral analysis. Individual embryos were placed into a 100-mm Petri dish arena filled with 10 mL system water and touch-elicited escape behaviors were recorded at 500 frames per second using a Fastec HiSpec Light (San Diego) high-speed digital camera attached to a Zeiss v12 AxioScope (Oberkochen, Germany). After a 30–60 s acclimation period, escape behaviors were induced by a gentle touch to the mid-dorsal region. The touch-evoked escape behaviors were assayed at 24 and 48 hpf time points for each fish. Duration of the recordings lasted from before the touch of the stimulus until a completion of escape behavior when the tail returned to a relaxed state.

We expected the touch stimulus to yield a C-bend response away from the stimulus at 24 hpf, and by 48 hpf, touch results in a C-bend away from the stimulus, followed by a series of counterbends, and eventually a coordinated swim. Spastic responses were identified as any escape response that deviated from the stereotyped response and included uncoordinated contractions, hesitation, bending toward the stimulus, repeated bends in the same direction, getting stuck in a bend, corkscrew (hypertrophied C-bend), or the inability to complete C-bends or counterbends. The time (ms) for each embryo to achieve a full coil was measured by analyzing recordings frame by frame.

Statistical analyses of behavior. To identify variance in escape behavior response times, the average time to complete an escape behavior was compared among treatment group in a one-way ANOVA ($\alpha=0.05$). *Post hoc* Tukey's tests allowed for pairwise comparison of average escape behavior time between each treatment. To avoid a type 1 error, a Bonferroni correction, for which $\alpha=0.01$, was applied to each pairwise comparison. When analyzing incidents of spastic behavior, the percentage of individuals displaying corkscrew (hypercoil) phenotype out of the total individuals per treatment were compared between treatments using *t*-tests ($\alpha=0.05$).

Excitatory/inhibitory balance in escape circuits: immunofluorescence. Following the 48 hpf escape behavior analyses, four separate groups, each containing 6–10 embryos from each treatment were euthanized and freshly embedded in OCT medium in cube foil molds. Embryos were then flash frozen in liquid nitrogen, then cryosectioned (12 μ m) on a Leica CM 1850 cryostat. Spinal cord sections were collected from behind the head and yolk sac, and tissues were affixed to positively charged slides, then fixed for 10 min in 4% paraformaldehyde.

Because the execution of escape behavior relies on balanced excitatory and inhibitory inputs onto motor neurons, spinal cord sections were then double antibody labeled using a protocol optimized to quantify expression of excitatory and inhibitory interneuron receptor proteins.³⁹ Primary antibodies against the inhibitory glycine receptor α -1 subunit (clone mAb4a, mouse IgG1, 1:100; Synaptic Systems Göttingen, Germany) and the excitatory NMDAR1 glutamatergic receptor protein (clone M68, mouse IgG1, 1:500; Synaptic Systems) were applied to tissues and counter labeled with Alexa 488 and 568-conjugated donkey anti-mouse secondary antibodies (1:2000; Life Technologies, Carlsbad, CA).

Labeled tissues were observed with a Zeiss LSM 700 laser scanning confocal microscope. Image stacks were taken of each cross-section using identical settings (exposure, gain). Average expression was quantified in the form of fluorescence arbitrary units (FAU) for both glutamatergic and glycinergic receptor proteins and was scored by manually defining the region of interest (spinal cord) on each cross-section using the Zeiss Zen Blue software image analysis tool. A negative control stain was prepared with the absence of the primary antibodies and also analyzed and subtracted from final treatment values to account for background signal.

Statistical analysis of excitatory:inhibitory balance. After labeling excitatory and inhibitory receptor proteins in spinal cord tissues with fluorescent antibodies, areas on these tissues were designated to analyze intensity of expression of fluorescent punctae representing excitatory (red) and inhibitory (green) receptor proteins. Expression of excitatory and inhibitory punctae was measured in FAU, and these expression data were compared among treatments using a one-way ANOVA ($\alpha=0.05$). Tukey's tests were used to identify differences in FAU expression between treatments ($\alpha=0.01$). All statistical tests were performed using Prism GraphPad Statistical Software, version 8.

Adult locomotor assays

Apparatus and method of D-amphetamine exposure. The effects of AMPH on adult locomotor behaviors were measured following a conditioned place preference (CPP) paradigm and apparatus adapted from the seminal study using zebrafish to assay drug-induced effects on locomotion.³⁶ Details of the CPP apparatus and the 11-day paradigm are outlined below and illustrated in Figure 2. The CPP testing

FIG. 2. Place preference paradigm. CPP paradigm to determine the effects of ingested D-amphetamine on experiential learning in the zebrafish. The 11-day experimental paradigm includes a preconditioning, IPP determination stage, a conditioning phase, and a final CPP determination stage. AMPH, amphetamine CPP, conditioned place preference; IPP, initial place preference.

tank (30 cm × 15 cm × 20 cm) was divided in half by distinct visual cues: one compartment colored white and the other half white with black dots.

Conditioning tanks were identical in design, including a clear mesh central divider that allowed the fish to see both sides and sense water in both compartments. The divider was placed once each fish established its preferred compartment and was treated according to the CPP paradigm. Water levels were kept at 17 cm from the bottom of the tank to minimize stress. AMPH was delivered to fish through the novel method of ingestion. All experimental fish were fed agarose cakes containing Zeigler zebrafish diet, and those fish in AMPH exposure groups were given agarose cakes identical to the control food, but with the addition of 40 µg D-amphetamine (Sigma-Aldrich). Ingestion of control and AMPH food was observed in preliminary tests using fish that were not included in the CPP experiment, and fish readily ate both control and AMPH-laced food.

Pretest: place preference determination. After an initial introduction into the testing apparatus, each fish was separately accommodated to the new environment for two full days (day 1 and 2). On the third day, fish were carefully netted from their home tanks and placed directly into the CPP apparatus. Home tanks of animals were placed in close proximity to experimental tank to minimize netting stress and hypoxia. The zebrafish was then permitted to explore the environment for 5 min, with the initial 5 min of exploration designated for acclimation to the environment. Once acclimated, behavior was recorded for 10 min, after which fish were returned to their housing tank. The preferred compartment was defined as the compartment in which a fish spent most of the time on day 3. This value was then expressed as a percentage, which served as each fish's baseline preferred compartment.

Conditioning: D-amphetamine-induced CPP. After place preference (PP) determination, zebrafish were randomly assigned to either Group A (control) or Group B (D-amphetamine). Zebrafish were fed agarose aliquots in housing tanks 30 min before being transferred to the experimental tank. On day 4, zebrafish were fed AMPH-laced food, then confined to the nonpreferred compartment for 45 min. Restriction to this compartment was achieved using a transparent divider such that visual contact to the preferred compartment was possible. The experimental tank and conditions were otherwise identical to the ones used for PP determination. After 45 min, the fish were removed from the experimental tank and placed back in its housing tank. On day 5, fish were fed control food then restricted for 45 min into the preferred compartment. AMPH food followed by restriction to the nonpreferred compartment was repeated on days 6, 8, and 10, and control food paired with restriction to preferred compartment was repeated on days 7 and 9. Final PP was then measured on day 11.

Quantification of D-amphetamine in the brain. Procedures for extraction and determination of D-amphetamine concentration in whole zebrafish brains by high-performance liquid chromatography (HPLC) were adapted from a previous study.⁴⁰ In this method o-phthalaldehyde (OPA) in conjunction with the reduced sulfhydryl group, 3-mercaptopropionic

acid (3-MA), reacts with primary amines to form fluorescent moieties. These derivatives of D-amphetamine are then measured using HPLC with fluorescence detection.

Instrumentation. HPLC determination of D-amphetamine in zebrafish brains was performed using an Agilent 1100 series HPLC system comprising an online degasser (G1379), a quaternary pump (G1379), thermostated autosampler (G1329A), and a thermostated column compartment at the Alabama A&M University. Fluorescent D-amphetamine derivatives were detected with an Agilent 1100 series diode array detector equipped with a 5 µL flow cell. Separations were performed using a 250 × 4.6 mm C18 column (Kinetex 5µ EVO C18 100A), and a guard column containing the same phase, both maintained at 4°C during analysis.

Collection of brain samples, sample preparation, and extraction. Subjects ($n=10$) were fed 10 µL aliquot AMPH spiked food and euthanized at varying time points following consumption (30, 45, 60 min). Brains were quickly dissected from the skull and chilled on a glass plate. Brains were also collected from fish fed a control diet ($n=4$) for blanks and standards. All tissues were stored at -80°C until analysis. D-Amphetamine, OPA, 3-MA, sodium tetraborate decahydrate, and all solutions were prepared from analytical-grade chemicals dissolved in Milli-Q water and HPLC-grade solvents (Sigma-Aldrich).

For fluorescence detection 27 mg OPA was dissolved in 500 µL ethanol, 5 mL of 0.1 borate buffer (pH 9.6), and 40 µL of 3-MA. This mixture was kept refrigerated and discarded after 48 h. Brains were pooled for each time point, weighed, and diluted with nine volumes of 0.1 M borate buffer (pH 10.6). After 20 s of ultrasonication, samples were centrifuged for 10 min at 18,000 *g*, then 100 µL of the supernatant was transferred to a clean centrifuge tube to which 200 µL of ethyl acetate was added. The mixture was vortexed for 2 min and placed on ice. After 10 min on ice, an additional 200 µL ethyl acetate and 200 µL of water were added to the mixture followed by brief vortex mixing to further facilitate extraction. The organic and aqueous phases were separated after 10 min centrifugation at 18,000 *g*, then 200 µL of the organic supernatant was transferred to a glass tube and dried at 45°C under nitrogen.

Samples were reconstituted in 80 µL KH₂PO₄ (0.05 M, adjusted to pH 2.6 with phosphoric acid), then after adding 20 µL of borate buffer (adjusted to pH 11.5 with sodium hydroxide), all samples were derivatized with 20 µL OPA reagent. OPA reagent was added to each sample at least 2 min before injection. Derivatization was performed in capped Eppendorf tubes, which were kept at 4°C until injection.

HPLC conditions. A linear gradient program (Table 1) was run at ambient temperature with mobile phase A consisting of 65% KH₂PO₄ (0.05 M, adjusted to pH 5.5 with potassium hydroxide) and 35% methanol, and mobile phase B with inverse proportions of buffer (35%) and methanol (65%). Fluorescent detector settings were: $\lambda_{\text{excitation}}$ 340 nm, $\lambda_{\text{emission}}$ 440 nm. Linear equation was calibrated with three AMPH solutions (2.5, 5.0, and 10 µM) prepared from AMPH detection standard (Sigma-Aldrich) in microdialysis buffer. Calibration curves were constructed by plotting the peak areas of OPA-3-MA-amphetamine (*y*, mAU) against the concentration (*x*, µM).

TABLE 1. CHROMATOGRAPHIC LINEAR GRADIENT CONDITIONS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF O-PHTHALDIALDEHYDE-3-MERCAPTOPROPIONIC ACID/AMPHETAMINE DERIVATIVES

Time (min)	Flow-rate (mL/min)	Buffer A (%)	Buffer B (%)
Initial (0)	1.5	100	0
0–3	1.5	100	0
4	1.5	0	100
18	1.5	0	100

Mapping tyrosine hydroxylase, DRD1, and DRD2 expression in the zebrafish brain. The fish were anesthetized with 0.1% MS-222. Brains were dissected and fixed in 4% paraformaldehyde at 4°C overnight. Fixed brains were then washed three times for 10 min with 1× phosphate-buffered saline (PBS) and then transferred into a solution of 30% sucrose in PBS (w/v) overnight or until tissue sank. On the next day, cryoprotected brains were transferred to Tissue-Tek O.C.T., froze using dry ice-chilled isopentane, and maintained in –80°C freezer until processing. Transverse sections through mesencephalon region of brain were taken at 20 µm and collected on charged slides.

Tissues were double stained using a previously optimized immunohistochemistry procedure³⁹ to show expression of tyrosine hydroxylase (TH) and type 1-like DA receptors (DRD1) throughout the mesencephalon. For TH expression, mouse anti-TH1 monoclonal antibody (Immunostar), was applied at a concentration of 1:500, then counterstained with super clonal Alexa Fluor 488 goat anti-mouse secondary antibody (green; Invitrogen) at 1:500 (2 ng/µL). DRD1 expression was highlighted with a 1:500 dilution (0.334 ng/µL) of rabbit anti-DRD1 monoclonal antibody (clone EP1560Y, rabbit IgG1, 1:500; Abcam, Cambridge, MA) and counter labeled with Alexa Fluor 568 goat anti-rabbit secondary antibody at (1:500 [6 ng/µL]; Molecular Probes, Eugene, OR) Rabbit anti-type 2-like DA receptors (DRD2) polyclonal antibody applied at (1:500 [6 ng/µL]; Immunostar, Hudson, WI), counterstained with Alexa Fluor 568 goat anti-rabbit secondary antibody (1:500 [6 ng/µL]; Molecular probes)-labeled expression of DRD2 in zebrafish brain.

Completed tissues were observed using a Zeiss LSM 700 laser scanning confocal microscope. Three to four repeated trials were used for each treatment with 6–10 tissue sections per slide. Average expression was in the form of FAU for DRD1 (red, 555 channel) and DAPI (blue, 405 channel) stains. Pictures were captured of each cross-section using identical gain (DRD1 = 756, DAPI = 757) and laser intensity (DRD1 = 6%, DAPI = 6.5%) settings with a z-stack of each tissue section. Total FAU was scored by manually circling the region of interest (posterior commissure [Cpost], dorsal zone of the periventricular nucleus of the hypothalamus [Hd], posterior tuberal nucleus, rostral tegmental nucleus [RT], and the vascular lacunae of the area postrema [VAS]) on each cross-section with the Zen Blue software imaging analysis tool. A negative control stain was prepared with the absence of the primary antibodies and also analyzed and subtracted from final treatment values to account for background signal.

Statistical analyses: initial PP versus CPP. For each individual, initial place preference (IPP) was identified by calculating the difference in time spent in the preferred versus the nonpreferred tank compartments on day 3. On day 11 of the CPP paradigm, the postconditioning PP was determined. The differences in IPP and CPP for each fish in each treatment group were analyzed with an *F*-test to compare for variances within treatments. Once equal variance was determined, we compared IPP time differences to CPP time differences with two-tailed paired *t*-tests ($\alpha < 0.05$) using Prism GraphPad Statistical Software (Version 8).

Statistical analyses: DRD1 and DRD2 expression. DRD1 expression was quantified in each region of interest and FAU averaged for controls and AMPH-fed tissues. A Chi-square analysis was performed ($\alpha < 0.05$) assuming no difference in average FAU per region. *t*-Tests ($\alpha < 0.05$) identified significant differences in average DRD1 expression in each region. DRD2 expression was not detected.

Results

Embryonic growth parameters and morphological anomalies

A significant number of newborns of mothers who abuse AMPHs during gestation were below the average birth weight.¹⁴ Likewise in other placental species, when offspring were exposed to AMPH during embryonic development, average birthweights were lower compared with controls.^{41,42} Our use of an anamnionic model, does not represent a parallel example of cross-placental AMPH exposure through the mother's blood stream. The zebrafish embryo, however, offers evidence that topical AMPH exposure across the chorion does have deleterious consequences on somatic growth and development of neural circuits necessary for survival that parallel those of placental mammals.

Zebrafish embryos generally wiggle free from their chorion between 48 and 72 hpf, a developmental milestone that allows the tiny embryo to escape from potential predators, while remaining contained in the chorion restricts movement. The early, spinal neuron-driven spontaneous coiling displayed between 17 and 21 hpf, and the more complex, touch-mediated coiling elicited by 21–27 hpf embryos undoubtedly contributes to the ability to hatch when escape behaviors are mature enough to increase survival. In the present study, AMPH-exposed embryos took significantly longer than controls to emerge from their chorion (Fig. 3A; $p < 0.001$). Although there was no difference among treatment groups in mortality after 24 hpf (Fig. 3B), latent hatching and measures of body length were indicative of delayed development in AMPH-treated embryos.

Significant variance in measures of length (Fig. 3C) was detected among all treatment groups by one-way ANOVA ($\alpha = 0.05$) at 48 hpf ($p = 0.0018$), 72 hpf ($p = 0.0015$), and 96 hpf (0.0001). Specifically, Tukey's *post hoc* comparisons detail that AMPH-treated embryos in the 10 µg group were significantly shorter than controls by 48 hpf ($p = 0.0043$), 72 hpf ($p = 0.0029$), and 96 hpf (0.0024), and 30 µg embryos differed significantly in length from controls at 96 hpf (0.0002). The 30 µg embryos did not differ significantly in length at 48 hpf ($p = 0.0919$) and 72 hpf ($p = 0.0431$). Controls and 30 µg

FIG. 3. Growth parameters. Embryonic growth parameters in D-amphetamine-exposed zebrafish embryos compared with controls. **(A)** All AMPH-treated embryos took significantly longer than controls to emerge from their chorion ($n=100$ /treatment; $p<0.001$, $\alpha=0.05$). Hatching rate is represented by a percentage of embryos emerged in each treatment per unit time (hpf). **(B)** Mortality was assessed after 24 hpf with embryos in the chorion emergence study ($p=0.0893$), revealing no differences in mortality among AMPH-exposed embryos and controls. **(C)** One-way ANOVAs ($\alpha=0.05$) of snout to tail tip measures identified significant differences in average body length among all AMPH-treated embryos compared with controls at 48 hpf ($p=0.0018$), 72 hpf ($p=0.0015$), and 96 hpf ($p=0.001$). Specific differences in average body length were identified using Tukey's pairwise comparisons (Bonferroni corrected $\alpha=0.01$) at 48 hpf between controls ($n=44$) and 10 µg AMPH ($n=43$) embryos ($p=0.0043$); at 72 hpf between control ($n=37$) and 10 µg ($n=40$) embryos ($p=0.0029$); at 96 hpf between controls ($n=36$) and 10 µg ($n=40$) embryos ($p=0.0024$) and control versus 30 µg ($n=39$) embryos ($p=0.0002$). **(D)** Curves comparing average length per treatment group per unit time did not differ in slope. **(E)** Common anomalies among AMPH-exposed embryos at 24 hpf include underdevelopment and severely bent (upcurved) tails, and at 48 hpf include tails mildly bent at the end, either up or down, blunted tails, edema, and underdeveloped eyes. ANOVA, analysis of variance.

embryos did not differ significantly in length from 20 µg AMPH embryos at any time point. The 10 µg embryos were shorter overall compared with 20 µg individuals, but did not differ significantly from this group at any time point. Because in the pairwise Tukey's tests, single data sets were used in repeated comparisons between other embryo groups, significance was Bonferroni adjusted ($\alpha=0.01$) to avoid a type 1 error.

Measures of somatic growth and comparisons of hatching time indicate that AMPH slows the timing to reach developmental milestones compared with controls. Aside from these deficits in growth, AMPH-exposed embryos had a higher percentage of morphological abnormalities than con-

trols. Common anomalies are illustrated in Figure 3D, including delayed development by 24 hpf, shortened, bent, or clubbed tails, cardiac edema, and hypertrophied yolk sacs. Curved trunk and/or tail was a common phenotype, with some of the upturned curvatures showing the accordion phenotype and others displaying an upcurved body with an abbreviated or clubbed tail. Others had down-curved tail ends, with the tail anomaly present only in the very posterior of the tail. Morphological anomalies were especially strong at the 24 hpf time point, including some embryos showing severely underdeveloped and misshapen bodies that were somewhat resolved by 48 hpf.

Timing of early escape behavior

Aside from delayed and anomalous somatic growth, AMPH exposure also affected the establishment of smooth, quick, and purposeful escape behaviors. Instead, AMPH-treated embryos displayed spastic behaviors that increased the time it took for fish to mediate a response to a touch stimulus. The ability for early embryos to respond to potentially harmful stimuli increases likelihood for survival, but spasticity delays or hinders escape, increasing the likelihood of predation.

To measure the effects of AMPH on early embryonic escape behaviors, embryos were assayed at 24 and 48 hpf time points in response to a touch stimulus. The timing of execution of this behavior, as well as the quantification of observed spasticity demonstrated the deleterious influence of AMPH on the development and signaling necessary for survival behaviors. A one-way ANOVA ($\alpha=0.05$) detected significant differences in the timing of escape response among treatment groups at both 24 hpf ($p<0.0001$; Fig. 4A) and 48 hpf ($p=0.0008$; Fig. 4B). Tukey's tests uncovered pairwise differences in escape behavior between treatment groups at each time point, with controls at 24 hpf taking significantly less time to execute an

escape response compared with 10 μg embryos ($p<0.0001$), and the 30 μg group ($p<0.0001$). The 20 μg embryos did not differ significantly in timing, but the margin for significance was very low ($p=0.0104$) in reference to the Bonferroni-corrected $\alpha=0.01$, increasing the confidence intervals to avoid determination of false significance.

Differences in timing of escape response at 48 hpf mirrored those at 24 hpf, with controls taking significantly less time to perform these survival behaviors compared with 10 μg embryos ($p=0.0057$) and the 30 μg group ($p=0.0014$). Controls and 20 μg embryos also did not differ in the timing of escape behaviors at 48 hpf ($p=0.0331$; $\alpha=0.01$).

Of the spastic behaviors observed (same side bends, getting stuck in a bend or while unwinding from a coil, making a C-bend toward the touch stimulus, erratic, unbalanced movements that are unproductive), the corkscrew or hypercoil C-bend behavior was most commonly observed among AMPH-treated embryos (Fig. 4C) compared with a smooth coil for which the embryo bends away from the touch stimulus with the tail wrapping up around the head. The corkscrew phenotype includes a tight winding of only the tail that would often result in the embryo spinning out of the coil and being disoriented. After observing 24 hpf, touch-induced

FIG. 4. Embryo behavior. AMPH influences embryonic escape response. (A) One-way ANOVAs ($\alpha=0.05$) detected significant differences in the average time it took for AMPH-treated embryos and controls to execute escape behavior at 24 hpf ($p<0.0001$). Tukey's tests ($\alpha=0.01$) indicated significant differences between controls ($n=40$) and 10 μg ($n=37$) embryos ($p<0.0001$) and controls versus 30 μg ($n=31$) embryos ($p<0.0001$). Controls were not significantly different compared with 20 μg ($n=32$) embryos ($p=0.0104$). (B, D) Significant difference in the time to execute escape behavior were also present at 48 hpf ($p=0.0008$), with Tukey's tests determining significant differences between controls ($n=33$) and 10 μg ($n=27$), $p=0.0057$ and between controls and 30 μg ($n=32$), $p=0.0014$. Control times did not differ significantly from 20 μg ($n=30$), $p=0.0331$. (C) Normal C-bend behavior and hypercoil or corkscrew escape behavior at 24 hpf. *t*-Tests ($\alpha=0.05$) indicate that control embryos ($n=39$) displayed significantly fewer corkscrew behaviors versus 10 μg ($n=36$; $p=0.0113$), versus 20 μg ($n=32$; $p=0.0329$), and versus 30 μg ($n=31$; $p=0.0255$).

escape response in 100 embryos per treatment, the corkscrew phenotype was present in 20.5% of controls, but incidents of corkscrew were more than double in 10 μ g (47.2%), 20 μ g (43.8%), and 30 μ g embryos (45.2%). *t*-Tests compared incidents of corkscrew behavior in AMPH-exposed embryos compared with controls ($\alpha=0.05$). Controls had significantly fewer embryos that displayed this spastic phenotype compared with 10 μ g ($p=0.0113$), 20 μ g ($p=0.0329$), and 30 μ g embryos ($p=0.0255$). The many spastic phenotypes observed among AMPH-treated embryos resulted in significant slowing of their escape response.

Effects of AMPH on excitatory and inhibitory signaling underlying escape response

AMPH deleteriously impacts the ability for zebrafish embryos to produce impactful escape responses such that the affected display spastic and slowed behaviors limiting their chances of survival. These behavioral data indicate that incidents of spasticity are more common among AMPH-exposed embryos, and that imbalanced signaling of excitatory and inhibitory inputs onto motor neurons innervating trunk muscles could underlie this spasticity. By 48 hpf, a touch stimulus elicits a C-shaped bend away from the stimulus, followed by counterbends that compile a coordinated swim. These maturing behaviors continue to result from afferent trigeminal or Rohon-Beard inputs onto Mauthner neurons that decussate in the hindbrain and simultaneously signal spinal glutamatergic excitatory or glycinergic inhibi-

tory interneurons that will activate trunk muscles or inhibit signal to reciprocal or opposing motor units, giving way to the trunk bending necessary for coiling and swimming. Because most of the escape behavior is modulated in these spinal circuits, and these efferent responses rely on balanced signals to carry out impactful escape behaviors, the present experiment quantifies the effect of AMPH on the expression of these excitatory and inhibitory inputs onto motor neurons at 48 hpf.

Primary antibodies against the excitatory glutamatergic receptor protein, NMDAR1, and inhibitory glycinergic receptor protein, GLRA1, were applied to spinal cord sections and labeled with fluorescent secondary antibodies (Fig. 5A). The presence of these proteins within the spinal cord was measured by quantifying fluorescent expression of secondary antibodies using FAU. Differences in NMDAR1 or GLRA1 expression were compared among treatment groups using a one-way ANOVA ($\alpha=0.05$), and pairwise comparisons of receptor protein expression identified specific differences in excitatory and inhibitory signaling between treatment groups.

Recall that the most profound delays in growth and escape behavior were measured in the 10 and 30 μ g embryos. A one-way ANOVA indicated a significant difference among treatment groups in inhibitory glycine receptor protein expression (Fig. 5B; $p=0.0041$; $\alpha=0.05$), with Tukey's tests ($\alpha=0.01$) identifying a marked increase in GLRA1 expression specifically between the 30 embryos and controls ($p=0.0074$) and 30 μ g and 20 μ g groups ($p=0.0085$). None of the other treatment groups yielded significant differences in GLRA1

FIG. 5. Excitatory/inhibitory balance. The SCs from cross-sectioned 48 hpf embryos reveal whether the balance of excitatory and inhibitory signaling onto motor neurons underlies spasticity. A representative cross-section through a control embryo (lower left) shows the SC region highlighted within a white square, the dorsal fin, and the notochord. SCs were labeled with antibodies against inhibitory glycine receptor alpha-1 protein, GLRA1 (green), and excitatory glutamatergic receptor protein, NMDAR1 (red). (A) Confocal image stacks showing GLRA1 expression (green punctae) in each treatment, (B) NMDAR1 (red punctae) in each treatment, and (C) merged images showing both GLRA1 and NMDAR1 punctae. (D) GLRA1 and NMDAR1 expression were measured using FAU and compared among treatments using one-way ANOVAs ($\alpha=0.05$), indicating a significant difference in average GLRA1 expression ($p=0.0041$) among experimental groups, but no significant differences in NMDAR1 expression among treatments ($p=0.2921$). Tukey's tests ($\alpha=0.01$) did identify significantly greater GLRA1 expression between controls and 30 μ g embryos ($p=0.0074$) and between 20 and 30 μ g embryos ($p=0.0085$). FAU, fluorescence arbitrary units.

expression when compared with embryos in the other treatments. Likewise, a one-way ANOVA showed that expression of the excitatory glutamatergic receptor protein, NMDAR1, did not differ significantly among treatments ($p=0.2921$; $\alpha=0.05$), nor did Tukey's pairwise comparisons yield significant differences in NMDAR1 expression between treatments.

Although imbalanced inhibitory inputs onto motor neurons may contribute to the spasticity displayed by 30 μg AMPH-exposed embryos, the degree to which spasticity hinders escape behavior in the 10 μg embryos at 24 hpf, as well as all AMPH-treated embryos at 48 hpf cannot be explained by imbalanced spinal interneuronal signaling. AMPH must influence another part of the escape circuitry to result in spasticity. Afferent detection and signaling pathways change

dramatically during the first 48 hpf, and with elongating descending inputs from the hindbrain and neuromuscular junction connections, the disparities in signaling must lie in one of these connections. These rapid changes in escape circuit complexity from 24 hpf through the larval period broaden the chances for AMPH to disrupt signaling at different circuit connections at different times during development.

Detection of AMPH in the zebrafish brain following ingestion

Because of the increase in AMPH prescriptions among people of all ages, along with the illicit use population ranging from young adult to adult, we assumed that the most common route of AMPH exposure in adults was through

FIG. 6. Detecting D-AMPH in brain tissue. HPLC measurement of D-AMPH in the brains of zebrafish at 30, 45, and 60 min following ingestion of AMPH-laced food. D-AMPH shows a peak between 16 and 17 min, and is indicated with *. (A1) Negative control OPA-3-MA reagent in microdialysis buffer ($n=2$). (A2) Positive control of D-AMPH standard (10 μM) indicated by a peak between 16 and 17 min. (A3) Control brain tissue ($n=4$). (B1) Brain tissue collected at 30 min postingestion of AMPH-laced food ($n=3$). (B2) Brain tissue at 45 min post AMPH food ingestion ($n=4$). (B3) Brain tissue at 60 min post AMPH food ingestion ($n=3$). *D-AMPH-OPA-3-MA derivative. Additional peaks before the AMPH peak represent unknown amounts of other biological amines contained in reagent and brain tissues. (C) Calculated amounts of D-AMPH present in brain tissue samples. *Theoretical dosage based on the assumption that 30% of the drug absorbed through ingestion. 3-MA, 3-mercaptopropionic acid; HPLC, high-performance liquid chromatography; OPA, o-phthalaldehyde.

ingestion. To understand whether acute AMPH exposure disrupts locomotor behavior in adults as well, zebrafish were fed AMPH-laced food, and AMPH was detected in the brains of these fish through HPLC at 30 min ($n=3$), 45 min ($n=4$), and 60 min ($n=3$) after ingestion.

In Figure 6A and B, chromatograms illustrate the presence of AMPH in brain samples and standards. D-Amphetamine is detected by a peak between 16 and 17 min. No AMPH was detected in control brains ($n=4$). Peaks detected before the AMPH retention time indicate the presence of biological amines derived from reagents and brain tissue. AMPH concentrations were quantified by measuring the area under the AMPH peaks (Fig. 6C). The relative standard deviation for retention time of AMPH derivative within the column was $<7.3\%$ and had an average of 5% , indicating an acceptable level of precision for the identification of AMPH derivative in analyte sample.

AMPH influences behavior in adults

Because we confirmed the presence of AMPH in the brain following ingestion, we could determine its influence on behavior. The sensory and motor networks modulating locomotor behavior change dramatically over the first 5 dpf of the embryonic period, becoming more complex by broadening sensory inputs that trigger coordinated escape and swimming responses. After 5 dpf, the main mechanosensory inputs are detected by the octavolateral system as Rohon-Beard neurons degenerate.²⁴ As the fish matures, olfactory and visual cues trigger responses from midbrain, dopaminergic reward circuits to rhythmic motor behavior nuclei that descend from the hindbrain onto spinal motor circuits.

When activated, these DAergic circuits drive locomotor behaviors of self-preservation, like seeking and avoidance, through modulation of one of two classes of G protein-coupled receptors. DRD1 and DRD2 both bind DA but vary in action and location on the neuron. DRD1 are exclusively located postsynaptically where receptors couple to stimulatory G proteins (G_s) to upregulate cAMP synthesis through the adenylate cyclase (AC) pathway, while DRD2 inhibit AC

action through inhibitory G protein (G_i) signaling on either pre- and post-synaptic sites. DRD1 activation of G_s influences transcriptional-level synapse modification. In contrast, the inhibition of the AC pathway by DRD2 to G_i coupling reduces cAMP synthesis, thus inhibiting transcription events in the nucleus.⁴² Addictive behaviors result from modification of DA signaling among these reward circuits.

Drugs of abuse, for instance, upregulate DA signaling among reward circuit neurons, triggering addiction behaviors like drug seeking, for which motivation to get the drug outweighs personal safety. We subjected AMPH-exposed adult zebrafish to a CPP test to determine whether AMPH alters the DAergic reward circuit signaling that governs conditioned or experiential learning. After fish determined their IPP (preferred compartment) in the experimental tank, fish were fed AMPH-laced food, then restricted to the nonpreferred compartment of the test tank. In a final PP (CPP) test following the conditioning paradigm, control fish showed no difference in time spent in the nonpreferred side compared with preferred side in IPP and CPP (Fig. 7A; $p=0.2549$; $\alpha=0.05$; $n=13$), but AMPH-fed fish spent significantly more time in the nonpreferred side of the tank versus the preferred side (Fig. 7B; $p=0.0031$; $\alpha=0.05$; $n=20$).

These AMPH-induced addiction behaviors were accompanied by increases in DRD1 expression in the midbrain areas compared with controls. Figure 8A defines the areas of the zebrafish midbrain analyzed for alterations in DAergic signaling. Immunofluorescence labeling with antibodies against TH, identified DA-communicating neurons (yellow) in the midbrain (Fig. 8B). In several of these TH-positive areas, AMPH-exposed fish showed significantly greater expression of DRD1 antibody staining (Fig. 8C) compared with controls (Fig. 8D). DRD1 expression was measured in controls and AMPH-exposed midbrains by quantifying the total fluorescence of DRD1 punctae in five specific midbrain areas, including the right vascular lacunae of the area postrema (VAS), left periventricular nucleus of the posterior tuberculum (TPp), left rostral tegmental nucleus (RT), left caudal zone of the periventricular hypothalamus (Hc), and the right dorsal zone

FIG. 7. D-Amphetamine ingestion influences CPP in adult zebrafish. Following an 11-day CPP paradigm. *t*-Tests ($\alpha=0.05$) compared the average time spent on the preferred tank compartment during IPP versus postconditioning (CPP). (A) Fish fed control diets ($n=13$) exhibited no difference in time spent in the preferred compartment at IPP versus CPP ($p=0.2549$). (B) Fish fed AMPH-laced food ($n=20$) during the conditioning period, however, showed a change in place preference from IPP to CPP, spending significantly more time in the AMPH-paired, nonpreferred compartment than during measures of IPP ($p=0.0031$).

FIG. 8. AMPH alters DA signaling. Increased DRD1 expression in diencephalon–midbrain areas following AMPH ingestion. **(A)** Atlas defining the area through the midbrain and diencephalon where DRD1 expression was measured, focusing on the VAS, PTN, RT, the Cpost, and the Hd. **(B1)** Confocal micrograph of the brain regions defined in A stained with anti-TH antibody (green) to highlight areas with catecholamine signaling (somata in blue). **(B2)** 40 $\times$ image of the ventromedial portion of **(B1)**, showing TH expression in the PTN and Hd regions. **(B3)** Control brain tissue stained with antibody against DRD1 (red) shows expression in several nuclei, including VAS, PTN, and Hd. Although DRD1 expression is detected in Cpost and RT, the red punctae are not apparent in this image. White arrowheads indicate areas within the analyzed nuclei where expression was detected. **(C1)** Brain tissue of an AMPH-exposed individual suggests hypertrophied DRD1 expression in several DA-communicating areas in the diencephalon/midbrain area, especially the VAS, PTN, and Hd. **(C2)** Close-up view of the ventromedial region of C1 shows high concentrations of DRD1 punctae in the PTN and Hd. **(C3)** Although DRD1 expression is brighter in this AMPH-exposed individual compared with B3, the intensity of DRD1 expression also varies from **(C1)**, suggesting variability among individuals, even though there is an overall increase in DRD1 expression in AMPH-fed embryos versus controls. **(D)** Measures of average fluorescence arbitrary units (FAU) indicate greater expression of DRD1 in the defined sampling areas. A Chi-square test confirms significantly greater DRD1 expression in AMPH-treated fish than in controls ($p = 0.0001$; $\alpha = 0.05$), and t -tests ($\alpha = 0.01$) reveal specific regions (Hd, PTN, and VAS), where DRD1 expression was significantly greater in AMPH-fed fish ($p < 0.0001$ for each region). Cpost, posterior commissure; DRD1, type 1 like-DA receptors; Hd, dorsal zone of the periventricular hypothalamus; PTN, posterior tuberal nucleus; RT, rostral tegmental nucleus; TH, tyrosine hydroxylase; VAS, vascular lacunae of the area postrema.

of the periventricular hypothalamus (Hd), highlighted in pink in Figure 8B. Chi-square tests of controls and AMPH-treated fish show a significant difference in DRD1 expression ($p < 0.0001$). In each of these brain areas, DRD1 expression was greater in AMPH-treated fish compared with controls, suggesting that acute AMPH exposure upregulates G_s -mediated DAergic signaling.

Discussion

From its most nascent stages, the vertebrate nervous system relies on precise signaling and tissue organization for proper connection and communication. These early circuits lay the groundwork for the integration of complex stimuli to produce relevant responses that, throughout an organism's lifetime, optimize survival and fitness. Zebrafish provide a window through which these earliest developmental processes can be assessed. During this first major critical period of development, tissues specialize with the addition of somites, and

by 15 hpf, the growing nervous system extends its connections to their neuromuscular junctions, and not long after that, the embryo practices its first, spontaneous behaviors.²³

As shown in Figure 1, the earliest activation of these circuits begins around 17 hpf when the embryo undergoes spontaneous coiling behaviors that result from local spinal interneuronal stimulation of primary motor neurons (PMN).⁴³ From this time, the essential circuitry in the spinal cord depends upon a balance of excitatory glutamatergic and inhibitory glycinergic interneuron inputs onto PMN that innervate trunk muscles.³⁹ These earliest connections, along with interneuronal signaling elements, become the initial framework on which the adult nervous system builds. The rapid growth in complexity of these circuits accompanies increasing coordination of escape behaviors mediated by the addition of stimulus detection from more sensory inputs and higher order efferent outputs to muscles.

With this unique window into nervous system development and signaling, the zebrafish also provides a translational

model showing how pharmaceutical perturbations by substances like AMPHs can alter the growth and connection of these survival circuits. Because both the prescriptive and illicit use of AMPHs continues to grow, so does the probability that these drugs can disrupt nervous system development and function at any time during an individual's life history.

The early embryonic stages in the zebrafish when the nervous system first becomes active, parallel human developmental periods in the first trimester when the nervous system is developing. The changes AMPH exerts on the established adult zebrafish nervous system also indicate neuroplastic events that disrupt signaling in the AMPH-exposed adult brain.⁴⁴ Thus, the results of the present study reflect on the consequences of ontogeny-specific AMPH exposure that yield measurable changes in the establishment of and communication among neural circuits in the zebrafish that govern survival.

By assaying development during these earliest critical periods, our study identifies how AMPH influences the growth and function of the most nascent neural circuits in the zebrafish that modulate behaviors necessary for survival. AMPH also delays overall growth in the zebrafish, mirroring lower-than-average fetal developmental parameters, birth weights, and childhood growth curves in children whose mothers abused AMPH during pregnancy.¹⁵

Like these children of AMPH-abusing mothers, the most profound effects of embryonic exposure in zebrafish were neurodevelopmental, specifically the extended time to reach milestone behaviors compared with controls. The ability for embryos to respond to potentially harmful stimuli with quick, smooth escape behaviors, even at these earliest life history stages, increases their likelihood of survival.²⁶ Instead, AMPH-exposed embryos exhibited ineffective, spastic behaviors that were significantly slower compared with controls. These data suggest that AMPH exposure during these nascent life stages influences nervous tissue organization, connection, and subsequent communication.

Within the first 48 hpf, wild-type escape behaviors are primarily directed by local spinal circuits, relying heavily on balanced signaling from excitatory and inhibitory interneurons onto motor neurons to elicit fast, coordinated axial bending necessary for movement.³⁹ The spastic escape responses in AMPH-exposed zebrafish embryos are most likely due to imbalanced signaling among these local spinal circuits. At 24 and 48 hpf, zebrafish models of hyperekplexia, an inherited disease marked by spinal seizures and hypercontracted muscle groups, displayed spastic escape behaviors mediated by reduced expression of glycine receptor α -1 subunit. Glycine receptor deficits alter the balance of excitatory and inhibitory signals onto opposing muscle groups, giving way to uncoordinated, spastic contractions that are hallmarks of hyperekplexia.³⁹

Spastic behaviors exhibited by AMPH-exposed embryos paralleled those of hyperekplexia models, although, after quantifying glutamatergic and glycinergic receptor expression, the only significant differences identified were greater inhibitory glycine receptor expression in high-AMPH-dose (30 μ g/mL) embryos compared with controls. These data imply that the signaling alterations slowing escape behavior are not rooted in the spinal interneuronal inputs onto motor neurons. AMPH-induced alterations in escape behavior must arise from elsewhere in the circuit.

Spontaneous coiling behavior begins at 17 hpf, peaks at 19 hpf, then declines as escape behaviors become more

complex and coordinated until average frequency ends around 30 hpf.¹⁷ Signaling among several somite-specific spinal interneurons to early PMN that innervate different parts of the somite's muscle group generate these coils,⁴⁵ thus the anomalous signaling could emerge from alterations in neuromuscular communication between a PMN and its somite muscle. Pharmaceutical perturbations by cannabinoids, delta 9-tetrahydrocannabinol (THC) and cannabidiol (CBD) during gastrulation appeared to target the neuromuscular junction communication. By 48 hpf, embryos exposed to both cannabinoids had reduced miniature endplate potentials at neuromuscular junctions, and expression of nicotinic acetylcholine receptors was also reduced, but only in the CBD-treated embryos. By 5 dpf, axonal elongation and branching onto muscle targets were stunted in only CBD-treated embryos.⁴⁶

Here, as with our study, anomalous signaling at the neuromuscular synapses is only partially explained by changes in receptor protein expression and axon elongation, because signaling deficits in THC-exposed embryos were not accompanied by decreased neuromuscular receptor protein expression. By 5 dpf cannabinoid-treated fish showed significantly fewer behavioral responses to acoustic stimuli than controls, but no differences in the number of response were detected after touch response between treatments. By 5 dpf escape behaviors are primarily induced by auditory stimuli modulated by hindbrain connections from the octavolateral receptor systems onto descending interneuron to motor neuron spinal circuits.^{20,47}

These previous examples of genetic and pharmaceutical perturbations on the earliest escape behaviors indicate that altered signaling in several circuit areas could underlie deficits in escape response. Because behavioral responses were stimulus specific at 5 dpf, and by 48 hpf, discrepant neuromuscular signaling components were CBD specific and not present in THC-exposed individuals, underscore that these developmental effects are going to be chemical- and timing specific. As far as differences in the afferent/stimulus detection components of the circuit as possible causes for spastic behaviors, in AMPH-exposed embryos the detection of touch stimulus, itself, did not slow escape response, rather the execution of behavior was spastic. Both studies highlight the many circuit components that could produce spastic escape behaviors in AMPH-exposed embryos.

An alternative explanation for these embryonic, AMPH-induced behavioral anomalies involves early DA signaling. Midbrain DAergic circuits will not be the primary drivers of escape responses until after 5 dpf, but special DAergic diencephalospinal descending neurons (DDN) are present at the diencephalon-midbrain junction by 24 hpf, and as they continue to extend from the midbrain, promote motor neuron growth until 4 dpf when they reach and innervate the spinal cord.⁴⁸ The development of the dopaminergic nuclei in the diencephalon begins with these DDN at only 8 hpf, with differentiation taking place around 16 hpf, and fibers innervating the spinal cord by 24 hpf.⁴⁹ These DDN are the singular source of DA in the spinal cord during development, and concentrations of DA from DDN are measurable in the spinal cord by 48 hpf where DA plays a major role in motor neuron growth.⁵⁰

In 33 hpf tyrosine hydroxylase 1 (TH1) morphants, TH expression was absent in the diencephalon, and there were significantly fewer spinal motor neurons.⁵¹ We did not quantify

changes in DAergic signaling until fish were adults and DA circuits played major roles in the execution of escape responses, but knowing that AMPH-exposed adults displayed altered DA signaling, along with data showing reduced motor neuron growth in early embryos following TH1 knockdown⁵¹ imply that disruptions in DDN-specific DAergic action may influence motor neuron growth and innervation of muscle during escape circuit establishment.

Aside from immediately recognizable escape behavior deficits in zebrafish larvae after early embryonic AMPH exposure, long-term effects of chronic AMPH treatment during adolescence in nonhuman primates limit proliferation of neuroprogenitor cells in the hippocampus where DA-driven neuroplasticity modulates learning and memory.⁵² Perhaps early effects on the growing DAergic circuits continue to hinder appropriate behavioral responses as the zebrafish reaches adulthood.

After 96 hpf (4 dpf), touch stimuli on the head region are detected by trigeminal nerve inputs, while trunk stimuli are still detected by Rohon-Beard interneurons.²⁴ Both trigeminal and Rohon-Beard detectors trigger Mauthner responses to spinal circuits for fast escape responses.⁴³ With the addition of sensory inputs from the head, the complexity of the escape circuit grows with descending efferent outputs extending from the hindbrain. At this developmental time point, DDN have reached and innervated the spinal cord, connecting dopaminergic midbrain efferents to the escape circuitry.⁴⁸ From 5 dpf into adulthood, these DAergic midbrain connections onto escape circuits integrate responses to olfactory, visual, and auditory-vestibular stimuli along with touch.⁵³

Specifically, dopaminergic nuclei that develop later than DDN and differentiate by 96 hpf integrate sensory signals from the olfactory bulb, retinal amacrine cells, striatum, preoptic, and pretectal areas, along with connections from nuclei in the tuberculum and the hypothalamus.^{49,54} In the zebrafish brain, the diencephalic-midbrain dopaminergic nuclei mirror the mammalian A11 DA-communicating neurons with efferents that extend to the spinal cord.⁴⁹ The increasing influence of these DAergic circuits becomes the main physiological driver of survival and escape circuit stimulation.⁵⁶ DA modulates responses to these sensory stimuli, and the action of these DAergic circuits also governs experiential learning and memory, so if the organism encounters the same stimuli in the future, it can respond in a way that maximizes fitness.³⁶

DA communication among these circuits increases in response to rewarding stimuli promoting seeking behavior, and during dangerous stimuli triggering avoidance behaviors. Long-term potentiation of these circuits increases with experience such that the stimuli and responses become learned behaviors.⁵⁷ Activation of DA circuits is modulated by DA receptors. DA binding to DRD1 triggers a G_s protein mechanism that upregulates cAMP production for a stimulatory effect, whereas DRD2 couples with G_i protein to produce an inhibitory effect by downregulating cAMP production.³³

The central role of the DAergic circuits for stimulating escape behavior leads us to understand what a powerful influence AMPH exerts on these survival responses. Although the most profound effects of AMPH exposure are thought to be during the earliest developmental stages when body plans are being laid down and neural circuits are making their first connections, the adult brain may be more sensitive to AMPH and, likewise, may exhibit stronger outcomes following acute

treatment.⁵⁸ Our behavioral data demonstrate that AMPH exposure in adult fish interferes with experiential learning, because AMPH influences PP. These CPP experiments reflect behaviors exhibited by addicted individuals who seek the addictive substance without regard for personal safety.⁵⁶ The AMPH-induced upregulation of DA signaling within these midbrain circuits underlies these aberrant behaviors.³⁵ Studies in rodent models affirm that AMPH exposure affects behavior, specifically targeting dopaminergic reward circuits, and that often these behavioral changes persist through adulthood after the stimulant-induced upregulation of neurochemicals during development has dulled signaling sensitivity and altered function.⁵⁹ Chronic AMPH exposure in nonhuman adolescent primates also yields neurotoxic outcomes, specifically in dopaminergic circuits.⁶⁰ Primate models were given oral AMPH formulations similar to commonly prescribed concentrations for adolescent humans (2.5–20 mg) over a 4-week period. Because posttreatment plasma AMPH concentrations in these experimental models were similar to those of AMPH-prescribed human adolescents, the significant (30–50%) reduction in striatal DA concentrations realistically reflects the consequences of AMPH exposure on the developing human brain.⁶¹ AMPH acts specifically on dopaminergic neurons in all mammal models, targeting the NS pathway's circuits that guide motivated and avoidance movement, coordination, and memory consolidation, and the mesolimbic pathway underlying addiction and reward.^{8,35} In Rhesus monkeys, AMPH treatment resulted in significant increases in DA release in the caudate of the NS pathway, and in the medial PC (mPC) of the MC pathway. AMPH-induced DA release was faster and increased to higher concentrations in the caudate compared with the mPC, and showed an AMPH dose-dependent sustainment of increased DA release.⁶² These animal models inform our understanding of the consequences of adolescent AMPH exposure, putting in perspective that alterations in dopaminergic signaling have persistent effects on behavior.

Conclusions

Our data show that even after acute exposure, AMPH exerts neuroplastic effects that have consequences for survival. Conflicting studies argue whether AMPH plays a positive or deleterious role in the recovery of locomotor function following a brain injury. After lesioning the sensorimotor cortex region of the brain, rats given a 3-week AMPH regimen combined with physical rehabilitative exercises, completed complex reaching tasks faster than controls and even surpassed their own prelesion performance abilities.⁶³ While a single AMPH dose in squirrel monkeys vastly improved postcortical lesion locomotor training.⁶⁴ Conversely, Wong and Stewart observed that a single dose of AMPH impaired recovery of locomotor behavior in rats compared with controls after spinal cord lesion.⁶⁵ In these studies, AMPH seems to play a role in locomotor task improvement when the injuries were cortical in nature, but not when the lesions were cord based. AMPH effects seem to follow a dose-dependent, inverted U pattern that mirrors AMPH- and cocaine-induced behaviors in mammal studies.³⁶ In particular, measures of locomotor activity in 6 dpf zebrafish larvae exposed acutely to a range of AMPH concentrations, revealed increased activity at low AMPH doses and hypoactivity at high doses,⁶⁶

supporting the notion that low stimulant concentrations may be therapeutic for upregulating locomotor recovery. These data, along with the findings in the present study recapitulate that the effects of AMPH on locomotor behaviors depend on the timing, magnitude, and duration of exposure at different stages of development and adulthood.

Perhaps the addition of AMPH, which upregulates DA communication, increases activity among circuits modulating experiential learning. What happens when the DA agonist AMPH is removed? In spinal cord injury experiments, perhaps the DA-driven signaling for locomotor responses is enhanced by AMPH, but since the efferent machinery executing the behavior is injured, there is no overall behavioral improvement. In developing zebrafish, when the spinal cord circuits are primary drivers of locomotion, AMPH exposure gives way to spasticity. Thus, these deficits in locomotor learning and accuracy after AMPH treatment may also be hindered by AMPH-induced spasticity. Our study did not identify the mechanisms by which AMPH treatment produced spasticity at early embryonic stages, but our future endeavors will assay other parts of the circuit, including the neuromuscular connections. Our data do show that AMPH targets the DA-driven reward circuits, modifying behaviors that help us meet our physiological need. Because AMPH affects DA-specific neurons in adults, perhaps a future direction of this study will investigate the role of DA from DDN in escape circuit connection. DA seems necessary to promote motor neuron elongation and connection to muscle targets, thus for AMPH to somehow alter signaling among DDN to motor neurons may have persisting consequences on escape circuit communication.

Learning, experience, and memory stimulate continued potentiation of neural circuits through both formative and adult stages, resulting in neuroplasticity of neurobehavioral circuits throughout an organism's lifetime. These constant changes also provide a template for the exposure to drugs like AMPH to alter the communication among these neurons. During the earliest stages of development, the differentiation and elongation of DDN show early influences on locomotor behaviors, but, as the organism grows and develops, the DAergic reward nuclei and circuits increase their modulation of sensorimotor behaviors, including those underlying survival.⁶⁷ Because AMPH specifically targets DAergic reward circuits, the degree to which AMPH affects behavior likewise depends on timing, duration, and magnitude of exposure. AMPH alters CPP in zebrafish by upregulating DA signaling within these highly conserved reward pathways, suggesting that these circuits are highly susceptible to substance-induced neuroplasticity, but also calling into question the permanence of these changes and their resultant influences on behavior. Indeed, reward pool genes encoding signaling among these pathways are activated by AMPH, resulting in CPP changes, and in a mutant line of zebrafish that are not susceptible to AMPH-induced stimulation of DAergic circuits, none of these genes were activated.⁶⁸ Reward pool genes encode the development, metabolism, and connectivity of these DAergic circuits, directly influencing signaling mechanisms among these neurons to optimize sensorimotor stimulus integration. Responses to olfactory, visual, auditory, and touch stimuli that activate either escape behaviors from a threat or fictive swims toward nutrients or other rewarding stimuli are governed by these genes and the many reward pool genes also

encoding transcription factors for reward gene modulation.^{69,70} The development of addiction behaviors is likely due to changes in signaling among these reward pool genes and likewise, suggests that drugs like AMPH can permanently alter these genetic mechanisms and perhaps be handed down epigenetically to offspring.⁷¹ Preexisting tendencies toward addiction and altered DAergic-based behaviors may be activated by addicting drugs like AMPH. Studies regarding the influence of social status in zebrafish support this hypothesis, showing that dominant individuals were better able to control their responses to various stimuli, while subordinate fish displayed escape behavior in response to most stimuli.⁷²

Although these DAergic reward circuits drive most adult behaviors in response to sensory stimuli,⁷³ the direct connection between octavolateral afferents onto Mauthner neurons mediate fast escape. Miller et al., suggest that socially dominant zebrafish are better able to temper responses to stimuli, because of differential excitability of DAergic circuits in charge of fictive behaviors and those governing escape, while subordinate fish rely on the simple hindbrain–Mauthner neuron connection for their behaviors.^{69,72,74} These observations imply that the influence of AMPH on communication among reward circuits as well as on the genes modulating the growth and neuroplasticity of the behavioral circuits can have lasting deleterious consequences on DA signaling for individuals exposed to AMPH during development and for offspring whose reward pool genes function according to AMPH-induced changes in parental reward circuits.

The variability by which AMPH influences behavior depends on the stage during the lifetime during which an individual is exposed, as well as the magnitude and duration of exposure. As the present study demonstrates, AMPH affects behavior during embryonic development, in larvae,⁶⁶ and in adults. Although the specific mechanisms by which AMPH exerts these effects have not been affirmed, early behavioral anomalies are not due to excitatory and inhibitory spinal signaling imbalances, as in adults, the involvement of DA signaling and motor neuron arborization and connection may underlie these aberrant behaviors. AMPH, by altering reward pool genes that govern growth of reward pathways and modulate communication among these DAergic neurons, can permanently and deleteriously affect behavior.

Vertebrate Animals Statement

The use of vertebrate animals was done in accordance with the official Kennesaw State University Institutional Care and Use Committee guidelines for ethical use of animals in research, protocol #16-006.

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Author Disclosure Statement

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Address correspondence to:

Lisa R. Ganser, PhD

Department of Molecular and Cellular Biology

Kennesaw State University

105 Marietta Drive, MD 1201

Kennesaw, GA 30144

Email: lisa.ganser@kennesaw.edu