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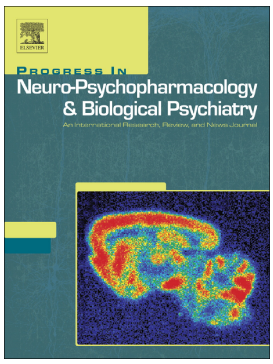

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Effects of acute and chronic arecoline in adult zebrafish: anxiolytic-like activity, elevated brain monoamines and the potential role of microglia

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Abstract

Arecoline is a naturally occurring psychoactive alkaloid with partial agonism at nicotinic and muscarinic acetylcholine receptors. Arecoline consumption is widespread, making it the fourth (after alcohol, nicotine and caffeine) most used substance by humans. However, the mechanisms of acute and chronic action of arecoline in-vivo remain poorly understood. Animal models are a valuable tool for CNS disease modeling and drug screening. Complementing rodent studies, the zebrafish (*Danio rerio*) emerges as a promising novel model organism for neuroscience research. Here, we assessed the effects of acute and chronic arecoline on adult zebrafish behavior and physiology. Overall, acute and chronic arecoline treatments produce overt anxiolytic-like behavior without affecting general locomotor activity and whole-body cortisol levels. Acute arecoline at 10 mg/L disrupted shoaling, increased social preference, elevated brain norepinephrine and serotonin levels and reduced serotonin turnover. Acute arecoline also upregulated early protooncogenes *c-fos* and *c-jun*, whereas its chronic treatment elevated brain expression of microglia-specific biomarker genes *egr2* and *ym1* (thus, implicating microglial mechanisms in potential effects of long-term arecoline use). These findings support high sensitivity of zebrafish screens to arecoline and related compounds, and reinforce the growing utility of zebrafish for probing molecular mechanisms of CNS drugs. Our study suggests that novel anxiolytic drugs can eventually be developed based on arecoline-like molecules, whose integrative mechanisms of CNS action may involve monoaminergic and neuro-immune modulation.

Keywords: zebrafish; arecoline; CNS; anxiety; genomic effects

1. Introduction

Arecoline (Fig. 1) is a naturally occurring nicotine-like psychoactive alkaloid from nuts of the areca palm (*Areca catechu)*, native of the Southeast Asia (Huang and McLeish, 1989). For millennia, arecoline has been used in the region for social, recreational and medical purposes due to its stimulant, anxiolytic and relaxing effects (Volgin et al., 2019). The clinical importance of this drug is reflected in its current role as the world's fourth most commonly used substance, after alcohol, nicotine and caffeine (Horenstein et al., 2019; Papke et al., 2015). With widespread consumption and over 600 million users globally (Papke et al., 2015), the growing use and abuse of arecoline necessitate further translational research into its CNS action (Volgin et al., 2019).

Readily crossing the blood-brain barrier (BBB), arecoline acts as a partial agonist of nicotinic and muscarinic acetylcholine receptors (Papke et al., 2015). In humans, depending on the dose and individual responsivity, arecoline produces cognition-enhancing, psychostimulant, euphoric, pro-arousal, aphrodisiac, anxiolysis and sedative effects (Volgin et al., 2019). In rodents, acute arecoline modulates locomotor activity, alleviates ethanol intoxication and potentiates behavioral sensitization induced by morphine (Liu et al., 2016; Volgin et al., 2019). The drug also produces discriminative stimulus control in rats (likely mediated via central muscarinic receptors) (Meltzer and Rosecrans, 1981) and, like nicotine, evokes withdrawal-like syndrome upon discontinuation in both humans and rodents (Volgin et al., 2019). However, the exact psychopharmacological profile of arecoline and molecular targets for its acute and chronic action remain poorly understood, warranting further preclinical testing.

Complementing rodent studies, the zebrafish is rapidly becoming a powerful tool for neuroscience and psychopharmacology (Cachat et al., 2010a; Kalueff et al., 2014). These fish demonstrate high genetic and physiological homology to humans (Barbazuk et al., 2000; Kalueff

et al., 2014; Stewart et al., 2014), including analogues of most structures of the human brain, and shared neurotransmitter and neuroendocrine systems (Panula et al., 2010). Models of acute and chronic stress have also been developed in zebrafish (Chakravarty et al., 2013; Fonseka et al., 2016; Fulcher et al., 2017; Song et al., 2018), displaying complex behaviors in major domains (e.g., locomotor activity, emotionality, reward, sociality and aggression) that are shared with humans (Norton and Bally-Cuif, 2010). Behavioral analyses of zebrafish are further empowered by their high-throughput capability and the availability of well-established screens and paradigms sensitive to major classes of CNS drugs (Cachat et al., 2010b; Cachat et al., 2011; Kysil et al., 2017; Rihel and Schier, 2012; Steenbergen et al., 2011). Capitalizing on this powerful vertebrate model system, here we evaluate pharmacological effects of arecoline on zebrafish behavior, neurochemistry, physiology and brain gene expression, aiming to evaluate potential mechanisms of its CNS effects (in a preclinical model) that may eventually serve as novel targets for future clinical applications.

2. Methods

2.1. Animal and housing

Adult (5-7 months-old) zebrafish of both sexes (~50:50 male to female ratio) of the wild type, short-fin outbred strain were obtained from a local commercial distributer (Eno Aquarium Technology Co., Ltd, Shanghai, China). All fish were given at least 15 days to acclimate to the laboratory environment, and housed in groups of 20-30 fish per a standard small 3-L tank (7-10 fish/L) in the Aquatic Benchtop Housing system (Jinshui Marine Biological Equipment Co., Qingdao, China). Water temperature was maintained at 27±1°C, pH at 7.0±0.1, dissolved oxygen at 6±0.5 mg/L, total ammonia at <0.01 mg/L, total hardness at 6+0.3 mg/L, and alkalinity at 22±2 mg/L $CaCO_3$. Illumination (960-980 lux) in the holding room was provided by ceiling-mounted fluorescent light tubes on a 14:10-h light/dark cycle (lights on 9:00 am), according to the standards

of zebrafish care (Avdesh et al., 2012). The strain selection for the present study was based on population validity considerations and their relevance for the present study. Briefly, while genetically controlled models (e.g., inbred zebrafish strains) can be a better reproducible and more reliable system for neurogenetics research, modeling CNS disorders, such as in the present study, involves 'real' human disorders affecting genetically heterogenous populations. Thus, using outbred populations of zebrafish (such as selected here) becomes a more populationally valid and translationally relevant approach for the purpose of this study. All fish used in this study were experimentally naïve at the beginning of the study, and fed twice a day with Tropical Fish Multi-Dimensional High-Protein Flakes (Beijing, China), a recommended food for small laboratory tropical fish, including zerbafish.

2.2. Behavioral testing and euthanasia

Behavioral testing was performed between 10.00 am and 17.00 pm, using tanks with filtered system water adjusted to the holding room temperature. All recorded behavioral endpoints fully corresponded to the established behavioral phenotypes described in the Zebrafish Behavioural Catalogue (Kalueff et al., 2013). In Experiment 1, we analyzed behavioral and physiological effects of acute (20-min) exposure to arecoline and areca nut extract at several doses. Experiment 2 tested these effects following a chronic 7-day exposure, whereas Experiment 3 examined CNS effects produced by acute 2-h withdrawal from 12-h arecoline treatment. To characterize arecoline effects in zebrafish, their behavior was analyzed in the 5-min novel tank test (NTT) and the light-dark box (LDB) tests, chosen here as the two most sensitive zebrafish anxiety tests (Colman et al., 2009; Kalueff and Cachat, 2011). Prior to testing, fish were pre-exposed in 0.5-L plastic beakers for 20 min to either drug-treated or drug-free water. For acute treatment, fish from the same batch were randomly divided (using the random allocation method and an online Research Randomizer tool,

www.randomizer.org/#randomize) into several groups (n = 14-15): drug-free control, arecoline- (0.1, 1, 10 mg/L) and areca nut extract-treated (3, 6, 12 g/L) zebrafish. Active acute doses for both experiments were chosen based on our pilot studies with these drugs, including the lack of impaired locomotor activity of zebrafish at 10 mg/L, with first signs of ataxia emerging only at 100-200 mg/L. The standard 20-min pre-treatment time for acute doses was chosen here based our prior experience with various CNS drugs (Riehl et al., 2011). The NTT apparatus consisted of an acrylic rectangle tank (18 cm height× 22 cm top × 23 cm bottom × 5 cm width), filled with water up to a 19-cm height, and divided into two equal virtual horizontal portions by a dashed line marking on the outside walls. Back and lateral sides of tank were pasted with white envelope to increase contrast during behavioral recording. In this test, increased top swimming indicates reduced anxiety-like behavior (Riehl et al., 2011). Trials were recorded by camera for further analyses, assessing distance traveled (cm), velocity (cm/s), as well as highly mobile (>80% of the individual fish average per trial) and mobile frequency (40-80% of the individual fish average) using the EthoVision XT11.5 software (Noldus IT, Wageningen, Netherlands)б based on the center point detection (Kysil et al., 2017). The fish used in all behavioral experiments belonged to the same baseline population (same batch housed in the same holding aquatic system) and were allocated randomly to the tested groups.

The LDB test utilized plastic apparatus divided into two compartments of equal size, one white and one black (30 cm length × 20 cm width × 20 cm height) filled with water. In this test, increased light activity typically reflects reduced anxiety (Kysil et al., 2017). Briefly, the fish were individually introduced into the dark section of the LDB test, and observed for 5 min, scoring the time spent in the light side (s), the number of entries to the light and the presence of freezing behavior for each fish was scored using the two-point scale (0 – absent, 1 – present). Manual observation was performed by two highly trained observers with inter-rater reliability > 0.85, as

assessed by Spearman correlation analyses of the raters' pilot data (collected from the same tested animals) during the standard observation and calibration pretrial experiments.

In the shoaling test, two cohorts of 24 zebrafish were each pre-exposed in 1-L plastic bakers for 20 min to 10 mg/L arecoline or drug-free water, and group-tested (4 groups, 6 fish per trial) in the observation tank, similar to the NTT apparatus. After a 5-min acclimation to the apparatus (necessary to establish normal group/shoaling behavior), zebrafish shoals were photographed each 10 s. A total of 30 photos (6 per each of the four 6-fish shoals) per drug /treatment were used for analyses in this study. Each photo was calibrated to the size of the tank and analyzed by trained observers, measuring distances (cm) between each fish in the group, using the Image Tool software (University of Texas Health Sciences Center, San Antonio, USA) and averaging them to obtain an average inter-fish distance for all photos per group, similar to (Canzian et al., 2017a).

The social preference test was performed in zebrafish acutely exposed to 10 mg/L arecoline by placing them individually in an open field-like white rectangular plastic container (13 height × 37 length × 29 width, cm), in which two 200-ml transparent plastic cups filled with water were placed in the opposite corners (fixed near the top of the tank by clips), with one cup containing a neutral stimulus zerbafish, and the other cup remaining empty. The tested fish were individually placed into the empty compartment, and recorded for 5 min to assess the number of entries to the conspectiifc half and time spent there (s), based on the center body point crossing of the virtual line dividing the apparatus into two compartments.

Following behaviioral testing, the animals exposed acutely in Experiment 1 were immediately euthanized in ice cold water, immediately dissected (to extract brains) and stored at -80°C for further analysis. In the chronic exposure Experiment 2, the animals were returned to their holding tanks and euthanized one day later, as described above. Although some prior zebrafish studies used

decapitation as a method for adult fish euthanasia (Chatterjee and Gerlai, 2009; Pan et al., 2012; Saif et al., 2013; Shams et al., 2015), here we selected a brief ice-cold water exposure as a fast, efficient and ethical method of fish euthanasia. This procedure also precluded highly stressful manipulations (excessive handling, immobilization, placement of fish in unusual environments) unavoidable during decapitation (Wilson et al., 2009). Other commonly used methods of fish euthanasia, such as the MS222 exposure, causes more distress than ice-cold water immersion (Wilson et al., 2009), are slower and act through the nervous system, that in turn may disturb neurochemical and genomic results. Thus, the ice-cold water was used in the present study as a recommended and approved method of fast (within seconds), less distressful euthanasia of zebrafish that provides little or no additional stimuli to the brain tissue that could confound behavioral, neurochemical and genomic data (Demin et al., 2017; Demin et al., 2020; Meshalkina et al., 2018; Wang, D. et al., 2020).

2.3. Whole-body cortisol and brain gene expression analyses

To analyze cortisol levels, the headless whole-body samples were homogenized in 1 mL of ice-cold 1× PBS buffer. The homogenizing rotor blade was then washed with an additional 500 mL of PBS and collected in a 2-mL tube containing the homogenate. Samples were transferred to glass tubes and cortisol was extracted twice with 5 mL of diethyl ether. After ether evaporation, the cortisol was reconstituted in 1 mL of 1× PBS. The levels of whole-body cortisol were assayed using the ELISA kits (Nanjing Herbal Source Biotechnology Co., Ltd), following the manufacturer instructions. The concentrations obtained were normalized to the weight of the extracted zebrafish brain tissue, and presented as ng/g, according to (Cachat et al., 2010b).

In Experiment 1, we used brains extracted previously (as described above) to evaluate the effects of acute arecoline exposure on brain expression of early protooncogenes (*c-fos, c-jun* and

egr1). Experiment 2 assessed the effects of chronic 7-day exposure to arecoline on mRNA expression of several brain microglia biomarker genes (*MHC-2, CD206, arg1, iNOS, egr2* and *ym1*). These genes were selected here as well-established, type-specific genomic and protein biomarkers of microglia, including the pro-neuroinflammatory M1 microglia biomarkers (*MHC-2, iNOS*), and the neuroprotective anti-inflammatory M2-microglia biomarker genes (*CD206, arg1, egr2* and *Ym1)* (Veremeyko et al., 2018)(Zhao et al., 2013). In all experiments, the expression was assessed by PCR (pooling two whole brains per sample), using specific primers listed in Table 1. Gene expression levels were normalized to the RNA expression of the housekeeping *β-actin* gene (relative quantification) with the △△CT correction (Wang, JiaJia et al., 2020a).

Because the gene expression depends largely on a chosen time scale after the induction (Hata et al., 2000; Sabban and Kvetňanský, 2001), several steps were taken to mitigate this potential factor here. In Experiment 1, we used fish immediately after acute 20-min treatment and a 5-min behavioral testing, applying the same conditions to both arecoline-exposed and control groups to compare their expression difference. Because 15-20 min is a sufficient time for early protooncogenes to become activated, this timeline was used in the present study for both groups, similar to (Wang, JiaJia et al., 2020a). Because Experiment 2 (chronic treatment) examined a wider spectrum of brain genes, which may have different time points for peaks after stimuli, we chose to focus only on baseline differences in their gene expression. For this, we performed the assay 1 day after the testing, i.e., removed fish directly from their holding tanks prior to immediate euthanasia. Accordingly, there were no stimuli in this experiment that could interfere with the temporal dynamics of expression changes of various genes assessed. The fast euthanasia in ice water, as discussed above, ensured zebrafish euthanasia in both experiments within 2-3 s, aiming to minimize any procedural impact on zebrafish gene expression findings.

2.4. Liquid Chromatography-mass spectrometry (LC-MS/MS) analyses of arecoline levels in the brain

In a separate group of drug-exposed zebrafish, the liquid chromatography-mass spectrometry (LC-MS/MS) was used to examine whether systemic administration of arecoline and areca nut extract in zebrafish results in detectable concentrations of arecoline in fish brain. Liquid chromatography was performed on Shimadzu LCMS-8030 chromatographer (Shimadzu Co., Kyoto, Japan) with a vacuum degasser, a binary pump and an auto sampler. The separation was performed using a C18 Shimadzu column (150 × 2.1 × 5 mm). The mobile phase consisted of methanol and 2 mM ammonium acetate containing 0.2% (vol/vol) formic acid (8:92, vol/vol) at an isocratic flow rate of 500 μL/min. The sample injection volume to the LC–MS/MS system was 20 μL. Isopropanol and water (50:50 vol/vol) mixture solution was used to wash the auto sampler injection needle to reduce carry-over effect. Applied Bio systems Shimadzu LCMS-8030 and the positive multiple reaction monitoring (MRM) modes under unit mass resolution were applied for mass spectrometric detection at m/z 156.2→53.2 for arecoline. The ion spray temperature and voltage were set at 400 °C and 5500V, respectively. The analyses were accurately weighted and separately dissolved in 50% methanol-water (1:1, vol/vol) to yield the 1-mg/mL stock solutions, which were stepwise diluted with methanol to provide working solutions (500 and 1000 ng/mL) of arecoline. Following a 20-min treatment, the animals were sacrificed in iced water, followed by decapitation, to extract the brain samples. Methanol (1 mL) was added to each sample and homogenized thoroughly with a glass homogenizer, followed by a 30-min sonication with ultrasonic Cleaner (Shimadzu LCMS-8030, Kyoto, Japan) and centrifugation at 10000 rpm for 10 min, to obtain the supernatant. Following an overnight evaporation, 0.3 ml of methanol was added

for reconstitution. All solutions were maintained at −20◦ C prior to using them for mass spectrometry analyses to measure arecoline signal by its UV absorption at 254 nm (Fig. 1).

2.5. ***Neurochemical analyses***

To assess the effect of acute arecoline on zebrafish neurochemical parameters, we utilized a separate group of drug-exposed zebrafish to analyze their whole-brain concentrations of norepinephrine, serotonin, dopamine and their metabolites 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) using the high-performance liquid chromatography (HPLC), as described earlier (Demin et al., 2017; Meshalkina et al., 2018). Briefly, the fish were euthanized in ice-cold water immediately after the 20-min drug exposure, and their brains dissected on ice and stored in liquid nitrogen prior to analyses. As already mentioned, ice-cold water immersion was utilized here as a fast, reliable and suitable method of zebrafish euthanasia that not only helps avoid unwanted stressful manipulations (e.g., handling, immobilization and placement during decapitation; (Wilson et al., 2009)), but additionally creates consistent temperature conditions for the samples as does the subsequent brain dissection on cold-ice, aiming to minimize concomitant changes in neurotransmission due to fish or sample handling.

On day of analyses, all samples were weighted and placed into 10 µL of ice-cold 0.1 M perchloric acid solution (this and other reagents: Sigma Aldrich, St. Louis, MO, USA) with 100 ng/mL DHBA (3,4-dihydroxybenzylamin, internal standard) per 1 mg of brain tissue for preservation of analytes. The samples were then sonicated for 10 s at half-power settings, cleared by centrifugation and filtered through a 0.22-µm Durapore Membrane hydrophilic PVDF filter (Merck Millipore, Billerica, MA, USA, Catalog number: UFC30GV00, R8EA70729). With the filter hold-up volume of only 5 µL, the PVDF membrane of this size is commonly used as the last step of sample preparation, aiming to avoid column contamination, using the HTEC-500

chromatograph (Eicom USA, Amuza, Inc., San Diego, CA, USA) in studies from other laboratories (Belov et al., 2020; Espinoza et al., 2020), as well as in our previous works (Demin et al., 2017; Demin et al., 2020; Meshalkina et al., 2018; Wang, D. et al., 2020). HPLC assays were performed using a CA-5ODS column for HTEC-500 chromatograph (Eicom USA) with a carbon electrode WE-3G using the +650 mV applied potential, and a standard 10-µL injected supernatant volume per run. The chromatography mobile phase consisted of 0.1 M phosphate buffer, 400 mg/L sodium octylsulphonate, 50 mg/L ethylenediamine-tetraacetic acid (EDTA), 17-% methanol and was adjusted to pH 4.5 by phosphoric acid (all reagents were purchased from Sigma Aldrich, St. Louis, MO, USA). The concentrations data were normalized using individual DHBA sample concentrations, and presented as pg/mg of brain tissue weight), as in (Demin et al., 2017; Meshalkina et al., 2018). Additionally, we assessed the ratios of 5-HIAA/serotonin, DOPAC/dopamine and HVA/dopamine, reflecting monoamine metabolism in the brain. A total of 12 fish per group was used for neurochemical analyses in this study (analyses of chronic effects of arecoline on zebrafish neurotransmission was beyond the scope of the present study, and may be addressed in subsequent follow-up studies). Brain monoamines and their metabolite levels may vary greatly between the published studies, largely depending on methods of sample collection, HPLC equipment, assays used, environmental conditions and the units reported (e.g., ng/mg of protein weight vs. whole brain tissue weight) (Chatterjee and Gerlai, 2009; Pan et al., 2012; Saif et al., 2013; Teles et al., 2013). In general, the neurotransmitter levels detected here (see the Results section further) were consistent with our previously published zebrafish studies (Demin et al., 2017; Demin et al., 2020; Meshalkina et al., 2018; Wang, D. et al., 2020) using the same methods of neurochemical analyses.

2.6. Statistical analyses and data handling

All data are expressed as group means ± SEM, and analyzed using the unpaired Wilcoxon-Mann-Whitney U-test or Kruskal-Wallis test followed by Dunn's post-hoc test, where appropriate, to compare the treatment (drug) and control groups. P was set as 0.05 in all experiments. Sample size for each experiment was based on previously published studies on zebrafish drug screening, gene expression and neurochemistry (Demin et al., 2017; Meshalkina et al., 2018; Volgin et al., 2018; Wang, JiaJia et al., 2020b), own pilot studies with arecoline and other drugs, as well as based on statistical power analyses of eta squared (η2) and Cohen's d, using the online calculator (www.psychometrica.de/effect_size.html), with the 0.8 (80%) power to detect a large effect size and a two-tailed significance level of 0.05. All fish tested were included in final analyses without attrition or exclusion, and all planned analyses were reported here. All experimenters were blinded to the treatment groups during behavioral testing and neurochemical analyses. Behavioral analyses were performed by two highly-trained experimenters blinded to the treatment (intra/inter-rater reliability >0.85, as assessed by Spearman correlation). Analyses of data were performed without blinding since all animals and samples were included in analyses, data were analyzed in an unbiased automated method, and the analysts had no ability to influence the results of the experiments. The study experimental design and its description here, as well as data analysis and presenting, adhered to the ARRIVE guidelines for reporting animal research and the PREPARE guidelines for planning animal research and testing.

3. Results

As shown in Fig. 1, chemical analyses of brain samples using LC-MS/MS successfully detected arecoline peaks following both acute drug- and areca nut extract exposure, but not in control zebrafish. In essence, this confirmed that arecoline: 1) entered zebrafish brain in detectable quantities following the acute 20-min treatment used in this study, 2) contributed to the observed

acute behavioral effects of the drug, and 3) was also responsible for the similar CNS effects reported here for the acute the areca nut extract exposure.

While automated analyses by video-tracking software indicate no overt neuromotor deficits produced by acute arecoline in the NTT (Fig. 2, inset), both arecoline and the areca nut extract acute exposure similarly increased top exploration in this test (Fig. 2, Experiment 1), indicative of an anxiolytic-like phenotype. Table 2 provides a summary of statistical analyses used in this study, supporting statistically significant treatment effects presented here. Reinforcing this anxiolytic profile, acute arecoline also increased time in light in the LDB test (Fig. 2), disrupted shoaling (increased the average inter-fish distance) in the shoaling test, and promoted social interaction in the social preference test (Fig. 3).

Although neither acute arecoline nor areca nut extract exposure affected whole-body cortisol (Fig. 3), an active anxiolytic dose of 10 mg/L arecoline potently modulated brain monoamines and their metabolites. Indeed, as shown in Fig. 4, this anxiolytic dose of arecoline elevated noradrenaline, serotonin and DOPAC levels, and lowered the 5-HIAA/serotonin ratio. Furthermore, arecoline also significantly upregulated the expression of early protooncogenes *c-fos* and *c-jun* (but not *egr1*) in zebrafish brain (Fig. 5).

In Experiment 2, chronic treatment with 1 mg/L arecoline for 7 days evoked similar anxiolytic-like behavioral effects in the NTT (Fig. 2), as did acute arecoline treatment mentioned above. Figure 3 shows that chronic arecoline exposure also upregulated brain expression of M2 microglial biomarker genes *egr1* and *Ym1*, but did not affect whole-body cortisol levels (Fig. 5).

Finally, in Experiment 3, zebrafish also demonstrated a significant withdrawal-like effect (Table 2), revealing an anxiogenic-like behavior (reduced top exploration activity) in the NTT, following a 12-h chronic 1 mg/L arecoline exposure 2-h discontinuation (Fig. 6).

4. Discussion

Currently representing the most understudied major addictive drug in the world (Horenstein et al., 2019; Papke et al., 2015), arecoline is an important CNS agent commonly used by humans (Volgin et al., 2019). Applying zebrafish as a powerful in-vivo system to analyze effects of arecoline on behavior and physiology, we aimed to evaluate its potential CNS mechanisms preclinically, with potential implications for novel drug targets and future clinical applications. Although zebrafish are a critical tool in neuroscience and CNS drug discovery (de Abreu et al., 2019; Kalueff et al., 2014), previous arecoline studies have only reported its developmental toxicity and motor retardation (Chang et al., 2004; Peng et al., 2015), or locomotion-evoked vibration signals (Wang, J et al., 2020). The present study is novel as the first evaluation of acute and chronic CNS effects of arecoline in adult zebrafish (a novel model organism itself), and the first report that comprehensively analyzed a wide range of behavioral, neurochemical, endocrine and genomic profiles evoked by this drug in-vivo.

Analyzing behavioral effects of acute arecoline, we first identified its dose-dependent NTT anxiolytic-like profile with characteristic peripheral 'slow' swimming near the NTT surface (Fig. 2). Strikingly resembling acute nicotine in adult zebrafish (Levin et al., 2007), this profile is also consistent with similar chemical structure and overlapping pharmacological activity of these two agents. After establishing the active dose range for arecoline, we next selected 10 mg/L as its 'reference' acute dose for further in-depth analyses. Reconfirming anxiolytic-like effect of arecoline in the LDB test, we further observed disrupted shoaling and increased social preference – the phenotypes consistent with the putative anxiolytic-like action in zebrafish. While zebrafish often increase shoaling when anxious (Canzian et al., 2017b; Maaswinkel et al., 2013), anxiety reduces social investigation in both rodents (de Angelis and File, 1979; File, 1980; Hölter et al., 2015) and

zebrafish (Giacomini et al., 2016). Furthermore, an anxiolytic profile of acute arecoline is also in line with our earlier findings (Wang, J et al., 2020) that acute 10 mg/L arecoline lowers 5-fish group locomotor-generated vibration in the small beaker, assessed as the height of peaks on vibration power frequency spectra (VPFS), indicating 'calming' behavioral profile of this agent (see Fig. 3 for an example).

Acute arecoline also up-regulated the brain expression of two main early proto-oncogenes *c-fos* and *c-jun* (Fig. 2, 3 and 5), suggesting behavior-activating profile of acute arecoline, as reported both clinically and in rodent models (Volgin et al., 2019). While we did not assess the expression of other CNS genes beyond fast-activated early proto-oncogenes in acute Experiment 1, we did observe robust neurochemical effects, including the activation of central monoaminergic neurotransmission (elevated norepinephrine and serotonin levels) and decreased turnover (lower metabolite/serotonin or metabolite/dopamine ratios, Fig. 4). Taken together, these findings suggest anxiolytic-like profile of acute arecoline in zebrafish (Fig. 2) is accompanied by altered monoaminergic neurotransmission (Fig. 4). Given similar anxiolytic effects of arecoline in humans and rodents (Volgin et al., 2019), our findings suggest shared, evolutionarily conserved behavioral effects of acute arecoline in zebrafish, also raising the possibility that potential other CNS effects of arecoline reported here (Fig. 3-6) may be translationally relevant. As we also tested acute effects of areca nut extract in zebrafish behavioral assays, their similar anxiolytic profile (Fig. 2) confirms the role of arecoline in the observed effects. Subsequent chemical analyses proved arecoline presence in zebrafish brain samples following acute treatment with both the drug and areca nut extract (Fig. 1). Given the ability of arecoline to quickly pass the BBB, these findings directly link the observed phenotypes in the present study to arecoline CNS action. Our findings also support high sensitivity of zebrafish as a screen for clinically relevant CNS effects of arecoline.

Zebrafish exposure to acute arecoline increased norepinephrine, serotonin and DOPAC brain levels, and decreased the 5-HIAA/serotonin ratio (Fig. 4). In line with these findings, acute arecoline increases dopamine levels in mouse cortex (Molinengo et al., 1986). Finally, the present study also paralleled behavioral and neurochemical analyses with genomic profiling. For example, acute arecoline evoked robust upregulation of early proto-oncogenes *c-fos* and *c-jun* in the brain, and this finding is consistent with the overall behavioral activation evoked by acute arecoline clinically (Volgin et al., 2019).

Following detailed characterization of acute arecoline profile, Experiment 2 next analyzed behavioral and physiological effects of its chronic, 7-day treatment. As chronic arecoline evoked similar anxiolytic effects to its acute treatment (Fig. 2), this clinically more relevant arecoline use also altered the expression of several brain immune genes, including the establisehd microglia biomarker genes early growth response 2 *egr2* and *ym1* (Fig. 5). Notably, both upregulated genes represent the activated M2 microglia subtype (that plays a protective role in the brain), as opposed to the M1 microglia that triggers neuroinflammation (Veremeyko et al., 2019). For examle, *egr2* is essential for the control of inflammation and antigen-induced proliferation of B and T cells, upregulating the expression of various cytokines (Li et al., 2012; Morita et al., 2016; Sumitomo et al., 2013) and modulating the sensitivity of M1 and M2 macrophages (Veremeyko et al., 2018). *Ym1* also regulates inflammatory responses by activated macrophages (Chang et al., 2001), and may play a similar role in microglia, controlling neuroinflammation (Zhao et al., 2013). On the one hand, well-known anti-inflammatory and immunosuppressant effects of arecoline may be due its agonism at α7 nicotinic acetylcholine receptors (Papke et al., 2015), especially since both nicotine and α7 receptors exert anti-inflammatory effects in the brain (Nizri et al., 2009; Reale et al., 2015). On the other hand, our present data suggest that chronic arecoline evokes M1->M2

shifts in microglia activity (Fig. 5), and that such neuro-immune modulatory effects may also contribute to long-term CNS action of this drug.

Finally, in Experiment 3, zebrafish also demonstrated withdrawal syndrome-like responses upon acute discontinuation of arecoline (Fig. 6). The ability of arecoline to induce withdrawal syndrome in zebrafish here is generally consistent with clinical data on drug abuse and withdrawal potential of arecoline (Volgin et al., 2019), thereby strengthening the overall validity of zebrafish models to study arecoline effects.

Clearly, there were several methodological and conceptual limitations in the present study. For example, no animal model can fully recapitulate complex CNS phenotypes and their pharmacological modulation in humans. The present study also did not assets individual, strain or sex differences in arecoline CNS effects. Given well-reported such differences in zebrafish responses for other neuroactive drugs (Demin et al., 2019; Genario et al., 2020), this line of research is important. Further studies may also investigate potential role of different cholinergic mechanisms of arecoline CNS action, as it acts as a non-selective agonist of nicotinic and muscarinic receptors, and they both may contribute to the complexity of arecoline CNS effects. Thus, analyses of activity and the expression of both types of acetylcholine receptors in zebrafish brain during arecoline exposure merit further scrutiny.

We also note that neurochemical and gene expression studies performed here used the whole-brain samples. This may be a rather general approach, less sensitive for detecting potential region-specific changes in brain neurotransmission and genomic responses. Thus, further region-specific studies may be needed to obtain a more detailed, nuanced profile of arecoline CNS effects. Finally, we only used a 7-day chronic arecoline exposure, which may differ from a long-term recreational use of arecoline and areca nuts in humans. Thus, future studies may assess different

protocols of chronic arecoline treatment in detail, as well as complement genomic findings with protein transcriptomic assays in zebrafish. Likewise, future immunohistochemical studies of microglia, as well as assessing their structrual M1-M2 transformation, may further support the putative role of microglia in chronic arecoline action.

In summary, robust anxiolytic-like behavioral effect of acute arecoline in zebrafish parallel those of the areca nut extract, and are accompanied by increased monoamine neurotransmission and brain expression of early proto-oncogenes *c-fos* and *c-jun*. Chronic arecoline evokes generally similar anxiolytic-like behavioral effects, accompanied by altered expression of several biomarker genes that implicate microglia and neuroimmune mechanisms in potential CNS effects of long-term arecoline use. Overall, these findings support high sensitivity of zebrafish screens to arecoline and related compounds, and reinforce the growing utility of zebrafish for probing molecular mechanisms of CNS drugs. Our study indicates the potential utility of arecoline as a potent anxiolytic upon both acute and chronic administration, and suggests that novel anti-stress drugs may eventually be developed based on integrative CNS mechanisms of arecoline-like molecules that can involve monoaminergic and neuro-immune processes.

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Figure 1. Arecoline and its qualitative chemical determination by mass spectrometry in zebrafish brains following a 20-min acute systemic administration of 10 mg/L arecoline and 1 g/L areca nut water extract vs. untreated control fish, n = 4 samples (each containing 4 brains) per group. Red arrows indicate arecoline peaks of UV absorption intensity at 253 nm, blue arrows denote the relative molecular weight of arecoline. Note significant treatment effect (Kruskal-Wallis H = 11, P< 0.006) with the presence of arecoline peaks in all treated fish, and their lack in all control fish (P<0.05 for both drug groups, Dunn's post-hoc test). The arecoline peak height, indirectly reflecting the drug level in the tissue, was also higher for arecoline (P<0.05 U-test), lower for areca nut extract and equal to zero in all control samples

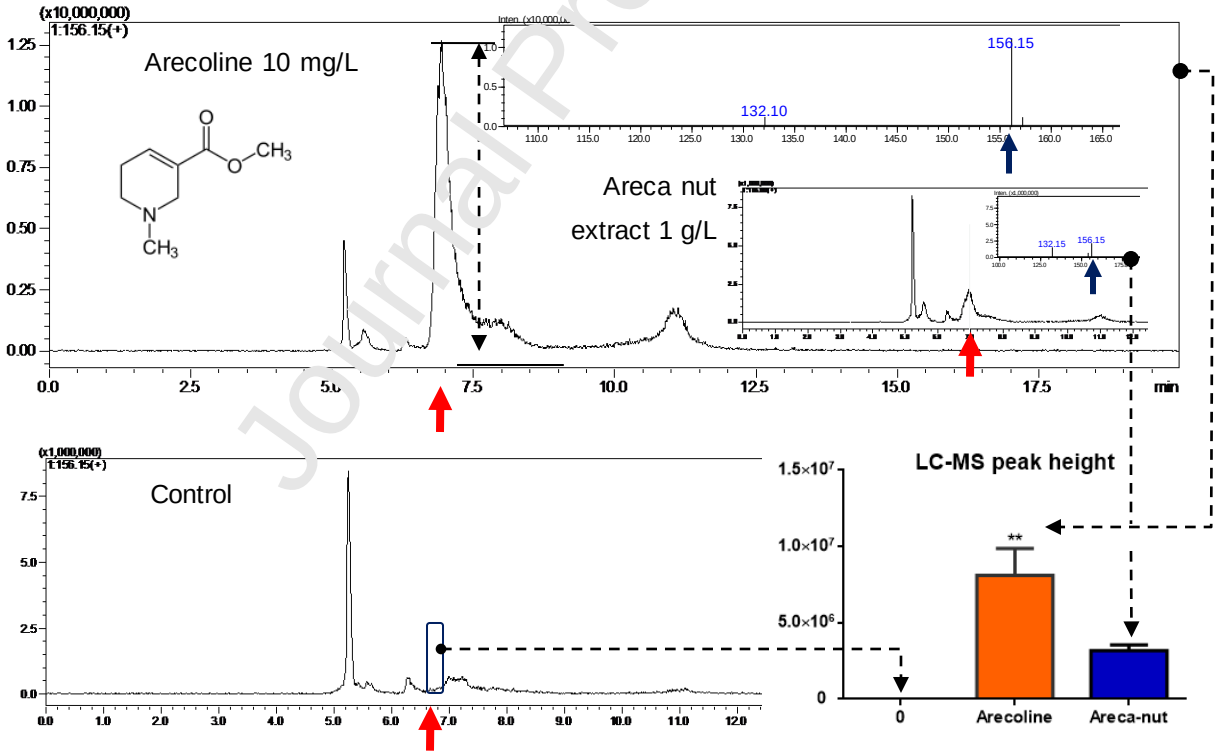

Figure 2. Behavioral effects of acute 20-min exposure to arecoline (0.1, 1 and 10 mg/L) and areca-nut extract (3, 6 and 12 g/L) in adult zebrafish tested in the 5-min novel tank (n = 15) and light-dark box tests (n = 12-13 per group, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, post-hoc Dunn's test for significant Kruskal-Wallis data (see Table 2 for summary of Kruskal-Wallis statistics). Inset: Automated behavioral endpoints for 10 mg/L arecoline exposure in the novel tank test, analyzed by the Ethovision XT11 software (P>0.05, NS, U-test).

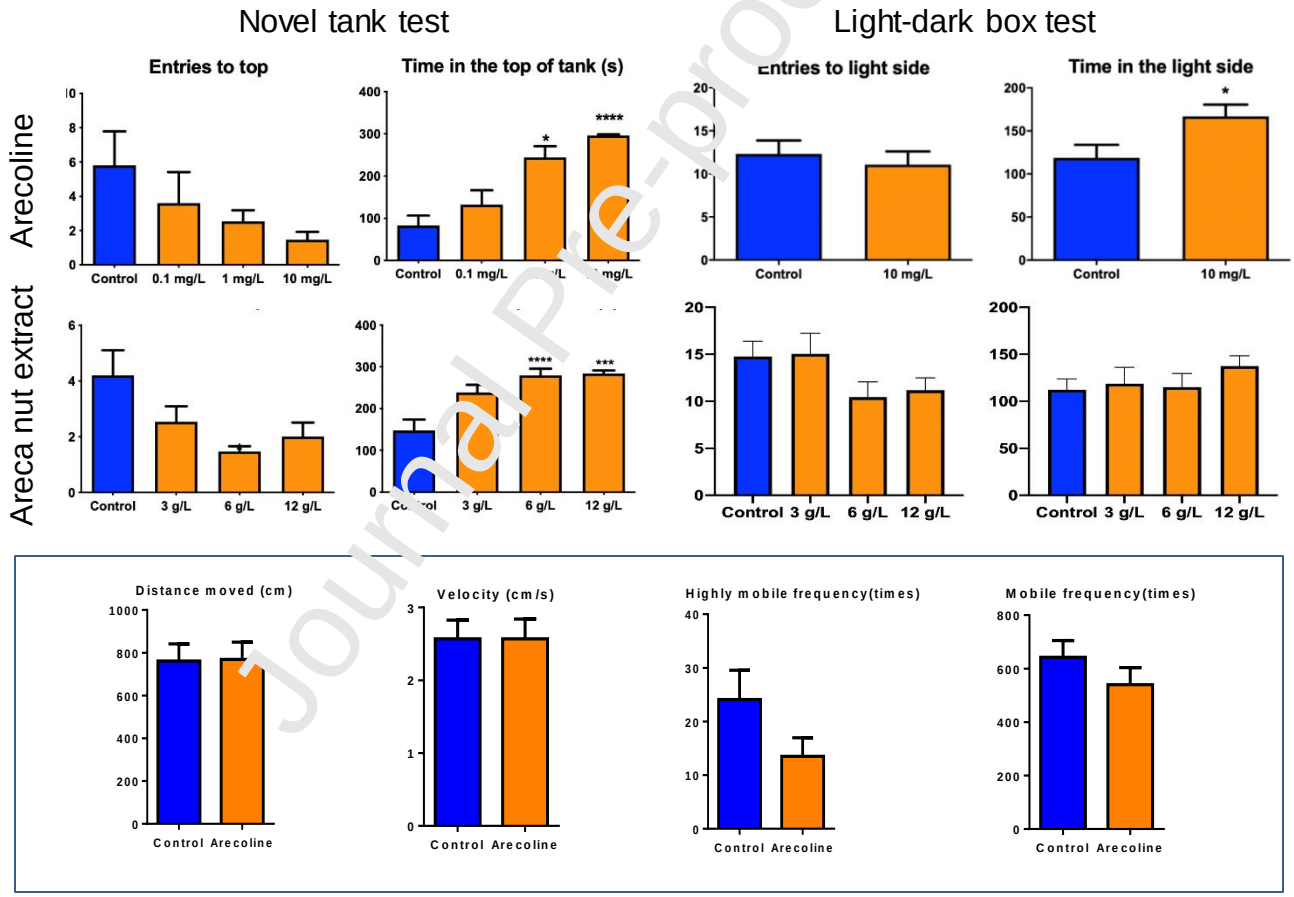

Figure 3. Effects of arecoline on zebrafish stress responses. Top panels represent acute arecoline (10 mg/L) effects on shoaling behavior (n = 24, left panel) and social preference (n= 15, right panel). Photos illustrate acute arecoline effects at 10 mg/L on zebrafish group behavior in the shoaling test. Bottom panels represent whole-body cortisol levels following (acute exposure, n = 13-14, chronic 7-day exposure to 1 mg/L, n = 18 per group). *P < 0.05, U-test vs. control. Insets: representative locomotion-generated vibration signals from a 5-fish control and acute 10-mg/L arecoline-exposed fish, assessed by the amplitude of 3-4-Hz peaks on vibration frequency power spectra (see (Wang, J et al., 2020) for details).

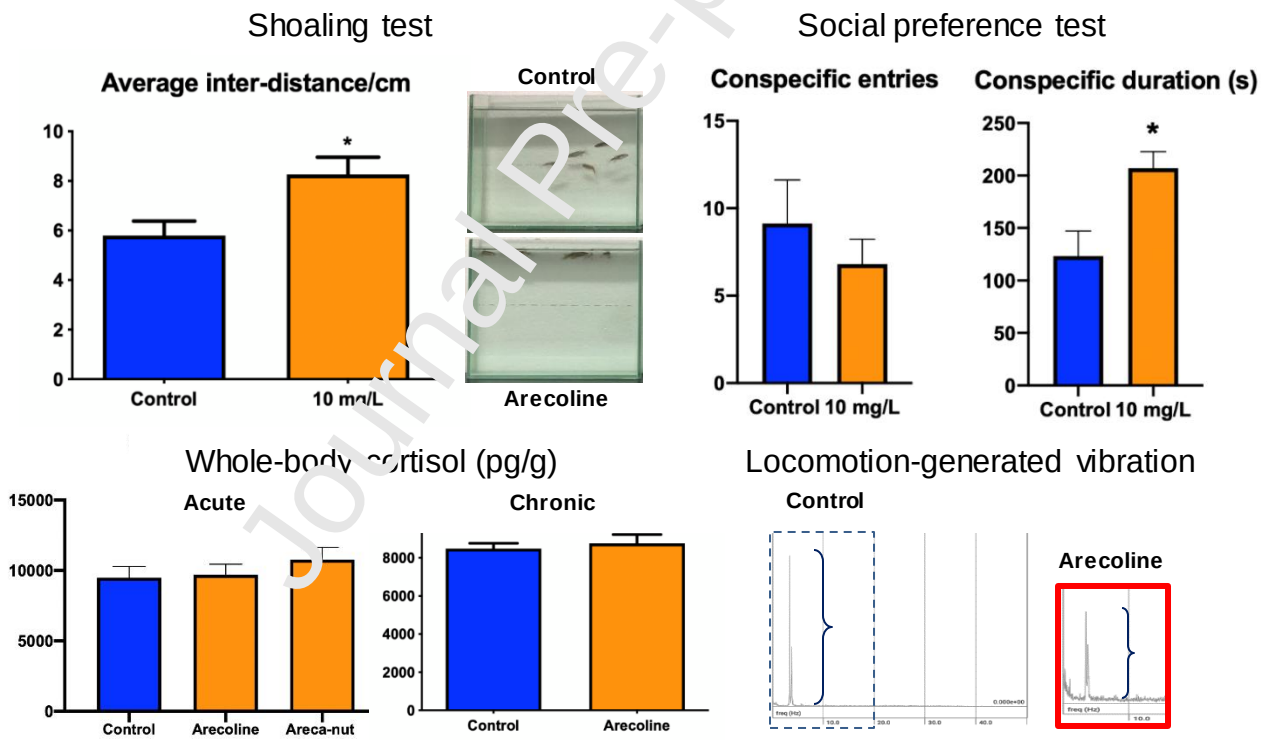

Figure 4. Effects of acute 20-min exposure to arecoline (10 mg/L) on brain monoamines norepinephrine (NE), serotonin (5-HT), dopamine (DA) and their metabolites 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), assessed by the high-performance liquid chromatography (HPLC); n = 12 per group, *P < 0.05, **P < 0.01 vs. control, U-test.

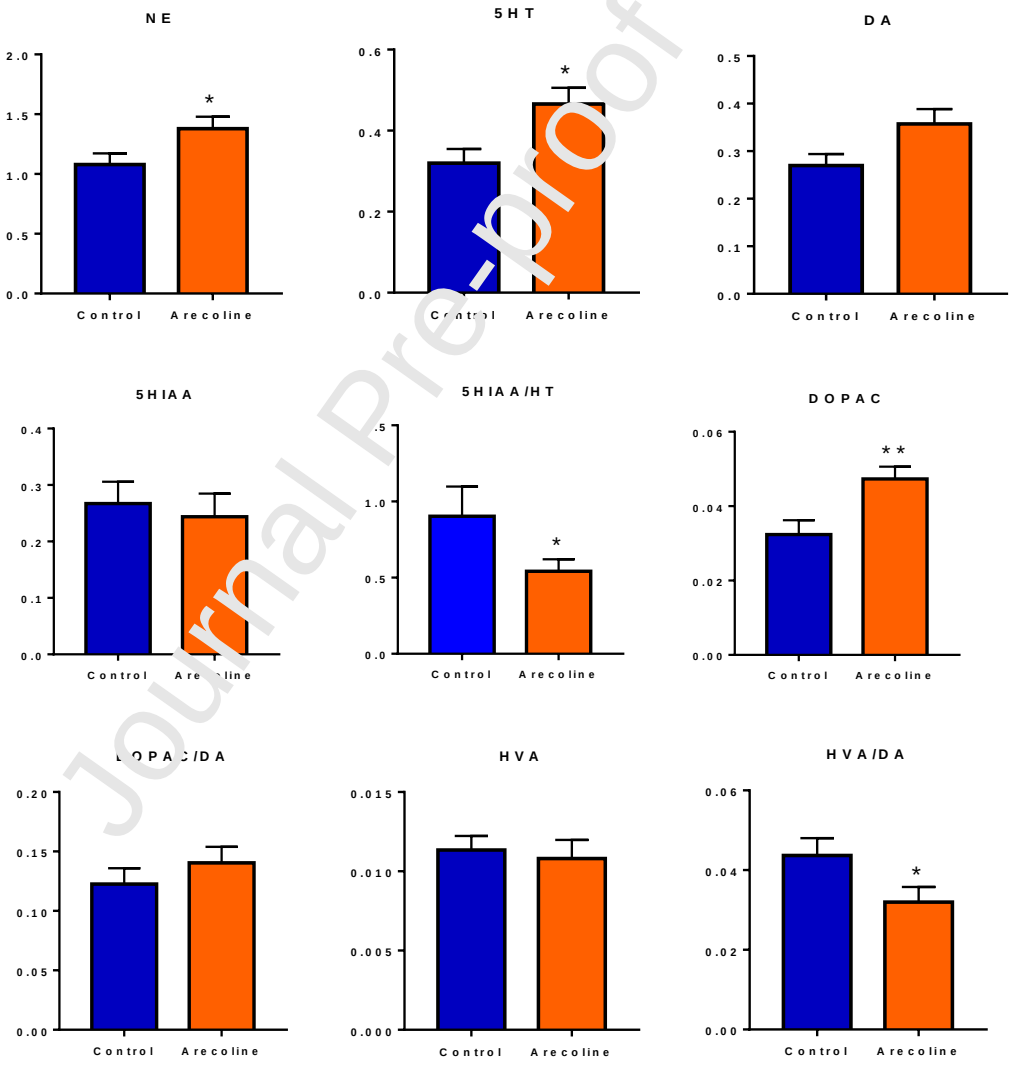

Figure 5. Effects of acute (10 mg/L) and chronic (1 mg/L for 7 days) arecoline exposure on brain expression of selected zebrafish genes (n = 9-11 per group, *P < 0.05, **P < 0.01 vs. control, U-test).

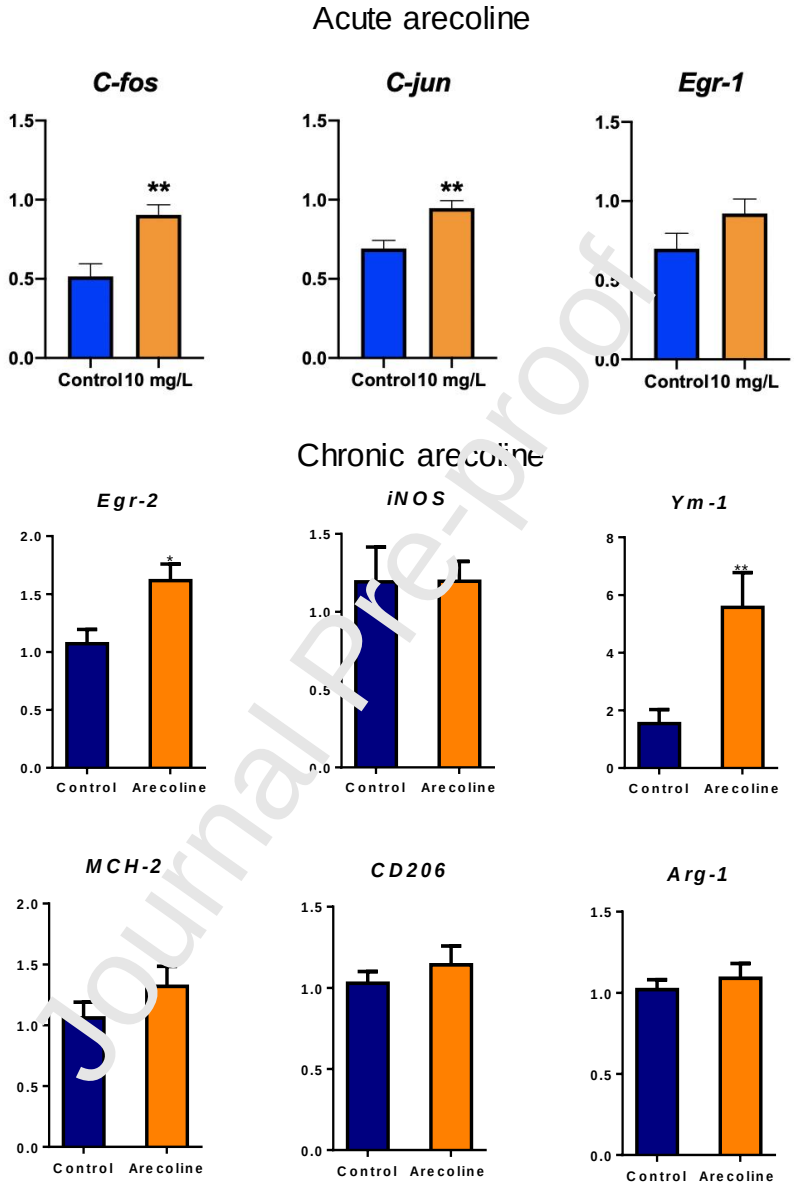

Figure 6. Effects of acute 2-h withdrawal from 12-h treatment with 1 mg/L arecoline (n = 15 per group), *P < 0.05, post-hoc Dunn's test for significant Kruskal-Wallis data (see Table 2 for summary of statistically significant treatment effects).

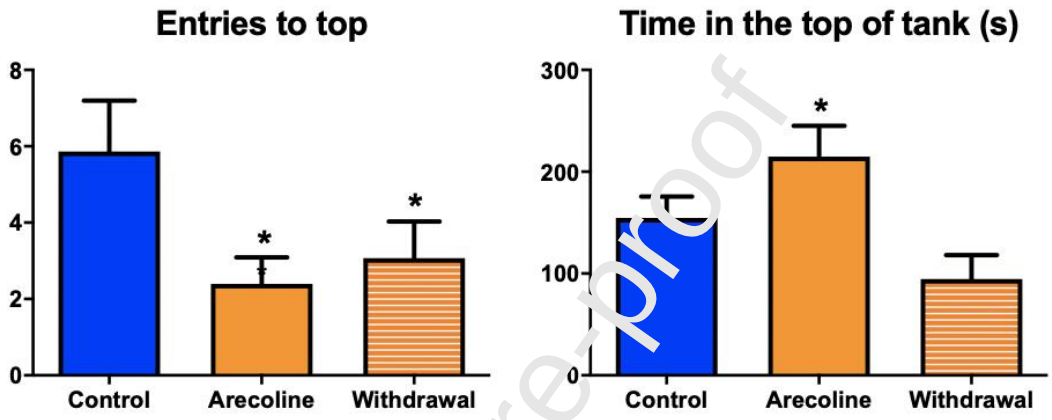

Table 1. Primers used in the present study (Sangon Biotech, Shanghai, China).

Genes	RNA sequence (F-forward, R-reverse)
β-Actin F	CATCAGGGTGTCATGGTTGGT
β-Actin R	TCTCTTGCTCTGAGCCTCATCA
C-fos F	TGAAACTGACCAGCTTGAGGAT
C-fos R	GTGTGCGGCGAGGATGAA
C-jun F	TGGATACAACCACAAGGCTCT
C-jun R	GTCACGTTCTTGGGACACAG
Egr1 F	CTAAGATCCACATGCGGCAGAAGG
Egr1 R	AGTAGCAGGAGTTGACTGGAGACG
MHC-2 F	CCGTCAAGAGCAAGAGCGTTCC
MHC-2 R	CGTTCACCAGCAGTGGCATACTC
iNOS F	AGGCACTCGTGGCTATCAATGTTG
iNOS R	ATGAAGGACTCGCTTGCGGAATG
Egr2B F	TCTGGATGCGGAGAGGTCTATCAAG
Egr2B R	AGTAGGATGGCGGAGGATATGAGATG
CD206 F	CGACACAGATGGCAGATGGAAGAC
CD206 R	ACGCTTCTTTGACTCAGGACAGTTC
Arg1 F	CACGCAGACATCAACACACCTTTAAC
Arg1 R	TGGAAGTTTGGGCATCTTGGACTG
Ym1 F	GCAAGAGGAAGTCCACCTGAAGAC
Ym1 R	ATACAGCAGCGGTCAGCATAAAGC

Table 2. The Kruskal-Wallis test data. NTT – the novel tank test, NS – no significant difference

Tests and endpoints	**Kruskal-Wallis H statistics**	**P value**
Acute arecoline (NTT)		
Time in top, s	H = 10.5	0.0001
Top entries	H = 5.5	0.14, NS
Acute areca nut extract (NTT)		
Time in top, s	H = 25.7	0.0001
Top entries	H = 8.8	0.03
Arecoline withdrawal (NTT)		
Time in top, s	H=7.6	0.023
Top entries	H=9.6	0.008

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COI statement

The authors declare no conflict of interest

Authors Credit Statement

Effects of acute and chronic arecoline in adult zebrafish: anxiolytic-like activity, elevated brain monoamines and the potential role of microglia

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Research highlights

- Arecoline is a naturally occurring psychoactive alkaloid agonist of nicotinic and muscarinic acetylcholine receptors.
- However, the mechanisms of acute and chronic action of arecoline in-vivo remain poorly understood.
- Here, we assessed the effects of acute and chronic arecoline on adult zebrafish behavior and physiology.
- Acute and chronic arecoline evoked anxiolytic-like behavior; acute arecoline elevated brain norepinephrine and serotonin levels in zebrafish.
- Chronic arecoline elevated brain expression of microglia-specific biomarker genes *egr2* and *ym1*.