Environmental Science and Ecotechnology 21 (2024) 100437

Contents lists available at ScienceDirect

Environmental Science and Ecotechnology

journal homepage: www.journals.elsevier.com/environmental-science-and-ecotechnology/

Original Research

Antagonistic interaction between caffeine and ketamine in zebrafish: Implications for aquatic toxicity

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ARTICLE INFO

Article history:
Received 25 August 2023
Received in revised form
6 June 2024
Accepted 6 June 2024

Keywords:
Caffeine
Ketamine
Systems toxicology
Antagonistic effect
GABAergic synapse

ABSTRACT

The coexistence of caffeine (CF) and ketamine (KET) in surface waters across Asia has been widely reported. Previous studies have implied that CF and KET may share a mechanism of action. However, the combined toxicity of these two chemicals on aquatic organisms remains unclear at environmental levels, and the underlying mechanisms are not well understood. Here we demonstrate that KET antagonizes the adverse effects of CF on zebrafish larvae by modulating the gamma-aminobutyric acid (GABA)ergic synapse pathway. Specifically, KET (10–250 ng L^{-1}) ameliorates the locomotor hyperactivity and impaired circadian rhythms in zebrafish larvae induced by 2 mg L^{-1} of CF, showing a dose-dependent relationship. Additionally, the developmental abnormalities in zebrafish larvae exposed to CF are mitigated by KET, with an incidence rate reduced from 26.7% to 6.7%. The competition between CF and KET for binding sites on the GABA-A receptor (*in situ* and *in silico*) elucidates the antagonistic interactions between the two chemicals. Following a seven-day recovery period, the adverse outcomes of CF exposure persist in the fish, whereas the changes observed in the CF + KET groups are significantly alleviated, especially with KET at 10 ng L^{-1}. Based on these results, it is imperative to further assess the environmental risks associated with CF and KET co-pollution. This pilot study underscores the utility of systems toxicology approaches in estimating the combined toxicity of environmental chemicals on aquatic organisms. Moreover, the nighttime behavioral functions of fish could serve as a sensitive biomarker for evaluating the toxicity of psychoactive substances.

1. Introduction

Drug abuse is a prominent issue of concern from pole to pole [1]. Drugs are excreted by the human body as parents or metabolites, enter sewage networks, and then contaminate surface water through effluent discharge [2]. In conjunction with the types of drugs rising and the number of abusers growing, the number of pharmaceuticals detected in aquatic environments is also increasing [3]. According to the anatomical therapeutic chemical (ATC) classification system, 502 in 4000 pharmaceuticals administered worldwide are psychoactive drugs [4]. Most share the same mode of action by altering the secretion and uptake of neurotransmitters in the brain, such as dopamine and gamma-aminobutyric acid (GABA) [5]. Considering that human neural and nervous architecture is conservative in evolutionary terms, psychopharmaceuticals designed for people may also successfully interact with nontarget organisms [6]. Regulatory authorities have identified the mixture effect as a major concern in the environmental risk assessment of organic pollutants [7]. Therefore, it is of utmost importance to estimate the combined toxicity of psychopharmaceuticals in aquatic organisms.

Caffeine (CF), a natural alkaloid, is the most widely consumed psychoactive substance as an additive in food products and many prescription drugs [8]. In China, CF is administrated as a class II psychotropic drug [9]. Statistically, CF abuse has increased rapidly in the northwest and northeast of China [10]. In particular, drug abusers frequently take CF with other illicit drugs (psychoactive substances), such as methamphetamine and ketamine (KET) [11].

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https://doi.org/10.1016/j.ese.2024.100437

The illegal use of KET as a recreational drug is widespread in China [1], and CF is often used as an additive in KET [12]. Consequently, the two psychoactive drugs have shown marked co-pollution in the rivers of China. The detected concentrations of CF and KET are up to 6167 and 9533 ng L^{-1}, respectively, in Taiwan [3], 3100 and 1100 ng L^{-1} in Haikou [13], and 56.9 and 3.7 ng L^{-1} in Wuhan [14,15]. Additionally, CF concentrations up to 0.7 and 1.1 mg L^{-1} have been detected in surface water in Costa Rica [16]. There have been many studies on the ecotoxicity of CF and KET in aquatic organisms. For example, changes in the growth of the Africa clawed frog have been observed after four days of exposure to CF at 0.11 μg L^{-1} [17]. A decrease in lysosomal membrane stability has been observed in mussels after exposure to CF at 500 ng L^{-1} [18]. Meanwhile, the growth of *Daphnia magna* is inhibited by KET at 1000 μg L^{-1} [19]. Exposure to KET at 100 ng L^{-1} has shown significant teratogenic effects on *Caenorhabditis elegans* [20]. In fish, neurotoxicity [21,22], developmental toxicity [23,24], and oxidative stress [25,26] have been reported after exposure to CF or KET. Hence, the widespread coexistence of these two chemicals in Asian aquatic ecosystems may pose a significant environmental risk. However, their combined toxicity and associated mechanisms remain unclear.

Whether the presence of CF and KET in aquatic systems has combined effects on organisms typically depends on the overlap between their mechanisms of action [27]. A previous study revealed that CF delayed the circadian clock of humans as a nonselective adenosine receptor antagonist [28]. Adenosine receptor A1 and A2α subtypes are mainly localized in striatopallidal GABAergic neurons of the brain [29]. Chronic injection of CF reduces the uptake of GABA and increases the release of GABA [30]. Intriguingly, convergent evidence suggests that KET features robust antidepressant effects by blocking N-methyl-D-aspartate receptors (NMDARs) on GABAergic interneurons [31]. Preclinical studies have associated KET's mechanisms of action with increased GABA levels [32]. Therefore, changes in GABAergic function might play a pivotal role in assessing the combined toxicity of CF and KET. Compared to other pollutants, psychoactive substances have the potential to mimic natural infochemicals in structure and disrupt intraspecies communication, including predator avoidance, navigation, and circadian rhythms [33]. Hence, behavioral functions are more sensitive than the classical biomarkers (i.e., morality, growth, and fecundity) as endpoints for assessing the ecological risks of psychoactive substances. Developing behavioral ecotoxicology would benefit the risk assessment of chemicals in aquatic environments [34]. However, such toxicological data, especially changes in the behavioral phenotypes at nighttime mediated by circadian rhythm, are limited [35]. The modulation of GABA levels in suprachiasmatic nuclei has been implicated in the synchronizing of circadian rhythms in mammals [36]. Zebrafish (*Danio rerio*) have well-developed GABAergic neurotransmission, and their response to GABAergic hypnotics is similar to that in mammals [37]. The release and uptake of the inhibitory neurotransmitter GABA have been demonstrated to be related to the regulation of circadian clocks in zebrafish [38]. This provides new insight to further evaluate the ecological effects posed by CF and KET, particularly in vertebrate models, such as zebrafish.

In this study, we use zebrafish larvae as model animals to assess the combined toxicity of CF and KET based on systems toxicological approaches. The behavioral (at nighttime), histological, morphological, and physiological (oxidative stress) indicators of fish are quantified as the toxicological endpoints. Then, the metabolomics profiles, molecular docking patterns between the chemicals and GABA receptor, and associated mRNA levels are determined to elucidate the underlying mechanism. After a seven-day withdrawal period, the biomarkers (i.e., locomotion, neurotransmitter levels, and gene expression levels) of larvae are analyzed to evaluate whether the effects posed by CF and KET are continuous. The results provide empirical evidence for assessing the joint toxicity of CF and KET in aquatic toxicity for the future study.

2. Materials and methods

2.1. Experimental design

Zebrafish maintenance and embryo collection were performed following standard procedures [39]. Briefly, wild-type (AB strain) zebrafish were cultured at 28 ± 0.5 °C in a photoperiod of 14 h light/10 h darkness and fed twice daily with *Artemia* spp. nauplii. Fertilized eggs (n = approx. 250) were obtained from adult fish by spawning in the morning, induced by the beginning of the light period. The embryos were rinsed several times using ultraviolet (UV)-sterilized water from our facility (28.5 °C, 200 mg L^{-1} instant ocean salt, and 100 mg L^{-1} sodium bicarbonate) before being randomly distributed [40]. Standards of CF (purity >99%) and hydrochloride salt of KET (purity >99.5%) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) and Sigma-Aldrich Co., Ltd. (St Louis Missouri, USA), respectively. Stock solutions (1 mg mL^{-1}) were prepared by diluting the liquid standards using water from our facility.

An overview of the experimental design is shown in Fig. 1a. Briefly, the exposure experiments were conducted in the semi-closed water system (200 mL) equipped with both mechanical and biological filtration. There were six groups set in this study, including control (water from our facility), CF (2 mg L^{-1}), and CF (2 mg L^{-1}) + KET (concentrations of 10, 50, 100, and 250 ng L^{-1}). 150 hatched larvae (five days post-fertilization [dpf]) per group were placed into three semi-closed water systems (50 individuals for each system). The exposure concentration of CF was selected based on the environmental levels (up to 1.1 mg L^{-1}) [16] and a previous publication in which 193.82 and 0.039 mg L^{-1} of CF were found as thresholds for the photo motor responses of *Danio rerio* and *Pimephales promelas* larvae, respectively, in the dark [21]. The range of KET concentrations was determined according to the threshold of behavioral function disorders in *Oryzias latipes* induced by KET and the levels detected in fresh water [22]. The exposure period lasted 21 days, and the exposure solution was entirely renewed every 24 h. During exposure, the morphology of each fish larva was recorded using a stereomicroscope. After exposure, 60 fish per group were transferred to a fresh container (200 mL of system water) to acclimate for 1 h and were then immediately used for biomarker testing. The remaining fish were placed in new beakers equipped with clean system water (no chemicals) for a seven-day recovery period. At the end of this stage, all the fish were used to assay the corresponding biomarkers. The concentrations of CF and KET in the exposure media were confirmed using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), as previously described [13]. The actual concentrations were 98.0−102.6% of the nominal values (Supplementary Material Table S1), and the details are shown in the Supplementary Materials.

2.2. Locomotion test

At the end of the exposure and recovery stages, the behavioral functions of the zebrafish larvae (11 individuals in each group) were evaluated using a system and software for animal movement tracking (EthoVison XT, Noldus Information Technology, the Netherlands), as previously described [38]. Briefly, the swimming trajectory was continuously recorded with an infrared-sensitive camera equipped with infrared light and a filter. The testing was

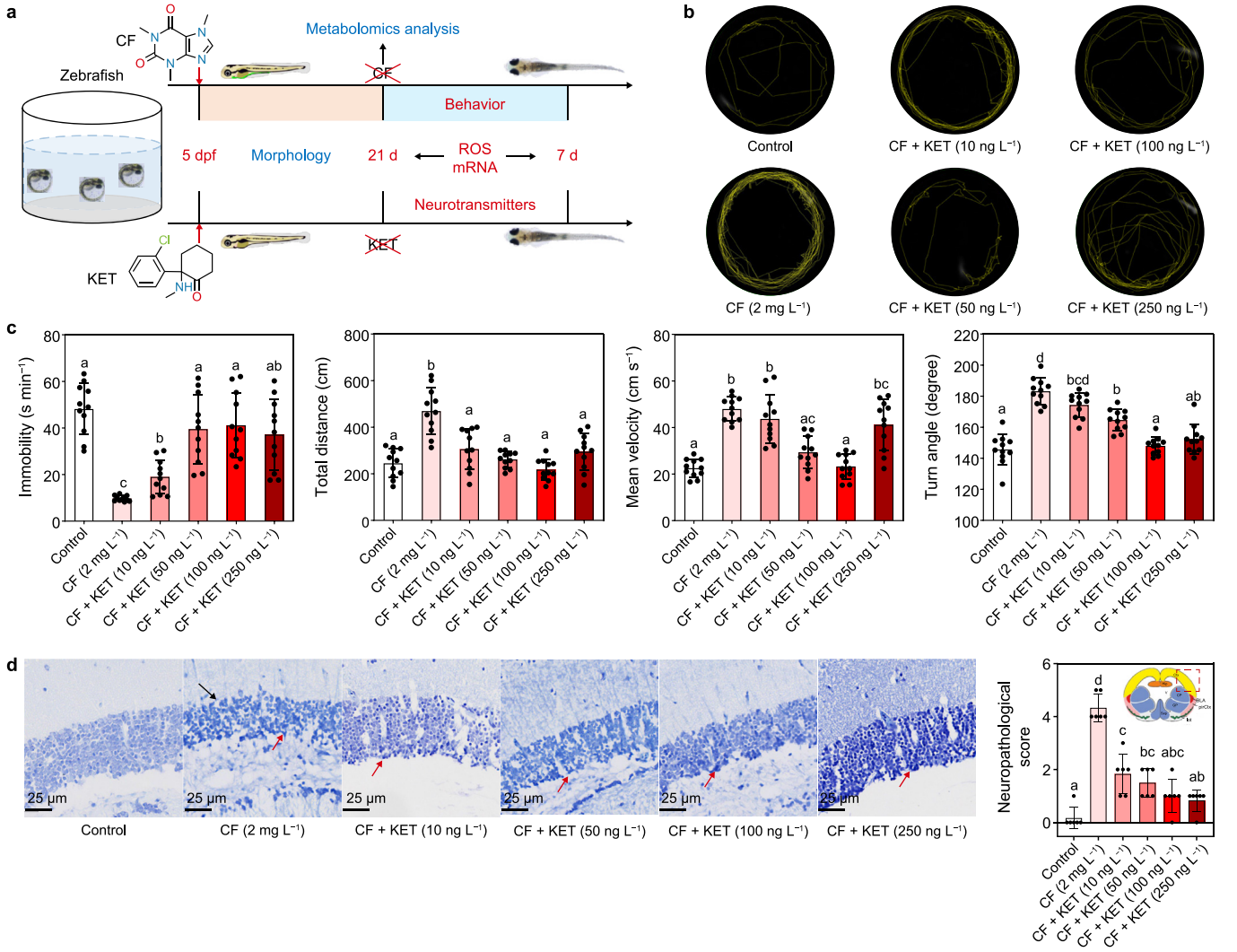

Fig. 1. Effects of caffeine and caffeine (CF) + ketamine (KET) on the swimming behaviors and histopathology in brain tissues of zebrafish larvae after 21 days of exposure. **a**, Schematic of the experiment. The larvae of five days post-fertilization were treated to CF at 2 mg L^{-1} or caffeine + KET (10–250 ng L^{-1}) for 21 days (salmon zone). The morphology was recorded daily. After exposure, the biomarkers were analyzed; part of the animals recovered for seven days (azure zone), and the same biomarkers were analyzed after recovery. **b**, The representative swimming trajectory of larvae from control and exposure groups. **c**, The quantitative results of the behavioral indicators from different groups, including immobility duration (s min^{-1}), total distance (cm), mean velocity (cm s^{-1}), and turn angle (mean degree). **d**, The histopathology changes of the gray zone (red dash line rectangle) localized in the optic tectum of zebrafish from different groups, and the pathological scores were calculated. Black arrow: haphazard sequence; Red arrow: apoptosis.

performed at night (9 p.m.–12 a.m.) without any disturbances. After analyzing the baseline, we selected a 10-min video segment where the fish had reached a steady state. This segment automatically analyzed behavioral parameters, such as immobility duration, mean velocity, total distance, and turn angle. Immobility duration (s min^{-1}) was calculated as the proportion of time during which the fish remained still out of the total measured time. Mean velocity (cm s^{-1}) represented the average distance traveled per unit of time. Total distance was the locomotor distance covered by the fish during the 10 min. Turn angle (degrees) measures the change in movement direction, either clockwise or counterclockwise.

2.3. Metabolic profiling determination

At the end of exposure, 25% of the fish larvae in the control and CF groups were collected. Subsequently, an accurate fish sample weight (60 mg) from each group ($n = 3$) was placed in a 2 mL centrifuge tube equipped with three steel beads, and 1 mL of tissue extracting solution (75% 9:1 (v:v) methanol:chloroform, 25% H_2O) was added. The tube was put into a high-throughput tissue grinder and ground for 60 s at 50 Hz, and this was repeated twice to blend the samples. The pretreatment for the mixtures, details on the instrument's conditions (HPLC-MS/MS), and data analysis are provided in the Supplementary Materials.

The metabolites involved in the GABAergic synapse, including GABA, oxoglutaric acid (α-KG), L-glutamic acid (Glu), and succinic acid (Suc), were quantitatively analyzed ($n = 3$) according to nontarget metabolomic analysis. The absolute concentrations in the fish in the control and CF groups were detected by ultra-performance liquid chromatography (UPLC; ExionLC™ AD, SCIEX, Framingham, MA, USA) equipped with tandem mass spectrometry (MS/MS; QTRAP®6500+, SCIEX, Framingham, MA, USA) in positive ionization mode. The parameters are shown in Supplementary Material Table S2, and the analysis details are shown in the Supplementary Materials.

2.4. Histopathological brain analysis

Given the small size of the larvae, the entire body of each fish was fixed in 4% paraformaldehyde (Aladdin Biochemical Technology Co., Ltd., Shanghai, China) for 24 h. The back side of the fish was taken as the embedded surface in the paraffin. The blocks were cut into slices of 5 μm, ensuring that the brain zone was fully exposed in the cross-section. Each slice was stained with 0.5% toluene blue (Nissl body staining of neurons). The changes in the histopathology in the brain tissues were observed and recorded with an optical microscope with a charge-coupled device camera ($n = 4$), and the pathological scores were calculated according to the criteria [41].

2.5. Molecular docking

Molecular docking analysis was performed using the "Discovering Active Sites of Homologous Proteins by Sequence Alignment" function module of Scigress (Ultra Version 3.0.0, Fujitsu) *in silico* [42]. The 3D structure of the ligand-binding domain between human GABA-A receptor α (GABAR; ID: 6D6T) and FYP (flumazenil) was downloaded from the Protein Data Bank website (http://www.rcsb.org.pdb). The sequence coding for amino acids in the protein of zebrafish GABAR (ID: AAI24698.1) was obtained from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/), and its 3D structure was predicted using the SWISS-MODEL online tool (https://swissmodel.expasy.org/), referring to the structure of human GABAR. The accuracy was evaluated using the SAVES v6.0 online tool (https://saves.mbi.ucla.edu/). On this basis, the binding affinities of CF and KET in the antagonist pocket of zebrafish GABAR were evaluated using the built-in program to discover active sites of homologous proteins via sequence alignment. The details are provided in the Supplementary Materials. The AutoDock score (ΔG, kcal mol^{-1}) of the top 20 ligand-protein structures were calculated.

2.6. Gene expression analysis

At the end of the exposure and recovery stages, the zebrafish larvae from each group ($n = 6$) were collected, and the total RNA of each whole fish was extracted with TRIzol® reagent and liquid nitrogen and treated with the Turbo DNA-free kit (Ambion Inc., TX, USA) to eliminate DNA contamination. The details of the subsequent reverse transcription (cDNA synthesis), quantitative polymerase chain reaction (qPCR) analysis method, primers (Supplementary Material Table S3), and instruments utilized are provided in the Supplementary Materials.

2.7. ROS Visualization and enzymatic activity measurements

After exposure, *in situ* ROS in the insomnia fish gut was detected by 2′,7′-dichlorofluorescein (H_2DCF) (Sigma-Aldrich Co., Ltd., St Louis Missouri, USA) following a previously described protocol [43]. Briefly, fish larvae ($n = 6$) were incubated with 10 μM H_2DCF for 15 min in the dark at room temperature and then anesthetized with tricaine (20 mg L^{-1}; Sigma-Aldrich Co., Ltd., St Louis, Missouri, USA) for 10 min. Fluorescent pictures of the larvae were taken using a fluorescence microscope (ZEISS Axio VertA1, Carl Zeiss AG, Oberkochen, Germany) immediately after rinsing three times in water from our facility. The excitation and emission wavelengths were 485 and 535 nm, respectively. The pictures were calibrated by Photoshop CC2015 (Adobe, USA) to eliminate background disturbances. Fluorescence intensities were quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, USA).

For measurements of enzymatic antioxidant activity, fish samples ($n = 6$) were randomly collected from each group and homogenized in PBS (pH = 7.2) on ice. The homogenate was centrifuged at 12,000×*g* and 4 °C for 30 min. The supernatant was pipetted into a fresh centrifuge tube used for the measurement of superoxide dismutase (SOD) and catalase (CAT) activities [44]. Nitroblue tetrazolium and hydrogen peroxide were the substrates used for SOD and CAT, respectively. The determination wavelengths were 560 nm for SOD and 240 nm for CAT. The parameters are shown in Supplementary Material Table S2.

2.8. Gamma-aminobutyric acid and melatonin quantification

At the end of the exposure and recovery stages, fish samples from each group ($n = 4$) were homogenized in PBS (pH = 7.2) on ice and then centrifuged (3000×*g*, 4 °C) for 10 min. Given that inhibitory neurotransmitter GABA and melatonin (MTN) secretion are associated with circadian clocks [45], the supernatant was used to determine the contents of GABA and MTN using ELISA kits (HEPENG Bio, Shanghai, China) according to the manufacturer's instructions. The parameters are shown in Supplementary Material Table S2, and the details are shown in the Supplementary Materials.

2.9. Statistical analysis

All data are shown as the mean ± SD. The normality and homogeneity of variance in the data were checked using the Shapiro-Wilk test and the Brown-Forsyth method, respectively. The differences in the biomarker levels of fish from different exposure groups were tested using one-way ANOVA followed by a post hoc Tukey's test (95% confidence interval). A $\chi 2$ test was performed to examine the difference in the incidences of fish with abnormal morphology between the control and exposure groups. The changes in the concentration-dependent relationship of the biomarker in the different exposure groups were assessed using the Jonckheere-Terpstra test. Differences with *p*-values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Changes in locomotion and histopathology

After 21 days of exposure, the swimming trajectory of the fish was determined, and the representative patterns from the different groups are shown in Fig. 1b. A relatively complex trajectory by the fish larvae was observed in the CF group compared to the control group featuring a simple track, which provided evidence of the stimulation of CF. Otherwise, because consistently swimming in circles is the primary behavioral phenotype of depressive zebrafish [46], the same pattern observed in the CF group suggests the potential depressive effects of CF on the fish. Intriguingly, the zebrafish from 50 to 250 ng L^{-1} of KET groups showed the same swimming pattern with the fish from control group, exploring the chamber with oblique turn and half-turn rotations [46]. The hyperactivity of zebrafish induced by CF (2 mg L^{-1}) was reported in a previous publication [47], while exposure to KET at 50 mg L^{-1} for 24 h has been shown to markedly increase the swimming distance of zebrafish larvae [24]. However, depression-like behaviors were not observed in the CF + KET group, which can be attributed to the sedative effects previously reported with joint CF and KET exposure [48]. As has been previously reported [38], the typical postures of zebrafish larvae in sleep, including floating with the head down and staying in a horizontal position close to the bottom, were observed in the control group. However, exposure to CF significantly disturbed the sleeping behavior of the zebrafish, and this phenomenon was mitigated with the combined treatment with KET. In summary, exposure to CF at high environmental concentrations

(2 mg L^{-1}) induced depression-like behavior in zebrafish, and the addition of KET at trace levels (10–250 ng L^{-1}) alleviated these outcomes.

Based on the behavioral criteria, the sleep state is defined in zebrafish larvae as more than 6 s of immobility in 10 s (60%) [49]. We statistically analyzed the corresponding behavioral indicators (Fig. 1c). The immobility duration reduced from 43.58 s min^{-1} (control) to 9.94 s min^{-1} after exposure to CF ($p < 0.0001$), indicating the interruption of sleep state by trace CF. Meanwhile, the values of total distance, mean velocity, and turn angle of the fish significantly increased in the CF-treatment group (4827.12 cm, $p = 0.0002$; 50.06 cm s^{-1}, $p < 0.0001$; and 189.24°; $p < 0.0001$, respectively) compared to the control group (2378.23 cm, 23.67 cm s^{-1}, and 151.58°, respectively). The immobility duration was prolonged for the fish in the CF + KET groups, and the locomotor activity (i.e., total distance, mean velocity, and turn angle) was suppressed. The changes in immobility duration ($p = 0.046$, 0–250 ng L^{-1}), mean velocity ($p = 0.048$, 0–100 ng L^{-1}), and turn angle ($p = 0.006$, 0–250 ng L^{-1}) were in a dose-response manner. In our study, the lowest observed effect concentrations in the presence of 2 mg L^{-1} of caffeine for immobility duration, total distance, mean velocity, and turn angle were 50, 10, 50, and 50 ng L^{-1}, respectively. The results highlight the antagonistic effects of CF and KET on the sleep behavior alterations of zebrafish at the environmental level. Notably, this effect was weakened at a high dose of KET (250 ng L^{-1}), implying the complexity of the combined toxicity of CF and KET.

Given the potential of CF for modification to cortical synapse and neuron networks in the brain [50], we assayed the changes in the histopathology of zebrafish brain. The periglomerular gray zone was selected as the target since the retinorecipient areas of zebrafish are mainly localized at the mesencephalon, which is responsible for the visually evoked behaviors (e.g., sleep and predation) (Fig. 1d) [51]. In comparison with the control (compact and regular granular cell layer), the cell layer showed a markedly haphazard sequence (black arrow) and neural apoptosis (red arrow) in the CF group. The detrimental effects on zebrafish brain from CF + KET groups (neural apoptosis) were obviously slighter than the CF group (disorganized granular cells + neural apoptosis). The degree of neuronal injury (i.e., neuronal necrosis and apoptosis) was scored according to three types: neuronal necrosis (grades 1–3), laminar necrosis (grades 4–6), and confluent infarct (grades 7–9). The score in the CF–exposed group (4.3) was much higher than in the other groups, and the addition of KET mitigated the pathological changes (ranging from 1.8 to 0.8; Fig. 1d).

3.2. Changes in the content of metabolites and the expression level of genes

The differences in metabolic profiles between the control and CF treatment groups were analyzed to further elucidate the underlying mechanism. 195 intersected metabolites, including 34 downregulated and 19 upregulated metabolites, were identified (absolute value of $\log_2$(fold change) > 1, $p < 0.05$) (Fig. 2a). Among them, the majority of the downregulated compounds were ketoleucin ($2^{-3.14}$-fold), GABA($2^{-1.34}$-fold), isobutyric acid ($2^{-3.40}$-fold), cortisol ($2^{-1.38}$-fold), nictotinic acid ($2^{-1.76}$-fold), and adenylsuccinic acid ($2^{-2.36}$-fold), while the upregulated chemicals included 3-dehydroshikimate ($2^{2.82}$-fold), 4-pyridoxic acid ($2^{2.32}$-fold), pyruvic acid ($2^{1.91}$-fold), and D-proline ($2^{1.50}$-fold). Typical metabolites were enriched in pathways such as the GABAergic synapse, alanine aspartate glutamate metabolism, nicotinate and nicotinamide metabolism, caffeine metabolism, and bile secretion (Fig. 2b). Existing studies have reported that neuronal hyperpolarization induced by the activation of the GABAergic pathway can facilitate sleep onset and extend sleep duration [52]. Well-developed GABAergic neurotransmission is found in the zebrafish brain, and many GABAergic hypnotics for mammals are strikingly effective for zebrafish [53]. Accordingly, the suppressed GABAergic pathway may be strongly responsible for the sleep disturbance of zebrafish by CF. Furthermore, upregulated CF metabolism and bile secretion has been associated with CF digestion in fish brains [54]. Further, the chemicals involved in the GABAergic synapse (map04727 in the KEGG database), including GABA, oxoglutaric acid ($2^{-0.66}$-fold), L-glutamic acid ($2^{-0.87}$-fold), and succinic acid ($2^{-0.85}$-fold) identified in the metabolic profile, were quantified. The concentrations of GABA, oxoglutaric acid, L-glutamic acid, and succinic acid in the fish in the CF-treatment group were 0.61, 1.54, 1.32, and 0.77 ng g^{-1}, respectively, which were significantly ($p < 0.05$) lower than the control (1.53, 2.44, 2.41, and 1.39 ng g^{-1}, respectively) (Fig. 2c). The results support the idea that the inhibition of the GABAergic pathway might be the reason for the adverse effect of CF.

Therefore, molecular docking (*in silico*) was performed to investigate the structural basis of the anti-GABAergic activity by CF using the "Discovering Active Sites of Homologous Proteins by Sequence Alignment" function module of Scigress (Ultra Version 3.0.0, Fujitsu). The 3D structure of GABA-A receptor α (GABAR) in zebrafish was modeled using SWISS-MODEL (Supplementary Material Fig. S1b), and this structure was well matched with the human GABAR template (Supplementary Material Fig. S1a). The sequence identity, global model quality estimate, and global QMEANDisCo score were 86.46%, 0.73, and 0.73, respectively. The Ramachandran plot derived from a PROCHECK analysis indicated over 90% of the dihedral angle involved in the most favored regions (crimson zone) (Supplementary Material Fig. S1c). Molecular docking was performed using FYP (flumazenil) as the template since it is a choosy and competitive GABA receptor antagonist for preventing benzodiazepine recognition action [55]. FYP, CF, and KET were found to fit well into the predicted antagonist pocket of GABAR in the zebrafish (Supplementary Material Fig. S1d). Most of the adjacent surfaces of the FYP (Supplementary Material Fig. S2a), CF (Supplementary Material Fig. S2b), and KET (Supplementary Material Fig. S2c) were hydrophilic (blue and purple-red), indicating amino and hydroxyl groups in the structure of the ligand. The core hydrophobic moiety was provided by ARG35, LYS89, PHE90, GLY91, SER92, TYR95, PRO96, MET97, ILE100, AIA101, TYR102, SER132, SER133, GLU134, ARG135, and LEU136. The hydrogen bonds between the CF and PHE90, and KET and ARG135 and LEU136 determined the interactions between CF and KET and GABAR (Fig. 2d). Meanwhile, the ΔG of FYP, CF, and KET binding to GABAR were −6.2, −4.3, and −4.9 kcal mol^{-1}, respectively.

Based on the metabolomics and molecular docking results, GABA may play an important role in fish exposed to CF and KET responses. A previous study elucidated that GABAergic amacrine cells are direct targets of melatonin (MTN) [56], and the administration of MTN has been found to impact behaviors by mediating the central GABAergic system [57]. Therefore, the relative contents of GABA and MTN (for the absolute contents, Supplementary Material Table S4), as well as the transcriptional levels of genes (i.e., *gabra1*, *kcnj3a*, *mntr1a1*, and *mntr1ba*) within GABAergic synapses, were determined. Compared with the control (as 100%), the relative levels (except *gabra1*) decreased after exposure to CF and gradually increased with the addition of KET. The changes in *kcnj3a* ($p = 0.034$, 0–250 ng L^{-1}) were concentration-dependent (Fig. 2e). The significant upregulation of the gene *gabra1* (*Danio rerio* gamma-aminobutyric acid type A receptor subunit α1) in the CF group ($p < 0.05$) indicated the blockade of CF for GABAR, which was evidenced by molecular docking. However, the GABA content of zebrafish larvae in the CF group significantly decreased. The

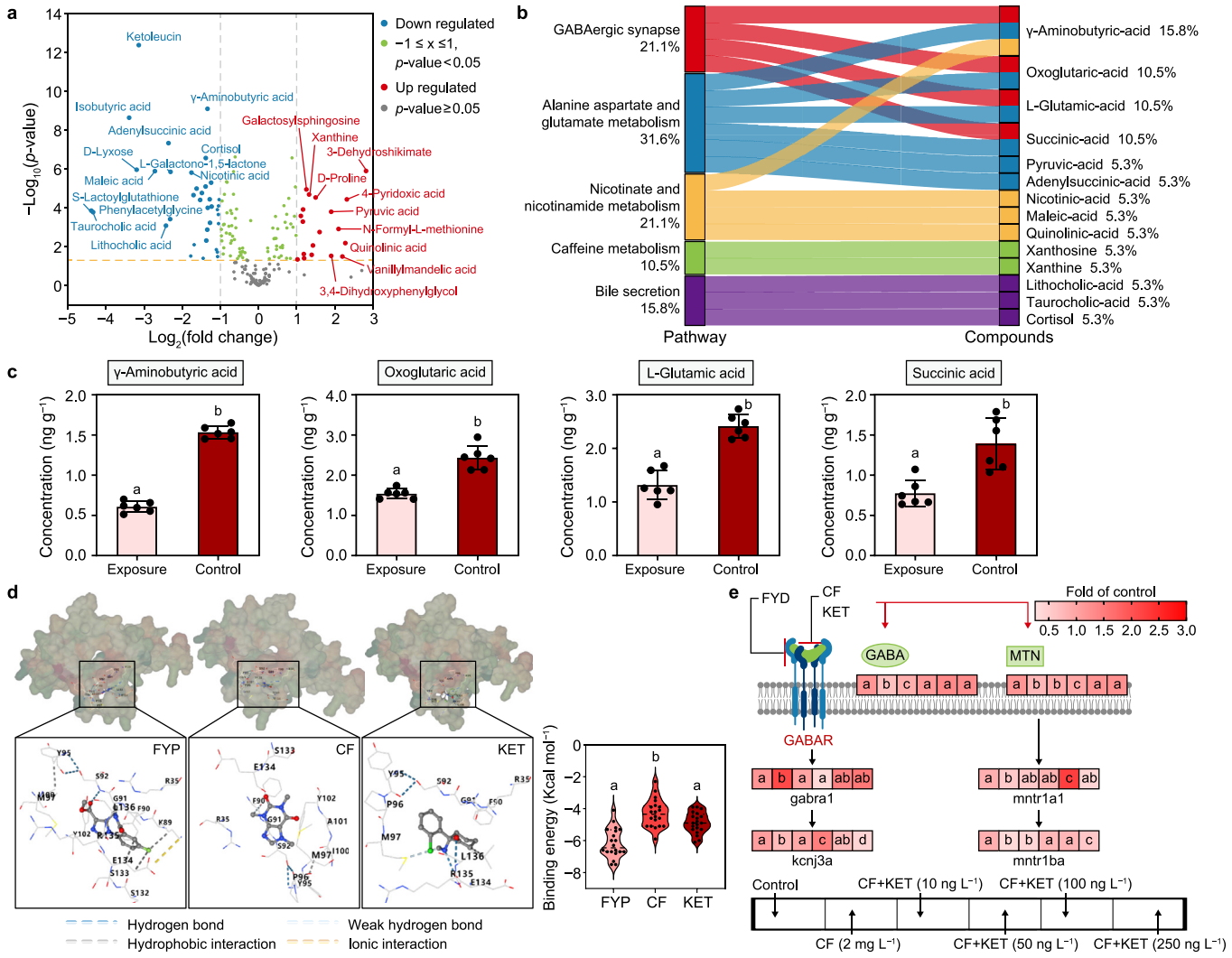

Fig. 2. The antagonistic effects of KET on CF through mediating the GABAergic synapse pathway. **a**, The volcano plot of the metabolites between the control and CF groups was done using metabonomic analysis. Yellow dash: $p = 0.05$; Gray dash: Absolute values of $\log_2$(fold change) = 1. **b**, The interaction network between the main metabolites and corresponding pathways. Primary metabolites were selected based on different metabolite profiles. **c**, The absolute concentrations of the chemicals involved in the GABAergic synapse pathway using LC-MS/MS analysis. **d**, Binding sites of FYP, CF, and KET to GABA-A receptor antagonist pocket, the interaction modes, and the binding scores. **e**, Changes of the relative contents of neurotransmitters GABA and MTN based on the absolute contents and the relative expression levels of genes, including *gabra1, mntr1a1, mntr1ba*, and *kcnj3a* from different groups (CF and CF + KET vs. the control).

contrary changes between gene expression and neurotransmitter secretions suggest that CF has an antagonistic effect on GABAR, triggering the compensation mechanism of the GABAergic system [58].

3.3. ROS accumulation and developmental malformations

To comprehensively assess the combined toxicity of CF and KET, it is crucial to identify individual adverse outcomes. Sleep loss has been found to alter the redox state of several rhythm-regulating neurons in fly brains and then impair waking locomotor activity [59]. However, oxidative stress in the brain does not appear to be significantly induced at a measurable level by rhythm disorders [60]. Hence, a search for signs of oxidative stress in other tissues was warranted. A previous study revealed that sleep deprivation may result in ROS accumulation in the guts of flies and mice and consequently cause the death or shortened lifespan of the organisms [43]. Similarly, in the present study, a visible accumulation of ROS in the larval guts was observed in individuals exposed only to CF compared to those in the control group (Fig. 3a). In addition, treatment with KET reduced this phenomenon, as the fluorescence area in the gut (the accumulation of ROS in the larval gut) was gradually lowered, while the area in the 250 ng L^{-1} group was larger than that in the 100 ng L^{-1} group (Fig. 3a). Statistically, the relative density of fluorescence in the gut (the density of the control was set as 100%) was significantly greater than that in the control group until the concentration of KET was increased to 100 ng L^{-1} ($p > 0.05$) (Fig. 3b). SOD and CAT are crucial enzymes that have been implicated in the neutralization of oxidative stress [61]. A previous study reported that exposure to environmental pollutants can induce oxidative stress in zebrafish embryos and trigger an increase in the activity of SOD and CAT. That increase will then counteract the ROS accumulation in the fish's body by eliminating surplus reactive radicals [62]. However, when xenobiotics have long-term stimulative effects, the upregulation of enzymatic activity and ROS accumulation may occur simultaneously [43]. Similarly, in the present study, the expression levels of the genes *sod1* and *cat* and the activities of SOD and CAT in zebrafish significantly increased in

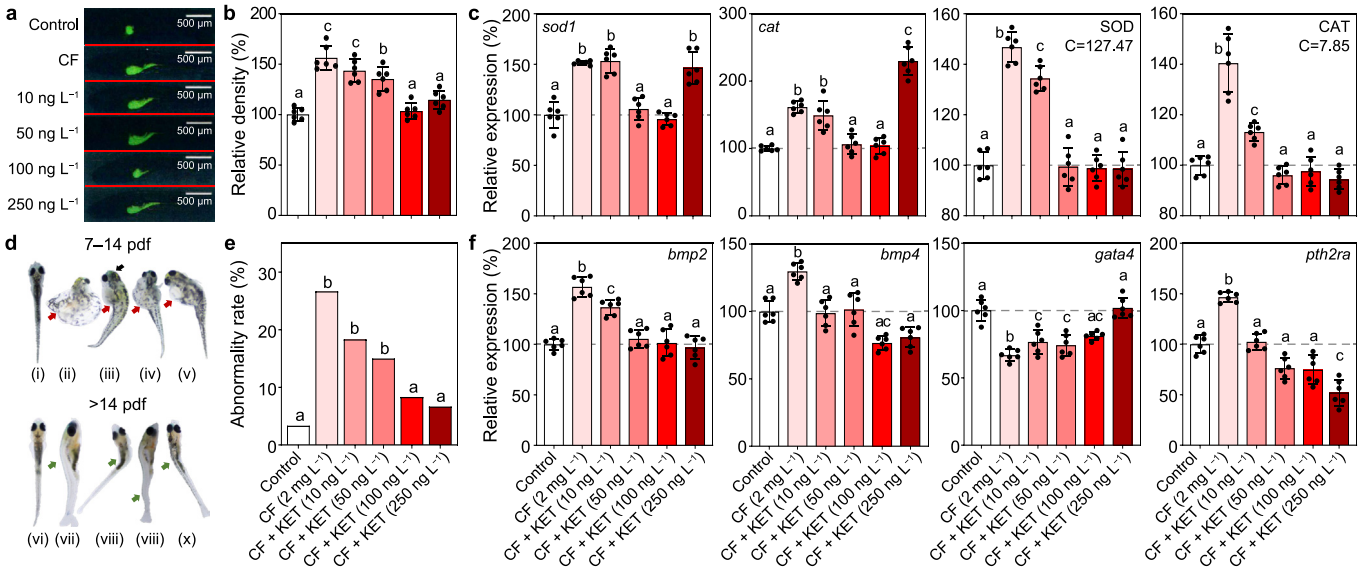

Fig. 3. ROS accumulation in the gut, developmental abnormalities, and the changes of relative expression of associated genes from different groups. **a**, The fluorescence area represents the ROS accumulation in the gut from control, CF, and CF + KET, respectively. **b**, Changes of relative intensity (% vs. the control) of the fluorescence. **c**, Changes of the relative expression levels of genes *sod1* and *cat* and the activities of SOD (control = 127.47 U mg^{-1} protein) and CAT (control = 7.85 U mg^{-1} protein). **d**, Abnormalities ((i)–(v): after 14 dpf; (vi)–(x): after 21 dpf) from different groups. Red arrow: hydro cardia; Black arrow: the single eye; Green arrow: skeleton deformity. **e**, The percentage of abnormalities in different groups. **f**, Changes in the relative expression levels of development-associated genes, including *bmp2*, *bmp4*, *gata4*, and *pth2ra*.

the CF group (Fig. 3c), which was consistent with the observed ROS accumulation in the gut (Fig. 3a). For the CF + KET groups, the mRNA levels of *sod1* and *cat* were downregulated in the 50 and 100 ng L^{-1} groups, as were the activities of SOD and CAT when compared to the group exposed solely to CF, while the transcription levels of the two genes in the 250 ng L^{-1} group were upregulated compared with those in the 100 ng L^{-1} group (Fig. 3c). These results indicate that the antagonistic effects posed by KET on the accumulation of ROS in the larval gut were triggered by CF. Alternatively, most ROS (~90%) is generated during adenosine triphosphate synthesis in the mitochondria, which correlates to apoptosis mediated via the *bax*-mitochondria-*caspase* protease pathway [63]. Likewise, we found that the transcriptional expression of apoptotic genes (Supplementary Material Fig. S3), including *tp53*, *aifm1*, *casp6*, and *casp9*, was significantly upregulated by CF in normal zebrafish larvae. However, the relative expression levels of the above genes were considerably decreased when KET was added and returned to the levels of the control group in the higher KET group (100–250 ng L^{-1}).

Sleep deprivation can reduce growth hormone secretion [64], alter host defense responses [65], and cause the breakdown of the skin and mucosal barrier functions of neonatal rats [66], which may consequently affect infant development. The adverse effects posed by ROS accumulation have been shown in the larval development of flies and zebrafish [67]. It has previously been reported that CF consumption may lead to developmental anomalies of *Danio rerio* [68]. In the present study, hydrocardia (Fig. 3d(ii)–(v)), single eye (Fig. 3d(iii)), and morphological deformation (Fig. 3d(v)) were observed in larvae at the early stage (7–14 dpf) compared with the controls (Fig. 3d(i)). Generally, developmentally retarded zebrafish larvae cannot survive. The mortality rate of larvae (7–14 dpf) in the CF exposure group was 10.3%, and no lethal effects were observed in the CF + KET groups. At the next stage (>14 dpf), abnormal spinal development (Fig. 3d(vii)–(x)) was found in larvae treated with CF compared to the normal morphology shown in Fig. 3d(vi), and the abnormal larvae showed obvious dyskinesia, although they were alive. As shown in Fig. 3e, the incidence of abnormalities increased by up to 26.67% and was significantly higher than that in the control group (3.35%, $p < 0.001$). Surprisingly, combined CF and KET exposure decreased the incidence (18.33% at 10 ng L^{-1} ($p = 0.003$), 15.00% at 50 ng L^{-1} ($p = 0.034$), 8.30% at 100 ng L^{-1} ($p = 0.068$), and 6.65% at 250 ng L^{-1} ($p = 0.072$)).

The expression levels of the associated genes, including *bmp2*, *bmp4*, *gata4*, and *path2ra*, were quantified to further elucidate the underlying mechanisms for the developmental disorder. Bone morphogenetic proteins (BMPs) have been identified by their bone-inducing activities as being members of the transforming growth factor beta (TGF-β) family [69]. Moreover, the skeletal deformation induced by triphenyltin has been attributed to the upregulation of BMP-related genes in medaka fish [70]. GATA4 (zinc finger transcription factor) is essential for the formation of the proepicardium in vertebrates [71], while parathyroid hormone-related peptide (PTHrP) plays a crucial role in craniofacial skeletogenesis in zebrafish [72]. The significant upregulation of the genes *bmp2*, *bmp4*, and *pth2ra* and the suppression of the gene *gata4* found in the CF group, compared with the control group, accounted for the teratogenic effect associated with the loss of sleep (Fig. 3f). KET alleviated the downregulation or upregulation of the genes induced by CF and then reduced the incidence of deformation.

3.4. Changes in biomarker levels in fish seven days post-recovery

A previous investigation using medaka fish as model animals treated with KET for 14 days found that the levels of ROS and SOD activity did not completely return to control levels after seven days of recovery [73]. Nevertheless, the ability of fish cotreated with CF and KET to recover is unknown. Hence, the associated biomarkers of zebrafish larvae exposed to CF or CF + KET were estimated after seven-day recovery. The movement trajectory (Fig. 4a) of the fish featured a similar pattern to Fig. 1b, implying that the stimulation posed by CF was prolonged beyond 21-day exposure and was still measurable after a seven-day recovery period. The immobility duration (9.44 s min^{-1}) and mean velocity (42.19 cm s^{-1}) of fish in the CF group were significantly different from those in the control

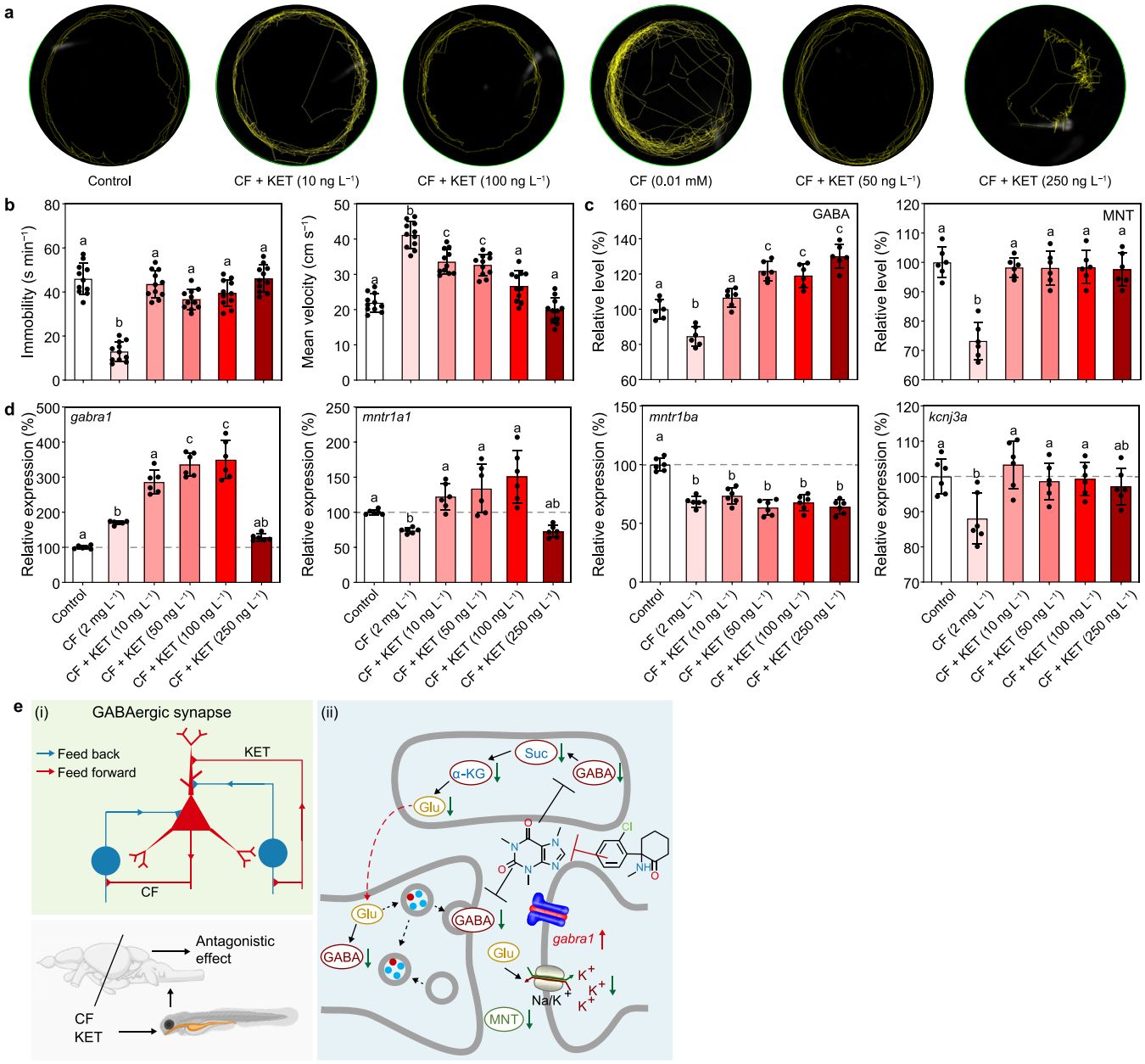

Fig. 4. The behavioral parameters and associated biomarkers of zebrafish larvae from different groups after seven-day recovery. **a**, The representative swimming trajectory of zebrafish larvae from different groups. **b**, The quantitative results of the behavioral indicators from different groups include immobility duration (s min^{-1}) and mean velocity (cm s^{-1}). **c**, The relative contents (% vs. the control) of neurotransmitters GABA and MTN are based on the absolute contents from different groups (CF and CF + KET vs. the control). **d**, The relative expression levels of genes including *gabra1, mntr1a1, mntr1ba*, and *kcnj3a* from different groups (CF and CF + KET vs. the control). **e**, The schematic of the underlying mechanism of the antagonistic effects posed by KET to neurotoxicity of CF in zebrafish.

group (50.90 s min^{-1} and 22.57 cm s^{-1}, respectively), with fish in the CF group showing a shorter immobility duration and a greater mean velocity (Fig. 4b). However, these parameters did not exhibit significant differences between the fish in the CF + KET groups. Those in the control group as the KET concentrations increased.

After a seven-day recovery, the changes in the relative levels of GABA and MTN (absolute data are shown in Supplementary Material Table S4) and the expression of genes (*gabra1*, *mntr1a1*, *mntr1ba,* and *kcnj3a*) in fish in the CF group, compared to those in the control group, showed similar patterns to those observed after 21 days of exposure (Fig. 4c and d). Meanwhile, the relative GABA content in the CF + KET groups (ranging from 50 to 250 ng L^{-1}) significantly increased after a seven-day recovery period (Fig. 4d). This indicates that the antagonistic effects of KET on the adverse outcomes of CF were still observable even after seven days of withdrawal. Notably, the relative expression levels of the *gabra1* gene in the zebrafish were significantly upregulated in the CF + KET groups (10–100 ng L^{-1}) compared to the control group, contrary to the results after 21 days of exposure (Fig. 2e). These findings further imply that the GABAergic synapse pathway may be crucial in mediating the combined toxicity of CF and KET.

The upregulation of development-related genes, including *bmp2*, *bmp4*, *gata4*, and *pth2ra4,* as well as the *sod1* and *cat* genes of larvae in the CF group, was still observed after seven days of recovery (Supplementary Material Fig. S4). There were no significant differences between the CF + KET and control groups. For the

apoptosis-associated genes, concentration-dependent down-regulation was found in the CF and CF + KET groups (Supplementary Material Fig. S4), demonstrating that apoptotic effects abated after recovery.

There are some limitations to consider. The changes in the indicators were evaluated after a short-term recovery stage (seven days) in this study, but the long-term effects (14 or 21 days) remain unclear. Notably, the persistence of the adverse effects induced by CF should be assessed based on long-term recovery. Hence, future investigations involving longer recovery durations and comparisons between withdrawal and continuous exposure experiments should be carried out.

3.5. Molecular mechanisms and environmental implications

Regulatory authorities have acknowledged that combined toxicity is a major concern based on environmental risk assessments of organic contaminants [7]. Chemical cocktails may have multiple effects on the physiological, behavioral, and genetic systems of organisms [74]. CF and KET are primary combined pollutants in surface water, with concentrations ranging from 3.7 to 9533 ng L^{-1}. Furthermore, CF and KET have circadian-disrupting effects on organisms by sharing an overlapping action mode [28,75]. Sleep behavior, regulated by the summation of circadian rhythms and homeostasis, should be considered when assessing the environmental risk of psychoactive substances. The circadian rhythm disorders in fish species posed by psychoactive substances (e.g., CF and KET) pouring into aquatic system should be a concern [76]. The results of the present experiments demonstrate that the duration of fish immobility during the dark period can efficiently reflect the phenotype of the alterations in sleep state induced by a combination of CF and KET, consistent with previously proposed criteria [49]. For the first time, we found that CF had adverse effects on sleep state and that KET mitigated this when added at trace levels. Hence, traditional risk assessment models are applied to estimate single chemicals [77], which might overestimate the realistic toxicity risks related to the co-occurrence of CF and KET in the natural environment.

Meanwhile, developing sensitive biomarkers to evaluate sleep behavior changes is crucial. According to the changes observed in this study, treatment with CF inhibited GABAergic synapse activity through the feedback pathway, while KET (10–100 ng L^{-1}) could mitigate suppression by irritating the nervous impulse (Fig. 4e(i)). CF can regulate GABA release through NMDAR activation [78]. Drinking regular caffeinated coffee causes a marked decrease in MTN metabolism through to the following nigh t [79]. Interestingly, the inhibition of the NMDAR by KET on GABAergic interneurons has been reported to lead to pyramidal cell disinhibition and the enhancement of glutamatergic neurotransmission [80]. The administration of an inverse agonist at the benzodiazepine binding site of the $GABA_A$ receptor promoted coherent network activity and exerted rapid antidepressant actions in some animal tests [81]. Evidence has shown that KET administration in mice selectively potentiates GABAergic synaptic inhibition and reverses behavioral despair [82]. Hence, the GABAR-associated pathway might be responsible for the antagonistic effects of CF and KET. Based on the metabolomic analysis, changes in the corresponding gene expression and neurotransmitter contents, and molecular docking, alternative mechanisms were postulated (Fig. 4e(ii)). Selectively blocking GABAR activity localized at the GABAergic cells in the periglomerular gray zone of mesencephalon induced a decline in the contents of GABA as well as neurotransmitters, including Suc, α-KG, and Glu. Subsequently, the lack of GABA and Glu in the synaptic cleft would reversely catalyze the upregulation of the expression of the *gabra1* gene and the sodium influx/potassium outflow. As a result, the normal circadian rhythm might be impaired by CF through this pathway, potentially inducing behavioral dysfunction in zebrafish at night. The subsequent addition of KET may reverse the changes above and ameliorate the adverse outcomes for fish. Overall, the neurotransmitters (i.e., GABA, MTN, Suc, α-KG, and Glu) are molecular candidate indicators for estimating the adverse effects of aquatic pollutants on the behavioral functions of fish at night.

In the aquatic environment, a variety of emerging pollutants, such as psychoactive drugs [83] and perfluorooctanoic acid [84], possess GABAergic-disrupting effects. Accordingly, fish behaviors at night are a valid indicator for assessing the environmental risk posed by the cocktail of psychoactive substances. This study provides new insight into the combined toxicity of CF and KET in aquatic systems, including physiological and molecular biomarkers.

4. Conclusion

This study, using system toxicological approaches, identified that exposure to caffeine (CF) markedly changes the behavioral functions of fish at night by disturbing their circadian rhythm and inducing obvious abnormalities during their development. Notably, ketamine (KET) at environmental levels could significantly mitigate the adverse effects of CF on fish by mediating GABAergic synapse activity. After a seven-day recovery period, the adverse effects posed by CF and the antagonistic effects of KET on CF could still be observed. Considering the coexistence of CF and KET in aquatic environments in Asia, the antagonistic effects may result in overestimations of the environmental risks posed by the monomers. Furthermore, future experiments should be conducted to test the effects of CF and KET in zebrafish throughout their life cycle, from embryos to adults, to better estimate the environmental risks.

CRediT authorship contribution statement

Zhenglu Wang: Investigation, Methodology, Visualization, Writing - Original Draft, Writing - Review & Editing. **Jindong Xu:** Investigation, Methodology. **Wei Du:** Methodology, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study received financial support from the National Natural Science Foundation of China (Grant No. 42007370), the Yunnan Major Scientific and Technological Projects (Grant No. 202302AO370001), the Fundamental Research Funds for the Central Universities (Grant No. YJ202315), and the Yunnan Fundamental Research Projects (Grant No. 202301AT070331).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ese.2024.100437.

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