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Behavioral and physiological effects of acute and chronic kava exposure in adult zebrafish

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ABSTRACT

Kava kava (*Piper methysticum*) is a medicinal plant containing kavalactones that exert potent sedative, analgesic and anti-stress action. However, their pharmacological effects and molecular targets remain poorly understood. The zebrafish (*Danio rerio*) has recently emerged as a powerful new model organism for neuroscience research and drug discovery. Here, we evaluate the effects of acute and chronic exposure to kava and kavalactones on adult zebrafish anxiety, aggression and sociality, as well as on their neurochemical, neuroendocrine and genomic responses. Supporting evolutionarily conserved molecular targets, acute kava and kavalactones evoked dose-dependent behavioral inhibition, upregulated brain expression of early protooncogenes *c-fos* and *c-jun*, elevated brain monoamines and lowered whole-body cortisol. Chronic 7-day kava exposure evoked similar behavioral effects, did not alter cortisol levels, and failed to evoke withdrawal-like states upon discontinuation. However, chronic kava upregulated several microglial (*iNOS*, *Egr-2*, *CD11b*), astrocytal (*C3*, *C4B*, *S100a*), epigenetic (*ncoa-1*) and pro-inflammatory (*IL-1*β, *IL-6*, *TNFa*) biomarker genes, downregulated *CD206* and *IL-4*, and did not affect major apoptotic genes in the brain. Collectively, this study supports robust, evolutionarily conserved behavioral and physiological effects of kava and kavalactones in zebrafish, implicates brain monoamines in their acute effects, and provides novel important insights into potential role of neuroglial and epigenetic mechanisms in long-term kava use.

1. Introduction

Kava kava (*Piper methysticum*) is a perennial plant native to the Pacific islands, with a long history of medicinal use in the region (Volgin et al., 2020). Extracts from kava roots play an important role in social rituals and traditional medicine (Shaver and Sosis, 2014; Singh, 1992), relaxing body and improving mood and sleep (Chua et al., 2016; Brown et al., 2007; Herberg, 1993; Paul et al., 2008; Singh, 2004). The main bioactive components of kava are a group of phenolic polyketones, kavalactones (Volgin et al., 2020; Wang et al., 2019), including six major pharmacologically active kavalactones - kavain, methysticin, 7,8-dihydromethysticin, yangonin, desmethoxyyangonin and 5,6-dihydrokavain (Jerome et al., 2011). Like kava extracts, kavalactones potently modulate human CNS, and can be used for alleviating anxiety, insomnia and pain (Volgin et al., 2020; Wheatley, 2010). Some of these effects can be attributed to central inhibition via positive modulation by kavalactones of gamma-aminobutyric acid (GABA)-A receptors (Chua et al., 2016).

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Table 1
Primers used in the present study (Sangon Biotech, Shanghai, China), F-forward, R-reverse.

Gene	RNA sequences	Gene	RNA sequences
β-Actin F	CATCAGGGTGTCATGGTTGGT	*BDNF F*	AACTCCAAAGGATCCGCTCA
β-Actin R	TCTCTTGCTCTGAGCCTCATCA	*BDNF R*	GCAGCTCTCATGCAACTGAA
C-fos F	TGAAACTGACCAGCTTGAGGAT	*CD11b F*	TCCTCGGATTCCAGAAACAC
C-fos R	GTGTGCGGCGAGGATGAA	*CD11b R*	AGCAGCACAAGTCCTCCAAT
C-jun F	TGGATACAACCACAAGGCTCT	*IL-4 F*	GCAGGAATGGCTTTGAAGGG
C-jun R	GTCACGTTCTTGGGACACAG	*IL-4 R*	GCAGTTTCCAGTCCCGGTAT
Egr-1 F	CTAAGATCCACATGCGGCAGAAGG	*IL-6 F*	TCAACTTCTCCAGCGTGATG
Egr-1 R	AGTAGCAGGAGTTGACTGGAGACG	*IL-6 R*	TCTTTCCCTCTTTTCCTCCTG
C3 F	TGATTCTGGCTCGCAGTGATGATG	*TNF-α F*	GTCGGGTGTATGGAGGGTGTTTG
C3 R	CATGGCTGAGGCTGGACAGTTATC	*TNF-α R*	CTGGGTCTTATGGAGCGTGAAGC
C4B F	CGCTCGTGCCACCAACTTCC	*Dnmt-3A F*	GGTTGTGCTCAGGCGGCTATTAG
C4B R	GTTCGTTCAGTGAGGCAGGTAGAC	*Dnmt-3A R*	AGGTCCAGTCATCTCTGCGTCTC
S100α F	CTGGATGCCAATGGAGACGG	*Dnmt-3B F*	ACCATTCCAGCAGCAGCAACAG
S100α R	ATCCACCCTGTAGCAAGCCT	*Dnmt-3B R*	ACCTCGGCCATCCTCGTATTCTG
EMP-1 F	TGCATAATGGTGGCTCTGTCAGTC	*Ncoa-1 F*	AATCTGGCGGCGATGGTGTTG
EMP-1 R	TGATGTAGGACCAGCCGTACCAG	*Ncoa-1 R*	TTGGATGCTTCTGGCGGTTTGG
IL-1β F	TTCCCCAAGTGCTGCTTATT	*HAT1 F*	TTGACTGCGTTGAGCCAGATGATG
IL-1β R	AAGTTAAAACCGCTGTGGTCA	*HAT1 R*	AGATCCTCTCCTGCCTCCACATTG
MHC-2 F	CCGTCAAGAGCAAGAGCGTTCC	*HDAC4 F*	CATCGCCCTCCCTTCCAAACATC
MHC-2 R	CGTTCACCAGCAGTGGCATACTC	*HDAC4 R*	GCACTGCCATCCTGTCACCATC
iNOS F	AGGCACTCGTGGCTATCAATGTTG	*KMT-2A F*	GCCTTGATGACTCCTCCTGTGATTC
iNOS R	ATGAAGGACTCGCTTGCGGAATG	*KMT-2A R*	CAGATGTTGGTGATGGTGGGTGAG
Egr-2B F	TCTGGATGCGGAGAGGTCTATCAAG	*MR F*	GGCAGCATGTCCATGTCCAGTC
Egr-2B R	AGTAGGATGGCGGAGGATATGAGATG	*MR R*	AGTCGTGCTCTCGGCTCTCAG
CD206 F	CGACACAGATGGCAGATGGAAGAC	*GR F*	ACAGCTTCTTCCAGCCTCAG
CD206 R	ACGCTTCTTTGACTCAGGACAGTTC	*GR R*	CCGGTGTCCTGTTTGAT
Arg-1 F	CACGCAGACATCAACACACCTTTAAC	*Caspase 3 F*	CCAGGGTCAACCATAAAGTAGC
Arg-1 R	TGGAAGTTTGGGCATCTTGGAGTG	*Caspase 3 R*	TCTTTGGTGAGCATTGAGACGA
Bcl-2 F	TGGATGACTGACTACCTGAAC	*Caspase 9 F*	GTGACCAAGCCAGGCAACT
Bcl-2 R	GTATGAAAACGGGTGGAAC	*Caspase 9 R*	AAATGACAGGAGGGCGATG
Bax F	GTGTATGAGCGTGTTCGTC		
Bax R	CGGCTGAAGATTAGAGTTGT		

Due to its unique and generally safe anxiolytic effects, recreational kava use is widespread worldwide (Baker, 2020; Volgin et al., 2020), showing anxiolytic, analgesic, anti-depressant and sleep-improving properties (Ooi et al., 2018; Savage et al., 2015; Shinomiya et al., 2005). However, its growing clinical and societal importance as a used and abused substance (tightly controlled in some countries worldwide) necessitates further translational research of kava CNS action (Volgin et al., 2020).

Various experimental (animal) models are a valuable tool in neuroscience and CNS drug screening (McArthur, 2010), and can be used to study neuroactive effects of kava. For example, strong sleep-enhancing and anxiolytic effects of kava have already been reported in rodents and chicks (Shinomiya et al., 2005; Smith et al., 2001), with similar action evoked by kavalactones (see (Volgin et al., 2020) for review). It is generally understood that kava lowers neuronal excitation and promotes inhibitory neurotransmitter signaling (Volgin et al., 2020). However, the exact pharmacological effects and specific molecular targets of kava and kavalactone remain poorly understood, necessitating further pre-clinical and clinical investigation.

Complementing rodent studies, the zebrafish (*Danio rerio*) has become a promising novel model organism in biomedical research due to genetic tractability, small size, easy maintenance, fast development and high genetic and physiological homology to humans (Volgin et al., 2019). Zebrafish are also an important tool in translational neuroscience research, possessing robust behavioral phenotypes and high sensitivity to stress and various genetic, epigenetic and pharmacological manipulations (Cachat et al., 2011; Kalueff et al., 2013; Kalueff et al., 2014; Stewart et al., 2014). Capitalizing on this powerful aquatic in-vivo vertebrate model system, here we assess pharmacological effects of kava and kavalactones on adult zebrafish behavior, neurochemistry, physiology and CNS gene expression, aiming to evaluate their potential mechanisms and targets for further clinical applications.

2. Materials and methods

2.1. Animals

The study was performed in adult (5–7 months) wild-type short-fin outbred zebrafish obtained from a commercial supplier (Eno Aquarium Technology Co., Ltd., Shanghai, China), with the 1:1 male-to-female ratio. Animals were kept 7-10 fish/L in the Benchtop Aquatic System (Jinshui Marine Biological Equipment Co., Qingdao, China) with water filtration system, the water temperature set at 28 °C, pH at 7.3-7.4, and a 14/10-h light/dark cycle (lights on 8.00 am). All animals were acclimated to the holding facility for at least two weeks prior to the experiments. All animals in this study were experimentally naive, and fed twice daily with Tropical Fish Multi-Dimensional High Protein Flakes (Beijing, China). Animal housing and husbandly fully adhered to the standards of zebrafish care.

2.2. Experimental design

In Experiment 1, cohorts of zebrafish were acutely exposed by immersion for 20 min to water extract from powdered kava roots (purchased from the Kava Bar, New Orleans, LA, USA) and pure kavalactones (commercially extracted from kava roots and sold as a powder mix online via Herbal Plant Health Workshop, Xian, China) at concentrations 10, 20 and 50 mg/L. The extracts were prepared in hot boiled water for 20 min, and then cooled to a room temperature. In Experiment 2, zebrafish were chronically exposed by immersion to kava extract for 7 days at 5 mg/L. In the withdrawal experiment, a 7-day chronic kava extract treatment at 5 mg/L was followed by a 12-h discontinuation: control cohort received water, chronic group continued to receive kava treatment, and the withdrawal group was treated with kava chronically (like the chronic group), but was withdrawn from kava

Table 2
Summary of one-way ANOVA statistics (factor - treatment) of behavioral endpoints used in the present study. NS – no statistically significant effects (see Fig. 1 for details).

Tests	Endpoints	F statistics	*P* value
Kava extract			
Novel tank test	Top entries	$F_{(3, 56)} = 3.74$	0.0160
(NTT)	Time in top (s)	$F_{(3, 56)} = 4.58$	0.0061
	Freezing frequency	$F_{(3, 56)} = 4.37$	0.0078
	Freezing duration (s)	$F_{(3, 56)} = 3.65$	0.0176
Light-dark box test	Light entries	$F_{(3, 56)} = 8.10$	0.0001
(LDB)	Time in light (s)	$F_{(3, 56)} = 6.67$	0.0005
Shoaling test	Average inter-fish distance (cm)	$F_{(3, 20)} = 19.27$	0.0001
	Aggression frequency	$F_{(2, 41)} = 14.10$	0.0001
	Aggression duration (s)	$F_{(2, 41)} = 13.07$	0.0001
Social preference test	Entries to conspecific area	$F_{(3, 59)} = 4.89$	0.0104
	Time near conspecific (s)	$F_{(3, 59)} = 4.19$	0.0093
Kavalactones			
Novel tank test	Top entries	$F_{(3, 56)} = 6.02$	0.0013
(NTT)	Time in top (s)	$F_{(3, 56)} = 4.94$	0.0041
	Freezing frequency	$F_{(3, 56)} = 9.48$	0.0001
	Freezing duration (s)	$F_{(3, 56)} = 3.46$	0.0224
Light-dark box test	Light entries	$F_{(3, 56)} = 3.38$	0.0248
(LDB)	Time in light (s)	$F_{(3, 56)} = 1.73$	0.17, NS
Aggression test	Average inter-fish distance (cm)	$F_{(3, 20)} = 4.89$	0.0104
	Aggression frequency	$F_{(2, 42)} = 3.16$	0.053, NS
	Aggression duration (s)	$F_{(2, 42)} = 11.62$	0.0001
Social preference test	Entries to conspecific area	$F_{(3, 60)} = 2.17$	0.10, NS
	Time near conspecific (s)	$F_{(3, 60)} = 10.22$	0.0001
Kava withdrawal			
Novel tank test	Top entries	$F_{(2, 46)} = 0.73$	0.4871, NS
(NTT)	Time in top(s)	$F_{(2, 46)} = 0.65$	0.5241, NS
	Freezing frequency	$F_{(2, 46)} = 0.33$	0.7171, NS
	Freezing duration (s)	$F_{(2, 50)} = 0.52$	0.5968, NS

and exposed to water for the final 12 h prior to testing. The animals were assigned to the experimental groups randomly (using an online randomization tool), and belonged to the same batch. All animals tested were included in the analyses without exclusion and attrition. All experiments were performed as planned, and all endpoints assessed were included in the analyses. Behavioral analyses were performed by highly-trained experimenters blinded to the treatment (intra/inter-rater reliability >0.85, as assessed by Spearman correlation). Statistical analyses of data, as well as neurochemical, genomic and cortisol assays, were performed offline 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.

2.3. Behavioral analyses

Behavioral testing was performed between 11.00 and 18.00 h using the 5-min novel tank test (NTT), light-dark box (LDB), shoaling, social preference and aggression tests, chosen here as highly sensitive and commonly used zebrafish behavioral paradigms (Colman et al., 2009; Kalueff and Cachat, 2011; Cachat et al., 2011). The animals were transferred from the holding room and acclimated to the testing room for 1 h prior to testing. The NTT apparatus represented a narrow rectangular glass container (20 height × 26 length × 5 width cm) virtually divided by a horizontal dashed marker line into the upper and lower halves, assessing the number of top entries, time spent in top (s), and the number of and duration (s) of freezing episodes (Demin et al., 2017). Visualization of zebrafish NTT locomotor tracks was performed using the Ethovision XT11 software (Noldus IT, Wageningen, Netherlands).

The LDB was a plastic 10-L box consisting of two chambers (dark and light, each 18 height × 14 length × 20 width cm), to assess the number of light transitions and time spent in light (s) (Cachat et al., 2011). Transition in all tests was defined as a mid-body point crossing the line. Behavioral endpoints in the NTT, LDB, shoaling and aggression test were recorded by highly trained experimenters, as assessed by a high, statistically significant correlation ($>90\%$) in the Spearman correlation test.

The shoaling test was performed in the same NTT apparatus, assessing social behavior of zebrafish. Briefly, groups of 6 zebrafish were pre-exposed to either kava or water (3 groups/$n = 18$ per treatment) for 20 min, and group-tested in the shoaling test. Zebrafish shoaling behavior was video-recorded for 6 min, and analyzed offline using 6 screenshots made every 10 s during the last half of the observation period. Each screenshot was properly calibrated and analyzed by trained observers, manually measuring the distances (cm) between each fish in the group, and then averaging this data to obtain an average inter-fish distance per screenshot, and then to generate its average index per group (Cachat et al., 2011).

The social preference test was performed in a plastic container (20 height × 26 length × 5 width cm), in which transparent 200-ml plastic cups were placed in two opposite corners, one cup containing a stimulus fish, and another being empty. The target zebrafish was placed into the empty cup-containing compartment. The marker line on the sides of the tank divided the container into two parts to record the number of entries to and time spent (s) near the 'conspecific' vs. the 'empty' halves. The aggression test utilized the same NTT apparatus, as fish were individually exposed to vertical 20 × 4.5-cm mirror attached to one of the side walls. For behavioral analyses, the tank was divided into two zones, based on the proximity to the mirror, assessing the time (s) fish spent in the 2-cm virtual zone near the mirror, reflecting higher aggression, and the number of attacks (bites) of the mirror during the session. Each test was performed in a separate group of fish, to avoid

the test battery effect. All zebrafish behaviors assessed here corresponded to the comprehensive Zebrafish Neurobehavioral Catalog (Kalueff et al., 2013).

2.4. Whole-body cortisol and quantitative real-time polymerase chain reaction (qRT-PCR) assays

Paralleling behavioral analyses, we also assessed whole-body levels of cortisol, an important glucocorticoid hormone involved in affective phenotypes in both humans and zebrafish (Demin et al., 2020; Lenze et al., 2011). Briefly, following the drug treatment, animals were sacrificed on ice, decapitated, and their body samples collected and homogenized in 1 mL of ice-cold 1 × phosphate-buffered saline (PBS) buffer. The homogenizing rotor blade was then washed with an additional 0.5 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 analyzed for both acute and chronic kava treatments using the fish cortisol enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Herbal Source Biotechnology Co., Ltd., Nanjing, China) commonly used in zebrafish assays, following the manufacturer instructions, as in (Cachat et al., 2010). Cortisol levels were normalized based on the weight of the respective headless body samples, and expressed as absolute cortisol concentrations (ng/g body weight).

Experiment 1 evaluated the effects of acute kava and kavalactone exposure on whole-body cortisol and brain expression of early protooncogenes (*C-fos*, *C-jun*, *egr-1*). To minimize the impact of handling and behavioral testing on cortisol levels and gene expression (especially critical for highly sensitive early protooncogenes), these studies were performed in separate sub-cohorts of fish that were treated with drug or water (similarly to those used in behavioral assays, as described above), but were not exposed to behavioral assays. Experiment 2 assessed the effects of chronic exposure to kava extract on whole-body cortisol and mRNA expression of brain genes encoding a key neurotrophin, the brain-derived neurotrophic factor (*BDNF*), astrocyte- (*C3*, *C4B*, *EMP-1*, *S100a*) and microglia biomarker genes (*CD11b*, *MHC-2*, *iNOS*, *Egr-2*, *CD206*, *Arg-1*), selected pro-inflammatory (*IL-1*β, *IL-6*, *TNF*-α) and anti-

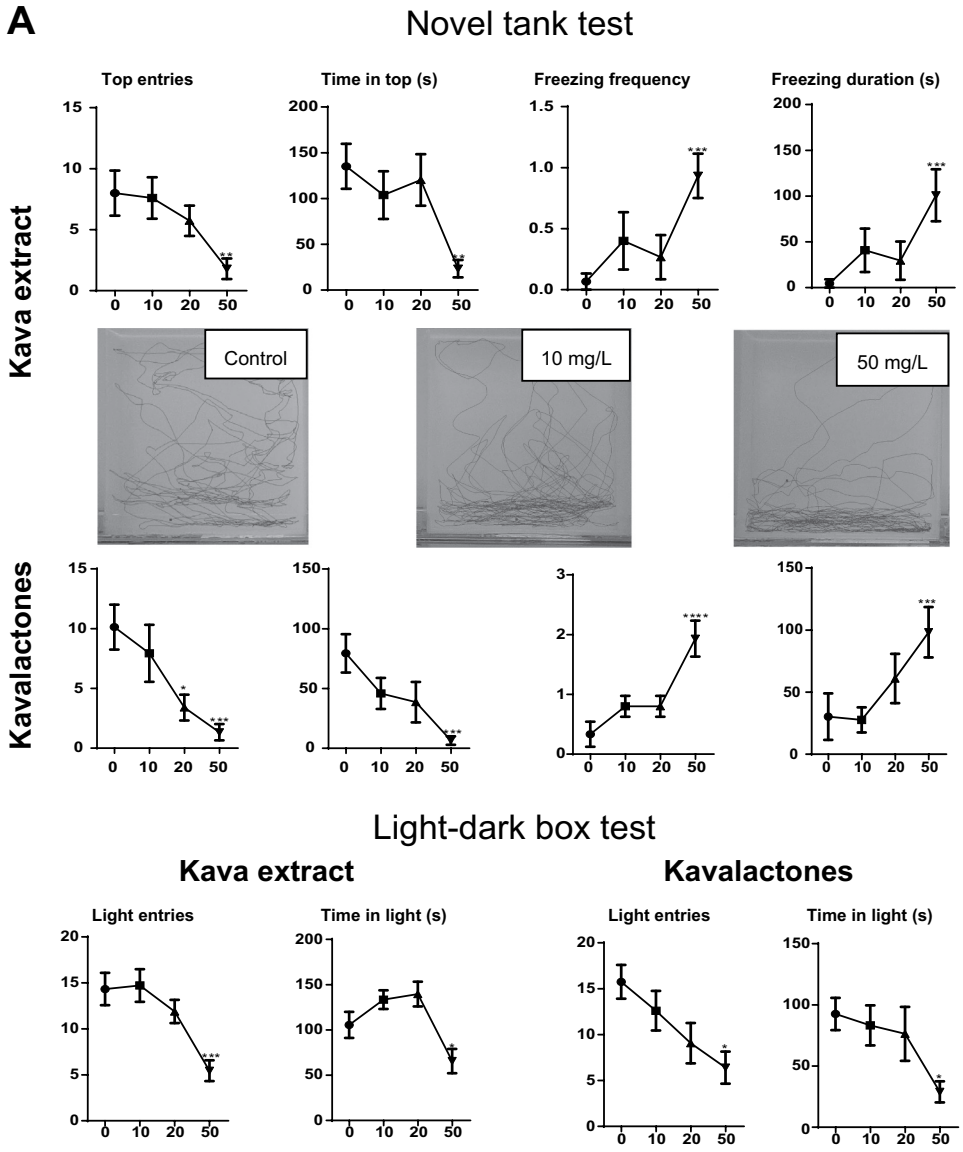

Fig. 1. Effects of acute kava extract and kavalactones on zebrafish anxiety-like behavior in the novel tank and the light dark box tests (Panel A), agonistic behaviors tested in the shoaling, aggression and social preference tests, and on endocrine responses (whole-body cortisol, 50 mg/L), $n = 15$–18 per group (Panel B). *$P < 0.05$, **P < 0.01, ***p < 0.001 vs. control, post-hoc Tukey test for significant one-way ANOVA data (Table 2) or *U* test (for cortisol data), where applicable. Photos in Panel A demonstrate representative locomotor tacks produced by kava extracts in zebrafish novel tank test, recorded by the Ethovision XT11 software.

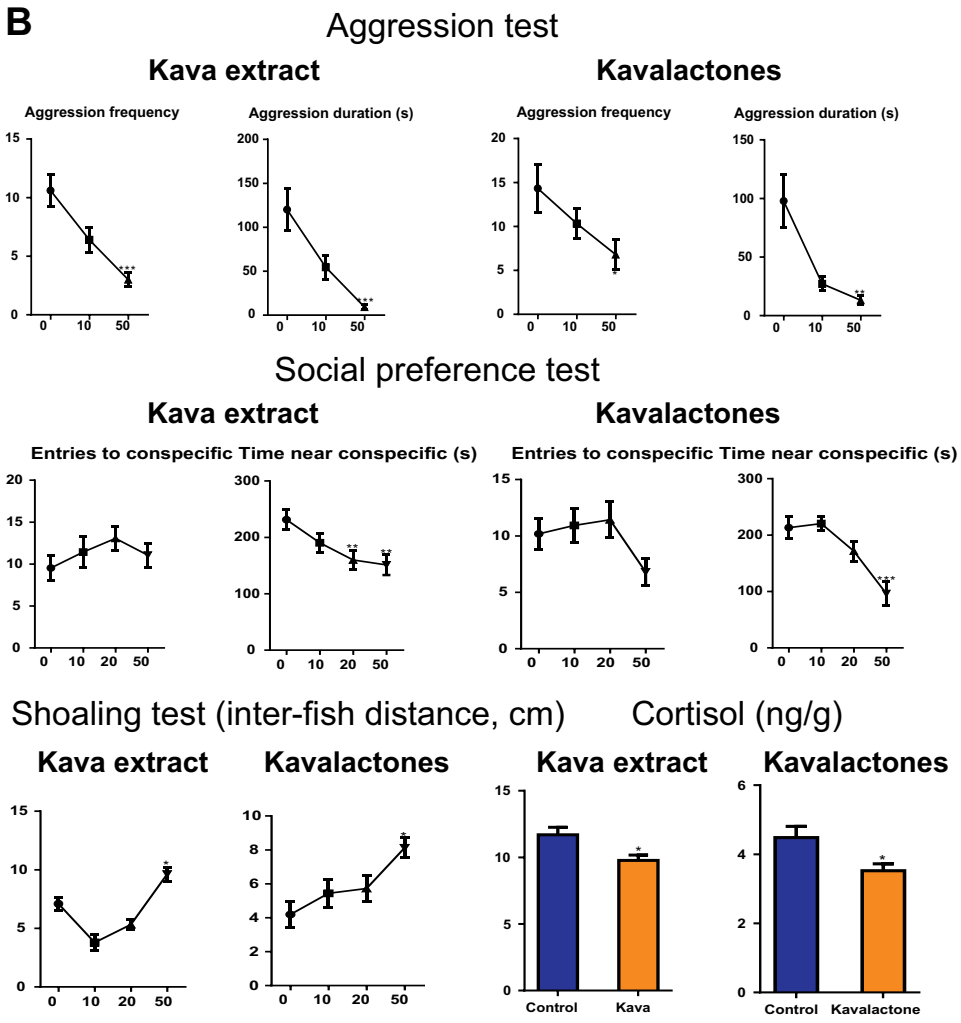

Fig. 1. (*continued*)

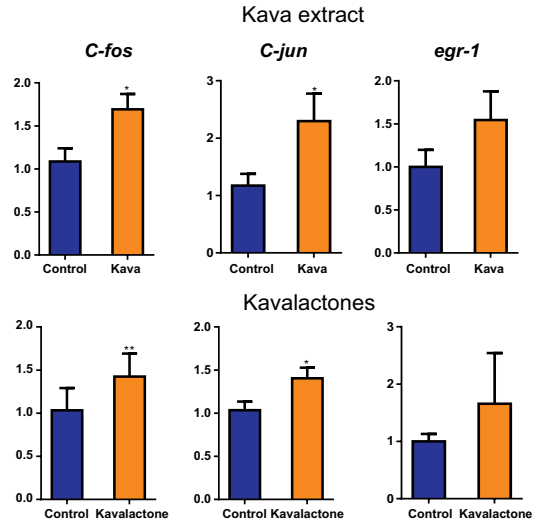

Fig. 2. Effects of acute exposure to kava (50 mg/L) and kavalactones (50 mg/L) on zebrafish brain mRNA expression of selected early protooncogenes (fold change, $n = 12$ per group). *$P < 0.05$ vs. control, *U* test.

inflammatory cytokines (*IL-4*), the glucocorticoid and mineralocorticoid receptors (*GR*, *MR*), as well as several apoptotic genes (*Caspase-3*, *Caspase-9*, *Bcl-2* and *Bax*). To avoid the immediate effects of handling and behavioral testing procedures on gene expression and whole-body cortisol levels, these studies were performed one day following behavioral testing in the NTT. 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 et al., 2020).

2.5. Neurochemical analyses

Concentrations of monoamines and their metabolites (dopamine, dihydroxyphenylacetic acid (DOPAC), norepinephrine, serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA)) in the brain were measured by high-performance liquid chromatography (HPLC) of whole-brain samples, as described earlier (Demin et al., 2017). To minimize the impact of handling and behavioral testing on neurochemical parameters, these studies were performed in separate sub-cohorts of experimentally naïve fish that were only treated acutely (20-min) with kava water extract or water. Following the treatment, the animals were sacrificed and their whole brains were dissected on ice, weighted and stored in liquid nitrogen for the analyses. Samples were thawed on ice and then placed into 5 μL of ice-cold 0.1 M perchloric acid (Sigma Aldrich, St. Louis, MO, USA) solution per 1 mg of brain tissue, to preserve monoamines from degradation. The samples were then sonicated on ice for 2 s at minimal power settings, cleared by centrifugation and filtered through 0.22 μm PVDF filter (Merck Millipore, Billerica, MA,

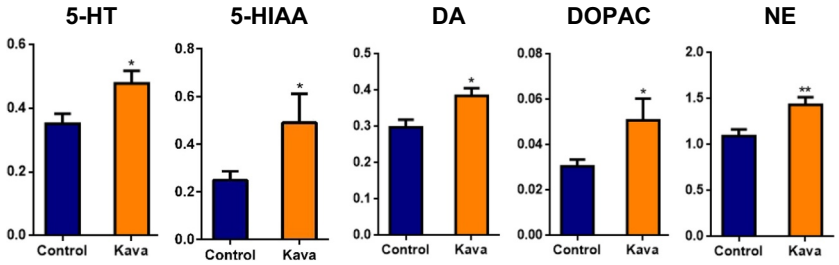

Fig. 3. Neurochemical effects evoked by acute exposure to 50 mg/L kava extract on whole-brain serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine, DOPAC and norepinephrine (NE) contents ($n = 10$ per group), $^{*}P < 0.05$ vs. control, *U* test.

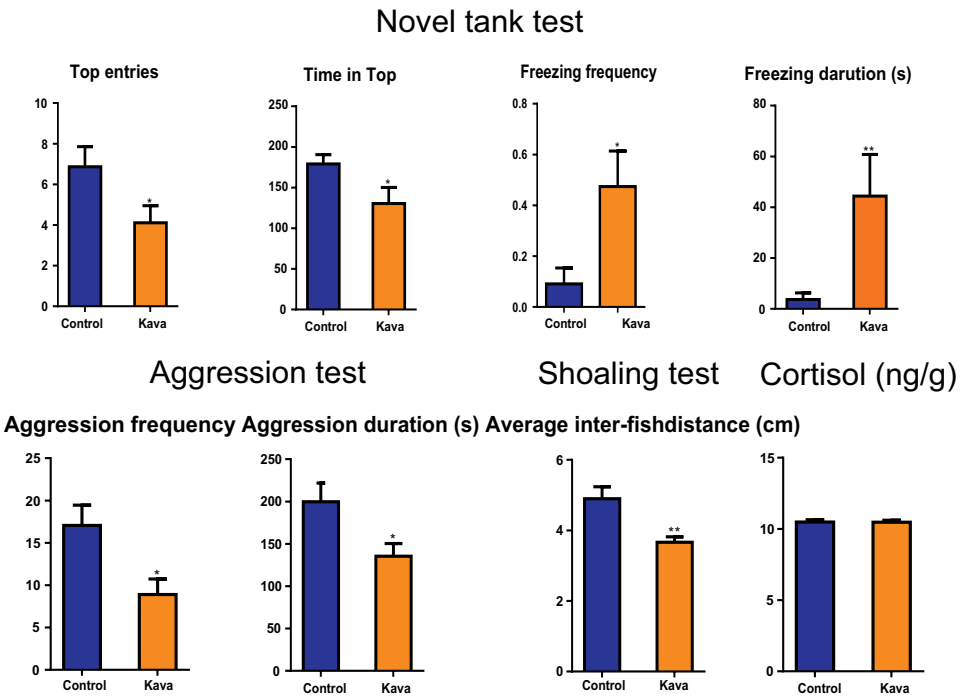

Fig. 4. Behavioral and endocrine (whole-body cortisol) effects of chronic 7-day kava exposure (5 mg/L) on zebrafish. Data are expressed in mean + SEM ($n = 17$–22 per group). *P < 0.05, $^{**}P < 0.01$, $^{***}p < 0.001$ vs control, *U* test.

USA). HPLC of brain extracts was performed on the CA-5ODS column with HTEC-500 chromatograph (Eicom, San Diego, CA, USA). Chromatography mobile phase contained 0.1 M phosphate buffer, 400 mg/L sodium octylsulphonate, 50 mg/L EDTA, 18% methanol and was adjusted to pH 4.4 by phosphoric acid (all reagents were purchased from Sigma Aldrich, USA). The concentrations obtained with chromatography were normalized to the weight of the extracted zebrafish brain tissue and presented as pg/mg.

2.6. Statistical analyses and data handling

All data are presented as Mean ± SEM, and analyzed using one-way ANOVA test (factor: treatment, Table 2), followed by post-hoc Turkey test for significant ANOVA data. Experiments with only two animal groups were analyzed using the unpaired Wilcoxon-Mann-Whitney *U* test. P was set as <0.05 in all experiments.

3. Results

Testing a wide dose range in a series of pilot experiments showed no effects of kava at lower doses (data not shown), and hence only higher doses (10-50 mg/L) were analyzed here in detail. Overall, in Experiment 1, acute 20-min kava and kavalactone exposure evoked similar dose-dependent sedative effects in zebrafish in the novel tank and the light-dark box tests (Fig. 1), reducing exploration activity in top and light sections, as well as promoting immobility (freezing) frequency and duration.

Likewise, acute treatment also caused global dose-dependent reduction of activity on the mirror test, social preference and shoaling tests, suggesting a global behavioral inhibition, likely reflecting sedation, in all tests used (Fig. 1). Moreover, both kava and kavalactones reduced cortisol levels and activated the expression of early proto-oncogenes *c-fos* and *c-jun* (Figs. 1 and 2). In addition, acute kava treatment at the behaviorally active 50-mg/L dose affected monoaminergic neurotransmission, elevating brain dopamine, DOPAC, norepinephrine, serotonin and 5-HIAA levels (Fig. 3).

Chronic 7-day kava exposure at 5 mg/L also caused overt behavioral inhibition in zebrafish NTT, aggression and shoaling tests (Fig. 4, Experiment 2). These behavioral effects generally paralleled the acute profile of kava at a higher dose in these tests, as described above (Fig. 1). Furthermore, chronic kava treatment also upregulated several biomarker genes, including microglia- (*CD11b*, *Egr-2*, *iNOS*) and astrocyte-specific genes (*C3*, *C4B*, *S100a*), as well as selected epigenetic (*ncoa-1*), pro-inflammatory (*IL1β*, *IL-6*, *TNFα*) and glucocorticoid receptor (*GR*) genes (Fig. 5). Chronic treatment with kava downregulated 'anti-inflammatory' biomarkers of microglia (*CD206*) and an anti-

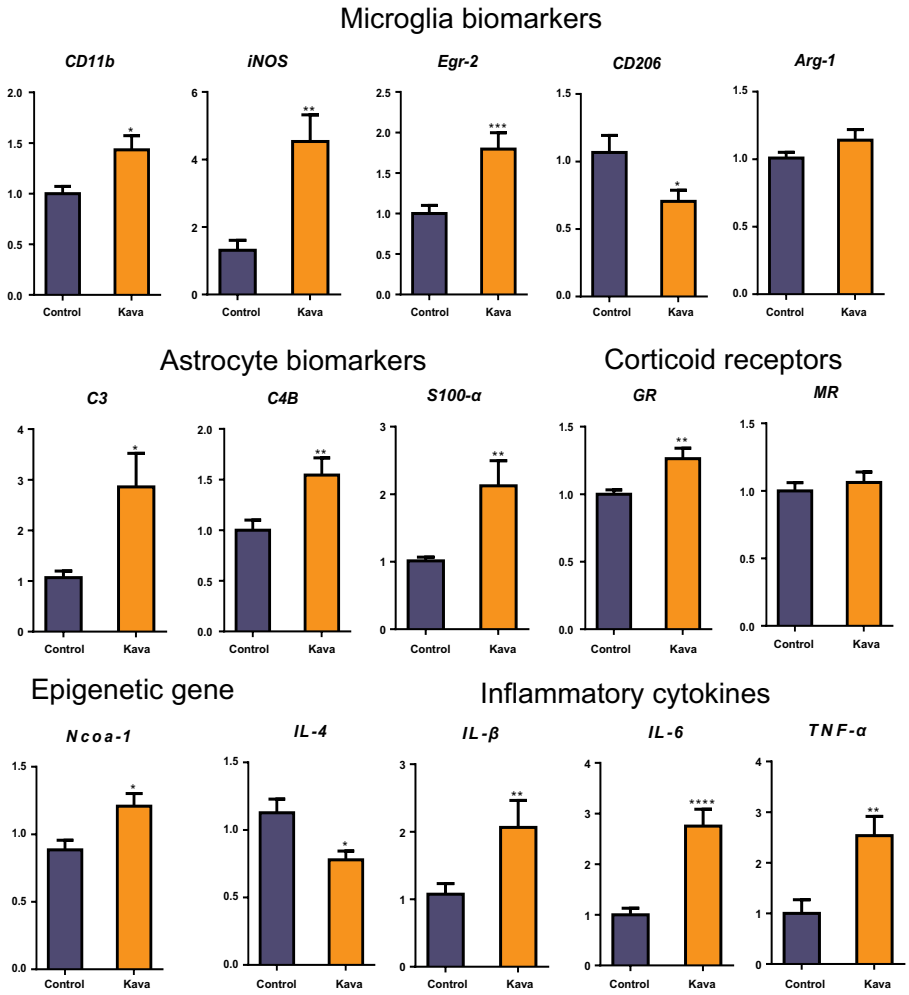

Fig. 5. Effects of chronic 7-day kava extract exposure (5 mg/L) on zebrafish whole-brain expression of microglia- and astrocyte-specific biomarkers, glucocorticoid (*GR*) and mineralocorticoid (*MR*) receptor-, as well as selected epigenetic and inflammatory cytokine genes (fold change, n = 10 per group). *P < 0.05, **P < 0.01, ***p < 0.001 vs. control, *U* test.

inflammatory cytokine *IL-4*, but did not affect brain apoptotic genes (*Bcl-2*, *Bax*, *Caspase-3*, *Caspase-9*) (data not shown) or whole-body cortisol levels (Fig. 4).

Finally, chronic kava treatment did not cause withdrawal-like behavioral effects in zebrafish NTT, yielding no overt behavioral differences from control or chronic groups, following a 12-h kava discontinuation in the withdrawal group (Fig. 6, Experiment 3).

4. Discussion

Although CNS effects of kava has been tested in both animals and humans (Volgin et al., 2020), the present study is the first to demonstrate its behavioral effects in zebrafish, and to parallel these behavioral findings with comprehensive analyses of a wide range of molecular biomarkers, including neurochemical, endocrine and genomic responses to kava and kavalactones in a complex in-vivo vertebrate model system. Overall, while both kava and kavalactones exerted overt sedative-like effects in zebrafish, and acute kava exposure modulated monoaminergic neurotransmission, its chronic administration also activated biomarkers of microglia and astrocytes (but not neuronal apoptosis), suggesting novel potential targets of long-term kava use that involve neuroimmune interactions. In line with this, neuroinflammation and epigenetic processes have recently been implicated in human and animal CNS pathogenesis (Blacker et al., 2019; Smith et al., 2019), whereas microglia and astrocytes - resident immune cells in the brain - play a key role in these pathogenetic mechanisms (Li et al., 2013; Greenberg et al., 2014; Munhoz et al., 2010).

Zebrafish acutely exposed to kava and kavalactones demonstrated dose-dependent sedative effects (Fig. 1) that are generally in line with CNS profiles reported for these treatments clinically (Smith and Leiras, 2018). This effect was also accompanied by reduced whole-cortisol levels, paralleling effects of another sedative, ketamine, in zebrafish (Riehl et al., 2011). Likewise, elevated brain *c-fos* and *c-jun* expression produced by kava here resembles *c-fos*-elevating effect of some sedative agents (e.g., propofol, ketamine) in rodents (Cui et al., 2011; Girgenti et al., 2017; Kidambi et al., 2010). Moreover, while chronic kava exposure did not alter cortisol levels here, it upregulated the expression of *GR* – an effect that may reflect anti-stress effects of kava, since stress levels in some zebrafish models negatively correlate with *GR* levels (Piato et al., 2011). Because cortisol plays an important role in stress response, further studies of potential effects of acute and chronic kava on cortisol and brain *GR* signaling may be necessary.

As already mentioned, acute exposure to kava increased dopamine, DOPAC, norepinephrine, serotonin and 5-HIAA (but not the dopamine/DOPAC or serotonin/5-HIAA ratios), suggestive of positive modulation of monoamines in zebrafish. As brain monoamines are important regulators of activity, emotionality and cognition in both humans and animals, these zebrafish findings are translationally relevant because they further link CNS effects of kava to brain monoaminergic mechanisms. Consistent with this notion, chronic stress reduces whole-

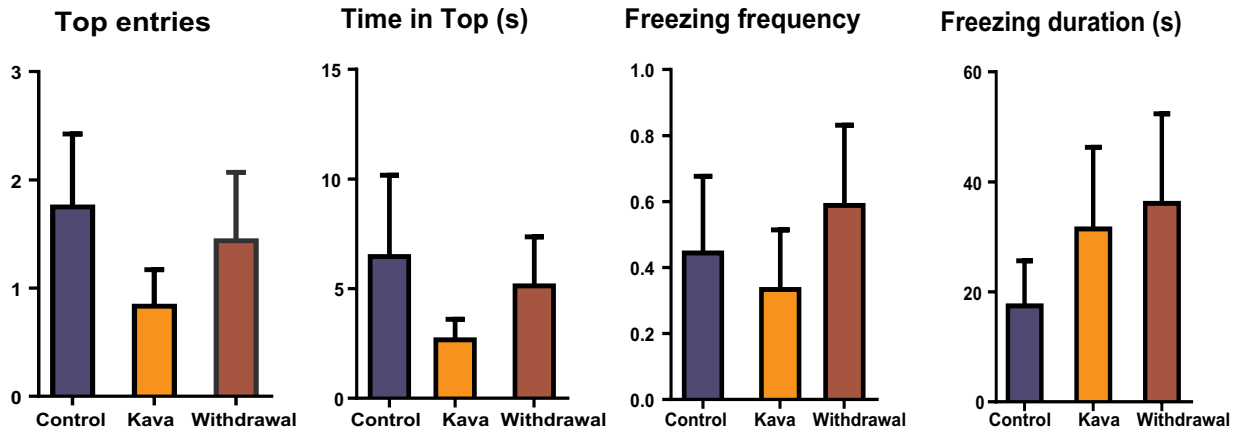

Fig. 6. Effects of kava withdrawal on zebrafish anxiety-like behavior in the novel tank test (n = 16–18 per group), $P > 0.05$, no significant differences by ANOVA (see Table 2 for ANOVA test results). The three groups utilized in this experiment were water-treated control fish, animals chronically treated with 5 mg/L kava for 1 week, and the chronically treated fish with a 12-h drug discontinuation (the withdrawal group).

brain levels of dopamine and 5-HIAA in zebrafish (Fulcher et al., 2017), whereas acute kava extract increases accumbal dopamine levels in rats (Baum et al., 1998). Together with our present findings (Fig. 3), this suggests potential monoamine-modulating CNS effects of kava and kavalactones in vivo.

Importantly, chronic kava exposure also upregulated several key CNS genes, including genes-biomarkers of microglia (*CD11b*, *Egr-2*, *iNOS*), astrocytes (*C3*, *C4B*, *S100a*), and the pro-inflammatory cytokines (*IL1*β, *IL-6*, *TNF*α; Fig. 5). In general, upregulated *Cd11b*, *Egr-2* and *iNOS* indicate microglia activation, neurodegeneration and neuroinflammation (González-Scarano and Baltuch, 1999; Rock et al., 2004; Stewart and Heales, 2003). Some rodent pro-inflammatory cytokines (e.g., IL-6, IL-1β, TNF-α) may also be raised by sedative drugs (e.g., ketamine) (Li et al., 2017). While microglia exist in two distinct forms (the pro-inflammatory M1 and the neuroprotective M2 phenotypes (Szabo and Gulya, 2013)), the M1-induced neuroinflammation and M1/M2 imbalance are common in clinical affective disorders (Wang et al., 2018). Here, chronic kava exposure upregulated a general microglial marker *CD11b*, the M1-specific markers *iNOS* and *IL1*β, and an M2 marker *egr-2*, while down-regulating an M2 marker gene CD206 and all three astrocytal biomarkers *C3*, *C4B* and *S100-a* (Fig. 5). These findings imply that chronic kava can alter the M1/M2 microglia and the microglia/astrocyte balances in the brain, implicating neuroimmune mechanisms as targets for long-term kava action.

In contrast, unaltered expression of brain apoptotic genes (*Bcl-2*, *Bax*, *Caspase-3*, *Caspase-9*) suggests that neuronal apoptosis may not be the main target for long-term effects of kava. Finally, since drug exposure can cause epigenetic modulation in the brain, this possibility should be considered here. For example, chronic kava exposure upregulated one zebrafish epigenetic gene (*ncoa-1*, encoding a nuclear receptor coactivator with intrinsic histone acetyltransferase activity), but not other major epigenetic biomarkers assayed (Fig. 5). Thus, although potential long-term kava effects on epigenetic mechanisms merit further scrutiny, they may be rather mild, given that only one epigenetic gene altered its expression here.

Clearly, the present study has several limitations. For example, animal models cannot fully reproduce complex effects of a psychotropic drug on humans, and zebrafish behaviors may not fully translate into complex human behavior. The present study also did not assess individual responses to kava, as well as age, sex or strain differences in its effects on zebrafish. We have also examined a mixture of extracted kavalactones, and further in-depth studies are therefore needed to differentiate potential effects of each individual kavalactone in the observed effects, as well as the potential impact of various important genetic (e.g., kava variety) and environmental factors (e.g., plant origin, cultivation and extraction methods) in this and similar in-vivo studies. Likewise, we only used a 7-day chronic kava exposure, which may differ from a 'real-life' long-term recreational or clinical use of kava in humans. Thus, future studies may apply different protocols of chronic kava treatment, perform genome-wide gene expression testing, and also complement genomic findings with protein transcriptomic assays in zebrafish.

Furthermore, drug combinations are also worth examining, as kava exerts synergistic effects with barbiturates and alprazolam, and such drug combinations can be effectively tested in zebrafish (Khedkar et al., 2018). It is also well-known that kava has a generally low addictive effect (Volgin et al., 2020), and testing this aspect in zebrafish may be warranted, aiming to identify vulnerability factors in kava addiction-prone fish individuals. At the same time, kava exerted no withdrawal effects here (Fig. 6), paralleling clinical data (Volgin et al., 2020) and reinforcing kava as a relatively safe drug devoid of withdrawal-like side effects, which are common among self-medicating abusers and chronically treated patients (Hughes et al., 1994; West and Gossop, 1994), and include headache, hallucinations and anxiety (Martinotti et al., 2008; Wu et al., 2009). In conclusion, this study supports evolutionarily conserved behavioral effects of kava and kavalactones in zebrafish, and also provides novel insights into potential role of monoamines, microglia, astrocytes and epigenetic mechanisms in CNS effects of kava.

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.

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