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Research Article

Sub-chronic Administration of Fearless Energy Drink Alters Hippocampal Expression of DRD2, COMT, and OPRM1 in Albino Rats

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ABSTRACT
Increasing consumption of energy drinks has raised concerns regarding their effects on neurobiological pathways involved in reward processing and addiction. This study evaluated the effect of sub-chronic administration of Fearless Energy Drink (FED) on hippocampal expression of dopamine D2 receptor (DRD2), catechol-O-methyltransferase (COMT), and mu-1 opioid receptor (OPRM1) genes in albino rats. Twenty-four adult albino rats were randomly assigned to four groups (n = 6). The control group received distilled water, while the treatment groups received FED orally at 7, 14, and 21 mL/kg body weight daily for 60 days. On day 61, hippocampal tissues were collected for gene expression analysis using quantitative real-time polymerase chain reaction (qRT-PCR). Relative gene expression was determined using the 2^-ΔΔCt method. FED administration altered hippocampal expression of all three genes. DRD2 expression was reduced in the low-, medium-, and high-dose groups, with fold-change values of 0.01, 0.02, and 0.61, respectively. COMT expression was also down-regulated across treated groups, with fold-change values of 0.005, 0.008, and 0.007. OPRM1 exhibited marked reductions, with fold-change values of 0.0002, 0.0018, and 0.0018 in the respective dose groups. Although all genes were down-regulated, the dose-response pattern was not consistently linear. Sub-chronic FED exposure was associated with down-regulation of DRD2, COMT, and OPRM1 expression in the hippocampus of albino rats. These findings suggest that prolonged energy drink consumption may influence gene-regulatory pathways linked to reward-related neurobiology. Further studies incorporating behavioural and protein-level assessments are warranted..

Keywords: Addiction-related genes; Albino rats; COMT; DRD2; Energy drink; Gene expression

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INTRODUCTION

Energy drinks are non-alcoholic beverages formulated to improve alertness, physical performance and mental stimulation. They commonly contain caffeine as the principal psychoactive ingredient, alongside varying quantities of taurine, guarana, ginseng, sugars, B vitamins and other herbal extracts (Mansy *et al*., 2017; Olatona *et al*., 2018; Tabassum *et al*., 2021). Although these products are widely marketed as performance-enhancing beverages, their increasing use among adolescents and young adults has generated public-health concern, particularly because their stimulant content may exceed recommended or expected levels in some formulations (Bano *et al*., 2020; Vargiu *et al*., 2021). In Nigeria, concerns have also been raised about the distribution and consumption of highly caffeinated energy drinks, especially where products are not appropriately registered or regulated (NAFDAC, 2023).

Energy drink consumption is common among young people and is often driven by perceived benefits such as improved wakefulness, enhanced endurance, increased concentration and reduced fatigue.

However, repeated exposure to highly caffeinated beverages may affect neural systems involved in reward, arousal and behavioural regulation (Howard & Marczinski, 2010). Caffeine primarily acts as an adenosine receptor antagonist, but its downstream effects may involve dopaminergic and other neurotransmitter systems that are relevant to reinforcement and addictive behaviour (Fiani *et al*., 2021). This has led to increasing interest in the possibility that heavy or prolonged energy drink intake may influence addiction-related biological pathways.

Addiction-related genes are genes whose variants or expression profiles may influence vulnerability to substance use, reinforcement, craving, dependence or relapse. Among these, DRD2, COMT and OPRM1 are frequently implicated in reward processing, dopamine regulation and opioid-mediated reinforcement (Bierut 2011). DRD2 encodes the dopamine D2 receptor, a key component of mesolimbic dopaminergic signalling. COMT encodes catechol-O-methyltransferase, an enzyme involved in catecholamine metabolism, including dopamine degradation. OPRM1 encodes the mu-opioid receptor, which is involved in reward, pain modulation, affective regulation and substance-related reinforcement (Kennedy *et al*., 2015; Noble, 2000; Picci *et al*., 2022; Simpson *et al*., 2014).

The hippocampus is essential for learning, memory and emotional processing, and it contributes to neuroplasticity associated with reward-related learning and substance exposure. Alterations in hippocampal gene expression may therefore provide insight into the neurobiological consequences of repeated stimulant-containing energy drink consumption (McEwen *et al*., 2015). Despite the growing literature on energy drinks and behavioural or biochemical outcomes, there is limited evidence on their effects on the hippocampal expression of addiction-related genes. The present study therefore evaluated the effect of sub-chronic FED administration on the expression of DRD2, COMT and OPRM1 in the hippocampal region of albino rats.

MATERIALS AND METHODS

Reagents and consumables

The reagents and consumables used included 10% formalin, cryovials, Bouin's fluid, TRIzol reagent, phosphate-buffered saline (PBS), chloroform, isopropanol, 70% ethanol, nuclease-free water, RNAlater, dimethyl sulfoxide, protease inhibitors, lysis buffers, reverse-transcription reagents, qPCR master mix, RNA/DNA extraction and purification kits, Luna one-step RT-qPCR kit, cDNA synthesis kit, primers and probes, and gene-specific primers for DRD2, COMT and OPRM1. Total RNA purification kits were obtained from Norgen Biotek Corporation, Thorold, Ontario, Canada.

Instruments and equipment

The instruments and equipment used included beakers, 1 ml and 2 ml syringes, nasogastric/oropharyngeal tubes, weighing balance, gloves, cotton wool, stopwatch, permanent markers, metre rule, threads, real-time qPCR machine, NanoDrop spectrophotometer, -80 °C refrigerator, centrifuge, homogeniser, micropipettes and tips, thermal cycler, reaction tubes or plates, microcentrifuge tubes, electrophoresis system, scissors, scalpel, forceps, dry ice, and Petri dishes on ice.

Ethics Approval/Consent to Participate and study site

Ethical approval for the study was obtained from the Animal Research and Ethical Committee on Animal Use. Faculty of Pharmacy, University of Maiduguri with approval No (FP/03/25/19/11/01/034), on March, 2025. The animal-exposure phase was conducted in the Pharmacology and Toxicology Laboratory, Faculty of Pharmacy, University of Maiduguri. Molecular analysis was performed at the Northeast Zonal Biotechnology Centre of Excellence, University of Maiduguri. Animal handling strictly followed the principles of laboratory animal care (NIH Publication No. 85-23, 1996).

Experimental animals and housing

Twenty-four adult albino rats of both sexes, aged 3-4 months and weighing approximately 90-120 g, were obtained from the Faculty of Pharmacy Animal House, Bayero University Kano, Kano State, Nigeria. The animals were transported to the Department of Pharmacology and Toxicology Animal House, Faculty of Pharmacy, University of Maiduguri, where they were acclimatised for two weeks before the experiment. The rats were housed in separate cages under standard laboratory conditions and were allowed free access to distilled water and grower's mash (TOPFEEDS, Premier Feed Mills Co. Ltd., Nigeria) throughout the study. Before treatment commenced, the animals were weighed and individually marked on the tail for identification.

Experimental design and administration of Fearless Energy Drink

The animals were randomly assigned into four groups of six rats each. Group A served as the control group and received distilled water. Groups B, C and D

received FED at low, medium and high doses, respectively. FED, a product of Rite Foods Ltd., Ososa, Ogun State, Nigeria, was purchased from retail outlets within the commercial area of the University of Maiduguri. The administered volumes were calculated based on the approximate intake of a 70 kg adult consuming one 500 ml bottle of FED, with one, two and three bottles corresponding to low-, medium- and high-dose exposures, respectively (Ferreira *et al*., 2004), and the doses were chosen following established dose-extrapolation approaches reported in the literature (Mansy *et al*., 2017). The assigned doses were 7 mL/kg (Caffeine: 2.17, Taurine: 2.65, Sugar: 0.84), 14 mL/kg (Caffeine: 4.34, Taurine: 5.30, Sugar: 1.68), and 21 mL/kg (Caffeine: 6.51, Taurine 7.95, Sugar: 2.52) for Groups B, C and D, respectively. Administration was performed once daily for 60 consecutive days by oral gavage using oropharyngeal tubes, with modification of previously described protocols (Adjene *et al*., 2010; Akande & Banjoko, 2011).

Table 1: Composition of Fearless Energy Drink

Composition	Concentration/Present
Water	Present
Carbohydrates (Sugars)	12 g/100 mL
Caffeine	0.031% (31 mg/100 mL)
Taurine	0.0378 % (37.8 mg/100 mL)
Carbon dioxide	Present
Citric acid (E330)	Present
Sodium citrate (E331)	Present
Inositol	Present
Niacin (Vitamin B3)	3 mg/100 mL
Vitamin B6	0.3 mg/100 mL
Vitamin B12	0.3 µg/100 mL
Ginseng extracts	Present
Flavouring agents	Present
Colouring agents	Present
Potassium sorbate (E202)	Present
Sodium benzoate (E211)	Present

The composition of FED was obtained from the product label and matched with published reports.

Tissue excision and hippocampal isolation

The hippocampal region works closely with the DRD2, OPRM1, and COMT genes to regulate memory, reward processing, and stress responses. Variations in these genes directly impact brain structure, synaptic plasticity, and emotional regulation (Otsuka *et al*., 2019: Gao *et al*., 2023). At the end of the 60-day administration period, the rats were anaesthetised by intraperitoneal injection of 0.5 ml ketamine hydrochloride and sacrificed by cervical dislocation on day 61. The brains were harvested following full organ dissection and rinsed in PBS. Each brain was placed in a Petri dish on ice, and the hippocampus was carefully isolated from surrounding brain regions. The dissected hippocampal tissues were transferred into microcentrifuge tubes containing cold PBS and gently rinsed to remove blood and debris. Samples were snap-frozen and stored at -80 °C until molecular analysis.

qRT-PCR Optimization, RNA Extraction, cDNA Synthesis and Gene Expression Analysis

Prior to analysis of experimental samples, qRT-PCR conditions were optimized to determine the most suitable annealing temperature and ensure specific amplification of target genes. Primer specificity was verified by melt curve analysis and agarose gel electrophoresis of PCR products.

Total RNA was extracted from hippocampal tissues using the Norgen Total RNA Purification Kit (Norgen Biotek Corp., Canada) according to the manufacturer's instructions. Briefly, approximately 30 mg of tissue was homogenized in 600 µL of Buffer RL. The lysate was centrifuged at 14,000 × g for 1 min and transferred to a purification column. The column was washed with 400 µL of Wash Solution A and centrifuged for 1 min; the washing procedure was repeated three times. RNA was eluted using 50 µL of Elution Solution A followed by centrifugation at 2,000 × g for 2 min and subsequently at 14,000 × g for 1 min. RNA concentration and purity were determined spectrophotometrically by measuring absorbance at

260 and 280 nm. Samples exhibiting A260/A280 ratios between 1.8 and 2.1 were considered acceptable for downstream applications. RNA integrity was further assessed by agarose gel electrophoresis through visualization of intact 28S and 18S rRNA bands.

First-strand cDNA synthesis was performed using (SuperScript IV First-Strand Synthesis System) according to the manufacturer's protocol. Briefly, 1 µg of total RNA was reverse transcribed in a 20 µL reaction mixture containing reverse transcriptase, reaction buffer, dNTPs, RNase inhibitor, and oligo(dT)/random primers. Reverse transcription was carried out at 42°C for 20 minutes, followed by enzyme inactivation at 85°C for 3 minutes.

Quantitative real-time PCR was performed using SYBR Green PCR Master Mix on a (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Each reaction contained (2× SYBR Green Master Mix, Forward primer (10 µM), Reverse primer (10 µM), Template cDNA, and Nuclease-free water). Thermal cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 5 min, annealing at 25°C for 5 min, and extension at 72°C for 20 min. A melt curve analysis ranging from 60°C to 95°C was performed at the end of amplification to confirm product specificity. Gene expression levels were normalized against the housekeeping (β-actin), which demonstrated stable expression across experimental groups. Relative gene expression was calculated using the 2^-ΔΔCt method.

Gene-expression analysis

The expression of DRD2, COMT and OPRM1 was quantified using qRT-PCR. Ct values for each target gene and the housekeeping gene were recorded. ΔCt was calculated by subtracting the Ct value of the housekeeping gene from the Ct value of the target gene for each sample. ΔΔCt was calculated by subtracting the mean ΔCt of the control group from the ΔCt of each treated group. Relative expression was calculated using the 2^-ΔΔCt method, with the control group normalised to a fold-change value of 1.00.

Data Presentation and Statistical Analysis

Quantitative real-time PCR (qRT-PCR) data were processed and expressed as cycle threshold (Ct) values, followed by normalization using the endogenous reference gene β-actin (ACTB) to generate ΔCt values. Relative gene expression was calculated using the ΔΔCt method and expressed as fold change (2^−ΔΔCt) relative to the control group. Each experimental group consisted of three biological replicates (n = 3). For each biological replicate, Ct values were obtained and processed individually prior to normalization. Group data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by appropriate post hoc comparisons to determine significant differences among groups. A p-value of < 0.05 was considered statistically significant.

RESULTS

Effect of FED on DRD2 expression

FED administration altered the hippocampal expression of DRD2 across the treatment groups. The control group had a ΔCt value of -13.48 and served as the reference condition. The low-, medium- and high-dose groups had ΔCt values of -6.17, -7.42 and -12.77, respectively. Relative to the control group, DRD2 expression was reduced in all treated groups, with fold-change values of 0.01, 0.02 and 0.61 in the low-, medium- and high-dose groups, respectively. These findings indicate treatment-associated down-regulation of DRD2 expression; however, the pattern was not strictly dose-dependent, as the high-dose group showed a less pronounced reduction than the low- and medium-dose groups (Table 2).

Effect of FED on COMT expression

The expression of COMT was reduced in all FED-treated groups relative to the control (Table 3). The control group had a ΔCt value of -13.520, while the low-, medium- and high-dose groups had ΔCt values of -5.870, -6.610 and -6.330, respectively. The corresponding fold-change values were 0.005, 0.008 and 0.007. These values indicate observable down-regulation of COMT expression across the treated groups. The changes were broadly consistent across doses, although they did not demonstrate a strictly linear dose-response relationship.

Effect of FED on OPRM1 expression

OPRM1 expression was appeared reduced in all FED-treated groups. The control group had a ΔCt value of -19.0550. The low-dose group had a ΔCt value of -6.4250, while the medium- and high-dose groups both had ΔCt values of -9.9700. The corresponding fold-change values were 0.0002 in the low-dose group and 0.0018 in both the medium- and high-dose groups. These results indicate substantial treatment-associated down-regulation of OPRM1 expression, with the strongest reduction observed in the low-dose group based on the available fold-change values (Table 4).

Table 2. Expression of dopamine D2 receptor gene (DRD2)

Sample	GOI (DRD2) A-Ct	HK A-Ct	ΔCt	ΔΔCt	2^-ΔΔCt
Control	26.46	39.93	-13.48	0.00	1.00
Low dose	23.47	29.64	-6.17	7.31	0.01
Medium dose	24.72	32.14	-7.42	6.06	0.02
High dose	21.96	34.73	-12.77	0.71	0.61

Note. GOI = gene of interest; HK = housekeeping gene; A-Ct = average Ct value; ΔCt = delta Ct; ΔΔCt = delta delta Ct; 2^-ΔΔCt = relative fold change.

Table 3. Expression of catechol-O-methyltransferase gene (COMT)

Sample	**GOI (COMT) A-Ct**	**HK A-Ct**	**ΔCt**	**ΔΔCt**	**2^-ΔΔCt**
Control	26.410	39.930	-13.520	0.000	1.000
Low dose	23.765	29.635	-5.870	7.650	0.005
Medium dose	25.530	32.140	-6.610	6.910	0.008
High dose	28.395	34.725	-6.330	7.190	0.007

Note. GOI = gene of interest; HK = housekeeping gene; A-Ct = average Ct value; ΔCt = delta Ct; ΔΔCt = delta delta Ct; 2^-ΔΔCt = relative fold change.

Table 4. Expression of mu-1 opioid receptor gene (OPRM1)

Sample	**GOI (OPRM1) A-Ct**	**HK A-Ct**	**ΔCt**	**ΔΔCt**	**2^-ΔΔCt**
Control	20.8750	39.9300	-19.0550	0.0000	1.0000
Low dose	23.2100	29.6350	-6.4250	12.6300	0.0002
Medium dose	22.1700	32.1400	-9.9700	9.0850	0.0018
High dose	24.7550	34.7250	-9.9700	9.0850	0.0018

Note. GOI = gene of interest; HK = housekeeping gene; A-Ct = average Ct value; ΔCt = delta Ct; ΔΔCt = delta delta Ct; 2^-ΔΔCt = relative fold change

DISCUSSION

This study examined the effect of sub-chronic FED administration on the hippocampal expression of DRD2, COMT and OPRM1 in albino rats. The findings show that 60-day exposure to FED was associated with reduced relative expression of all three target genes compared with the control group. Because DRD2, COMT and OPRM1 are involved in dopaminergic and opioid signalling pathways, the observed alterations indicate that prolonged FED exposure may influence molecular pathways associated with neurotransmitter regulation. However, the present study assessed transcript abundance only and did not evaluate the functional consequences of these changes.

DRD2 expression was reduced in all FED-treated groups. The D2 receptor is a major component of dopaminergic neurotransmission and has been widely implicated in dopamine-mediated neuronal signalling (Noble, 2000). Reduced DRD2 expression may reflect altered transcriptional regulation following repeated exposure to constituents of FED. Caffeine-containing products can influence dopaminergic function indirectly, largely through antagonism of adenosine receptors and subsequent modulation of downstream neurotransmitter activity. The present finding is consistent with reports that psychostimulant exposure can alter dopamine-related gene expression (Gonzalez *et al*., 2015). However, the DRD2 pattern in this study was not strictly dose-dependent, because the high-dose group showed a fold-change value closer to control than the low- and medium-dose groups. This non-linear pattern may reflect compensatory molecular responses, biological variability, or limitations arising from the use of group-level Ct summaries rather than individual-level replicates.

COMT expression was also reduced across all treated groups. COMT encodes catechol-O-methyltransferase, an enzyme involved in the metabolism of catecholamines, including dopamine. Down-regulation of COMT may indicate altered regulation of catecholamine-metabolizing pathways within the hippocampus (Kennedy *et al*., 2015; Simpson *et al*., 2014). Although reduced COMT expression could potentially affect dopamine metabolism, the present study did not assess neurotransmitter concentrations, enzyme activity, or downstream physiological effects. Therefore, the findings should be interpreted as evidence of altered COMT transcript abundance rather than confirmation of altered dopaminergic signalling. The treated

groups showed broadly similar reductions rather than a clear monotonic dose-response pattern, suggesting that the molecular response may not be strictly dose-dependent under the conditions of this study.

OPRM1 showed the most marked reduction in relative expression across the treated groups. The OPRM1 gene encodes the μ-opioid receptor, which is involved in opioid neurotransmission and several neurophysiological processes, including nociceptive and affective regulation (Picci *et al*., 2022). Reduced OPRM1 expression may therefore indicate transcriptional changes affecting opioid-related molecular pathways. However, because receptor protein expression, receptor activity, and behavioural outcomes were not assessed, the functional significance of the observed reduction remains uncertain. The similar fold-change values observed in the medium- and high-dose groups may indicate a plateau effect, although this interpretation requires confirmation using individual biological replicates and protein-level validation.

Taken together, the findings suggest that prolonged FED exposure can alter the hippocampal expression of genes associated with dopaminergic and opioid neurotransmission. This is biologically plausible because energy drinks contain caffeine and other bioactive components, including taurine, guarana and herbal extracts, which may interact with neurotransmitter systems and cellular regulatory pathways (Al-Basher *et al*., 2018; Zeidan-Chulia *et al*., 2013). Previous studies have reported that caffeinated or carbonated beverages can induce oxidative stress and alter brain-related gene expression (El-Terras *et al*., 2016), while other work has suggested that caffeine and coffee-related compounds may influence epigenetic regulation (Ding *et al*., 2023). Nevertheless, caffeine primarily acts through adenosine receptor antagonism; therefore, the observed changes in DRD2, COMT and OPRM1 should be interpreted as molecular alterations in gene expression rather than evidence of specific neurobiological, behavioural, or functional outcomes.

This study has important limitations. First, the analysis was based on group-level Ct summaries, which limits the ability to conduct inferential statistics or estimate biological variability. Second, the study measured mRNA expression only; therefore, the findings do not confirm corresponding changes in protein expression, receptor abundance, enzyme activity, or neurotransmitter function. Third, behavioural assays were not included, so no conclusions can be drawn regarding reward-related behaviours, reinforcement, addiction susceptibility, or other neurobehavioural outcomes. Finally, because FED contains multiple active ingredients, the specific contribution of caffeine relative to other constituents could not be isolated. Future studies should include individual-level biological replicates, validated reference genes, protein assays, neurotransmitter measurements, behavioural endpoints, and ingredient-specific experimental arms to better characterize the biological significance of the observed transcriptional changes.

CONCLUSION

Sub-chronic administration of FED for 60 days altered the hippocampal expression of DRD2, COMT and OPRM1 in albino rats. All three genes showed reduced relative expression in the treated groups when compared with the control group. However, the available fold-change values do not support a strictly dose-dependent pattern across all genes. The findings suggest that prolonged consumption of stimulant-containing energy drinks may influence neurobiological pathways associated with reward regulation and addiction vulnerability. Further mechanistic and behavioural studies are required before definitive conclusions can be drawn regarding the implications for addictive behaviour.

List of Abbreviations

AREC, Animal Research and Ethical Committee; COMT, catechol-O-methyltransferase; Ct, cycle threshold; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; DRD2, dopamine receptor D2; ED, energy drink; FED, Fearless Energy Drink; GOI, gene of interest; HK, housekeeping gene; NFW, nuclease-free water; OPRM1, opioid receptor mu-1; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; RNA, ribonucleic acid.

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Author Contributions

YHAG conceived the study. YHAG and FSD carried out the laboratory animal handling and treatment. GUS supervised the work. FSD analysed the data. YHAG and FSD prepared the initial manuscript draft. GUS critically revised the manuscript. All authors reviewed and approved the final version for submission.

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Availability of Data and Materials
The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

Competing Interests
The authors declare that they have no competing interests.

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