Alterations in MicroRNA-132/212 Expression Impairs Fear Memory in Goldfish *Carassius auratus*

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Keywords

Goldfish · Contextual fear conditioning · Nicotinamide · MicroRNAs-132/212 · Immediate-early genes · Brain-derived neurotrophic factor

Abstract

Background: Earlier, we showed that nicotinamide (NAM) treatment impairs spatial memory through the downregulation of CREB-Sirt 1-brain-derived neurotrophic factor (*Bdnf*) signaling cascade. **Purpose:** In this study, we examine whether NAM treatment alters CREB-regulated genes through microRNAs. **Method:** To test this hypothesis, goldfish (*Carassius auratus*) were divided into 2 groups: (i) vehicle group (VEH; double distilled water intra-peritoneally [i.p.]) (ii) nicotinamide group (NAM, 1,000 mg/kg, i.p.) and again divided into VEH untrained/trained, NAM untrained/trained. One hour after receiving VEH or NAM, individuals were subject to contextual fear conditioning (CFC) training. After 24 h, both the groups were tested for contextual fear memory. Subsequently, miR-132/212 levels, regulated immediate-early genes (IEGs: C-fos and EGR-1) and *Bdnf* but not its receptor, TrkB1 were examined following 0' and 60' min after training, and compared with the untrained group. **Results:** We observed that NAM treatment significantly impaired fear memory. Further,

the analysis showed that miR-132 level was not altered, but miR-212 level was significantly upregulated after CFC training only in NAM-treated fish. We also found that NAM treatment downregulated IEGs and *Bdnf* but not its receptor TrkB1. **Conclusions:** Present study suggests that NAM-treatment impaired fear memory and control IEGs, *Bdnf* and TrkB1 expression by differentially regulating miR-132 and 212.

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Introduction

Fear learning plays a major role in the survival of an animal. It is highly influenced by the contextual and social information that helps individuals to recognize, avoid or respond to predators [1]. Immediate-early genes (IEGs) are known to be rapidly induced by behavioral experience at respective regions of the brain. IEGs' expression has been considered a marker to measure neuronal activity [2]. IEGs act as transcription factors that induce the expression of late-response genes, which drive long-term potentiation and synaptic plasticity [3, 4]. Several studies demonstrate that IEGs such as c-Fos [4, 5] and egr-1 [4, 6] are critically involved in the process of long-term synaptic changes and memory formation [2, 7].

Induction of IEGs is transduced via the extracellular-signal-regulated kinase-1/2 (ERK-1/2) and cyclic adenosine monophosphate response element binding (CREB) protein pathway [8]. However, expressions of CREB-regulated genes are tightly controlled by transcriptional and translational regulators, including microRNAs (miRs) [9–11]. miRs can post-transcriptionally suppress a gene expression by specifically recognizing and cleaving the mRNA transcripts [12]. Earlier studies have documented that miRs play a critical role in different biological functions including neurogenesis, dendritic spine morphogenesis, neuronal plasticity, and learning and memory [13, 14]. Interestingly, miR-132 and miR-212 are known to have contrasting functions in neurons [15, 16]. Both miR-132 and 212 tightly control the expression of CREB-regulated genes [9, 17] including the neurotrophic factor. Upregulation of brain-derived neurotrophic factor (BDNF; exon IV) transcript in hippocampus has been linked with enhanced fear memory-consolidation and its knockout leads to the impairment of fear memory [18, 19]. Interaction of BDNF with Tyrosine-Kinase family-B (TrkB) receptor has been established as a fear-response and fear memory circuit [18, 20, 21]. These precedents suggest that BDNF-TrkB downstream signaling is essential for fear memory.

Earlier, we showed that nicotinamide (NAM) treatment downregulated the CREB-SIRT1-*Bdnf* signaling cascade, which impaired spatial learning in goldfish *C. auratus* [22]. In the present study, we predict that miRs-132/212 participates in CREB-regulated IEGs' expression, and that they may respond differentially to NAM treatment. To test this, goldfish were subject to the contextual fear conditioning (CFC) task following NAM treatment. Then, we examined whether miR-132/212 regulates positively or negatively on CREB-regulated IEGs, *Bdnf*, and its receptor TrkB1.

Material and Methods

Animals

Naïve goldfish *C. auratus* (mean body length: 6.5–7.5 cm) were purchased from a local pet shop. Fish were housed in groups ($n = 10/\text{group}$) at home tanks (circular tank with diameter 40 cm and height 30 cm) with sufficient aeration at standard condition (RT: $25 \pm 2^\circ\text{C}$; 12:12 Light:dark cycle). Dry food pellets (Taiyo Pet Products Pvt. Ltd., India) were provided thrice a day (9:00, 14:00 and 18:00 h) at their home tank, which was cleaned and replaced with fresh water every alternative day to remove debris. Individual fish were identified with phenotypic differences.

Fig. 1. Diagram showing the experimental apparatus used to test contextual fear condition. Electrical shocks are provided at the stimulus zone (STZ) through the thin copper sheet (C) with the power source (P). To avoid the electrical shock, fish must escape from STZ to the safe zone (SFZ).

Experimental Apparatus

Goldfish received CFC training in a glass experimental tank ($60 \times 30 \times 30$ cm), which consisted of 2 compartments – stimulus zone (STZ; $15 \times 30 \times 30$ cm) and safe zone (SFZ; $45 \times 30 \times 30$ cm; Fig. 1). The SFZ was completely covered with black sheet on all sides (not shown in Fig. 1). STZ was enabled with daylight. Thus, the STZ with daylight and dark SFZ itself perform as context. The movement of fish between STZ and SFZ is facilitated by 2 circular openings (diameter 6.5 cm each) at different positions. Copper sheets (C; 15×15 cm, 1.0 mm thick) were positioned on the opposite flanks at the STZ connected with a step-down eliminator with timer (P; Conel, DC power, model 120, New Delhi, India) to deliver a $\sim 10\text{V}$ current. The 10V power supply was ensured before every trial by using a multimeter (Digital multimeter, DT 830D).

Behavioral Study

Drug Administration

NAM is an inhibitor of Sirtuin1-a class III NAD $^{+}$ -dependent histone deacetylase [23]. NAM (#BCBD0222V, Sigma, USA) was dissolved in autoclaved double distilled water (DD-H $_2$ O) and administered intra-peritoneally (i.p.; 1,000 mg/kg; 10 μL) to goldfish using a Hamilton syringe with a 3-mm needle [22, 23].

Experimental Groups

Fish were randomly grouped into (i) NAM group ($n = 12$) that received NAM; (ii) vehicle (VEH) group ($n = 14$) that received DD-H $_2$ O. Individuals received NAM or VEH i.p. an hour before the behavioral training, and the line diagram (Fig. 2) shows the complete experimental procedure.

Contextual Fear Conditioning

Conditioned stimulus (CS – a specific context-here STZ with day light) paired with an unconditioned stimulus (US-an electric shock) was used to test the effect of NAM. The individuals learn to associate the CS with US, by which they tend to avoid the context. The CFC is a modified protocol according to earlier reports [24, 25].

Behavioral Analysis

Goldfish were allowed to get acquainted with the laboratory conditions at their home tank for 5 days. Prior to training, exploration starts with the complete shoal and is then gradually reduced to

Fig. 2. Schematic procedure used to test the effect of NAM in CFC. CFC, contextual fear conditioning; NAM, nicotinamide; VEH, vehicle; UT, untrained.

individual fish on the final day of exploration. This gradual reduction of shoal size will help to minimize the stress. During training, fish were individually introduced into the STZ and allowed to explore for a period of one-min without any stimulus (pre-stimulus). After the pre-stimulus, an electric stimulus (10V; 5 s/stimulus) was given to individuals continuously for 6 min; with an inter-stimulus interval (ISI) of 10 s. Totally 20 stimuli were delivered during the training with concomitant ISIs in between every stimulus. Stimulus was immediately stopped after the fish moved to the SFZ from STZ, and considered escape response. After behavioral training, the fish were transferred to their home tank and left undisturbed. Retention test was conducted 24 h after training using the same procedure followed in the training session without electric stimuli. The entire behavior process was video recorded and the individual's response latency (time taken by fish to enter the SFZ from STZ), thrashing or escape response (highly uncountable inconsistent attempts), the time spent inside the STZ, and SFZ were measured.

Gene Expression Study

Sample Preparation

Fish grouped into VEH or NAM were again categorized as untrained groups – vehicle untrained and NAM untrained – that were sacrificed 1 h after VEH/NAM treatment without CFC training; and trained groups that were subjected to CFC training 1 h after VEH/NAM treatment and sacrificed immediately (VT-0' and NT-0') or 60 min after training (VT-60' and NT-60'). Fish were anesthetized by immersing in water containing tricaine methanesulfonate (1:20,000). Once the fish was completely anesthetized (absence of any body movements), it was placed on the surgical board where it was fixed in position by lateral holders. The dorsal skin and skull were removed carefully and the telencephalon region was dissected out [26]. TRIzol (Merck Specialties Pvt. Ltd., India) was used to isolate the total RNA that was stored with the RNase inhibitor (Merck Specialties Pvt. Ltd., India) at -70°C . Total protein was also extracted from experimental groups by homogenizing the tissue in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.1% v/v NP-40, 1 mM DTT, 0.2 mM sodium orthovanadate, 0.23 mM PMSF) with protease inhibitor cocktail (Sigma-Aldrich, USA). The homogenates were incubated in ice (30 min) and then centrifuged at 10,000 g for 30 min at 4°C . The clear supernatant was again centrifuged at 12,000 g for 30 min at 4°C and the final supernatant was stored as aliquots at

-80°C . The protein sample's concentration was estimated by measuring the absorbance at 595 nm using Biophotometer plus (Eppendorf Inc., Germany).

Quantitative Real-Time PCR

cDNA was synthesized from total RNA (1.0 μg /sample) with random/oligo-dT primers (iScriptTM cDNA Synthesis Kit, Bio-Rad Laboratories Inc.). The level of specific genes expression was analyzed by quantitative real-time PCR (qRT-PCR) in RT mixture (10 μL) (SSoAdvancedTM SYBR[®] green supermix, Bio-Rad Laboratories Inc.) with specific primers (10 μM) and cDNA (0.4 μg). The qRT-PCR was performed following standard conditions: initial denaturation (92°C , 30 s); denaturation (92°C , 5 s), annealing (primer-specific, 5 s), extension (72°C , 5 s), with 39 cycle repeats and melt-curve analysis (65 to 95°C , 0.5°C increment for 0:05 s). Specific primers were miR 132 (67°C ; For: 5'-CCTCCGGTCCCCA CAGTAACAA-3' and Rev: 5'-CCGCGTCTCCAGGGC AAC-3'), miR 212 (56.1°C ; For: 5'-GTCAGTGCATCAATACCTTG-3' and Rev: 5'-TAGA CTGTTACTGTACTTTC-3'), TrkB1 (66.1°C ; For: 5'-CCGAGCTGTTGACCAATCTG-3' and Rev: 5'-CATCAG GACCATGAGCCCTT-3'), Bdnf (58°C ; For: 5'-TCCTGGAGA AGG TCCC-3' and Rev: 5'-CGAAGTGCTATCCGTATAAAC-3'), β -actin (60°C ; For: 5'-ACC GAGAGGGCTACAGCTT-3' and Rev: 5'-TCCCATCTCCTGCTCGAAGT-3') and U6 SnRNA (58°C ; For: 5'-CTCGCTTCGGCAGCACA-3' and Rev: 5'-AAC GCTTCACGAATT TGCGT-3') were used as internal control for respective reactions. Triplicate reactions were performed with threefold serial dilution of cDNA. Amplification of the PCR product was confirmed by monitoring the dissociation curve followed by melting curve analysis. The expression level of specific genes was analyzed by comparing the mean *C_q* value. Data are presented as mean fold change of the normalized expression with the respective vehicle group (CFX ManagerTM version 2 software; CFX-96 TouchTM Real-time PCR Detection System Bio-Rad Laboratories Inc.).

Western Blot Analysis

Protein sample (20 μg) mixed with loading buffer (100% glycerol, 125 mM Tris-HCl pH 6.8, 4% SDS, 0.006% bromophenol blue, 2% mercaptoethanol) was resolved in 10% polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Millipore India Pvt. Ltd., India) through semi-dry electrophoresis. The membranes

Fig. 3. Behavioral responses of *C. auratus* during CFC training and retention test. Fish either received NAM or saline (VEH) 1 h before CFC. **a** NAM group showing a significantly higher response latency than VEH group during test but not during training. **b** NAM group showing more escape response than VEH group at STZ. CFC, contextual fear conditioning; NAM, nicotineamide; VEH, vehicle; STZ, stimulus zone. Data are shown as mean $\pm$ SEM, * significant difference between groups are indicated by (^a VEH vs. NAM during training, * $p < 0.05$; ^b VEH vs. NAM during test, *** $p < 0.001$).

were blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 3 h at room temperature. Membranes were then incubated for 6 h with a specific primary antibody: affinity purified rabbit polyclonal-anti-EGR-1 (SC-189, 1:250) or anti-C-fos (SC-52, 1:2,000) antibody and anti- β -actin (SC-130656; 1:200) antibody as internal control for each sample. Alkaline phosphatase conjugated with goat anti-rabbit (Cat #621100180011730; 1:2,000; MERCK, Bangalore, India) was used (4 h) to detect membrane-bound primary antibodies. The Molecular Imager XRS+ System was used to acquire images, and the sample's trace quantity was measured using Image Lab 2 software (Bio-Rad Laboratories, Inc., USA). Data are presented as mean fold change of the normalized expression with the respective vehicle group.

Statistical Analysis

Values are presented as mean $\pm$ SEM and plotted with KyPlot (version 1.0) for graphical representation. One-way analysis of variance was used to examine the differences between groups using SPSS software (version 22.0).

Results

NAM Treatment Impairs Contextual Fear Memory

We tested whether the treatment of NAM impairs contextual fear memory. When we tested for fear learning, there was no significant difference between VEH and NAM groups ($F_{1,25} = 0.860$, $p > 0.05$), but during retention the NAM group showed significantly higher response latency than the VEH group ($F_{1,25} = 186.29$, $p < 0.001$; Fig. 3a). The NAM group exhibited more escape response in the STZ than VEH group during training ($F_{1,25} = 6.29$, $p < 0.05$) and retention ($F_{1,25} = 39.51$, $p < 0.001$; Fig. 3b).

Similarly, VEH and NAM groups spent equal time in STZ ($F_{1,25} = 0.314$, $p > 0.05$) during training, but the NAM group spent significantly more time ($F_{1,25} = 71.40$, $p < 0.001$) in the STZ than the VEH group during retention

(Fig. 4a). Consistent with the above results, both the groups spent the same time ($F_{1,25} = 0.314$, $p > 0.05$) in the SFZ during training, whereas during retention the NAM group spent significantly lesser time than the VEH group in the SFZ ($F_{1,25} = 71.40$, $p < 0.001$; Fig. 4b). This implies that only fear memory is affected by NAM treatment and not the learning process.

NAM Treatment Alters Training-Induced Expression of miR-132/212

Further, we analyzed whether NAM treatment alters the expression profile of miR-132/212 after the individuals were trained in CFC (Fig. 5). We observed a significant upregulation of miR-212 expression in trained individuals (after 60 min: T 60') than 0 min after training (T 0'); ($F_{1,5} = 40.24$, $p < 0.005$) and in T 0' than UT ($F_{1,5} = 75.42$, $p < 0.001$). In comparison, we estimated significantly higher expression of miR-212 in T 60' than UT ($F_{1,5} = 230.76$, $p < 0.001$; Fig. 5a). Indeed miR-132 expression levels in individuals showed no significant difference after the CFC retention test. We observed that there was no significant difference between UT and T 0' ($F_{1,5} = 0.00$, $p > 0.05$); UT and T 60' ($F_{1,5} = 1.47$, $p > 0.05$) or between T 0' and T 60' ($F_{1,5} = 0.93$, $p > 0.05$; Fig. 5b). Results described that NAM treatment suppressed miR-132 expression but upregulated miR-212 expression.

NAM Treatment Alters Training-Induced IEGs Expression

We sought to examine whether NAM treatment alter the expression level of IEGs through miRs. It was evi-

Fig. 4. Behavioral responses of *C. auratus* during contextual fear condition training and retention test. **a** NAM group spent significantly more time at STZ than VEH during retention test but not during training. **b** Consistently, NAM group spent significantly lesser time in SFZ than VEH during retention test. CFC, contextual fear conditioning; NAM, nicotinamide; VEH, vehicle; STZ, stimulus zone. Data are shown as mean $\pm$ SEM, * significant difference between groups are indicated by (^b VEH vs. NAM during test, *** $p < 0.001$).

Fig. 5. Expression pattern of miR-212 (**a**) and miR-132 (**b**) after the individuals (VEH/NAM treated) were subjected to CFC training. Expression level of NAM treated group's (UT; trained: after 0 min – T 0' and 60 min – T 60') miRs (132/212) were normalized with respective vehicle-treated groups. miRs, microRNA; CFC, contextual fear conditioning; NAM, nicotinamide; VEH, vehicle; STZ, stimulus zone; UT, untrained. Data are shown as mean $\pm$ SEM, * significant difference between groups are indicated by (^a UT vs. T 0'; ^b UT vs. T 60'; ^c T 0' vs. T 60'; ** $p < 0.01$; *** $p < 0.001$).

dent from the western blot analysis that NAM treatment suppresses the training-induced expression of EGR-1 (Fig. 6a). However, significant difference was not found between UT and T 0' ($F_{1,5} = 2.60$, $p > 0.05$); UT and T 60' ($F_{1,5} = 2.67$, $p > 0.05$) or between T 0' and T 60' ($F_{1,5} = 0.18$, $p > 0.05$) in EGR-1 expression (Fig. 6b). C-fos expression was significantly less in T 60' than UT ($F_{1,5} = 145.74$, $p < 0.001$) and TR 0' ($F_{1,5} = 110.54$, $p < 0.001$). In comparison, C-fos expression level in T 0' was slightly higher than UT, but the difference was not significant ($F_{1,5} = 1.72$, $p > 0.05$; Fig. 6c). This observation demonstrates that NAM treatment suppresses the activity-dependent expression of IEGs.

NAM Treatment Alters *Bdnf* and *TrkB1* Expression

Further, we assayed BDNF and its receptor TrkB1 expression pattern. We found that the difference between UT and T 0' ($F_{1,5} = 2.60$, $p > 0.05$) and T 0' and T 60' ($F_{1,5} = 4.32$, $p > 0.05$) was not significantly different in the *Bdnf* expression. Interestingly, individuals 60' after behavioral training (T 60') showed significantly lower expression than UT group ($F_{1,5} = 9.78$, $p < 0.05$; Fig. 7a). This showed that NAM treatment has suppressed the training-dependent expression of *Bdnf*, which is required for memory formation. Subsequently, we also analyzed the expression of TrkB1, a BDNF receptor. We found that the expression of TrkB1 in T 0' was significantly lower than that of the UT group ($F_{1,5} = 44.50$, $p < 0.005$). However, the expression

Fig. 6. Expression profile of selected IEGs after the individuals (VEH/NAM treated) were subjected to CFC training. **a** Representative western blot showing the expression pattern of IEGs (EGR-1, C-fos and internal control β -actin). Activity-dependent expression of IEGs **(b)** Egr-1; **(c)** C-fos downregulated after CFC training in the NAM-treated group (UT; trained: after 0 min- T 0' and 60 min - T 60'). The expression level of the NAM group was normalized with the respective vehicle-treated groups. IEGs, immediate early-genes; CFC, contextual fear conditioning; NAM, nicotineamide; VEH, vehicle; STZ, stimulus zone; UT, untrained. Data are shown as mean $\pm$ SEM, * indicates the significant difference between groups indicated by (^b UT vs. T 60'; ^c T 0' vs. T 60'; *** $p < 0.001$).

was significantly increased 60 min after behavioral training than T 0' ($F_{1,5} = 53.00, p < 0.005$) and UT ($F_{1,5} = 11.44, p < 0.05$; Fig. 7b). This observation demonstrates that NAM-treatment suppresses the training-induced expression of *Bdnf* but not its receptor TrkB1.

Discussion

Earlier studies described that NAM acts as a potent inhibitor of Sirt 1 [22, 27, 28]. SIRT 1 is a NAD⁺-dependent deacetylase and is known to be involved in a diverse cellular process including hippocampal-dependent memory. Previously, we showed that NAM-treatment impaired spatial memory formation in goldfish downregulate the expression status of ERK1/2- CREB-Sirt 1-*Bdnf* signals [22]. In this study, we predict that NAM treatment possibly impairs contextual fear memory, and may alter the IEGs and miRs 132/212 that are involved in synaptic plasticity. Regarding avoidance learning, animals can learn to associate CS with US and during testing respond to CS to avoid the US. Our behavioral analysis showed that individuals who received NAM responded to the stimulus and learn to avoid during training, but they showed higher response latency and spent more time at STZ during testing. These

observations suggest that infusion of NAM did not impair contextual fear learning but significantly inhibits the formation of contextual fear-memory. Similar to the rodent model, our data showed that NAM-treated fish displayed higher “escape response” in the STZ than control [10]. Thus, it was proposed that NAM treatment impairs contextual fear memory but not the fear learning in CFC.

Further, we speculate that the expression of *Bdnf* might be controlled by miRs such as miR-132/212 not to mention that miR-132/212 are transcripts from the same non-coding region and located in a same intergenic region [15]. However, it was demonstrated that miR-212 and miR-132 function in opposite directions following reward-conditioning [17]. Interestingly, we observed that NAM treatment suppresses the CFC induced miR-132 expression but not miR-212. Therefore, these results suggest that NAM treatment differently regulate the miR-132 and 212 during fear learning and memory formation.

Behavioral tasks, including CFC known to induce IEGs expression in neurons are distributed across the brain regions [29–32]. Similar to the results reported in the earlier study, CFC facilitated the activation of IEGs in telencephalon of goldfish, whereas NAM infusion suppressed the CFC-induced IEGs expression. Suppression of IEGs expression in telencephalon of goldfish may dis-

Fig. 7. Expression profile of *Bdnf* and its receptor TrkB1 after the individuals (VEH/ NAM treated) were subjected to CFC training. Expression of (a) *Bdnf* was significantly downregulated; but its receptor (b) TrkB1 was significantly upregulated after CFC training in the NAM-treated group (UT; trained: after 0 min T 0' and 60 min – T 60'). Expression of the NAM treated group was normalized with the respective vehicle-treated groups. *Bdnf*, brain-derived neurotrophic factor; CFC, contextual fear conditioning; NAM, nicotinamide; VEH, vehicle; STZ, stimulus zone; UT, untrained. Data are shown as mean $\pm$ SEM, * indicates significant difference between groups are indicated by (^a UT vs. T 0'; ^b UT vs. T 60'; ^c T 0' vs. T 60'; * $p < 0.01$; ** $p < 0.001$).

integrate the neuronal circuits involved in fear memory formation [33] and may lead to the impairment of fear memory. Observed results indicate that NAM suppresses the IEGs expression, and IEGs expressing neurons in distinct brain regions may be directly or indirectly critical for fear memory formation/recalling.

Activity-dependent expression of the *Bdnf*, reduction in the expression of *Bdnf* (variant IV) impaired synaptic plasticity, and behavioral flexibility have been characterized earlier [34–36]. In this study, the level of *Bdnf* (T 60') was lesser than T 0' and suggests that NAM-treatment suppresses the activity-dependent *Bdnf* expression, which is similar to SIRT1 deletion in the rodent model [10]. On the other hand, BDNF's high-affinity receptor TrkB 1 was significantly elevated following CFC, which is similar to earlier reports [18]. Different fear conditioning protocols showed that *Bdnf* and TrkB1 coordinately upregulated [37] and their induction is critical for fear memory, whereas deletion of *Bdnf* and TrkB1 inhibition leads to the impairment of fear memory [38]. Interestingly, our analysis demonstrates that NAM treatment only suppresses the expression of *Bdnf* but not its receptor TrkB1. This finding suggests that *Bdnf* may act as a master switch in fear learning and memory in different biological contexts. Further, *Bdnf* and TrkB1 expression may be regulated by independent and region-specific mechanism.

In conclusion, the present study showed that goldfish can learn contextual fear memory through the coordinated function of miR-132/212, IEGs, *Bdnf*, and its receptor TrkB1. NAM-treatment impaired contextual fear memory but not learning. Further, the CFC failed to induce the expression of miR-132, IEGs, and *Bdnf* but not that of miR-212 and TrkB1 in the NAM-treated group. Thus, our

results suggest that NAM treatment differently regulate miR-212, 132 and *Bdnf* and its receptor TrkB1 possibly through an independent mechanism during contextual fear memory formation. In addition, our study suggests that goldfish *C. auratus* can be used as a model to study the fear memory and associated molecular mechanism.

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Ethical Approval

The experimental protocol was approved by Institutional Animal Ethical Committee, Bharathidasan University, Tiruchirappalli. Care was taken to minimize pain and the number of animals used.

Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

Author Contribution

K.E.R. conceived, designed, and prepared the manuscript. S.T. and V.S. performed the experiment and analyzed the data. M.M. and K.R. contributed to the analysis of data. All the authors read and approved the final manuscript.

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