Intermittent Calorie Restriction Ameliorates Behavioral Changes and Tau Hyperphosphorylation in Chronic Immobilization Stress in Rats

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Abstract

Background: Chronic stress has been linked to mood and anxiety disorders and alteration of cognitive functions, such as memory. Intermittent calorie restriction (ICR) is a repeated mild stress that enhances the cell ability to combat more severe stress. **Aim**: Examination of the effects of ICR on rat behaviour and Tau hyperphosphorylation in chronic immobilization stress (CIS) rat model. *Methods*: 32 rats were divided into 4 equal groups: control, ICR (subjected to ICR protocol), CIS (subjected to CIS protocol), and CIS+ICR (subjected to both CIS and ICR protocols). After performing behavioural (open field and Barnes maze) tests, serum level of corticosterone, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were determined, and hippocampal tissue level of phosphorylated Tau (phospho-Tau), noradrenaline, and serotonin were measured. *Results*: We found that, ICR significantly altered the stress-induced anxiety-like behavior and memory disturbances (P<0.001). Rats exposed to both ICR and CIS protocols had significantly lower levels of phospho-Tau, corticosterone, and TNF-α, and enhanced levels of noradrenaline and serotonin than those subjected to CIS alone (P<0.01). *Conclusion*: our results suggest that ICR protects against chronic stress-induced anxiety-like behaviour, memory disturbance and Tau hyperphosphorylation, possibly through inhibition of hypothalamic pituitary adrenal (HPA) axis, anti-inflammatory effects, and enhancement of noradrenaline and serotonin hormones.

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INTRODUCTION

Stress is a serious phenomenon in the modern world that acts as a risk factor for the development of several diseases in the long term [1]. Stress is associated with neuropsychiatry disorders such as anxiety and major depression [2]. Stressor exposure induces various physiological responses to maintain integrity; stress activates hypothalamic-pituitary-adrenal (HPA) axis and compensatory increase in the brain serotonin and noradrenaline to cope with stress demands [3, 4]. The hippocampus, the main part of the brain relevant to memory and learning, is very sensitive to corticosterone and cytokines. Prolonged exposure to corticosterone impairs hippocampal and amygdala circuits [1]. Hippocampal Tau is a major cytoskeletal microtubule-associated protein that promotes microtubule assembly and stabilization. Tau has an essential role in memory and learning [5, 6]. When Tau is hyperphosphorylated, it accumulates in the cells leading to Tau missorting at dendritic spines and incompetency in performing its functions resulting in cognitive and mood deficits [2, 5]. Time restricted feeding protocols were frequently used in behavioural studies, as the behaviour of the organism is in accordance with 24 hour light/dark cycles [7]. Intermittent calorie restriction (ICR) is a food deprivation period, which is repeated between fasting and non-fasting duration, with no malnutrition effects [1]. ICR was evidenced to have a good impact on various health measures [8]. It has a repeated mild beneficial stress effects on the cell similar to that induced by ischemic preconditioning [9]. In the brain, ICR enhanced the adaptive stress responses to cope with more severe stress and increase resistance to oxidative and metabolic stress [8, 10]. We aimed to investigate the possible protective effects of ICR on rat behaviour and hippocampal Tau hyperphosphorylation in CIS rats, and demonstrate the possible associated changes in systemic corticosteroids and inflammatory mediators and hippocampal tissue level of stress hormones.

Material and Methods

Animals: Thirty two male Wistar albino rats (100-150 g) were used in this study. The animals were housed in the animal house of Faculty of Medicine, Menoufia University, Egypt in groups of 4 per cage. They were kept at room temperature, 26±1 °C, on artificial light/dark cycle of 12 hour with water and food available adlibitum. The animals were acclimatized to laboratory conditions one week before the beginning of the experiment. The experimental procedures were performed in accordance with the internationally accepted ethical guidelines for the care and use of laboratory animals and were approved by the ethical committee of the Faculty of Medicine, Menoufia University, Egypt (Institutional Review Board approval No. is 191219PHYS59).

Experimental Design and Collection of Samples

Rats were randomly divided into 4 equal groups (n=8). The control group: rats were not underwent any protocols for 12 week duration, the ICR group: rats underwent the ICR protocol for 12 weeks, the CIS group: rats were not underwent any protocols for 6 weeks then, they underwent the CIS protocol daily for the next 6 weeks of the experiment duration, and CIS+ICR: rats underwent ICR protocol for 6 weeks followed by both CIS and ICR protocols for the next 6 weeks. After the 12 week duration, behavioral tests were performed. Then, body weight was measured and fasting

retro-orbital blood samples were collected via heparinized microcapillary tubes. Blood was left to clot for 30 minutes, then, centrifuged at 2000 rpm for 10 min. The clear supernatant serum samples were stored at -20 °C until use. Rats were sacrificed by cervical decapitation and the adrenal glands were rapidly removed and weighed. Brain was rapidly dissected, removed, immersed in ice-cold saline and separated into two hemispheres. Hippocampi from both hemispheres were dissected and immediately stored at -80 °C for further analysis.

Intermittent Calorie Restriction Protocol

Rats from ICR and CIS+ICR groups were deprived of food for 24 h every other day between 10:30 and 11:00 AM for 12 weeks [11].

Chronic Immobilization Stress (CIS) Protocol

Rats from the CIS and CIS+ICR groups underwent the CIS protocol daily, as previously described [12, 13] at 9:00 AM for 90 minutes for 6 weeks starting from the 7th week of the experiment. In brief, rats were put on a wooden plate with their trunks wrapped. Thus, the movement of the trunk was prevented while the head and limbs move freely. It is a simple and a suitable method for induction of physiological and psychological stress in rodents.

Behavioral Tests

Rats' behavior was recorded at 8:00 AM by investigators who were unaware of the treatment groups. The tests were performed in the following order: (1) open field test (OFT). (2) Barnes maze test. Rats were observed on alternate basis from the 4 studied groups to avoid circadian influences.

Open Field Test (OFT)

The anxiety-like behavior in rodents is usually measured in the OFT by assessing animal locomotor and behavioral activity in an open field. The apparatus adopted is wooden, open topped box (100x100 cm) with 40 cm high walls. The floor is divided into 25 squares (20x20 cm), defined as 9 central and 16 peripheral squares. At the beginning of the test, the rat was placed in the centre of the apparatus and its behavior was recorded in 10 minutes for further analysis. The time spent in the central area (TCA) and number of returns to the center (NRC) were quantified. The central area of a novel environment is perceived to be anxiogenic and aversive. The TCA and NRC are therefore an indicator of anxiety and emotional reactivity. The rearing (standing on hind limbs), grooming (licking body and paws, and face washing), and defecation (fecal droppings) numbers were also recorded which indicates the anxiety state of the animals. 70% ethyl alcohol is used to clean the walls and floor of the apparatus between each examination.

Barnes Maze Test

For assessment of spatial learning and memory we used the Barnes maze method [14-16]. The maze consists of a circular platform (90 cm in diameter) with 18 equally spaced holes (5 cm diameter; 7.5 cm between holes) along the perimeter. The maze was elevated 100 cm above the floor. A small dark recessed chamber is present below the surface of the platform called the escape box (28x22x21cm) which can be reached by the rat through the corresponding hole on the surface of the platform. The test depends upon rats' inherent aversion to open spaces and seeking for a quiet space in the escape box. The animals were firstly trained (3 trials/day with 15 minute interval for 4 consecutive days); the rat was placed in the centre of the maze, adapted for 10 seconds then, a buzzer (90dB) was

switched on to induce escape behavior, and the rat was allowed to explore the maze. Once the rat entered the target escape box, the buzzer was switched off, the hole was covered, and the rat was allowed to stay in it for 1 minute. The animal was gently guided by the experimenter if it failed to reach the box within 3 minutes. The trial concludes when the rat enters the escape box or 3 minutes elapses. Spatial visual cues should be maintained fixed throughout the experiment, including the position of the experimenter. One piece of clean tissue paper was used for each rat trial and the apparatus and the escape box were cleaned with 70% alcohol after each trial. The position of the escape box remained at fixed location relative to the spatial cues for the duration of the experiment. This was called the acquisition phase, which indicates the spatial acquisition or learning of the animal. This process typically took 4.8 min per rat and was done with 2 rats at a time.

Twenty four hours after the final session of acquisition training, rats undergo probe trial phase. The phase was conducted to determine if the animal remembers the location of the target hole for assessment of short term spatial memory retention. The probe trial was conducted in a similar manner to the acquisition trials. The animal was placed in the centre of the platform and left to explore the maze for only 90 seconds. All steps were recorded with a Sony video digital camera. The recorded variables included escape latency (the interval of time to reach the target box in seconds) and number of errors (number of head deflection into the incorrect holes) during both the acquisition phase and the probe phase.

Tissue Homogenate Preparation and Analysis

The hippocampi were homogenized in PBS (0.1mg tissue:1µl PBS), PH 7.2-7.4 (Biodiagnostic company, Egypt) by a glass homogenizer. The samples were then centrifuged at 3000 rpm for 20 min at 4C° (Narco-Bio system, UK). The supernatant was taken and kept at -80 C° for further analysis of hyperphosphorylated Tau protein (phospho-Tau), noradrenaline, and serotonin.

Measurement of Serum Corticosterone, Tumor Necrosis Factor-α (TNF-α), and Interleukin-6 (IL-6), and Hippocampal Tissue Level of Phospho-Tau

Serum level of corticosterone (Assaypro LLC, St. Charles, MO, USA), TNF-α and IL-6 (Quantikine® ELISA, R&D Systems Inc., MN, USA), and tissue level of phospho-Tau at Ser 199 site (Sigma-Aldrich, UK) were measured by enzyme linked immunosorbent assay (ELISA) technique according to manufacturer's instructions. An automatic optical reader (SUNRISE Touchscreen, TECHAN, Salzburg, Austria) was used and the absorbance was taken at 450 nm.

Measurement of Serotonin and Noradrenaline Levels in Hippocampus

Hippocampal serotonin and noradrenaline levels were determined by high performance liquid chromatography (HPLC) method with diode array detector (Agilent Technologies 1200 series), as previously described [17]. In brief, the homogenate sample was injected into the Zorbax Extend C18 column (150x4.6 mm, 5 µ). The mobile phase consisted of 97:3 phosphate buffer (20 mM, pH 3)/acetonitrile with a flow rate of 1.5 mL/min. UV detection was at 270 nm. Separation

of serotonin and noradrenaline were done after 12 minutes. The concentration of each monoamine in the sample was measured by comparing the chromatogram of the sample to that of the standard curve that was made by the Eurochrom HPLC Software, version 1.6.

Statistical analysis:

Data were expressed as mean ± SD. All statistical analysis was done using SPSS software version 16.0. The Mann–Whitney U test was used for testing significance. The probability value < 0.05 was considered statistically significant.

Results

The ICR group showed insignificant changes in all parameters as compared to control group (P>0.05)

Δ Body Weight and Relative Weight of the Adrenal gland

Delta body weight was calculated and insignificant change was observed when the control, CIS, and CIS +ICR groups were compared to each other (P>0.05) (Table 1).

A significant increase in the relative weight of the adrenal gland and serum corticosterone level was observed in CIS group when compared to control group (P<0.001). The CIS+ICR group showed a significant decrease in the relative adrenal weight and serum corticosterone level when compared to CIS group (P<0.001 and P=0.002 respectively) to a level that was still significantly higher than control group (P<0.001) (Table 1).

Animal Behavior

Rats in CIS group spent more time away from the opened central area evidenced by a significant decrease in the TCA (P=0.001) and NRC (P<0.001) as compared to control group. There was also a significant increase in grooming (P=0.005), rearing (P=0.003), and defecation

(P=0.007) as compared to control. Rats in CIS+ICR group showed a significant increase in TCA (P=0.002) and NRC (P=0.001) and a significant decrease in the number of grooming (P=0.003), rearing (P<0.001), and defecation (P=0.001) as compared to CIS group, and these changes in CIS+ICR group were insignificant when compared to control group (P>0.05) (Table 2).

The behavioral and locomotor performance of rats in the Barnes maze test were recorded (Figure 1). The learning curve for each group was descending during the 4 days of the acquisition phase. However, CIS group showed significant increase in the mean number of errors during days 1, 2, 3, 4 of the acquisition phase when compared to control group (P<0.001). The CIS+ICR group showed significant decrease in the mean number of errors during days 1, 2, 3, 4 of the acquisition phase when compared to CIS group (P=0.003, P=0.002, P=0.022, and P<0.001 respectively) and the same group showed significant increase in the mean number of errors in those days when compared to control group (P=0.005, P=0.044, P=0.002, and P=0.008 respectively). The mean time of escape latency was also insignificantly changed when ICR group was compared to control group in days 1, 2, 3, and 4 of the acquisition phase (P>0.05). The CIS group showed significant increase in the mean time of escape latency during days 1, 2, 3, 4 of the acquisition phase when compared to control group (P=<0.001). The CIS+ICR group showed significant decrease in the mean time of escape latency during those days when compared to CIS group (P<0.001, P<0.001, P=0.002, and P=0.006 respectively) and significant increase when

compared to control group (P4=0.01, P=0.01, P=0.025, and P=0.003).

During the probe phase, the mean number of errors and the mean time of escape latency were significantly increased in CIS group as compared to control group (P<0.001), and a significant decrease in both parameters was observed in CIS+ICR group when compared to CIS group (P=0.004 and P<0.001 respectively) to a level that was still significantly higher than that in control group (P=0.001 and P=0.002 respectively) (Figure 1).

Hippocampal Phosphorylated Tau Protein and Serum TNF-α, and IL-6

Levels of phospho-Tau, TNF-α and IL-6 were significantly increased in the CIS group when compared to the control group (P<0.001). The CIS+ICR group showed significantly lower levels of phospho-Tau, TNF-α and IL-6 when compared to the CIS group (P<0.001, P=0.002 and P<0.001 respectively), but still significantly higher when compared to the control group (P=0.043, P=0.004 and P=0.034 respectively) (Figure 2).

Table 1. Effect of ICR on Δ body weight (g), relative adrenal weight (%), and serum corticosterone (ng/ml) in all study groups.

	Control	ICR	CIS	CIS +ICR
Δ Bogy Weight (g)	149.5±18.02	131.7±30.9	135.8±27.2	143.6±29.1
Relative Adrenal Weight (%)	0.012±0.007	0.016±0.004	0.061±0.016*	0.029±0.012#*
Serum Corticosterone (ng/ml)	2.2±0.39	2.6±0.42	8.8±0.55*	5.2±0.31#*

Data are expressed as mean±SD (n=8). *: Significant difference as compared to control group. #: Significant difference as compared to CIS group. ICR: intermittent calorie restriction; CIS: chronic immobilization stress; CIS+ICR: combined chronic immobilization stress and intermittent calorie restriction group.

Table 2. Intermittent CR improves behavioral and locomotor performance of rats in the OFT in the different studied groups.

	Control	ICR	CIS	CIS +ICR
TCA (seconds)	11.4±5.08	9.7±4.24	4.7±2.67*	10.2±4.26#
NRC	5.6±2.12	5.7±2.26	1.8±.79*	5.4±2.46#
Grooming	5.1±2.28	5.7±2	8.7±2.5*	4.9±1.97#
Rearing	4±2	4.7±1.7	7.3±1.7*	3.1±1.45#
Defecation	3.3±1.25	3.4±1.35	5.5±1.65*	2.4±1.26#

Data are expressed as mean±SD (n=8). *: Significant difference as compared to control group. #: Significant difference as compared to CIS group. ICR: intermittent calorie restriction; CIS: chronic immobilization stress; CIS+ICR: combined chronic immobilization stress and intermittent calorie restriction group. OFT: Open field test, TCA: time spent in the central area, NRC: numbers of returns to the center.
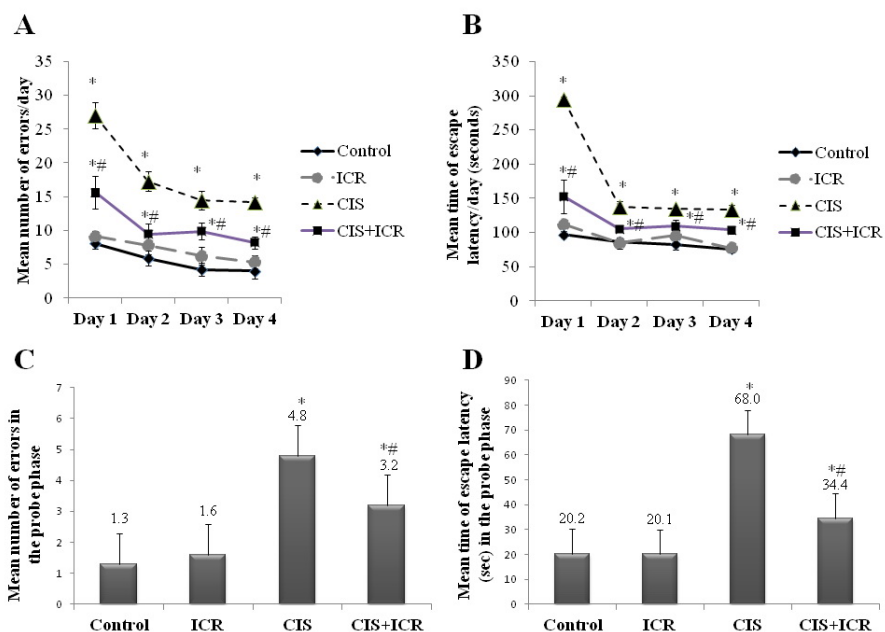
Figure 1. Intermittent CR restores the mean number of errors/day and the mean time of escape latency/day (seconds) during the acquisition phase [A, B] and the probe phase [C, D] of Barnes maze test in CIS in rats. Data are expressed as mean±SD (n=8). *: significant difference as compared to the control group. #: significant difference as compared to the CIS group. ICR: intermittent calorie restriction; CIS: chronic immobilization stress; CIS+ICR: combined chronic immobilization stress and intermittent calorie restriction group.
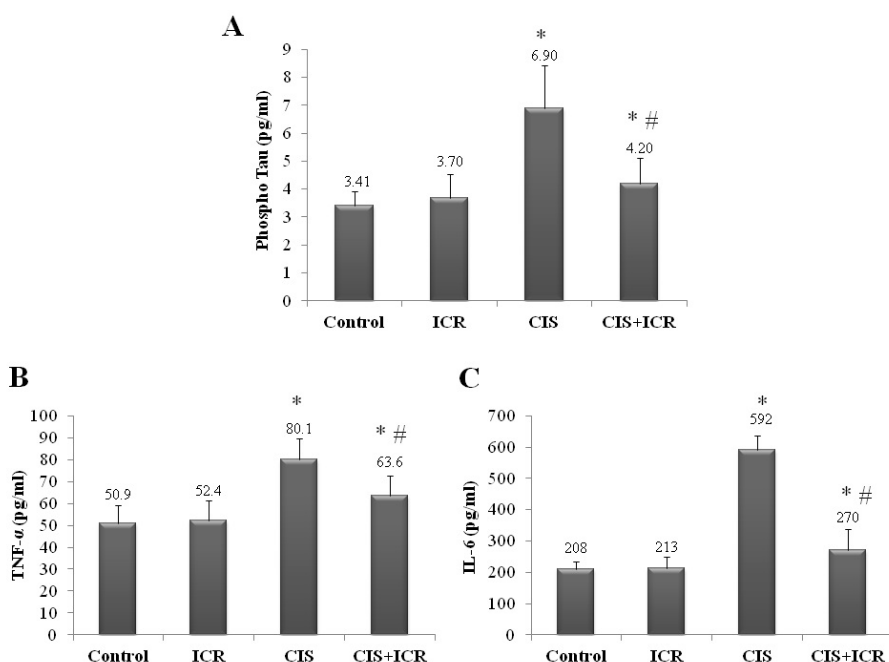

Figure 2. Intermittent CR restores hippocampal tissue level of phospho-Tau (pg/ml) [A], and serum level of TNF-α (pg/ml) [B] and IL-6 (pg/ml) [C] in CIS in rats. Data are expressed as mean±SD (n=8). *: significant difference as compared to control group. #: significant difference as compared to CIS group. ICR: intermittent calorie restriction; CIS: chronic immobilization stress; CIS+ICR: combined chronic immobilization stress and intermittent calorie restriction group; phosphor Tau: phosphorylated Tau protein; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6.

Serotonin and Noradrenaline Levels in the Rat Hippocampus

The CIS group showed significantly decreased level of hippocampal serotonin and noradrenaline when their values were compared to the corresponding values in the control group (P=0.001 and P<0.001 respectively). Both serotonin and noradrenaline were significantly increased in CIS+ICR group to a level that was significantly changed from the CIS group (P=0.013 and P<0.001 respectively) and insignificantly changed from the control group (P>0.05) (Figure 3).
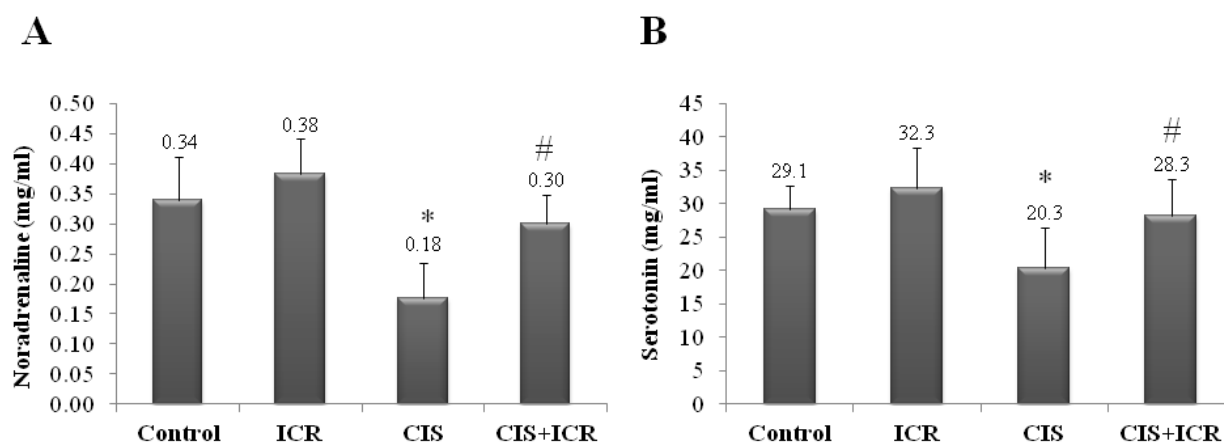
A

B

Figure 3. Intermittent CR increases hippocampal tissue levels of noradrenaline (mg/ml) [A] and serotonin (mg/ml) [B] in CIS in rats. Data are expressed as mean±SD (n=8). *: significant difference as compared to control group. #: significant difference as compared to CIS group. ICR: intermittent calorie restriction; CIS: chronic immobilization stress; CIS+ICR: combined chronic immobilization stress and intermittent calorie restriction group.

Discussion

Chronic stress is associated with amnestic, cognitive and anxiety disorders. ICR is a positive stressor that improves cognitive function [1]. In the present study, ICR improved the anxiety-like behavior and the impairment of memory and learning that were observed in CIS rats without significant loss of body weight. This was associated with a significantly decreased level of phospho-Tau, relative adrenal weight, serum corticosteroid, TNF-α, and IL-6, and significantly increased levels of serotonin, and noradrenaline.

In the present study, we observed an anxiety-like behavior in CIS rats in the open field test. The pathways were concentrated in the corner areas with decreased locomotor activities with significant increase in the number of grooming, rearing and defecation which is indicative of the development of a state of emotional stress in rats. Similar results were shown in other reports using different models of stress [18, 19].

Rats subjected to Barnes maze test in our study, and consistent with Woo et al. [20], showed impaired learning and memory in the CIS group indicated by a significant increase in the mean number of errors/day and the mean time of escape latency during both acquisition and probe phases. That increase was maximum on the first day of the acquisition phase and a descent was noticed in the learning curve in all groups the later days which is likely not due to spatial learning, but rather due to habituation to the environment resulting in

decreased anxiety and increased the motivation to escape over repeated trials [21], ICR could significantly improve the depressive behavior and learning and memory functions in chronic stress conditions [1].

Elevated serum corticosterone level and relative adrenal weight in the CIS rats, in our study, was an indicator of a successful model of stress in rats, because stress induces activation of the HPA axis [22]. Glucocorticoids are potent stress hormones that facilitate short-term and long-term adaptation to stressful conditions [23]. Prolonged glucocorticoid exposure increases apoptosis in the hippocampus, thereby causing memory decline, mood and behavior changes, and anxiety-like features [24]. Chronic corticosteroid exposure promotes hippocampal Tau hyperphosphorylation and accumulation [25]. ICR could reduce plasma level of stress hormones (corticosterone) [1]. Recently, Tau was proposed as a critical mechanism of induction of neuropathological and brain structure changes by chronic stress and glucocorticoids [2]. Consistent with Carroll et al. [26], CIS significantly increased phospho-Tau that was related to anxious behavior and memory impairment [27]. And ICR could significantly reduce phospho-Tau in the CIS+ICR group. Supporting our result, ICR upregulates Sirtuin-1 that decreased Tau phosphorylation in a rat model of diabetes [28].

Glucocorticoids enhances inflammatory processes [29]. TNF-α and IL-6 are key inflammatory cytokines that play an important role in the pathogenesis of anxiety and depression in stress [30, 31]. Dysregulation of TNF-α or IL-6 induces Tau hyperphosphorylation and impairs cognitive ability [32]. ICR decreased TNF-α and IL-6 in CIS

in rats. ICR messages the brain to decrease circulating inflammatory cytokines during stress conditions [1, 33, 34].

Intermittent CR can modulate monoamine neurotransmitters, serotonin and noradrenaline [35]. Serotonin and noradrenaline are stress hormones that regulates emotion, cognition, motivation and social interactions [36, 37]. Noradrenaline and serotonin release increased in the acute response to stress however, chronic stress exposure leads to degeneration of noradrenergic neurons and attenuation of serotonergic neurotransmission which is associated with long persistent cognitive and mood changes [38, 39].

Noradrenergic or serotonin depletion results in increased level of accumulated hyperphosphorylated Tau and suppression of neurogenesis. This was linked to depression, impaired spatial memory, and decreased hippocampal inhibition of the HPA axis exacerbating stress axis overactivity [38, 40]. Significantly increased noradrenaline and serotonin content in the hippocampus of CIS+ICR rats was observed. ICR significantly enhanced the serotonin and norepinephrine levels in the brain of rats which are crucial in the regulation of adult hippocampal neurogenesis and activation of neurogenic precursors and stem cells in the hippocampus [38, 41]. Conclusion: ICR is a short-term regimen that can partially protect against behavioral changes associated with stress through inhibition of HPA axis, decreased Tau hyperphosphorylation, decreased inflammatory markers and increased hippocampal noradrenaline and serotonin neurotransmitters.

Conflict of Interests: The authors report no conflict of interests.

Abbreviations

CIS: chronic immobilization stress

HPA: hypothalamic pituitary adrenal axis

ICR: intermittent calorie restriction

IL-6: Interleukin-6

NRC: number of returns to the center

OFT: open field test

Phospho-Tau: phosphorylated Tau protein

TCA: time spent in the central area

TNF-α: tumor necrosis factor-α

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