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Comparative evaluation of adolescent repeated psychological or physical stress effects on adult cognitive performance, oxidative stress, and heart rate in female rats

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ABSTRACT

Multiple adult health problems are associated with adolescent stress. As the brain discriminates physical and psychological stressors by activation of different neural networks, we hypothesized that behavioral and physiological performance would be modulated differently based on the nature of the stressors. Thus, we studied the comparative effects of adolescent repeated physical and psychological stresses on adult cognitive performance, pro-oxidant-antioxidant balance (PAB) and heart rate in female rats. The aim was to differentiate disparate potency of chronic psychological and physical stresses leading to long-term behavioral and physiological alterations. Twenty-one female rats were divided randomly into three groups of seven rats each: control, physical, and psychological stress. Experimental rats were exposed to the stressors for five consecutive days (10 min daily) via a two-communication box. After verifying stress induction by serum corticosterone measurement, the rats were returned to their home cage for 6 weeks, until adulthood, elevated plus maze (EPM), forced swimming test (FST), Y-maze, object recognition task (ORT), and passive avoidance test (PAT) were used as five different behavioral tests to evaluate cognitive performance of each group. Serum PAB and heart rate were measured to assess long-term stress-induced physiological disorders. The results showed exposure to adolescent psychological stress resulted in a larger set of significant changes (in behavioral variation, oxidative stress, and elevated heart rate) 6 weeks post-stress compared to adolescent physical stress. Hence, mental health care in adolescence and therapies targeting PAB and heart rate could be prevention and treatment approaches to confront persistent adolescent stress-induced disorders.

Lay summary

The aim of our study on female laboratory rats was to differentiate disparate potency of chronic psychological and physical stresses in adolescence leading to long-term behavioral and physiological alterations. The results suggest that psychological stresses result in a greater extent of changes compared to physical stress. Adolescent chronic psychological stress may reveal itself in the form of certain behavioral and physiological variations in adulthood. Therefore, mental health care in adolescence could be a valuable prevention approach to confront a variety of adult stress-induced disorders.

Introduction

Stress can be considered as a critical factor that influences general health. This may be acute or chronic, of which the latter is more detrimental to health. A wide assortment of diseases such as hypertension, cardiovascular, and various metabolic disorders can be consequences of stress (Berntson, Patel, & Stewart, 2017; Bomhof-Roordink et al., 2015; Rosmond, 2005; Selye, 1955; Wolff, 1950; Yan et al., 2003).

Based upon previous studies on the effects of chronic stress on males and female rats, significant sex differences have been observed in stress-induced neurobiological changes showing increased vulnerability in females (Campbell, Lin, DeVries, & Lambert, 2003; Pyter, Kelly, Harrell, & Neigh, 2013; Takahashi et al., 2017; Toledo-Rodriguez & Sandi, 2011). Studies on stress exposure during adolescence or adulthood in female rats have revealed that adolescents are more prone to stress and later development of psychophysiological disorders (Ver Hoeve, Kelly, Luz, Ghanshani, & Bhatnagar, 2013). Augmented susceptibility to stress in adolescence can be explained by the finding that major biological transitions such as cerebral development take place during this critical period of life (Cunningham, Bhattacharyya, & Benes, 2002; Spear, 2000).
One of the direct results of stress exposure is activation of the hypothalamic pituitary adrenal (HPA) axis pathway. An early stress-induced physiological change caused by HPA activity is elevated glucocorticoid secretion by the adrenal cortex (Tsigos & Chrousos, 2002). It is widely accepted that increased blood glucocorticoid concentration would affect cognitive performance in animals and humans (Belenoﬀ, Gross, Yager, & Schatzberg, 2001; Roozendaal, 2000). To test effects of stress in animal models, cognitive performance is evaluated by various noninvasive behavioral tasks (Di Liberto et al., 2016; Joshi, Leslie, & Perrot, 2017; Kavushansky, Ben-Shachar, Richter-Levin, & Klein, 2009). Injection of an excessive amount of glucocorticoid or its receptor agonist into the rat hippocampus results in weakened memory retrieval (Roozendaal, 2002). Several studies have reported devastating effects of glucocorticoid on memory, however, positive effects have also been demonstrated (Roozendaal, 2002).

Another pathway which is activated upon exposure to stress is the sympathetic nervous system (SNS), resulting in increased catecholamine secretion. Elevated plasma catecholamines increase blood pressure and heart rate. At this point, baroreceptors get activated in order to decrease blood pressure back to its previous level. It has been demonstrated that continuous exposure to stress can decrease responses of baroreceptors to a stimulus (Bristow, Honour, Pickering, Sleight, & Smyth, 1969; McCubbin, Green, & Page, 1956).

Both HPA and SNS pathways signiﬁcantly increase the blood-glucose concentration (hyperglycemia) by stimulating gluconeogenesis (Fagerholm, Haaparanta, & Scheinin, 2011) or glycolysis (Ullrich & Wollheim, 1984), respectively. Excessive exposure to circulating glucose could stimulate generation of reactive oxygen species (ROS), which leads to oxidative stress. This is the most notable mechanism through which hyperglycemia causes tissue damage (Halliwell & Gutteridge, 2015).

Although some recent studies have investigated the life-long effects of adolescent stressors (Barha, Brummelte, Lieblitch, & Galea, 2011; Burke, Renner, Forster, & Watt, 2010; Nishi, Sasagawa, & Horii-Hayashi, 2017), there is no sufﬁcient differentiating information about long-term consequences of different types of stressors on behavioral and physiological performance (Isgor, Kabbaj, Akil, & Watson, 2004; Traslaviña, de Oliveira, & Franci, 2014). It has been established that the brain discriminates physical and psychological stressors by activation of different neural networks which lead to explicit health problems (Dayas, Buller, Crane, Xu, & Day, 2001). Thus, we hypothesized that behavioral and physiological performance would be modulated in a different way based on the nature of stressors. Accordingly, we decided to evaluate the comparative effects of adolescent chronic psychological and physical stresses on adult cognitive performances, pro-oxidant-antioxidant balance (PAB) and heart rate in female rats. The aim of our study was to differentiate disparate potency of chronic psychological and physical stressors leading to long-term behavioral and physiological alterations. A two-compartment box system was utilized for exposing rats to physical and psychological stresses (Ramsey & Van Ree, 1993; Van den Berg, Lamberts, Wolterink, Wiegant, & Van Ree, 1998). Physical stress was induced by exposure to electric foot shocks. Psychological stress was induced in rats that witnessed physical harm and their consequent discomfort in their neighboring counterparts in a two-compartment box system. Six weeks later, five different behavioral tests (Elevated plus maze [EPM], forced swimming test [FST], Y-maze, object recognition task [ORT], and passive avoidance test [PAT]) were performed to evaluate cognitive performance and emotionality of each experimental group. Furthermore, serum PAB and heart rate were measured to assess long-term stress-induced physiological changes.

**Methods**

**Animals**

Twenty-one female Wistar rats (~100 g), on postnatal day (PND) 21, were obtained from the animal house of the University of Tehran (Tehran, Iran). The rats were housed under standard environmental conditions on a 12 h/12 h d/night cycle (light on from 07:00 h. to 19:00 h.) and in a temperature-controlled room (21–24°C). Each group of seven rats (body weight range 100–200 g) was housed in a cage 70 × 40 × 30 cm (floor area 70 × 40 cm, 2800 cm², more than twice the 1290 cm² recommended for eight rats of this body weight range (given as 199.5 inches² in Albus, 2012).

All the rats had free access to standard food and water. The experimental procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the institutional guidelines of the University of Tehran (Tehran, Iran). During all the experimental procedures, maximum eﬀort was made to reduce the number of animals used and their suﬀering.

**Rat estrous (reproductive) cycle phase determination**

In order to minimize the effects of variations in levels of the female rat’s gonadal hormones on the physiological and behavioral parameters, all the rats were chosen in accordance with identical estrous (reproductive) cycle phase. Thus, on PND21, 68 rats from the animal house were placed in the colony room to habituate for two weeks. These rats were housed in a large cage (70 × 320 × 30 cm, formed from 8 interconnected cages, each 70 × 40 × 30 cm). On the first day of the third week (PND 35), estrous cycle phases of all the rats were determined by utilization of vaginal smear cytology during 1 h to select 21 rats that were at the same diestrus phase. In brief, vaginal samples were collected with a plastic pipette ﬁlled with 0.1 ml of normal saline (NaCl 0.9%) by inserting the tip gently into the rat vaginal orifice at a depth of approximately 5–10 mm. A small drop of the sample was then placed on a glass slide and allowed to air-dry. Unstained collected vaginal smears were observed under a light microscope to select rats which were at the same phase assessed by the proportion of cell types (Marcondes, Bianchi, & Tanno, 2002). Twenty-one rats in diestrus were chosen and the remainder was returned to the animal house. The diestrus smears displayed overall dispersed cells containing neutrophils, small and large nucleated cells in low to moderate densities, and also anucleated epithelial cells in low numbers.
Moreover, prior to each test in this investigation, estrous cycle phase determination was performed again. In this regard, a few of the rats that were in a different phase were tested a few hours earlier or later in order to ensure identical phase for testing and data collection.

**Experimental design**

The rats were divided randomly into three groups (seven rats in each cage) consisting of control, physical stress and psychological stress. Each group was housed in a cage (70 cm × 40 cm × 30 cm, as above) and at the time of assessment, each individual rat was taken to the experimental room. After testing a rat in the control group, its corresponding counterparts from physical and psychological groups were subsequently subjected to the same treatment. Hence, waiting time for all the groups was the same. At the first day (PND 21), as stated above, all the rats were placed in the colony room for a two-week habituation. In the third week (PND 35–39), two groups were exposed to physical or psychological stress and the control group was only placed in the two-communication box without any electric foot shock in five successive days. Immediately after the stress exposure of the last day, blood samples were collected for measurement of serum corticosterone concentration to verify stress induction. The rats were placed back into their home cages without further stress for 6 weeks (till PND 80). Then, five different behavioral paradigms, serum PAB, and heart rate were assessed (PND 80–90) to evaluate cognitive performance and emotional behavior, oxidative stress and SNS activity in adult rats (Figure 1). All the experiments were performed between 08:00 – 13:00 h including stress induction and the various assessments.

**Physical and psychological stress induction**

A two-compartment box (30 cm × 25 cm × 30 cm) with two equally sized chambers separated by a porous transparent Plexiglas plate, as described by Ramsey and Van Ree (1993) and Van der Berg et al. (1998), was used for induction of repeated physical and psychological stress on five successive days of the study (Ramsey & Van Ree, 1993; Van den Berg, et al., 1998). The porous transparent partition allows the rats of the psychological stress group to have visual, auditory, and olfactory sensation from the peer rat of the physical stress group in the adjacent compartment. One of the chambers was insulated by a sheet of cardboard to provide the non-foot shock compartment for psychological stress. The other chamber was connected to an electric stimulator (Tajhiz Gostar Omide Iranian Co., Tehran, Iran) to apply foot shocks (0.25 mA, 50 Hz, 1 s-duration, 10 shocks in a 10 min-period with irregular intervals). Physical stress was induced by 10 foot-shocks with irregular intervals in a 10 min-period, every day for 5 d. Psychological stress was induced at the same time in the neighboring rat by exposure to emotional signals such as smelling, hearing shrieks, and observing the jumping responses of the physically stressed rat. Stress induction was performed between 08:00 and 10:30 h on 5 successive days. A 3-min adaptation period was determined before starting each stress session. The control rats were placed pairwise in the two-communication box without any foot shock for the same period of 10 min. After each session (each par trial), the box was entirely cleaned with 70% ethanol and the cardboard sheet in the insulated chamber was replaced. The stress induction was confirmed after the last session of stress exposure by serum corticosterone measurement.

**Serum corticosterone measurement**

In order to verify stress induction, serum corticosterone was measured by blood sampling after the last session of stress exposure (between 19:00 and 21:00 h, at proestrus). Rats were immobilized by placing on a flat bottom restrainer (clear acrylic plastic; 6 cm × 15 cm; with an adjustable tail piece and front breathing holes) without anesthesia. A 3-min adaptation period was applied before blood sampling. After taking 0.1 ml blood sample each with an insulin syringe (1.0 ml, needle: 0.33 mm × 12.7 mm) via a lateral tail vein of the rat, serum was separated by centrifugation (1500 g, 10 min at 4°C) and stored at –80°C. The serum corticosterone measurement was carried out in duplicate aliquots with a commercial ELISA kit (DRG international, Springfield Township, NJ; Cat. No. EIA-5186) according to the manufacturer’s instructions. The detection limit of the assay was 4.1 ng/ml. The intra-assay coefficient of variation (CV) ranged from 2.8 to 8.3% with a mean of 5.3% and inter-assay CV ranged from 4.8 to 12.4% with a mean of 8.2%.

**Behavioral tests**

To evaluate how adolescent psychological and physical stresses lead to adult cognitive and emotional behavior changes, five different behavioral tests were performed from PND 80 to 88. Each behavioral task was performed simultaneously for all three groups by using three adjacent sets of apparatus. The behavior of each rat was recorded using a video camera for future behavioral scoring and scored

![Figure 1](image.png) Schematic diagram of experimental design. After classification of rats into three groups (each n = 7) and two weeks habituation (PND 21–35), physical and psychological stress induction was done on five consecutive days (postnatal day, PND, 35–39). After verification of stress induction by serum corticosterone measurement on PND 39, the rats were housed for 6 weeks. In adulthood, behavioral performance, serum PAB, and heart rate were evaluated (PND 80–90). H: habituation; SI: stress induction (physical or psychological); C: corticosterone measurement; EPM: elevated plus maze; FST: forced swimming test; YM: Y-maze; PAB: passive avoidance test; ORT: object recognition test; R: rest; PAB: pro-oxidant-antioxidant balance assessment; HR: heart rate measurement.
manually with stopwatches by an observer blind to the study. Notably, after each trial, the apparatus was completely cleaned with 70% ethanol; in the case of the FST, the water in the swim tank was changed.

**Elevated plus maze (EPM)**

To evaluate adult anxiety-like behaviors, the rats were tested using the EPM on PND 80. The EPM task was performed according to the method previously described by Di Liberto et al. (2016) and Joshi et al. (2017) with minor modification. In brief, the EPM consisted of two open arm (50 × 10 cm each), two closed arm (50 × 10 × 40 cm each) and a central platform (10 × 10 cm), arranged in a way such that the two arms of each type were opposite to one another. The wooden maze was elevated to 75 cm above the floor. Exploration of the open arm was encouraged by testing under indirect dim light (100 lx). Each rat was placed on the central platform facing one of the open arms. During a 5-min test period, the following parameters were recorded: (a) number of open arm entries, (b) number of closed arm entries, (c) time spent in the open arm, and (d) time spent in the closed arm. Entry by a rat into an arm was defined as the condition in which the rat placed all four paws in that arm (Di Liberto, et al., 2016; Joshi, et al., 2017).

**Forced swimming test (FST)**

To assess adult depressive-like behaviors, the rats were tested using the FST on PND 82–83. The FST task was performed according to the method previously described by Di Liberto et al. (2016) and Cryan and Lucki (2000) with minor modification. Briefly, the FST was performed by placing a rat in a transparent plastic cylinder (30 cm diameter ×50 cm height) filled with water (24 ± 1°C) to a depth of 35 cm. At this depth, the rats could not touch the bottom of the cylinder with their tails. The rats were exposed to a pre-swimming session for 15 min, one day before the experiment. Twenty-four hours after their first exposure, the rats were then subjected to a 5-min FST. Duration (seconds) of struggling, swimming, and the number of dives was evaluated as active behaviors; duration of immobility was regarded as a passive behavior. The rats were judged to be immobile when they remained in the water without struggling or making only those movements necessary to keep the head above the water. Struggling behavior was determined as intense upward directed movements of the forepaws along the side of the swim chamber; while swimming behavior was considered as movements throughout the swim chamber including crossing into another quadrant. Immobility behavior was calculated as the length of time in which the rats did not show any escape responses. Finally, the rat was dried in a towel and placed back in its home cage (Cryan & Lucki, 2000; Di Liberto, et al., 2016).

**Y-maze**

To evaluate adult spatial memory, the rats were tested by the Y-maze, which exploits the rat’s natural tendency to explore novel environments (PND 85). Recognition of the novel arm from the other two familiar arms is considered as a spatial recognition memory performance. Rats which discriminate the unfamiliar arm show exploratory behavior. In addition, the total number of arm entries was used as an indicator of locomotor activity to rule out interference of variations in motility with learning and memory processes. The Y-maze task was accomplished based on the method described by De Luca et al. (2016) with slight modification. In short, the Y-maze was a wooden three-arm maze with equal angles between all the arms (50 cm long × 17 cm wide ×32 cm high) and differently shaped paper cues were put on the walls of the arms. The rats were habituated to the maze twice on consecutive days for 5 min. On the test day (day 3), the rats were allowed to explore the maze for 10 min, having access to two of the three arms. Afterward, they were returned to their home cages for a 4 h inter-trial interval (ITI) and the maze was cleaned. The rats were then placed back into the maze for 5 min, this time having access to all three arms (De Luca et al., 2016).

**Object recognition task (ORT)**

In order to examine adult object-recognition memory, the rats were tested by the ORT on PND 88. The ORT task was performed based on the method described by Liu et al. (2015) and D’avila et al. (2017) with slight modification. Briefly, the rats were introduced into two sessions of 5-min habituation to an empty open field arena (an opaque Plexiglas box, 65 cm × 65 cm × 65 cm) on the days preceding the test. On the testing day, a 5-min acquisition trial in the same arena with two identical (red plastic ball, 1.5 cm diam) objects was given to the rats. The objects were placed in the center of the box with equal distances from all sides. Following a 1 h ITI, the rats were returned to the arena with one familiar object and one novel (glass block, 2 ×1×1 cm) object for a 5-min retention test. Results from the retention phase were expressed as a Discrimination Index (DI) calculated as time spent interacting with the novel object divided by the overall exploration time of both familiar and novel objects in second. (D’avila et al., 2017; Liu et al., 2015).

**Passive avoidance test (PAT)**

To examine adult fear memory, the rats were tested using the PAT on PND 86–87. The PAT task was carried out according to the method described by Sarkaki et al. (2014) and Bargi et al. (2017) with minor modification. In brief, the passive avoidance memory test was evaluated by a shuttle-box apparatus. The shuttle box apparatus (27 × 14.5 × 14 cm) was divided into one illuminated and one dark compartment separated by a sliding door. The floors of the chambers were covered with stainless steel bars (2 mm in diameter) with 1 cm distance between each. First, each rat was placed in the illuminated chamber for 15 s. and after that, the sliding door was opened for 10 min without the electric shock to habituate to the apparatus. On the second trial, the rats were placed individually in the illuminated compartment for 15 s.
and then the door was raised. The delay for each rat to enter the dark chamber was recorded as initial latency (IL). Subsequently, the sliding door was closed and a single electric shock (50 Hz, 0.2 mA, 3 s) was delivered through the grid floor by an isolated stimulator. Following 2 min (with the aim of consolidation), the rats were removed from the dark compartment. In order to test the fear memory, 24 h later, each rat once more was placed in the illuminated chamber and after 15 s, the sliding guillotine door was opened, and the delay to enter the dark compartment was recorded again as memory latency (step-through latency). The maximum time considered in the PAT was 300 s and if a rat avoided entering the dark chamber, a latency of 300 s was recorded (Bargi et al., 2017; Sarkaki et al., 2014).

Serum PAB

The long-lasting effect of adolescent chronic psychological and physical stresses on ROS production was determined by adult serum PAB measurement. For serum PAB assessment, blood sampling was performed on PND 89 (between 11:00 and 12:00 h). Rats were immobilized by placing on the flat bottom restrainer (as above) without anesthesia. A 3-min adaptation period was applied before blood sampling. A 0.1 ml blood sample was taken as above and serum separated. Serum samples were analyzed for PAB based on the method described by Alamdari. In brief, in PAB assessment, oxidant and antioxidant activity are measured simultaneously by two different reactions; one chromogenic enzymatic reaction by peroxidase oxidation (3,3′,5,5′-Tetramethylbenzidine (TMB) is oxidized to a colored cation), and one chemical reaction by antioxidants (cationic TMB is reduced to a colorless compound) which provides a redox index (Alamdari et al., 2007). PAB measurement was carried out in duplicate aliquots manually by a commercial ELISA kit (Pars Azmoon Co., Tehran, Iran) based on the manufacturer’s instructions. The detection limit of the assay was 48.3 pg/ml. The intra-assay %CV ranged from 5.8 to 9.6%, with a mean of 7.5%. The inter-assay %CV ranged from 5.7 to 12.8%, with a mean of 8.9%.

Heart rate

The long-lasting effect of adolescent chronic psychological or physical stress on SNS activity was determined by adult electrocardiogram (ECG) recording on PND 90. Rats were anesthetized with a mixture of ketamine and xylazine (ketamine: 80 mg/kg; xylazine: 8 mg/kg; dosage: 0.88 ml/100 g; IP) and then were placed in the supine position on the test desk. After connecting three electrodes of a Powerlab data acquisition system (Powerlab, AD Instruments, New South Wales, Australia), the ECG of each rat (5 min after anesthesia) was recorded for 6 min using a standard limb lead II electrode configuration for mean heart rate measurement.

Statistical analysis

All data were analyzed by GraphPad Instat statistical package (GraphPad Software, Inc., La Jolla, CA) using one-way ANOVA and mixed ANOVA followed by Tukey’s post-hoc test. p Values <.05 were taken as statistically significant differences. DF is the degree of freedom for each calculation. Data from all the experiment results are expressed as mean ± SEM.

Results

Serum corticosterone concentration was significantly increased by more than two-fold for both acutely physically (2270 ± 287.8 ng/ml) and psychologically (2212 ± 131.4 ng/ml) stressed groups compared to their control counterparts (1105 ± 100.1 ng/ml) which verified stress induction (F(2,18) =11.753, p =.0005) (N = 7 per group).

Behavioral tests

EPM

According to one-way ANOVA, adolescent psychological stress had significant effects on anxiety-like behaviors at an older age which were: (a) increased proportion of time spent in the open arm to the total duration occupied in either closed or open arm (30.7 ± 4.98, psychological stress; 11.8 ± 2.31, physical stress; 13.6 ± 1.21, control) (F(2,18) =10.287, p =.0010; Figure 2(A)); (b) a rise in the number of entries into an open arm (F(2,18) =5.167, p =.0169; Figure 2(B)); (c) increase in total locomotion events, which consists of the number of open and closed arm entries (F(2,18) =14.895, p =.0002; Figure 2(C)) (N = 7 per group).

FST

All the rats in each group were immobile (without swimming or struggling) for a certain amount of total time spent in the FST task. The time was approximately 21 and 24% for the control and physically stressed group, respectively (Figure 2(D)). The immobilization time for the psychologically stressed group was only 2.5%, which was statistically significant compared to the physically stressed group (F(2,15) =3.93, p =.042; Figure 2(D)). Moreover, two-factor mixed ANOVA revealed significant intragroup differences between swimming, struggling, and immobility behaviors in psychologically stressed rats, which spent significantly more time swimming (about 53.5%) and struggling (44%) in comparison to the control rats, which exhibited no diving. The psychologically stressed group displayed statistically significant more diving events compared to the control group (F(2,15) =5.97, p =.0124; Figure 2(E)) (N = 6 per group).

Y-maze

The duration and total number of arm visits are presented in Figure 3 (A,B)). Statistical analysis of the data demonstrated that the psychologically stressed group exhibited a significant decrease in duration of the novel arm visits in comparison to control and physically stressed groups. Hence, percentage time for visits on the novel arm relative to the total time was
Figure 2. Anxiety-related and depressive-like behaviors assessment using EPM (A–C) and FST (D,E), respectively. (A) The percentage time rats spent in an open arm: ***p < .01 psychological stress vs. control and physical stress. (B) The number of open arm entries: *p < .05 psychological stress vs. control. (C) The total number of entries into the open and closed arm: ***p < .01 psychological stress vs. control and ***p < .001 psychological stress vs. physical stress (N = 7 per group). (D) Duration of struggling, swimming, and immobility: * p < .05 immobility of psychological stress vs. physical stress. ***p < .01 within-group differences in psychological stress for immobility vs. struggling and ***p < .001 within-group differences in psychological stress for immobility vs. swimming. (E) Number of diving events: *p < .05 psychological stress vs. control. Data are expressed as mean ± SEM. Data were analyzed by two-factor mixed ANOVA followed by Tukey’s post-hoc test (N = 6 per group). EPM: elevated plus maze; FST: forced swimming test.

Figure 3. Spatial memory, object-recognition memory and fear memory performance evaluation using Y-maze (A,B), ORT (C) and PAT (D), respectively. (A) Duration of novel arm visits: *p < .05 novel arm time for psychological stress vs. control and physical stress. (B) The number of novel arm visits: no statistically significant differences between groups were found (N = 6 per group). (C) Discrimination index: ***p < .001 psychological stress vs. control. *p < .05 psychological stress vs. physical stress (N = 6 per group). (D) PAT: ***p < .001 latency times for acquisition days, physical stress vs. control. *p < .05 latency times for retention days, physical stress vs. control. &p < .05 within-group differences in control group for latency times of retention day vs. acquisition day, and &&&p < .001 within-group differences in psychological stress group for latency times of retention day vs. acquisition day. Data are expressed as mean ± SEM. Data were analyzed by two-factor mixed ANOVA followed by Tukey’s post-hoc test (N = 6 per group). YM: Y-maze; ORT: object recognition test; PAT: passive avoidance test.
about 20% in the control group, which was reduced to 7% in the psychologically stressed group (F(2,15) = 4.89, p = .0231; Figure 3(A)) (N = 6 per group).

**ORT**
The proportion of time spent on exploring the novel object, represented by the DI, was decreased in the psychologically stressed group. One-way ANOVA revealed a significant reduction in DI of psychologically stressed rats in comparison with control and physically stressed rats (F(2,15) = 10.78, p = .0008; Figure 3(C)) (N = 6 per group).

**PAT**
According to statistical analysis, the latency time for acquisition day in the physically stressed group was significantly longer than that of the control and psychologically stressed groups (F(2,15) = 264.3, p < .0001; Figure 3(D)). It was approximately equivalent to the retention day of the psychologically stressed group. Furthermore, none of the physically stressed rats entered the dark chamber on the retention day within the 300-s cutoff time. This exemplifies a statistically significant difference on the retention day of between physically stressed rats and that of control rats (F(2,15) = 4.162, p = .0365; Figure 3(D)) (N = 6 per group).

**Serum PAB**
One-way ANOVA indicated a significant increase in PAB values of both experimental groups in comparison with control (F(2,15) = 21.33, p < .0001; Figure 4(A)). Meanwhile, there was a notable difference between PAB values of psychologically and physically stressed rats (N = 6 per group).

**Heart rate**
One-way ANOVA revealed that heart rate in the psychologically stressed group was significant increased compared with the control group (F(2,15) = 6.62, p = .0087; Figure 4(B)) (N = 6 per group).

**Discussion**

Previous investigations reported that several adult psychophysiological disorders could be associated with adolescent chronic stress exposure. The purpose of our study was to evaluate long-term effects of physical and psychological stresses in adolescent female rats by measurement when adult of various indicators such as behavioral and physiological alterations. Upon the last stress exposure, both physical and psychologically stressed groups exhibited a strong increase in serum corticosterone concentration compared to their control counterparts that verified proper stress induction (Chrousos & Gold, 1992; Jafari, Salehi, Zardooz, & Rostamkhani, 2014). Although the corticosterone concentration could also reflect the imposed stress by the 3-min of the restraining period, our results indicated a significant difference between control and experimental groups regardless of the inevitable artifact of the restraint procedure for blood sampling. According to previous studies, corticosterone concentration depends on several factors such as sex, phase of the estrous cycle, blood collection methodology, level of stress exposure and, most importantly, diurnal rhythm (Atkinson & Waddell, 1997; Kalil et al., 2013). Hence, late afternoon sampling during the proestrus phase of female rats resulted in higher possible variations.

Upon exposure of repeated stress during adolescence, a broad spectrum of behavioral changes was observed in adulthood. Our findings indicated that adolescent psychological stress had significantly reduced either anxiety-like or depressive-like behavior. Thus, emotional development appeared to have been altered to a greater extent by psychological compared to physical stress. Regarding cognitive development, comparative assessment of exploratory behavior in the novel environment utilizing the Y-maze, indicated that adolescent psychological stress resulted in reduced spatial memory at an older age. With regard to results from novel-object exploration using the ORT, significant DI reduction in the psychologically stressed group revealed a notable decline in object-recognition memory compared to the
control and physically stressed groups. Based on the PAT results, both experimental groups displayed intensified fear memory in comparison to the control group. The results for the acquisition day demonstrate that psychologically stressed rats are fearless, as they showed risk-taking behaviors before exposure to danger; however, physically stressed rats consistently showed danger anticipation from the start of the task. In summary, our results acquired by five different behavioral tests indicated that adolescent psychologically stressed rats displayed more behavioral changes in adulthood compared to their physically stressed counterparts. These findings are in accordance with studies reporting variations in locomotor activity (Pijlman, Wolterink, & Van Ree, 2003), risk-taking decisions (Starcke, Brand, & Kluge, 2016), and memory performance (Klein Selle et al., 2017; Traslaviña et al., 2014) upon stress exposure depending on the type of stress. Moreover, our study was also consistent with previously reported persistent stress-induced behavioral changes (Nikiforuk & Popik, 2011) and also diverse cognitive performance due to activation of different neuronal pathways (Kavushansky et al., 2009).

In respect of stress-induced physiological variations, several studies concluded an increase in ROS level (Jafari et al., 2014) and heart rate (Van Den Buuse, Acker, Fluttert, & Kloet, 2001; Vrijkotte, Van Doornen, & De Geus, 2000); however, their long-term persistence from adolescence to adulthood was not extensively investigated. It appears that oxidative stress and SNS activity are both involved in the persistence of stress-induced health problems due to substantial increases in adult serum PAB and heart rate of adolescent psychologically and physically stressed rats in our investigation. Moreover, comparative evaluation of serum PAB and heart rate in the two experimental groups revealed a higher concentration of oxidizing agents and elevated heart rate in the psychologically stressed group in comparison to its physically stressed group counterpart. This result further supports our behavioral analysis. Oxidative stress, by producing a high amount of ROS, is considered to play a critical role in the pathogenesis of several diseases and disorders through oxi-do-neuroinflammatory pathways. ROS serve as crucial secondary messengers in signal transduction which could significantly affect inflammatory pathways (Halliwell & Gutteridge, 1999; Waris & Ahsan, 2006), and may result in an impaired immune system that could negatively alter neuronal functions in the hippocampus (Xiong, Zhang, & Liu, 2017). Furthermore, sustained SNS activity by elevating heart rate ultimately increases pro-inflammatory cytokine levels (Slavich & Irwin, 2014). Thus, it seems that evaluation of ROS concentration and heart rate following psychologically stressed adolescence may be beneficial for monitoring of long-term stress-induced physiological disorders.

It is notable that in the physically stressed rats, anticipation of the possibility of subsequent electrical shock may also result in emotional distress. Hence, physical stress which is actually a mixture of both stresses surprisingly resulted in a lower amount of physiological variation in comparison to psychological stress. Consequently, it appears that different emotional and coping responses between physically and psychologically stressed rats lead to differential stress-related physiological changes. This is in accordance with an investigation that reported diverse emotional coping reactions are associated with different physiological responses (Höhne et al., 2014).

It should be mentioned that our experimental design has some limitations that require consideration. First, exposure of control rats to repeated testing could possibly be more stressful for these rats as they were never exposed to any form of stress prior to testing. Second, considering carryover effects of multiple testing, the results do not necessarily represent what would be observed in a naive animal. In order to elude cumulative carryover effects of multiple testing, each experiment must be limited to a specific group in order to avoid successive testing. However, to limit use of experimental rats, it was decided to evaluate tests on each group in sequential order. In order to minimize artifact, the PAT, which involves electrical foot shock and has the largest carryover, was executed last. The first two tests, EPM and FST would also have induced stress in the rats. Thus, performing these tasks may have impacted on performance in the subsequent memory tasks even in the control group.

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