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Social stress during adolescence in Wistar rats induces social anxiety in adulthood without affecting brain monoaminergic content and activity

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Abstract

Adolescence has been described as an important period to acquire social competences required for adult life. It has been suggested that early stress experiences could affect the development of the brain at different levels. These changes in the brain during adolescence may be related with the development of psychopathologies such as depression and social anxiety in adulthood. In the first experiment, we examined long-term effects of repeated social stress during adolescence on adult social approach–avoidance behavior. For that purpose, adolescent male Wistar rats were exposed twice at postnatal day (Pnd) 45 and Pnd48 to the resident–intruder paradigm followed by three times psychosocial threat with the same resident. Three weeks after the last psychosocial threat experience the animals were behaviorally tested in a social approach–avoidance test. Socially stressed animals spent less time in the interaction zone with an unfamiliar male adult rat. These data suggest that animals exposed to social stress during adolescence show a higher level of social anxiety in adulthood. In the second experiment, we investigated whether these long-term effects of social stress during adolescence on behavior draw a parallel with changes in brain monoamine content, biosynthesis and turnover. Using the same experimental design as in the first experiment, HPLC analysis of various brain regions showed that there were no differences in monoamine content, monoamine biosynthesis and monoamines activity in the prefrontal cortex, hippocampus, hypothalamus and striatum in adulthood. These results indicate that long-lasting changes in social behavior following social stress during adolescence are not accompanied by changes in brain monoamine content, biosynthesis and turnover.

Keywords: Social stress; Bullying; Adolescence; Serotonin; Dopamine; Noradrenaline; Social behavior; Social anxiety; HPLC; Rats

1. Introduction

Adolescence has been defined as an important period for developing social competences required for adult life and it has been characterized by changes at distinct neurobiological levels [1]. Particular similarities have been found in terms of behavior in that period of life in different species such as rodents and humans with respect to increasing peer directed social interactions, novelty seeking and increase of risk-taking behavior [2–4]. The adolescence period in male rats extends from postnatal day 21 until postnatal day 60. Some human studies have reported that adverse social experiences such as bullying in childhood and adolescence may affect social behavior functioning but may also increase the vulnerability to develop mental diseases such as depression, social phobia and anxiety in adulthood [5–8]. During this period monoaminergic systems in the brain are still developing as shown by changes in the densities of monoaminergic transporters, monoamine levels, dopamine receptors, dopamine and serotonin transporters in several brain areas [9–13]. It is unknown whether social experiences during adolescence interfere with the development of the monoaminergic brain systems which might contribute to the vulnerability to develop certain mental diseases. Many studies have tried to establish a connection between alterations in monoaminergic systems and vulnerability to develop major depression [14]. According to the monoamine-deficiency hypothesis, depressive symptoms are associated with reductions in monoamine neurotransmission, particularly serotonin and noradrenaline. This hypothesis is supported by the successful treatment of depressive

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disorders with compounds enhancing monoaminergic transmission [15].

Additionally, stress has been demonstrated to be an important modulator of monoaminergic systems [16]. Numerous studies in rodents have shown that different stress paradigms such as predictable and unpredictable shock, chronic variate stress, chronic intermittent stressor regimen, novelty stress, and forced swimming stress produce changes in the monoamine levels in different brain structures [17–22]. The effect of stress on monoaminergic systems depends on factors such as intensity, duration and predictability [23]. Few studies have examined the long-term consequences of adolescent social stress on monoaminergic content and activity. Stressful life events have been described as one of the main factors affecting the vulnerability to suffer from depression [24,25]. In addition, distinct behavioral and neurobiological differences have been found between adolescent and adult animals in response to stress [26,27]. The social defeat paradigm has been considered as an animal model to study bullying, victimization and social subjugation [28]. Numerous studies in rodents have demonstrated that acute and chronic social defeat experience produce short- and long-term changes in physiological, neuroendocrine, neurobiological and behavioral parameters [29–32]. The effects of social stress on brain, behavior and physiology vary in the temporal dynamics depending on parameters studied [34]. Some studies have shown that antidepressant treatment may reverse long-term behavioral and endocrine effects of social stress. The importance of the monoaminergic system in response to social stressors but also in the performance in different social stress paradigms has been widely demonstrated. For instance, in adult rats acute social defeat induces an increase of 60% in the release of 5-HT in the hippocampus [34] and also alters dopamine release in nucleus accumbens and prefrontal cortex [35]. Furthermore, single social defeat produces changes in the density of hippocampal serotonin transporters [36]. In addition, studies have shown that the number of serotonin-immunoreactive varicosities in the anterior hypothalamus and in the lateral septum in subjugated hamsters during puberty increases with 20% [37]. The main objective in our first experiment was to investigate the long-term effects of repeated social stress during adolescence in Wistar rats on social anxiety. Social interaction-based tests such as social approach–avoidance tests and social interaction tests have been widely used to measure social anxiety. In the second experiment the long-term effects of repeated social stress during adolescence in Wistar rats on monoaminergic contents, synthesis and turnover were examined using HPLC.

2. Materials and methods

2.1. Animals

For the two experiments 32 male Wistar rats (Harlan, NL) were used. The animals arrived at postnatal day 24 (Pnd24) weighing 61.75±0.92. Rats were socially housed in groups of 4 in standard clear Plexiglass cages (42×26×15 cm) until the first social defeat at postnatal day 45 (Pnd45). Animals were subsequently housed individually in similar cages. Food and water were given ad libitum. The rats were maintained under standard conditions with a 12 hour reversed light/dark cycle (lights on at 23:00 h) at constant temperature of 22 °C. All procedures were approved by the Groningen University Committee on Animal Experiments.

2.2. Experimental design

2.2.1. Stress protocol

For the social defeat procedure, the resident–intruder paradigm was used. The residents (Wild-type Groningen rat) were housed in large cages (84×56.5×40 cm) each with a female Wild-type rat. Residents were trained with the objective to elicit reliable levels of aggressive behavior. The attack latency was measured in every training session and only residents with an attack latency under 150 s on the last day of training were selected for the experiments. Females were removed 15 min before the first social defeat. Animals were socially stressed during adolescence 5 times (Pnd45, Pnd48, Pnd51, Pnd54. Pnd57). At Pnd45 and Pnd48, rats were exposed to the residents for 10 min allowing direct physical contact with the resident, after 10 min each experimental animal was separated from the resident by placing the animal in a wire mesh cage (31×15×14 cm) to avoid injuries until 1 h was completed. Due to the high levels of aggression of the residents, experimental animals were placed in the resident cage for 15 min without physical contact protecting them from severe injuries by placing the rats in the wire mesh cage allowing psychosocial threat of being attacked at Pnd51, Pnd54, and Pnd57. The control animals were put in a clean cage at Pnd45 and Pnd48 for 1 h and for 15 min at Pnd51, Pnd54 and Pnd57.

2.2.2. Experiment 1. Long-lasting effects of repeated social stress during adolescence on social approach–avoidance behavior

In experiment 1, 18 rats were assigned randomly to one of the two conditions: stress ($n=8$) or control ($n=10$). Animals were behaviorally tested at Pnd78, which was 3 weeks after the last social stress experience. A modification of the social approach–avoidance test as described previously was used [38]. The social approach–avoidance test was performed in a black wooden cage (84×57×40 cm) which was placed in an unfamiliar room. In the wooden cage a wire mesh cage was located at the side wall of the cage (Fig. 1). The test was performed in the dark period under 1.5 lux light intensity as measured in the center of the cage. Each experimental rat was transferred to the test room and introduced into the cage and was allowed to freely explore the cage for 2 sessions of 2 min and 50 s. Between the sessions, experimental animals were replaced in their home cages for 1 min. In the first session, the animals were placed in the test cage with no animal in the wire mesh cage ("no target"). In the second session, the animals were again placed in the test cage, but now the wire mesh cage contained an unfamiliar male conspecific ("target ": unfamiliar Wild-type Groningen rat). Ethovision (Noldus, NL) was used to register different behavioral parameters: time spent in the interaction zone (s), number of entries in the interaction zone, latency to enter in the interaction zone (s), velocity (cm/s) and total distance traveled (cm).

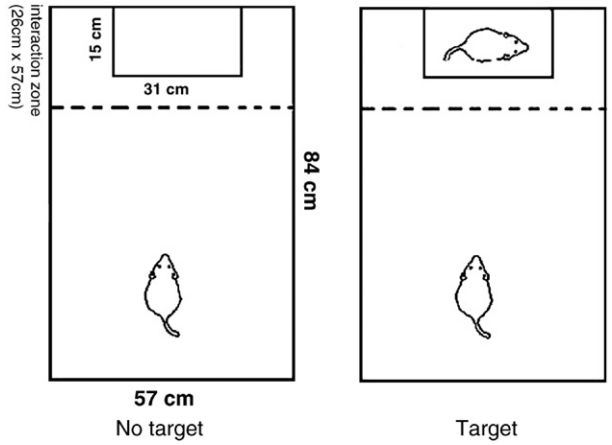

Fig. 1. Schematic representation of social approach–avoidance test.

2.2.3. Experiment 2. Long-lasting effects of repeated social stress during adolescence on central nervous monoaminergic content and activity

In experiment 2, 14 rats were assigned randomly to one of the two conditions: stress ($n=6$), control ($n=8$). In order to study long-term consequences of repeated social stress during adolescence on monoamines biosynthesis, turnover and content, experimental animals from both the stress and control groups were decapitated 3 weeks after the last psychosocial stress experience at postnatal day 78. Animals were briefly sedated (<30 s) with CO_2 by placing them in a box filled with dry ice and subsequently decapitated [41]. From each rat, the brain was rapidly extracted and dissected on ice into four areas: prefrontal cortex, hippocampus, striatum and hypothalamus. The brain structures were frozen in liquid nitrogen immediately after the dissection and stored at −80 °C. For determination of monoamine contents and monoamine metabolites such as: 5-hydroxytryptamine (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and noradrenalin (NA) in these brain areas, High Performance Liquid Chromatography (HPLC) was used. For that purpose brain samples were homogenized in 0.5 ml 1 M perchloric acid and centrifuged at 14,000 rpm for 10 min at 4 °C. Supernatant was removed and assayed for 5-HT, 5-HIAA, DA, DOPAC and NA by injecting 100 µl onto a reversed phase Gemini C18 column (150×4.6 mm, 5 µm particle size), connected to a detector (ESA coulochem model 5100A). A difference in potential of 340 mV was set (cell 5011). The mobile phase consisted of 62.7 nM Na_2HPO_4, 40.0 nM citric acid, 0.27 mM EDTA, 4.94 mM HSA, 10% methanol at pH 4.1 with a flow of 0.5 ml/min. Known amounts of 5-HT, 5-HIAA, DA, DOPAC, HVA, (Sigma Chemicals), and NA (Research Biochemicals International) were run throughout the whole procedure for standardization. Monoamine concentrations in the samples were calculated and expressed as ng/g wet tissue. The monoaminergic activity (or turnover) of the dopaminergic system and the serotoninergic system were calculated as DOPAC/DA, HVA/DA and 5-HIIAA/5T ratios. The monoaminergic biosynthesis was calculated as DOPAC+DA, HVA+DA, 5-HTIAA+5-HT.

2.3. Statistics

Statistical analyses were performed using Statistica 7.0. Behavioral parameters from the social approach–avoidance test such as time in the interaction zone, frequency of entries in the interaction zone, latency to enter in the interaction zone, velocity, total distance traveled were analyzed using repeated measures ANOVA with two between-subjects factors; social stress (social stress vs control) and a within-subjects factor; target (target vs no target). Fisher LSD-post hoc comparisons were calculated when required. Monoamines levels (NA, DA, DOPAC, 5-HT) and their metabolites levels (5-HIAA, DOPAC, HVA), biosynthesis parameters (DOPAC+DA, HVA+DA, 5-HIAA+5-HT) and

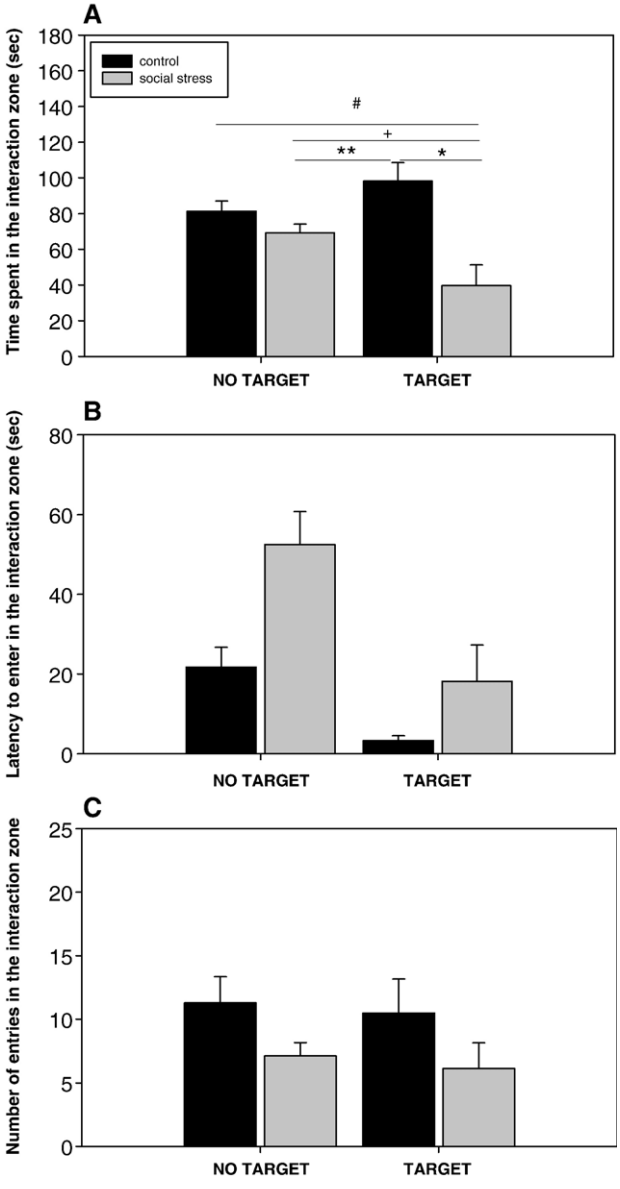

Fig. 2. Social approach–avoidance behavior of adult male rats that were socially stressed during adolescence in comparison with controls. A: Time spent in the interaction zone. B: Latency to enter in the interaction zone, C: number of entries in the interaction zone. *: Stress target vs control target ($p<0.01$), #: stress target vs control no target ($p<0.05$), **: stress no target vs control target ($p<0.05$), +: stress target vs stress no target ($p<0.05$). Data are expressed as mean±s.e.m.

Table 1
Long-term effects of repeated social stress during adolescence on monoamines (NA, DA, 5-HT) and monoamine metabolites (DOPAC, 5-HIAA, HVA) in brain regions (prefrontal cortex, hippocampus, striatum, hypothalamus)

	NA	DOPAC	DA	5-HIAA	HVA	5-HT
PFC						
Control	276.33±16.64	12.66±0.65	47.61±1.30	188.53±9.74	16.66±1.59	433.61±28.49
Social stress	291.74±14.78	13.29±0.74	51.50±2.65	194.17±9.73	16.31±1.60	452.61±25.73
Hippocampus						
Control	410.61±30.20	6.00±0.34	13.89±1.26	260.15±23.29	5.23±0.34	296.15±29.91
Social stress	450.51±27.29	5.04±0.37	12.52±0.65	268.04±16.98	4.45±0.55	311.76±28.61
Striatum						
Control	56.20±8.92	2408.91±251.19	16699.78± 1528.78	473.12±44.69	1337.54±126.03	491.42±51.42
Social stress	58.75±4.04	2373.34±138.05	18595.51±493.51	525.84±30.99	1402.22±101.91	570.26±19.90
Hypothalamus						
Control	1741.22±91.24	68.43±5.79	356.30±19.04	386.81±5.75	14.11±1.61	774.02±18.17
Social stress	1649.84±78.90	65.53±4.30	362.16±18.26	371.50±16.62	14.58±1.09	795.53±26.56

Concentrations of monoamines and monoamines metabolites are expressed in ng/g per tissue (mean±s.e.m.).

turnover of monoamines (DOPAC/DA ratio, HVA/DA ratio, 5-HIAA/5-HT ratio) in four brain areas (prefrontal cortex, hippocampus, striatum and hypothalamus) were analyzed using multivariate ANOVA with one between factors: social stress (social stress vs control). Significance was set at $p<0.05$ for all analyses and values are expressed as mean±s.e.m.

3. Results

3.1. Experiment 1. Long-term effects of repeated social stress during adolescence on social approach–avoidance behavior

3.1.1. Time spent in the interaction zone (s)

ANOVA showed an interaction between the factors stress and target $F(1,16)=11.469$; $p\leq0.01$. LSD-post hoc analysis demonstrated that animals, socially stressed during adolescence, spent less time in the interaction zone compared to controls in the "target" session in the adulthood (39.65 vs 98.18); $p\leq0.002$, but no difference was observed between stress and controls animals in the situation that there was only an empty cage ("no target") (69.22 vs 81.36); n.s. Additionally, animals which were socially stressed during adolescence decrease the time spent in the interaction zone in the "no target" situation in comparison with the "target" session (69.22 vs 39.65); $p\leq0.05$. Furthermore, stressed animals spent less time in the interaction zone in the "target" situation in comparison with controls in the "no target" situation (39.65 vs 81.36); $p\leq0.05$ (Fig. 2A).

3.1.2. Latency to enter in the interaction zone (s)

ANOVA indicated that the stress factor was significant, animals which were socially stressed showed a higher latency to enter in the interaction zone in comparison with controls regardless whether there was a male rat in the wire mesh cage or not $F(1,16)=11.37$; $p\leq0.01$. ANOVA also showed that the target factor was significant; animals in the "target" situation presented a higher latency to enter the interaction zone in comparison with animals in the "no target" session $F(1,16)=22.89$; $p\leq0.001$. No differences were found in the interaction

Table 2
Long-term effects of repeated social stress during adolescence on monoamines biosynthesis (DOPAC+DA, HVA+DA, 5-HIAA+5-HT) and monoamine turnover (DOPAC/DA, HVA/DA, 5-HIAA/5-HT) in brain regions (prefrontal cortex, hippocampus, striatum, hypothalamus)

	DOPAC/DA	HVA/DA	5-HIAA/5-HT	DOPAC+DA	HVA+DA	5-HIAA+5-HT
PFC						
Control	0.27±0.01	0.35±0.03	0.45±0.04	60.28±1.82	64.28±2.56	622.14±33.31
Social stress	0.26±0.01	0.32±0.03	0.43±0.02	64.79±3.06	67.81±3.39	646.77±30.97
Hippocampus						
Control	0.44±0.02	0.39±0.03	0.88±0.03	19.90±1.52	19.13±1.44	556.31±52.27
Social stress	0.40±0.01	0.34±0.03	0.87±0.05	17.56±1.00	16.97±1.18	579.80±43.41
Striatum						
Control	0.14±0.005	0.08±0.001	0.97±0.03	19108.69±1762.80	18037.33±1650.63	964.54±94.12
Social stress	0.12±0.004	0.07±0.004	0.92±0.05	20968.86±619.63	19997.73±569.98	1069.10±39.99
Hypothalamus						
Control	0.19±0.01	0.03±0.003	0.50±0.007	424.73±23.50	370.41±19.93	1160.84±22.90
Social stress	0.18±0.001	0.04±0.003	0.47±0.02	427.70±21.10	376.75±18.42	1167.03±32.05

Values are presented as mean±s.e.m.

between stress and target in the latency to enter the interaction zone (Fig. 2B).

3.1.3. Number of entries in the interaction zone

No differences were found in any factor (target) or interaction (stress × target) in the number of entries in the interaction zone (Fig. 2C).

3.1.4. Distance moved (cm)

No differences were found in any factor (stress, target) or interaction in the distance moved (cm) (stress × target) (data not shown).

3.1.5. Velocity (cm/s)

No differences were found in any factor (stress, target, time) or interaction in the velocity (stress × target) (data not shown).

3.2. Experiment 2. Long-term effects of repeated social stress during adolescence on monoaminergic activity and metabolism

No differences were found between stressed animals and controls on monoamines (5-HT, DA, NA) (Table 1), monoamine metabolites (5-HIAA, HVA, DOPAC) (Table 1), monoamine biosynthesis (5-HIAA + 5-HT, DOPAC + DA, HVA + DA) (Table 2) and monoamine turnover (5-HIAA/5-HT, DOPAC/DA, HVA/DA ratio) (Table 2) in the prefrontal cortex, hippocampus, hypothalamus and striatum.

4. Discussion

In the present study, it was shown that animals exposed to social stress during adolescence exhibited higher levels of social anxiety in adulthood as measured by a striking decreasing time spent in the interaction zone when an unfamiliar Wild-type Groningen rat was presented as a target. Interestingly, no differences were found between socially stressed animals during adolescence and controls on monoamines contents (5-HT, DA, NA), monoamine turnover (5-HTIAA/5-HT ratio, DOPAC/DA ratio, HVA/DA ratio), monoaminergic biosynthesis (5-HTIAA + 5-HT, DOPAC + DA, HVA + DA) in different areas in the prefrontal cortex, hippocampus, hypothalamus and striatum, exactly 3 weeks after the last social stress experience.

Social interaction-based tests such as social approach–avoidance test have been described as good indicators to measure social anxiety after stress in rodents [40–43]. Several of these studies with rodents have reported the short- and long-term effects of social stress during adulthood and the influence on later social behavior functioning. Meerlo et al. [44] demonstrated that a single social defeat in adulthood decreases the time spent in social interaction with a non-aggressive Wistar rats 2 days after the last social stress experience. Not only acute but also repeated social defeat experiences during adulthood decrease the time in the interaction zone when the animals were placed in an open field with dominant rodents. This effect lasted up to 4 weeks after the last defeat, demonstrating a long-lasting social anxiety [45,46]. One study found contradictory data on the effects of repeated social stress on social behavior [47]. Our data demonstrate that social stress experiences during adolescence also produce effects on adult social behavior. It confirms the idea that social stress has important consequences on later social behavior. Adolescence has been characterized as being an important period in the development of social skills required for the adult life [48]. The resident–intruder paradigm may be a valid animal model to study neurobiological and behavioral consequences of bullying [49]. Several studies in humans have shown that being bullied during childhood and adolescence not only produces changes in social functioning but also correlates with the development of adult psychopathologies such as depression, anxiety and social phobia [50]. Our behavioral data indeed support the idea that the rodent resident–intruder paradigm may be a validate model to study long-term consequences of bullying as previously reported.

On the other hand, adolescence has been described as a period between childhood and adulthood characterized by important changes in several systems including monoaminergic system [51–55]. Some theories have suggested that alterations in brain development induced by stress during adolescence may contribute to development of several psychopathologies in adulthood [56]. Alterations in the monoaminergic system have been widely implicated in the development of various psychopathologies. Numerous experiments have shown that stress using different paradigms such as immobilization stress, forced swimming test and chronic restraint stress produces changes in monoaminergic activity [57–62]. Also, social stress induces changes in monoaminergic activity. Studies using the social defeat paradigm found that mice exposed to a single social defeat increased DOPAC concentrations in the hypothalamus and decreased DA levels in the prefrontal cortex [63]. In addition, chronic social stress induced by colony housing in the visible burrow system (VBS) increases the 5-HT metabolite 5-HIAA in several brain areas of subordinate animals but has no effect on 5-HT levels and DA metabolites [64]. The majority of these experiments have focused on the short-term effects of stressors (30 min, 2 h and 24 h and immediately after the stress) but not many studies have considered the long-term consequences of social stress on monoaminergic activity in rodents.

Several hypotheses can be raised that may explain the absence of any effect in the monoaminergic systems between stress and controls animals. First, one may argue that the social stress was rather mild. This explanation seems unlikely in view of the observed behavioral effects and the strong and consistent reduction in body weight and food intake that is generally observed using the same stress protocol (Vidal in prep). Second, using HPLC for detecting long-term changes in the monoaminergic signaling is a crude measure of monoaminergic activity in various brain regions. Therefore, our results cannot be interpreted as a conclusive negative result. After all, total content and turnover measurements based on HPLC analysis are only one aspect of the subtle changes that may occur in monoaminergic neuronal signaling. For example, chronic social stress induced by colony housing in the VBS induced long-term indirect changes in the dopaminergic activity by decreasing dopamine transporter density and increasing the dopamine D2 receptors [65]. In addition, Berton et al. [38] reported a reduction

of the density of hippocampal serotonin transporters 24 h after a single social defeat without affecting hippocampal 5-HT_{1A} and cortical 5-HT_{2A} receptors and serotonin synthesis and metabolism [38]. Finally, it may be that the repeated social stress has induced a temporal change in monoaminergic activity either during or after the stress period that has recovered at the time of adulthood. Subsequently, more detailed and focused experiments are required to determine the possible effects of adolescent stress on adult monoaminergic activity.

In summary, social stress during adolescence induces social anxiety in adulthood in Wistar rats. Adverse social stress experiences in humans such as bullying in children correlate with the vulnerability to develop several psychopathologies including social anxiety and depression. Several studies using other animal models of depression suggest that the behavioral disturbances following stress may be associated with a reduction in 5-HT content and an increase in 5-HIAA in the brain. Although no accompanying changes were observed in long-term changes in the monoaminergic activity in the current experiment, further research in the resident–intruder paradigm in adolescent rats may provide better insight in the neurobiological mechanisms involved in the development of adult psychopathologies following adolescent social stress.

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