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Research report

# Effect of chronic stress during adolescence in prefrontal cortex structure and function

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## HIGHLIGHTS

- Chronic stress impairs the development of the adolescent brain.
- Chronic stress delays the maturation of extracellular structures called perineuronal nets, which stabilize synaptic contacts on inhibitory neurons, and prefrontal cortex-associated behavior.
- These negative effects of chronic stress on the prefrontal cortex can theoretically occur in humans.

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## ABSTRACT

Critical periods of plasticity (CPPs) are defined by developmental intervals wherein neuronal circuits are most susceptible to environmental influences. The CPP of the prefrontal cortex (PFC), which controls executive functions, extends up to early adulthood and, like other cortical areas, reflects the maturation of perineuronal nets (PNNs) surrounding the cell bodies of specialized inhibitory interneurons. The aim of the present work was to evaluate the effect of chronic stress on both structure and function of the adolescent's rat PFC. We subjected P28 rats to stressful situations for 7, 15 and 35 days and evaluated the spatial distribution of histochemically-labeled PNNs in both the Medial Prefrontal Cortex (MPFC) and the Orbitofrontal Cortex (OFC) and PFC-associated behavior as well. Chronic stress affects PFC development, slowing PNN maturation in both the (MPFC) and (OFC) while negatively affecting functions associated with these areas. We speculate upon the risks of prolonged exposure to stressful environments in human adolescents and the possibility of stunted development of executive functions.



## 1. Introduction

Many neuropsychiatric conditions are diagnosed during adolescence [1,2], when the brain goes through an intense period of synaptic remodeling, which is reflected in a loss of grey matter (GM) volume and concurrent increases in white matter (WM) volume [3]. Chronic stress induces long-lasting changes in the brain and could contribute to an increased vulnerability to mental illness, especially during the so-called critical periods of cortical development [4,5]. Unfortunately, many adolescents are exposed to stressful situations around the world, including war [6] and extreme poverty (UNICEF, 2005). Not surprisingly, epidemiological studies estimate that up to 20% of children and adolescents worldwide suffer from a disabilitating mental illness [7].

Stress is the response of an organism to a threat, either explicit or not, which includes physiological or behavioral

*Abbreviations:* MPFC, medial prefrontal cortex; PFC, prefrontal cortex; OFC, orbital prefrontal cortex; PNN, perineuronal net; SAAT, spontaneous arm alternation test; Vv, *Vicia villosa* lectin.

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compensatory adaptations to maintain bodily homeostasis [8,9]. The body response to the stressor event proceeds through two distinct but interrelated phases, 1) a rapid fight or flight response due to sympathetic activation of the adrenal medula and the subsequent release of catecholamines in the bloodstream, and 2) a slow response that depends on the activation of the hypothalamic-pituitary-adrenal axis, resulting on the release of glucocorticoids (cortisol in humans and corticosterone in other animals) by the adrenal cortex in the blood circulation [10–15].

Under normal conditions, the stress response is deactivated when the threat is no longer present. This is implemented by an inhibitory feedback mechanism that is controlled by the levels of stress hormones in the circulation. However, when the level of hormones remains elevated for a long period of time, the central nervous system can be cumulatively damaged by the exposure [10,16]. For instance, chronically elevated levels of cortisol can impair the development of the prefrontal cortex (PFC) [14], which has a large density of glucocorticoid receptors [17], causing dendrite retraction and a decline in the number of dendritic spines; both effects leading to an overall decrease in synaptic contacts [18–20].

In humans, the PFC is situated in the anterior portion of the frontal lobe and includes Brodmann areas (BA) 8, 9, 10, 11, 13, 14, 44, 45, 46 and 47/12 [21]. In rodents, the PFC is comprised by the MPFC, which includes the medial precentral, anterior cingulate, prelimbic and infralimbic areas, and the OFC, which is subdivided into medial, ventrolateral, and lateral orbital areas [22,23]. Injuries to the MPFC and the OFC cortex are closely associated with working memory disorders [24,25] and poor judgment and maladaptive decisions due to an inability to anticipate actions' consequences [26], respectively.

Those effects are more evident during the critical period of plasticity of the PFC [14]. Critical periods of plasticity are defined, among other things, by the maturation of GABAergic neurons [27], which make up about 25% of all cortical neurons in primates [28,29]. Although this fraction of GABAergic interneurons in the cortex is relatively constant from neurogenesis to adulthood [30], during postnatal development a subgroup of these cells expressing the $Ca^{++}$-binding protein parvalbumin gradually accumulate extracellular matrix components around the cell body, such as chondroitin sulfate proteoglycans, forming a lattice-like structure called perineuronal net (PNN). PNNs mature slowly and help stabilize synaptic contacts under environmental constraints [31–35]. Their maturation signals the closing of the critical period of plasticity in several cortical regions [31,32,36,37]. PNNs can be easily revealed histologically with plant lectins with high affinity to N-acetylgalactosamine amino sugars, such as *Wisteria floribunda agglutinin* and *Vicia villosa* [35,38–40].

In the present work, we study the effects of chronic stress on the structure and function of the rat's PFC during the critical period of plasticity. More specifically, we address the effects of chronic stress both on the spatial-temporal distribution of $PNN^{+}$ neurons in the PFC and on tests of executive function during adolescence. To test maladaptive executive function associated with deficits in working memory and bad judgment in decision making we used the spontaneous alternation [41–46] and the open field test [47], respectively.

## 2. Materials and methods

### 2.1. Experimental design

All experimental procedures were approved by the Animal Ethics Committee of the Federal University of Pará (BIO069-12). We used 48 male Wistar rats (*Rattus norvegicus*) aged 28 postnatal days (P28). The animals were bred and raised in the University's animal facility and were housed in standard cages with a 12/12 light/dark cycle with free access to food and water. The animals were randomly assigned to either the control (n = 24) or experimental group (n = 24). Each group was subsequently divided into three sub-groups, depending on the length of exposure to either the stressful or the control environment: 7 (n = 8), 15 (n = 8) or 35 (n = 8) days. Behavioral tests were carried out on the last day of exposure to each environment and the animals were immediately sacrificed, after blood was collected to measure corticosterone levels. Researchers tasked with rating behavior were blinded to the groups animals belonged.

### 2.2. Chronic stress paradigm

Animals of the stressed group were housed in standard cages (0.40 × 0.30 × 0.20 m) with 2 or 3 rats per cage and were subjected to chronic stress once a day according to a protocol similar to the one proposed by Duccotet and coworkers [48]. The stressing events were chosen randomly from the following procedures: forced restraint in a plastic tube for 2 h without access to food or water; forced bath in a cylindrical tank (0.60 m height x 0.30 m diameter) for 30 min in water at 32 °C; pairing with another stressed animal in wet sawdust (18 h); inversion of the light/dark cycle; crowded housing (8 rats living in a cage for 24 h); tail pinch for 10 min; hot air (approx. 38.00 °C) blown from a hair dryer for 10 min [48–53].

### 2.3. Behavioral tests

Behavior was evaluated with two tests: spontaneous alternation in the elevated plus maze and thigmotaxis. Animals were tested in a single session on the last day of exposure to either the experimental or control environment. Three days before the tests, the experimenter handled the rats for 5 min/day. Thirty minutes before the test the animals were brought to the experimental room. The test apparatuses were cleaned after each session with alcohol 70%. The experimental procedures were videotaped for later analysis.

Thigmotaxis refers to a specific behavior of animals when exploring an open space: they tend to stay close to walls. Thigmotaxis is associated with fear of open spaces and is a valid measure of anxiety and risk evaluation [47]. Thigmotaxis was evaluated in a wooden apparatus (0.60 × 0.60 × 0.35 m) and the dependent variable was the amount of time the animal spent away from a 0.05 m wide strip close to the wall. The animals were placed in the center of the apparatus and their movement was recorded for 5 min [54,55].

Rats spontaneously alternate among arms when exposed to an elevated maze with closed arms. The spontaneous arm alternation test (SAAT) is used to measure spatial working memory in laboratory animals [41–46]. The SAAT was performed in a wooden maze with four closed arms (0.47 × 0.16 × 0.34 m) and an open central space (0.16 × 0.16 m) located 0.5 m away from the floor. The rats were placed individually at the center of the apparatus and observed for 10 min. The alternation behavior was defined as the number of consecutive entries into all four arms without repeated entries and was expressed as percentage of total arm entries [45].

### 2.4. Corticosterone blood levels

Immediately after the behavioral tests the animals received a mixture of xylazine (Kenzol, Konig, 9 mg/kg) and ketamine chloride (Vetanarcol, Konig, 72 mg/kg) (i.p.) for perfusion and 1 mL of blood was collected by cardiac puncture in order to measure corticosterone levels [56,57]. The biological material was maintained at 37 °C and the blood was centrifuged at 2300 RPM during 7 min. The resultant blood plasma was separated and stored at −30 °C until

analysis of the plasma concentration of corticosterone hormone with an enzymatic test (Oxford Biomedical Research). All samples were run in duplicate. The plate was read at 450 nm with SoftMax® Pro V. 4.0 (Life Sciences Edition). The intra- and inter-assay coefficient variations for the corticosterone measurements were 8.0% and 4.5%, respectively.

### 2.5. Histological analysis

After blood collection, the animals were perfused transcardially with PBS followed by 4% paraformaldehyde in phosphate buffer (PB, pH 7.4, 0.1 M). The brains were removed from the skull, post-fixated overnight, cryoprotected for one day, and frozen-blocked in Tissue-Tek® (Sakura, Japan) to be cut into serial, 20 or 50 μm-thick coronal sections with a cryostat (Leica, Germany). The brain slices were mounted on glass slides and processed histochemically with biotinilated *Vicia villosa* lectin (Vv).

The brain slices were washed three times for 20 min in PB and once in a solution of 3% Triton X-100 in PB before being incubated for 16–24 h at 20 °C in PB containing 0.5% biotinylated *Vv* (Vector, USA) and 6% Triton X-100 (Sigma, USA). Control sections were incubated only in PB without the lectin. After three washes in PBS, all sections were incubated with the avidin-biotin-peroxidase complex (ABC, 1:200, Vector, USA) and peroxidase was revealed using the diaminobenzidine reaction intensified with nickel ammonium sulfate [58]. Finally, sections were dehydrated and cover slipped with Entellan (Merck, Germany). Microphotographs of serial sections were taken with an Axiocam digital camera (HR series, Zeiss, Germany) using the Axiovision software (Zeiss, Germany) and had their brightness and contrast adjusted with the Canvas XII software (ACD Systems of America, Inc.). Alternate Vv+ sections were also drawn and all positive (Vv+) neurons were plotted under direct microscopic examination at 20X magnification. All cells with clear profiles were counted in each cerebral hemisphere at the area corresponding to the prefrontal medial cortex (cingulate anterior cortex and pre-limbic cortex) and prefrontal ventral cortex (orbito-ventral cortex). The number of Vv+ cells in each region of interest was counted using a 40X objective lens and an ocular grid of 0,0625 mm2 coupled to an upright bright field microscope (Nikon Optiphot-2, Japan).

### 2.6. Statistical analysis

Results are expressed as mean ± standard error (SE). Data from experimental groups were compared using two-way analysis of variance (two-way ANOVA, environment x time) followed by Tukey's post hoc test to determine specific differences among experimental groups. Statistical significance was set at 0.05.

## 3. Results

Table 1 summarizes the main effects of environment and the length of exposure to chronic stress on behavior, corticosterone blood levels and PPN maturation.

Analysis of corticosterone blood levels revealed a main effect of environment ($F_{1,19} = 15.56$, $p = 0.0008$). In the control group, the average corticosterone blood levels after 7, 15, and 35 days of exposure were, respectively, 216.00 ± 191.91, 545.25 ± 338.82 and 257.50 ± 219.97. In the stressed group, the average values for the same exposure periods was 85.00 ± 57.47, 43.25 ± 11.87 and 62.00 ± 64.29, respectively. Statistical analysis revealed significant differences ($p < 0.05$) in the control group between 7 and 15 days of exposure. Differences between groups were significant for 15 days of exposure ($p < 0.01$). Thus, chronic stress reduces corticosterone blood levels and decreases hormonal variability during maturation (Fig. 1).

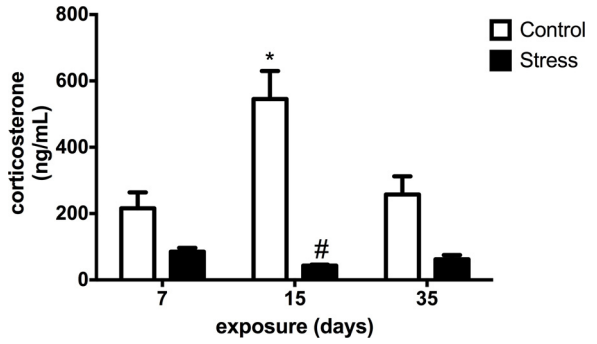


**Fig. 1.** Average blood corticosterone levels of adolescent rats submitted to different periods of stress and matched controls. Symbols indicate statistically significant difference in the average values: * between 7 and 15 days of exposure, ** between 7 and 35 days of exposure, *** between 15 and 35 days of exposure, # between control and stressed rats.

### 3.1. Effects of chronic stress in the spatial temporal distribution of perineuronal nets in the prefrontal cortex

Vicia villosa labeled PNNs around PFC neurons during development (Fig. 2). Analysis of $PNN^+$ neuron density in the MPFC revealed that there was a significant interaction between environment and exposure ($F_{2,12} = 118.5$ $p < 0.0001$) and a main effect of environment ($F_{1,12} = 23.58$; $p = 0.0004$) and exposure ($F_{2,12} = 84.08$; $p < 0.0001$). In the control group, we see an increase in the number of PNNs during maturation. The number of $PNN^+$ neurons after 35 days in the control environment (74.00 ± 2.65) is higher than after 15 days (33.33 ± 1.45) ($p < 0.001$), which is higher than after 7 days (P35: 22.00 ± 1.53) ($p < 0.01$). In stressed animals, however, we found a different maturation dynamics. The number of $PNN^+$ neurons decreases as the animal spends more time under chronic stress (7: 45.33 ± 2.67; 15: 25.33 ± 2.19; 35: 34.33 ± 1.33) ($p < 0.01$). Interestingly, seven days of chronic stress increases the number of $PNN^+$ neurons compared to controls (45.33 ± 2.67 versus 22.00 ± 1.53) ($p < 0.001$). However, extending the duration of chronic stress exposure by 35 days causes a decrease in the number of $PNN^+$ neurons compared to controls (34.33 ± 1.33 versus 74.00 ± 2.65) ($p < 0.001$) (Fig. 3A).

In the OFC, there was also a significant interaction between environment and exposure ($F_{2,12} = 19.43$; $p = 0.002$) and a main effect of environment ($F_{1,12} = 106.10$; $p < 0.0001$) and exposure ($F_{2,12} = 10.83$; $p = 0.0021$) on the number of $PNN^+$ neurons. Basically, the result is qualitatively like the one above for the MPFC. The number of $PNN^+$ neurons increases with increasing time spent in the control environment ($p < 0.001$), with the average number of $PNN^+$ neurons after 35 days (41.33 ± 0.887) greater than after both 7 (22.00 ± 4.00) and 15 days (23.00 ± 2.52) ($p < 0.001$). Chronic stress, on the other hand, seems to stunt maturation of $PNN^+$ neurons, with the number of neurons being significantly smaller than in controls after both 15 (Control: 23.00 ± 2.52; Stress: 11.00 ± 0.58; $p < 0.01$) and 35 (Control: 41.33 ± 0.88; Stress: 10.00 ± 0.58; $p < 0.001$) days of chronic stress. Different from the MPFC, however, we did not see an increase in the number of $PNN^+$ neurons in the OFC after 7 days of chronic stress at the beginning of adolescence (Control: 22.00 ± 4.00; Stress: 14.33 ± 0.88; $p > 0.05$) (Fig. 3B).

### 3.2. Effects of chronic stress on behavior

Analysis of the SAAT revealed a significant interaction between environment and exposure ($F_{2,42} = 34.76$; $p < 0.0001$) and a main effect of environment ($F_{1,42} = 19.73$; $p < 0.0001$), but no effect of

**Table 1**
Effects of chronic stress on corticosterone blood levels and behavior (two-way ANOVA).

| | | Environment | | | Time | | | Interaction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | df | F | p | df | F | p | df | F | p |
| corticosterone | | 1 | 15.56 | 0.0008 | 2 | 1.76 | 0.1975 | 2 | 2.67 | 0.937 |
| $PNN^+$ neurons | MPFC | 1 | 23.58 | 0.0004 | 2 | 84.08 | <0.0001 | 2 | 118.50 | <0.0001 |
| | OFC | 1 | 106.10 | <0.0001 | 2 | 10.83 | 0.0021 | 2 | 19.43 | 0.0002 |
| Behavior | SAAT | 1 | 19.73 | <0.0001 | 2 | 1.82 | 0.1743 | 2 | 34.76 | <0.0001 |
| | Thigmotaxis | 1 | 4.78 | 0.0342 | 2 | 14.32 | <0.0001 | 2 | 19.82 | <0.0001 |

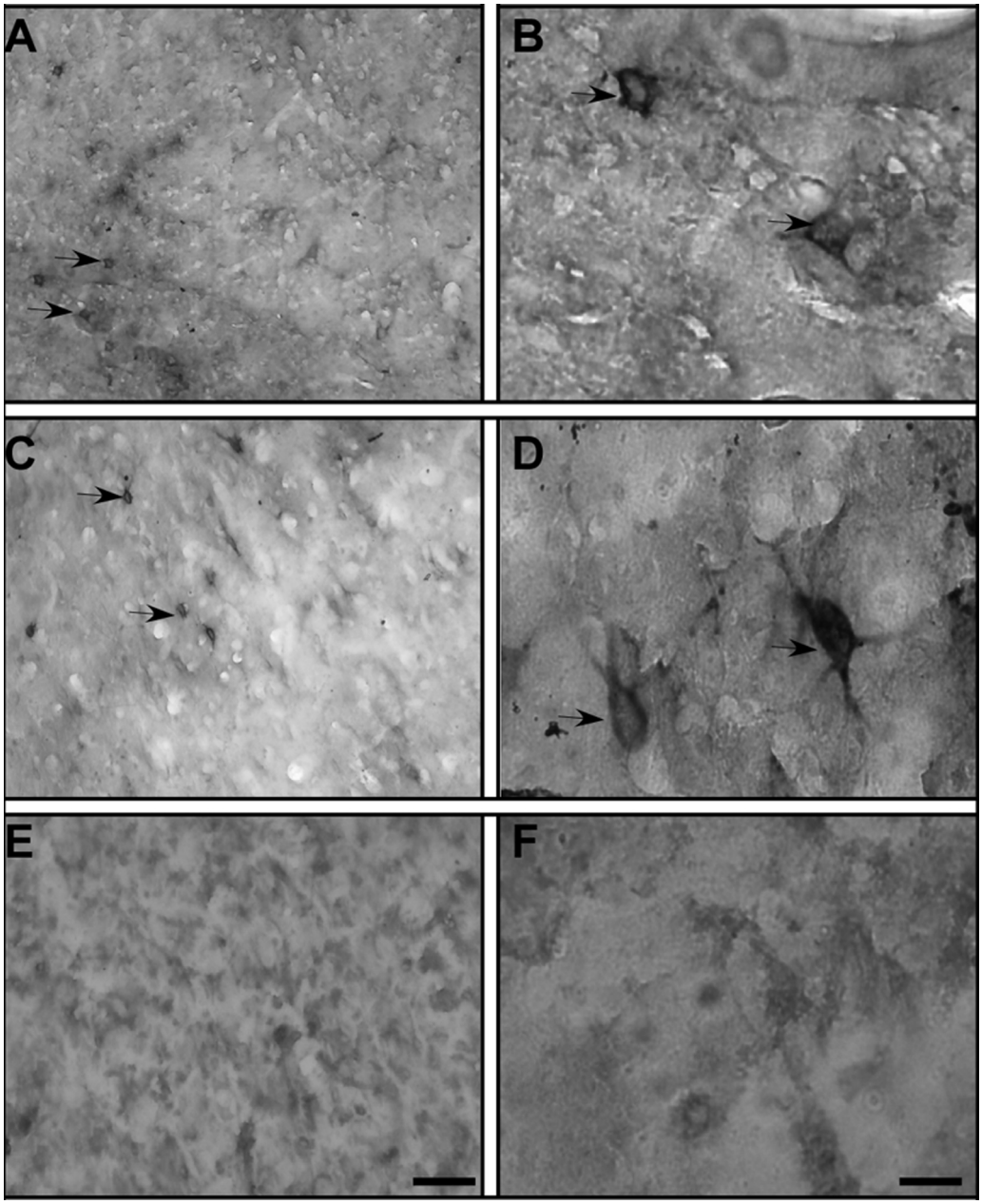


**Fig. 2.** Photomicrographs of coronal sections of the PFC of adolescent rats exposed to a chronically stressful environment labeled for the *Vicia villosa* lectin. Exposure periods: 7 (A), 15 (C), and 35 (E) days (magnified in B, D, and F, respectively. The arrows indicate $Vv^+$ cells. Scale bar: 100 μm.

exposure ($F_{2,42} = 1.822$; $p = 0.1743$). Animals in the control group showed an improvement in alternation rates with developmental maturation (Fig. 4). Animals in the stressed group, on the other hand, had a different developmental pattern (Fig. 4A). After being submitted to stress for 7 days, average alternation rates were significantly better than controls (Fig. 4A). After 15 days of chronic stress, however, the average alternation rate was smaller than controls (Control: $62.83 \pm 3.05$; Stress: $39.97 \pm 1.70$; $p < 0.001$). The same occurred after 35 days of chronic stress (Control: $55.06 \pm 1.56$; Stress: $39.72 \pm 1.94$; $p < 0.001$). These behavioral results parallel the findings for the number of $PNN^+$ neurons in the MPFC (see Fig. 3A).

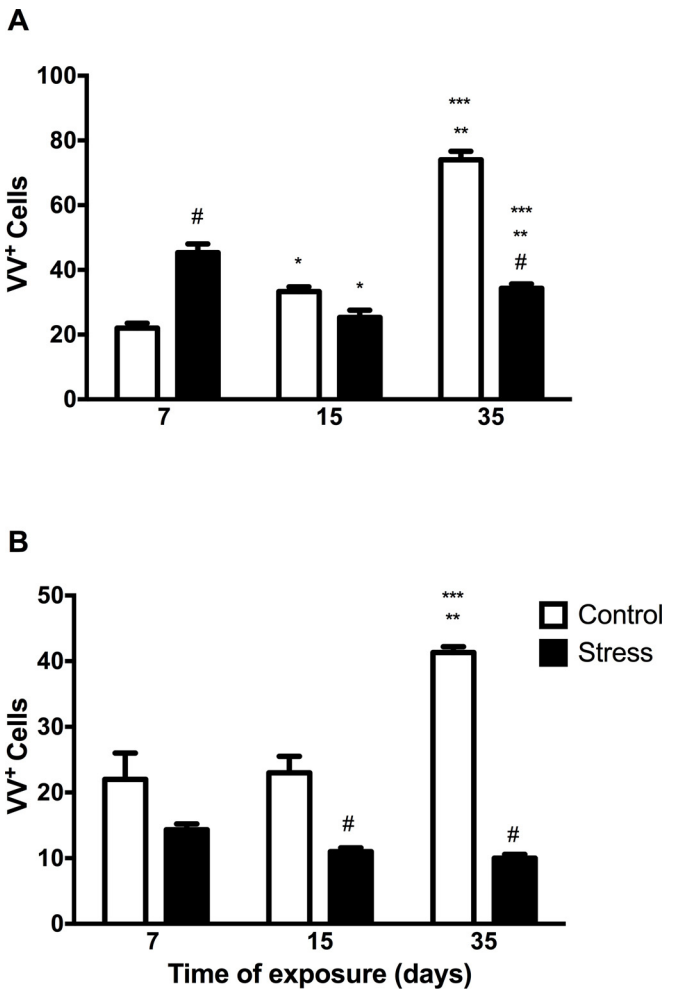


**Fig. 3.** (A) Average number of $Vv^+$ cells in the MPFC of control and stressed animals. Symbols are like Fig. 1. (B) Average number of PNN cells in the OFC. Symbols are like Fig. 1.

In the open-field test, there was a significant interaction between environment and exposure ($F_{2,42} = 19.82$; $p < 0.0001$) and a main effect of environment ($F_{1,42} = 4.795$; $p = 0.0342$) and exposure ($F_{2,42} = 14.32$; $p < 0.0001$) on thigmotaxis. The apparently stunting effect of chronic stress on development revealed by the SAAT also appear on the thigmotaxis scores, though less evidently (Fig. 4B). There is an increasing tendency for increased thigmotaxis in control animals as they develop during adolescence, probably suggesting the maturation of the neural pathway between the amygdala and the prefrontal cortex [59]. Performance after 35 days of exposure to the control environment ($97.55 \pm 0.49$) was better than after 15 days ($88.99 \pm 1.02$) ($p < 0.001$). However, thigmotaxis decreased from 7 ($92.55 \pm 1.61$) to 15 ($88.99 \pm 1.02$) days in the control environment ($p < 0.05$). In stressed animals, on the other hand, thigmotaxis decreased from 7 ($94.78 \pm 0.07$) to 15 days ($90.00 \pm 0.20$) ($p < 0.001$) in the stressful environment and stabilized afterwards (Fig. 4B).

## 4. Discussion

Our results show that subjecting rats during the critical period of plasticity of the PFC to stressful situations for an extended period interferes with maturation of perineuronal nets and this finding is correlated with changes in associated behavior, compared to control animals. Though we did not test the effect of chronic stress exposure in other developmental periods, our findings corroborate other studies indicating that the prefrontal cortex is particularly sensitive to stress exposure during infancy and adolescence [60,61]. Chronic stress can be induced in the laboratory by continuously submitting animals to stressful situations, such as physical immobilization, and observing the effects on brain and/or behavior. For instance, several studies have used this approach to evaluate morphological changes caused by chronic stress on pyramidal neurons of the hippocampus, amygdala and prefrontal cortex [10,17,62–64]. Other studies have shown effects on neurogenesis, synaptogenesis and myelination, as well as the availability of neurotransmitters and neurotrophic factors [65,66]. Chronic stress also interferes with the function of the sympathetic nervous system and the HPA axis [10,14,52]. However, since several studies have demonstrated that animals adapt to the predictability of a single stressor agent [12,57], in our study we used different stressors in a non-predicable way [49,50,63].

Plasma corticosterone levels were reduced in animals submitted to chronic stress (Fig. 1). This could be due to the action of a negative feedback loop from the HPA axis triggered by changes in glucocorticoid levels caused by stress. This situation is commonly observed in studies of post-traumatic stress disorder [9].

As expected, the number of $PNN^+$ neurons increased in the prefrontal cortex of control animals during adolescence, signaling the maturation of inhibitory circuits [67]. This pattern of PNN maturation had also been demonstrated in other developing cortical regions, such as the primary somatosensory cortex [38], the primary visual cortex [31,68] and subcortical regions including the amygdala [69] and the striatum [70]. This increase in $PNN^+$ neuron density is supposed to be associated with maturation of inhibitory circuits and to signal the closure of the critical period of plasticity of particular areas [71,72].

We demonstrated that chronic stress during adolescence has negative effects on the maturation of $PNN^+$ neurons. Chronic stressor agents cause a decrease in the amount of labeled $PNN^+$ neurons both in the medial and orbital regions of the prefrontal cortex. Other studies had already demonstrated the effects of chronic stress on the morphology of pyramidal neurons, such as structural modifications on dendritic arborization and decrease in the number of dendritic spines [18,20], probably due to an increase in the number of excitatory amino acids (glutamate) induced by greater amounts of glucocorticoids in the bloodstream [50,56,63]. Chronic stress also increased the expression of genes that regulate neuronal metabolism and synaptic changes in glutamate receptors [10,73]. In addition to excitatory neurons, inhibitory neurons are also vulnerable to the effects of chronic stress. These changes may be related with compensatory responses to excessive excitatory aminoacid release [74] and helps to prevent further damage to the central nervous system due to over stimulation of the HPA axis caused by environment stressors [14,75].

Another important finding is the negative effect of chronic stress on performance in tests associated with executive functions and the dependence of these effects on duration of exposure. The SAAT has been used before as a test of spatial working memory in rats and lesions of the prefrontal cortex impair performance on this task [41–46]. Our results showed an improvement in test performance in animals exposed to stress for seven days. This could be explained by short-term activation of stress adaptation mechanisms, increasing alertness and other coping mechanisms [14,62,76]. The negative effects observed in animals exposed to stressful situations for longer periods, such as 15 and 35 days, may reflect a desensitization of the HPA axis [77,78] as observed elsewhere [79].

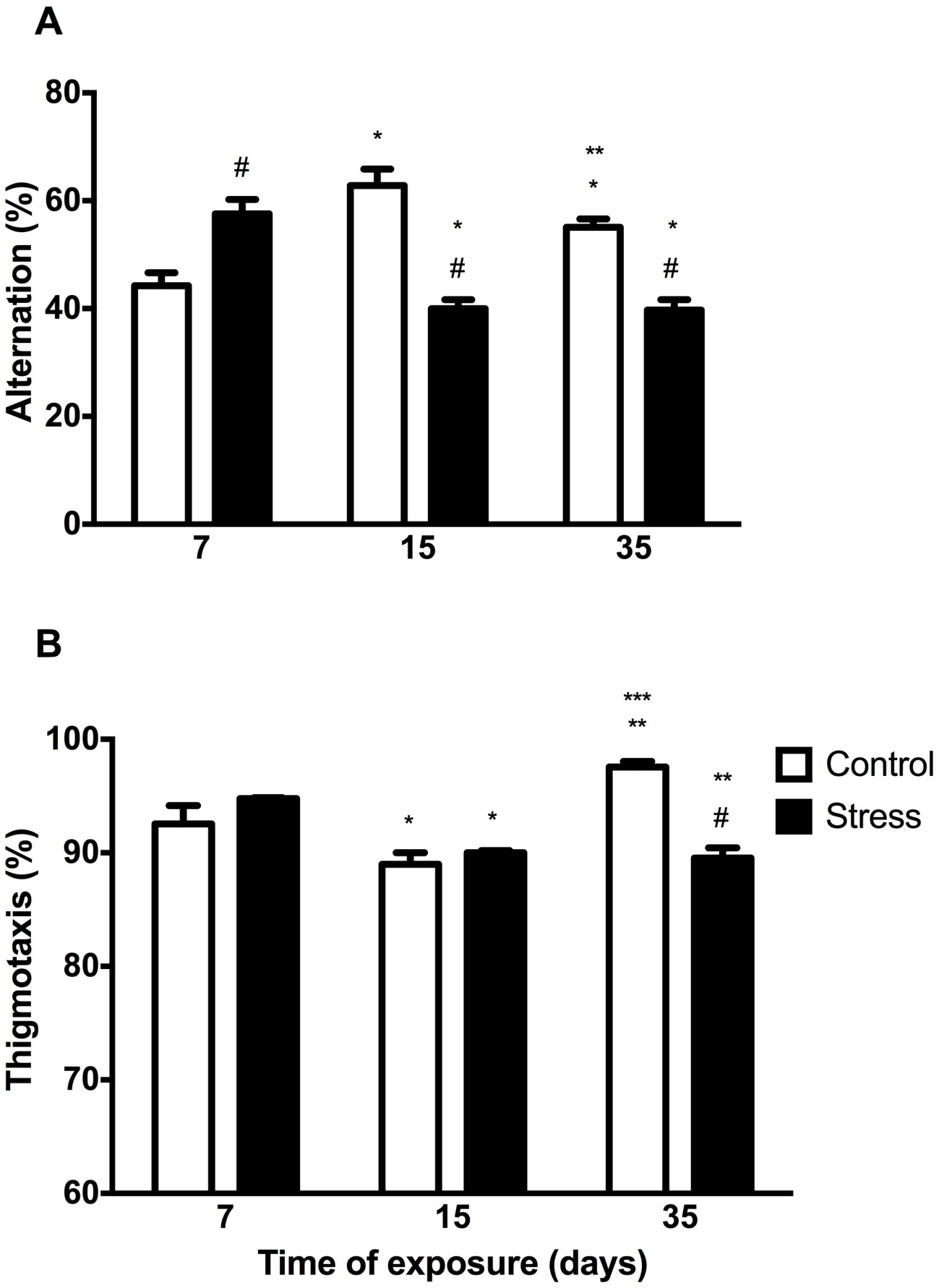


**Fig. 4.** (A) Spontaneous alternation behavior in the plus maze of stressed and control animals. Average results are shown as the percentage of total alternation. Symbols are like Fig. 1. (B) Thigmotaxis of stressed and control animals in the open field. Symbols are like Fig. 1.

## 5. Conclusions

Our results suggest that life in chronically stressful environments can impair the synaptic maturation of prefrontal cortex, with negative effects on behavior controlled by these circuits. In practice, sustained stress can effectively arrest prefrontal cortex development, leaving it in an immature state. It is tempting to conclude this is part of a mechanism that works to protect developing prefrontal circuits from negative influences of a bad environment. Since this could also happen to the human brain in identical situations, these dramatic results underscore the need to protect the adolescent brain from prolonged stressful situations, as is sadly the norm in many parts of the world.

## Conflict of interest

The authors declare no conflict of interest.

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