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## ORIGINAL ARTICLE

# Peripubertal stress-induced behavioral changes are associated with altered expression of genes involved in excitation and inhibition in the amygdala

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Early-life stress is a critical risk factor for developing psychopathological alterations later in life. This early adverse environment has been modeled in rats by exposure to stress during the peripubertal period—that is, corresponding to childhood and puberty—and has been shown to lead to increased emotionality, decreased sociability and pathological aggression. The amygdala, particularly its central nucleus (CeA), is hyperactivated in this model, consistent with evidence implicating this nucleus in the regulation of social and aggressive behaviors. Here, we investigated potential changes in the gene expression of molecular markers of excitatory and inhibitory neurotransmission in the CeA. We found that peripubertal stress led to an increase in the expression of mRNA encoding NR1 (the obligatory subunit of the *N*-methyl D-aspartate (NMDA) receptor) but to a reduction in the level of mRNA encoding glutamic acid decarboxylase 67 (GAD67), an enzyme that is critically involved in the activity-dependent synthesis of GABA, and to an increase in the vesicular glutamate transporter 1 (VGLUT1)/vesicular GABA transporter (VGAT) ratio in the CeA. These molecular alterations were present in addition to increased novelty reactivity, sociability deficits and increased aggression. Our results also showed that the full extent of the peripubertal protocol was required for the observed behavioral and neurobiological effects because exposure during only the childhood/prepubertal period (Juvenile Stress) or the male pubertal period (Puberty Stress) was insufficient to elicit the same effects. These findings highlight peripuberty as a period in which stress can lead to long-term programming of the genes involved in excitatory and inhibitory neurotransmission in the CeA.
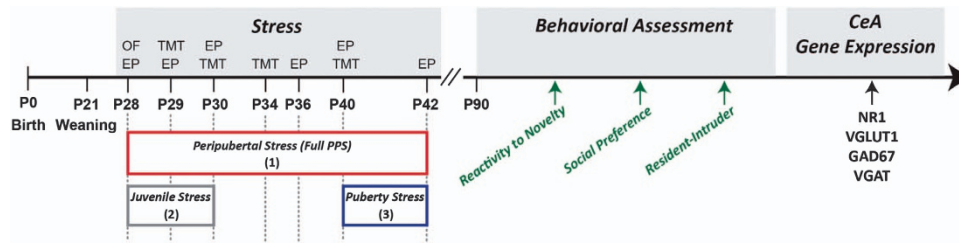
## INTRODUCTION

Although substantial evidence indicates that exposure to early-life stress is a critical risk factor for developing psychopathological alterations later in life,[1–5] including anxiety disorders,[2,5] impaired social behavior and increased aggression,[3,6] the underlying neurobiological mechanisms are largely unknown. In light of this lack of knowledge, the recent emphasis placed on the development of animal models of psychopathology triggered by early adversity is essential to explore mechanistic questions.[7,8]

Childhood and adolescence are periods known to be sensitive to the long-term programming effects of stress.[7,9–12] Our laboratory has developed a model that involves exposing rats to stressful experiences during the peripubertal period—consisting of childhood and puberty—leading to alterations in several behavioral domains during adulthood, including decreased sociability, increased anxiety and pathological aggression.[13,14] These behavioral changes are observed in parallel with hyperactivity in the amygdala both under basal conditions and after an aggressive challenge.[14] The relevance of this enduring effect of peripubertal stress (PPS) on the activation of the amygdala is supported by the mounting literature implicating this brain region in emotional regulation,[15,16] social behaviors[17,18] and aggressive behaviors[19,20] as well as by the well-known hyperactivity of the amygdala in post-traumatic stress disorder, other anxiety and personality disorders[21–24] and aggressive behaviors.[25] Importantly, some studies have related aspects of an imbalanced excitation/

inhibition ratio in the amygdala with alterations observed in the brain of individuals who present psychopathological alterations involving marked social deficits.[26–28] In rodents, acute alteration of the excitation/inhibition balance in the prefrontal cortex was shown to reduce social exploration.[29] Furthermore, differences in the GABAergic system have been proposed to be involved in the differential amygdala responsiveness in stress-related anxiety disorders,[30,31] and a recent study indicated reduced GABAergic activity in our PPS model in rats.[32]

Studies that have examined the contribution of the different nuclei of the amygdala have highlighted the central nucleus (CeA) as being particularly implicated in the regulation of social[33–35] and aggressive behaviors.[14,36,37] Note that the main projections from the amygdala to the lateral hypothalamus, the latter structure being critically implicated in the emergence of aggressive behaviors,[36,38] stem from the CeA.[39] Importantly, in our peripuberty stress model of psychopathology, effective connectivity analyses applied to data from a c-Fos experiment revealing neuronal activity in a large number of brain regions following an aggressive challenge, found that activity in the CeA was the only predictor of aggressive output.[14] Evidence for a role of the CeA in anxiety has also been reported.[40,41]

Here, with the goal of gaining insight into the mechanisms underlying the alterations induced by PPS exposure in the CeA, we investigated potential changes in the gene expression of molecular markers for excitatory (the obligatory subunit of *N*-methyl D-aspartate (NMDA) glutamate receptors, NR1, and the

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**Figure 1.** Experimental design. All animals were weaned at P21 and subsequently assigned to Control or Stress groups. Three different cohorts of rats (numbered in brackets on the schema) were subjected to either the full extent of the Peripubertal Stress (PPS) protocol (P28-42) (1), the early phase (P28–30; representing early adolescence or juvenility) (2) or the late phase of the protocol (P40–42; representing puberty) (3). The stress protocol consisted of exposure to an open field (OF) on P28 and then to an elevated platform (EP) and predator odor (trimethylthiazoline), with stressors presented in a variable schedule on the days depicted in the schema. The behavioral testing started at P90. Control animals were handled briefly on the days on which their experimental cohorts were exposed to stress.

vesicular glutamate transporter 1 (VGLUT1)) and inhibitory neurotransmission (glutamic acid decarboxylase 67 (GAD67) and the vesicular GABA transporter (VGAT)).

The PPS protocol consisted of exposing rats to fearful experiences (that is, exposure to an elevated platform and to a synthetic fox odor) using an unpredictable schedule on 7 specific days, across postnatal days 28–42 (P28-42), to cover the equivalent of 'childhood' (P28–P30) and puberty (P34, P36, P40 and P42) periods in the rat; note that puberty in male Wistar rats typically occurs on P41 ± 1 day. Given that this protocol includes repeated exposure to stressors across different developmental periods, a second goal of the current study was to investigate whether the full extent of the stress included in the PPS protocol (designated Full Peripubertal Stress) was required for its behavioral and neurobiological impact or whether exposure to the corresponding stressors during either childhood/prepuberty (P28-–P30; designated Juvenile Stress) or during the male puberty period (P40, P42; designated Puberty Stress) would lead to similar or no alterations in adulthood.

## MATERIALS AND METHODS

### Animals
The experimental subjects were the offsprings of Wistar Han rats (Charles River Laboratories, L'Arbresle, France) bred in our animal facility. Twelve breeding pairs were established for each cohort of animals. In each cohort, 10–11 litters were finally used as, typically, 1–2 females per cohort did not become pregnant after the cohabitation with the male. Only the male offspring were tested at adulthood. At weaning on P21, male rats from different litters were mixed throughout the different home cages by placing an equivalent number of animals from each litter into the stress or control groups and by avoiding placing siblings in the same home cage. In addition, cage mates were matched by weight. The rats were housed two animals per cage and maintained on a 12-h light–dark cycle (lights on at 0700 hours). Food and water were available ad libitum. All procedures were conducted in conformity with the Swiss National Institutional Guidelines on Animal Experimentation and approved by a license from the Swiss Cantonal Veterinary Office Committee for Animal Experimentation.

### Experimental design
After weaning at P21, animals were randomly assigned to control or stress groups, with animals in the same cage always assigned to the same experimental condition. Separate experiments (using different cohorts of animals) were performed to include the various lengths and time of exposure to stress around the peripubertal period. Control rats were briefly handled on the days that the corresponding experimental cohorts were exposed to stress.

The stress protocol procedure began on P28 (for the first two cohorts) or P40 (for the third cohort). The first cohort was exposed to stress during the peripubertal period as previously described.[14] Animals in the second cohort, representing the Juvenile Stress group, were subjected to only the early phase of the PPS protocol on P28, P29 and P30 (Figure 1). Finally, a third cohort, representing the Puberty Stress group, was exposed to stress according to the last 2 days of the protocol on P40 and P42 (Figure 1).

The cohort exposed to the entire duration of PPS (that is, from P28–P42) is termed Full Peripubertal Stress with their respective control cohorts called CTRL Full. The second cohort, stressed during P28–P30, is referred to as Juvenile Stress, with their respective control cohorts called CTRL Juvenile. Similarly, the third cohort, stressed on P40 and P42, is referred to as Puberty Stress with their respective controls called CTRL Puberty. For each cohort, the behavioral assessment and characterization began at adulthood (P90; Figure 1).

### Stress protocol
The PPS protocol, also referred to here as the Full Peripubertal Stress protocol, was based on exposure to fearful situations.[14] Briefly, following exposure to an open field for 5 min on P28, two different fear-inducing stressors were presented in a variable schedule in the span of 7 days across the P28–P42 period: the synthetic fox odor trimethylthiazoline (PheroTech, Delta, BC, Canada) administered in a plastic box ($38 \times 27.5 \times 31$ cm) and exposure to an elevated platform (EP; $12 \times 12$ cm; Figure 1). Each stressor lasted for 25 min. Following each stress session, the animals remained separated for 15 min before rejoining their cage mates.

### Reactivity to novelty
The open field and novel object tests were performed to assess the animals' exploratory/locomotive behavior, as well as their reactivity upon novelty exposure. The test was performed as previously described.[42] For the open-field test, a circular open arena was utilized (1 m in diameter, 40 cm high). Each rat was placed near the wall and allowed to freely explore and habituate to the apparatus for 10 min. For analysis, the floor of the open field was considered to be divided into three parts: the center zone in the middle of the arena with a diameter of 25 cm, an intermediate zone with a diameter of 75 cm and the remaining peripheral zone along the walls of the arena. At the end of the 10-min period, an object (white plastic bottle) was introduced into the center of the arena for the novel object test. Each animal was allowed to explore the arena and object for 5 min. The center zone of the arena, where the object was placed during the novel object phase, was considered as the exploration area and was of the same dimensions for both the open-field and novel object tests. The behavior was monitored using a video camera and analyzed with a computerized tracking system (Ethovision XT 8, Noldus IT). The entries and the time spent in the exploration area (center) and the periphery (wall), as well as the distance traveled in the arena, were recorded automatically. The apparatus was cleaned with a 1% acetic acid solution and dried thoroughly after each animal.

### Social preference test
The social preference test was adapted from the protocol described by Crawley and co-workers[43] to investigate social affiliation in male mice using the same conditions previously described.[14,44] The apparatus was a rectangular three-chambered gray opaque polycarbonate box, consisting of a center ($20 \times 35 \times 35$ cm), left and right compartment ($30 \times 35 \times 35$ cm). The dividing walls had retractable doorways allowing access to each chamber. The left and right compartments contained a central transparent Plexiglas cylinder (15 cm diameter) with small holes, where either a social stimulus (unfamiliar juvenile rat of 30 ± 2 days old) or a non-social stimulus (yellow plastic bottle) was placed. The cylinder permitted visual, tactile, auditory and olfactory communication. The juvenile rats were first

habituated to the three-chambered apparatus by placing them individually into the box within the Plexiglas cylinder for 10 min each day during the three consecutive days preceding the social test. On the test day, the experimental rat was first placed in the middle chamber and allowed to habituate for 5 min. The doorways into the two side chambers were closed during this habituation phase. After the habituation period, the unfamiliar juvenile was placed in one of the side chambers and the object placed in the other chamber. The locations of the juvenile and the object in the left and right side chambers were counterbalanced. Next, both side chamber doors were carefully removed, and the experimental rat was allowed to explore the entire apparatus for a 10-min session. Each trial was video-recorded (MediaCruise, Canopus, Kobe, Japan) and scored offline manually by an experimenter blind to the conditions. The time the rats spent sniffing each cylinder was scored to evaluate the level of preference for the unfamiliar juvenile compared with the object. The rats were considered to explore the object and the juvenile when they approached the cylinders within less than ~ 2 cm, with their nose oriented toward the contents of the cylinders. The social preference ratio was calculated as follows: time exploring the juvenile/ (time exploring the juvenile + time exploring the object). The entire apparatus was cleaned with 1% acetic acid solution and dried thoroughly between each trial.

## Resident–intruder test

The resident–intruder protocol was adapted from Veenema et al.[45] and performed following the same conditions as those employed in the previous studies from our laboratory.[14,44] Briefly, each rat (resident) was housed in an experimental cage ($40 \times 29 \times 20$ cm) together with a naive adult female Wistar rat for 10 days. Following 10 days of cohabitation with the female rat, the RI test was performed. Thirty minutes before the test, the female was removed from the resident's home cage and was returned after the test was over. The test was conducted during the dark cycle. The resident control or stressed male rat was exposed in his home cage to a slightly smaller (~5–10% body weight difference), unfamiliar male Wistar rat, the intruder, for a 30-min encounter. The tests were videotaped and the behavioral scoring was performed offline using The Observer XT 10 (Noldus IT, Wageningen, The Netherlands). Resident and intruder pairs in which neither the resident nor the intruder exhibited any offensive behavior were excluded from the analysis (3 out of 92 pairs). In addition, the data for this test from one Full Peripubertal Stress animal was lost due to a technical problem with the video-recording. The following parameters related to aggression were scored in the experimental animals: duration of attack, lateral threat, offensive upright posture and keeping the intruder down. The definitions of these behaviors followed the ones described previously.[46] More specifically, 'attack' was counted when the experimental animal oriented to the other on very rapid rolling, jumping and biting. 'Lateral threat' was scored when it was standing or moving slowly in a sideways direction toward the opponent. 'Offensive upright' was scored when it was standing on its hindlegs facing the opponent, in occasions touching the other animal with its forepaws. 'Keeping down' was scored when the animal was standing over the opponent, keeping it down by pushing against the cage floor or walls. In addition, these four behaviors were summed up to provide an index of the total aggressive behavior displayed by each experimental animal.

## Brain punching

Two weeks after the last behavioral test, the rats were decapitated under basal conditions. After decapitation, the brains were snap-frozen in isopentane at − 45 °C and stored at − 80 °C until further processing. The brains were sectioned using a cryostat, and 200-µm-thick slices were mounted on slides in order to punch and remove the region of interest. Bilateral punches of the central amygdaloid nucleus (CeA) were conducted using a 1-mm puncher according to the atlas coordinates indicated in Paxinos et al.[47] The tissue was collected in RNAase-free tubes and maintained in a − 80 °C freezer until further processing for RNA extraction and isolation.

## Gene expression analysis

Total RNA from the CeA was isolated using the RNAqueous Micro kit (Ambion, Life Technologies, Austin, TX, USA), and complementary DNA was synthesized using the Superscript VILO kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the supplier's recommendations. For quantitative polymerase chain reaction (PCR), PCR reactions were performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies, Warrington, Florida, USA) in an ABI Prism 7900 Sequence Detection system (Applied Biosystems, Life Technologies, Singapore). Two genes were used as internal controls: gamma-actin (actg1) and eukaryotic elongation factor-1 (eef1). Primers for the genes of interest were designed using the Assay Design Center software from Roche Applied Science, except for NR1.[48] Primer sequences are listed in Table 1. Gene expression was analyzed with the qBase 1.3.5 software using the comparative cycle threshold method. The genes investigated were NR1, VGLUT1, GAD67 (glutamic acid decarboxylase 67) and VGAT.

## Statistical analysis

The data were analyzed with independent samples t-tests using the statistical package SPSS version 17.0 (SPSS, Chicago, IL, USA). If Levene's test for equality of variances was significant, equal variance was not assumed, and the altered degrees of freedom were rounded to the nearest whole number. All bars and error bars represent the mean ± the s.e.m., and the statistical significance was set at $P < 0.05$. The P-value was considered tending toward significance when $0.05 \leqslant P \leqslant 0.1$. For these cases, the specific P-values are reported on the respective graphs. The graphs were created using GraphPad Prism 5 (San Diego, CA, USA).

## RESULTS

### Reactivity to novelty

First, we evaluated whether animals exposed to the different stress protocols showed changes in their behavioral responses compared with the corresponding controls in the open-field and novel object reactivity tests. Although no data had been collected for these tests during adulthood in previous studies examining peripubertal stress effects, a study performed during the immediate post-puberty stress period, at adolescence, found no changes in locomotion but increased reactivity to a novel object in the stressed animals.[49]

In the current study, for animals tested as adults, we found no significant differences between CTRL Full and Full Peripubertal Stress during the open-field phase of the test as demonstrated by entries in the center (exploration area) (Figure 2a; $t_{18} = 1.260$, n.s.), time in the center and the distance traveled (data not shown; time in the center: $t_{28} = 0.735$, n.s., distance moved: $t_{28} = -1.128$, n.s.). Juvenile Stress animals showed fewer entries in the center than CTRL Juvenile rats during the open-field test (Figure 2b; $t_{28} = 2.019$, $P = 0.05$), but no differences were observed between Juvenile Stress and CTRL Juvenile for the time spent in the center and the distance traveled (data not shown; time in the center: $t_{28} = 0.492$, n.s., distance moved: $t_{28} = 0.148$, n.s.). Puberty Stress rats showed no difference compared with CTRL Puberty for the entries in the

**Table 1.** Primer sequences for quantitative PCR

| Gene | Forward primer (5′-3′) | Reverse primer (5′-3′) | RefSeq (NCBI) |
| --- | --- | --- | --- |
| NR1 | CGCATCACGGGCATCA | CAAGTTCATCTACGCAACTGTAAAGC | NM_017010.1 |
| VGLUT1 | GTCATGACTATCATCGTACCCATC | GTAGCTTCCATCCCGAAACC | NM_053859.1 |
| GAD67 | TACAACCTTTGGCTGCATGT | TGAGTTTGTTGGCGATGCTT | NM_017007.1 |
| VGAT | GGGCTGGAACGTGACAAA | GGAGGATGGCGTAGGGTAG | NM_031782.1 |
| EEF1 | TGTGGTGGAATCGACAAAAG | CCCAGGCATACTTGAAGGAG | NM_175838.1 |
| ActinG1 | TAGTTCATGTGGCTCGGTCA | GCTGGGGACTGACTGACTTT | NM_001127449.1 |
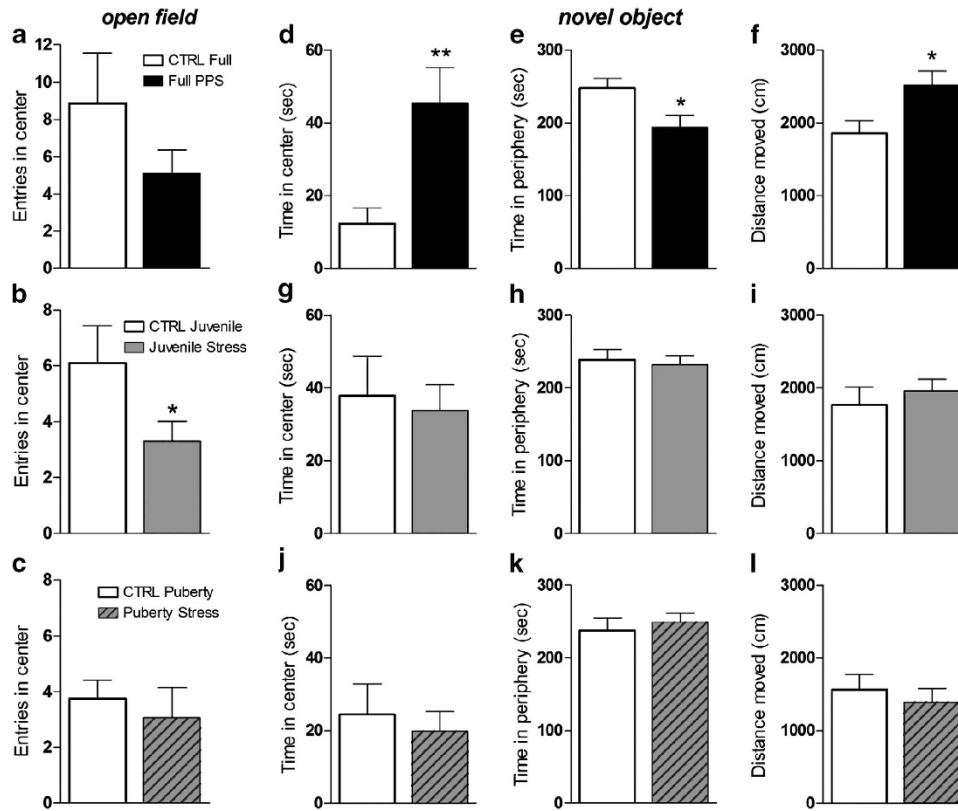
**Figure 2.** Open field and novel object tests. Rats exposed to the entire stress protocol (Full PPS or Peripubertal Stress) showed no differences in the entries in the center zone (exploration area) during the OF test (**a**). Juvenile Stress animals performed fewer entries in the center as compared with their corresponding CTRL rats (**b**). Puberty Stress rats showed no differences for their entries into the center zone compared with CTRL Puberty rats (**c**). When an object was placed in the exploration area of the OF, Full PPS animals spent more time in the center of the arena (**d**), less time in the periphery (**e**) and an increased distance traveled in the arena compared with CTRL Full animals (**f**), suggesting an increase in object exploration. However Juvenile Stress (**g–i**) and Puberty Stress (**j–l**) animals showed no differences in the parameters of the novel object exploration test compared with their CTRL cohorts. (N: CTRL Full = 14, Full PPS = 16, CTRL Juvenile = 10, Juvenile Stress = 20, CTRL Puberty = 16, Puberty Stress = 16), *$P < 0.05$, **$P < 0.01$ versus CTRL, results are the mean $\pm$ s.e.m.
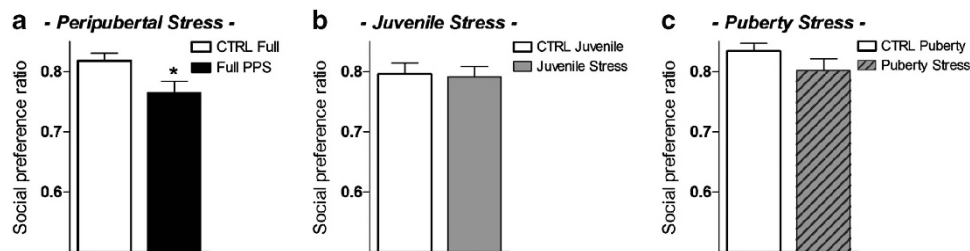


**Figure 3.** Social preference test. Rats exposed to the entire stress protocol (Full PPS or Peripubertal Stress) displayed a lower sociability ratio (juvenile exploration/total exploration of juvenile and object) compared with CTRL Full rats (**a**). However, Juvenile Stress (**b**) and Puberty Stress (**c**) groups exhibited no differences when compared with their respective CTRLs in the social behavior ratio. (N: CTRL Full = 14, Full PPS = 16, CTRL Juvenile = 10, Juvenile Stress = 20, CTRL Puberty = 16, Puberty Stress = 16), *$P < 0.05$ versus CTRL, results are the mean $\pm$ s.e.m.

center zone during the open-field phase (Figure 2c; $t_{30} = 0.542$, n.s.) and time in the center but traveled a significantly shorter distance in the arena (data not shown; time in the center: $t_{30} = 1.312$, n.s., distance moved: $t_{30} = 2.860$, $P < 0.01$).

When a novel object was placed in the center of the arena (novel object test), Full Peripubertal Stress animals exhibited increased reactivity to novelty, as indicated by the increased duration spent in the center around the object (Figure 2d; $t_{21} = -3.073$, $P < 0.01$), the decreased time spent in the periphery (wall zone) of the arena (Figure 2e; $t_{28} = 2.503$, $P < 0.05$) and the increased distance traveled (Figure 2f; $t_{28} = -2.525$, $P < 0.05$). No differences were observed during the novel object exploration phase of the test between the Juvenile Stress rats and their control cohort (Figure 2g; time in center: $t_{28} = 0.328$, n.s.; Figure 2h; time in

periphery: $t_{28} = 0.338$, n.s., and Figure 2i; distance traveled: $t_{28} = -0.671$, n.s.). Similarly, no differences were observed during this phase of the test between the Puberty Stress animals and their control cohort (Figure 2j; time in center: $t_{30} = 0.458$, n.s.; Figure 2k; time in periphery: $t_{27} = -0.567$, n.s. and Figure 2l; distance traveled: $t_{30} = 0.611$, n.s.).

These results indicate that Full Peripubertal Stress resulted in increased reactivity upon exposure to a novel object, whereas stress during only juvenility or puberty did not. In addition, during the open-field exploration, animals that were stressed during juvenility exhibited decreased exploration of the center zone, whereas rats that were stressed during puberty showed decreased locomotor activity upon exposure to a novel environment,

findings that may indicate an augmentation of anxiety-like behavior.

## Social behavior

To assess the animals' motivation for social exploration, we employed the social preference test. Previously, animals subjected to the Full Peripubertal Stress have been shown to exhibit reduced social exploration when tested in adulthood.[14] As expected, Full Peripubertal Stress rats exhibited a decreased social preference ratio compared with CTRL Full (Figure 3a; $t_{28} = -2.265$, $P < 0.05$), whereas no difference was observed for their total exploration time (data not shown). Compared with CTRL Juvenile animals, Juvenile Stress rats did not exhibit any differences in their motivation for social exploration (Figure 3b; $t_{28} = 0.165$, n.s.) or for their total time spent exploring the juvenile rat and the object (data not shown). Similarly, Puberty Stress animals did not exhibit any alterations in their sociability (Figure 3c; $t_{30} = 1.342$, n.s.) or the time spent exploring both stimuli (data not shown) as compared with their respective CTRL rats. Thus, these results suggest that exposure to stress for the entire peripubertal period is necessary to observe alterations in social preference because exposure to either the early or late phase of the protocol alone resulted in no relevant alterations.

## Aggressive behavior

To evaluate the effects of stress exposure in aggressive behavior, we used the resident–intruder test. In previous studies from our laboratory, exposure to peripubertal stress augmented aggressive behavior.[13,14] Full Peripubertal Stress animals exhibited increased amount of time spent keeping down the intruder compared with relevant CTRLs, with no significant differences in other types of offensive behavior (Figure 4a; attack: $t_{13} = 1.276$, n.s.; offensive upright: $t_{24} = -1.600$, n.s.; lateral threat: $t_{14} = -1.227$ n.s.; keep down: $t_{17} = -2.273$, $P < 0.05$). When the sum of all offensive behaviors was calculated, Full Peripubertal Stress animals exhibited an increase in total aggressive behavior compared with CTRL Full (Figure 4b; $t_{17} = -2.122$, $P < 0.05$). Juvenile Stress animals exhibited an increase in the duration of offensive upright behavior and a tendency for an increased duration in keeping down behavior (Figure 4c; attack: $t_{12} = 0.552$, n.s.; offensive upright: $t_{23} = -2.243$, $P < 0.05$; lateral threat: $t_{28} = -0.390$, n.s.; keep down: $t_{22} = -1.734$, $P = 0.097$). No difference was observed between Juvenile Stress and CTRL Juvenile in the total duration of their aggressive behaviors (Figure 4d; $t_{28} = -1.257$, n.s.). Compared with CTRL Puberty, Puberty Stress rats tended to increase the duration of time they kept down intruders, with no differences in the other aggressive behaviors (Figure 4e; attack: $t_{30} = -0.148$, n.s.; offensive upright: $t_{30} = -1.425$, n.s.; lateral threat: $t_{21} = -1.536$, n.s.; keep down: $t_{23} = -1.911$, $P = 0.069$). For total aggression, $t$-tests revealed a trend for augmented aggression in the Puberty Stress rats compared with the respective CTRL rats (Figure 4f; $t_{22} = -1.926$, $P = 0.067$). These data reveal that, although stress exposure throughout the extent of our protocol augments total aggressive behavior, exposure to stressors during early or mid-adolescence mildly enhances aspects of aggressive behavior.

## Gene expression in the central amygdala

To address the long-term changes in gene expression after exposure to stress in the different conditions examined here, we performed quantitative PCR. Genes related to excitation/inhibition balance were examined in the CeA of Full Peripubertal Stress, Juvenile Stress and Puberty Stress animals and compared with their respective control groups. Peripubertal stress induced an increase in the expression levels of the gene encoding NR1 in the CeA of Full Peripubertal Stress rats (Figure 5a; $t_{13} = -4.345$, $P < 0.01$) and a trend toward an increase in the expression levels

of the gene encoding VGLUT1 (Figure 5b; $t_{23} = -1.742$, $P = 0.095$). Moreover, a reduction in the expression levels of the GAD67 gene was found in the CeA of Full Peripubertal Stress rats compared with CTRL Full rats (Figure 5c; $t_{25} = 2.740$, $P < 0.05$). Finally, peripubertal stress did not lead to alterations in the VGAT gene expression levels (Figure 5d; $t_{23} = 1.584$, n.s.). Importantly, Full Peripubertal Stress animals showed an increased VGLUT1 to VGAT ratio (Figure 5e; $t_{23} = -2.125$, $P < 0.05$). No differences were observed between Juvenile Stress animals and their respective controls (Figures 5f–j; NR1: $t_{28} = 0.505$, n.s.; VGLUT1: $t_{28} = 0.002$, n.s.; GAD67: $t_{28} = -0.435$, n.s.; VGAT: $t_{28} = 0.067$, n.s.; VGLUT/VGAT: $t_{28} = -0.080$, n.s.). Similarly, stress during puberty did not induce any CeA expression alterations in the expression of the genes studied (Figures 5k–o; NR1: $t_{25} = 0.554$, n.s.; VGLUT1: $t_{28} = 0.264$, n.s.; GAD67: $t_{28} = 0.830$, n.s.; VGAT: $t_{28} = -1.073$, n.s.; VGLUT1/VGAT: $t_{28} = 0.259$, n.s.). This evidence indicates that peripubertal stress leads to long-lasting alterations in the expression of genes used as markers of excitation/inhibition balance, and that exposure to the early or late phase of our protocol alone was insufficient to induce similar alterations.

## DISCUSSION

We showed here that exposure to peripubertal stress in rats resulted in long-term alterations in the expression of genes related to excitatory and inhibitory neurotransmission in the CeA, with a bias toward enhanced excitation. These molecular alterations were present together with increased novelty reactivity, sociability deficits and increased aggression, consistent with previous findings.[14,49] Interestingly, the concomitant modulation of aggression and novelty seeking by peripuberty stress is in line with rat studies showing a co-selection of these behaviors in rats selectively bred for their differential reactivity to explore in novel environments.[50] Likewise, children and adolescents that score highly in novelty seeking parameters often display antisocial behavior and elevated levels of aggression.[51,52]

The peripubertal stress protocol (Full Peripubertal Stress) covers different periods throughout the juvenile and pubertal developmental stages in the rat. Previous studies in which rats were exposed to stress during the prepuberty period (that is, from P27/28-P29/30 and thereby equivalent to the Juvenile Stress cohort in the present report) displayed increased emotionality at adulthood.[53–56] However, the opposite findings were reported when animals were tested in the aftermath of stress exposure during the adolescence period.[49,56–58] Our present results also showed that the full extent of the peripubertal stress protocol was required for the observed behavioral and neurobiological effects because exposure corresponding only to the period of rat childhood/prepuberty (P28–P30; Juvenile Stress) or male puberty (P40, P42; Puberty Stress) alone was insufficient to elicit the same effects. Given that our study had a main focus on investigating the impact of the repetitive nature of stressor exposure throughout the peripuberty period in our protocol, our results specifically address questions related to the chronicity of stress exposure across childhood and puberty. This was at the expense of interrogating the differential vulnerability of the early versus the late period comprised in the protocol. Examining the latter question would require to expose animals to the same stress protocol at each of the two developmental periods. In this context, it is noteworthy that the reported behavioral changes were not observed when the Full Peripubertal Stress was given in adult animals and behavior measured 2–3 months afterwards (similar to the delay included in the experiments in which animals are stressed in the peripubertal period).[14] These findings support the view that, in addition to a role for the repeated exposure to stress throughout childhood and puberty reported in the current study, timing at which stressors are delivered during the individual's lifespan is probably as well a major factor in determining the
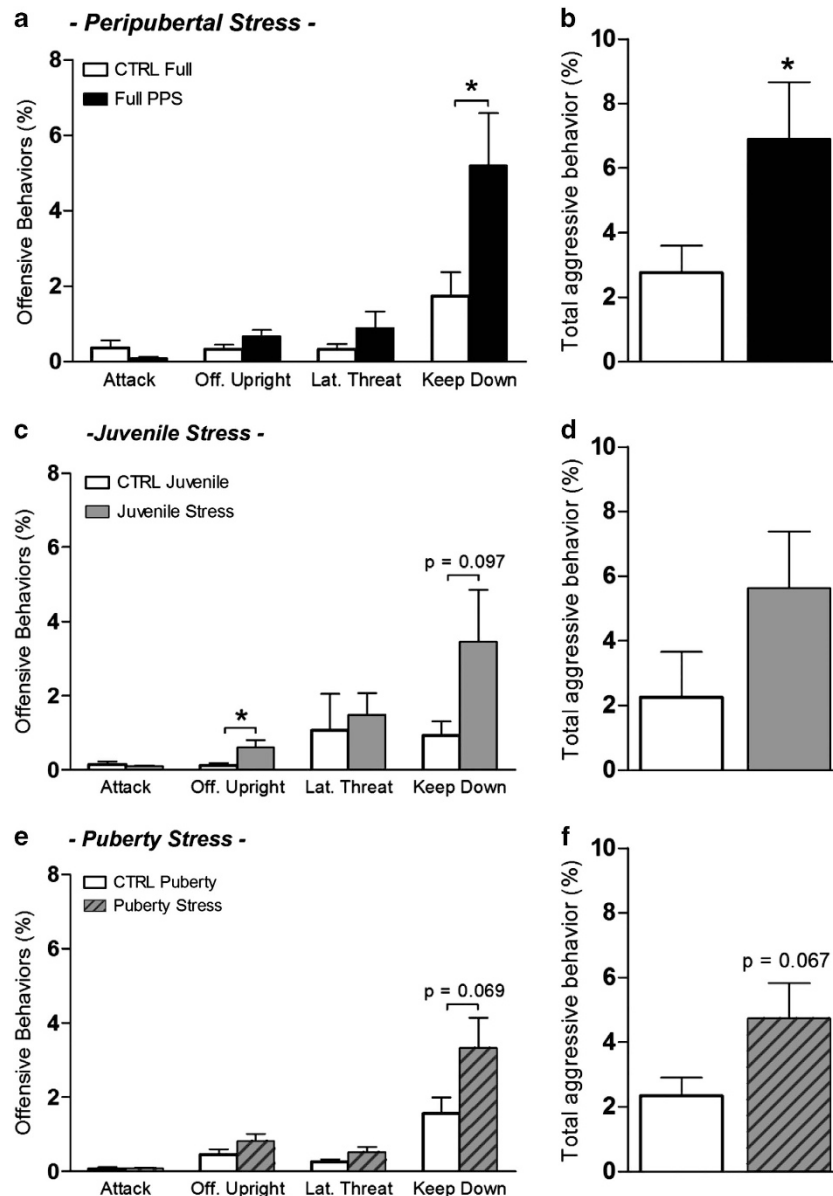
**Figure 4.** Resident–intruder test. Rats exposed to the entire stress protocol (Full PPS or Peripubertal Stress) showed an increased duration of keeping down behavior (**a**) and an overall increase in total aggressive behavior (**b**) when compared with CTRL Full animals. Juvenile Stress rats exhibited an increase in the duration of offensive upright behavior and a trend toward increased keeping down behavior (**c**), whereas no significant difference was evident in the total duration of offensive behavior (**d**). Puberty Stress rats exhibited a trend toward increased keeping down behavior (**e**) and increased total aggression (**f**). (N: CTRL Full = 13, Full PPS = 13, CTRL Juvenile = 10, Juvenile Stress = 20, CTRL Puberty = 16, Puberty Stress = 16), *$P < 0.05$ versus CTRL, results are the mean ± s.e.m.

impact of stress. An additional point to mention is the fact that our study was performed only in male, not in female rats. For this reason, we did not explicitly include a cohort exposed to stress during the intermediate period comprising P34 and P36, which corresponds to the onset of puberty in female rats. However, as female rats exposed to the Full Peripubertal Stress also develop increased aggression,[59] it will be important in future studies to investigate potential sex differences linked to the timing and chronicity of stress exposure during the developmental periods under study.

The amygdala is a brain area highly sensitive to the effects of early-life stress.[5,12] The CeA has been implicated in anxiety[40,41] as well as in the regulation of social[33–35] and aggressive behaviors.[14,36,37] Our previous work showed an enhanced activation of the CeA in adult rats subjected to the Full Peripubertal Stress protocol, and effective modeling established a key role for the activity in this brain region to predict aggressive behavior.[14] Here, we found that the same Full Peripubertal Stress protocol increased NR1 and reduced GAD67 mRNA expression as well as increased the VGLUT1/VGAT ratio in the CeA. Although a limitation of the current study is that the reported changes were made at the gene expression but not at the protein level, the reduction observed in GAD67 mRNA is in agreement with our recent observations showing reduced GAD protein expression across different amygdala nuclei (including the CeA) in rats submitted to the Full Peripubertal Stress.[32]

Given the well-known impact of stress in glutamatergic transmission,[60–62] the upregulation of NR1—the obligatory sub-unit of the NMDA receptor—found in the CeA of Full Peripubertal Stress animals might be secondary to increased glutamatergic
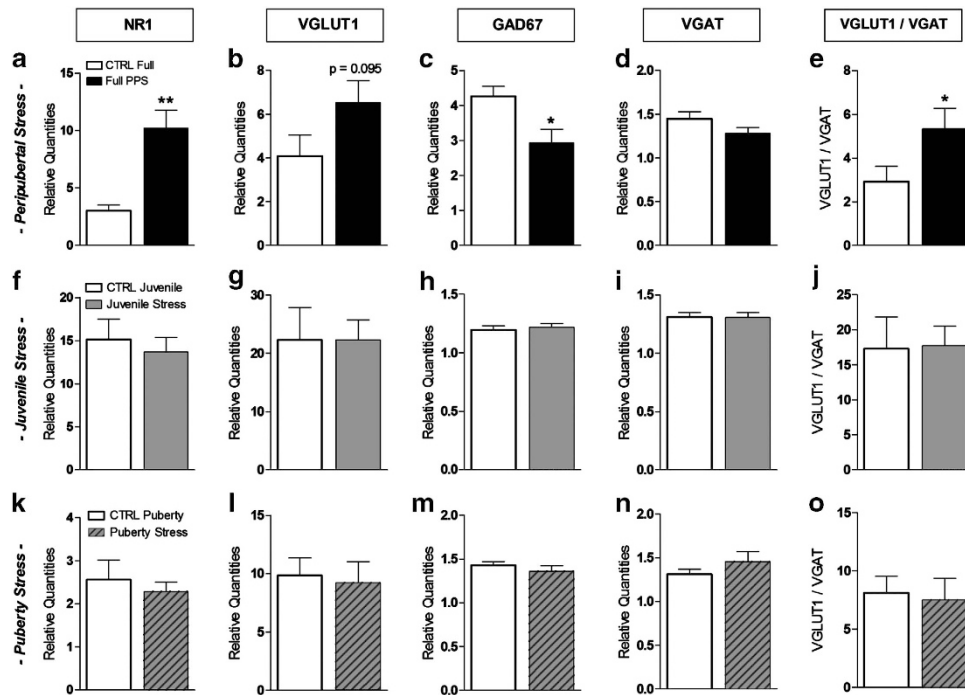
**Figure 5.** Gene expression analysis in the central amygdala. Compared with their respective CTRLs (CTRL Full), rats exposed to the entire stress protocol (Full PPS or Peripubertal Stress) showed an increase in the expression of NR1 mRNA (**a**), a trend toward an increase in VGLUT1 mRNA levels (**b**) and a decrease in GAD67 mRNA levels (**c**). Furthermore, no difference was found in the expression of VGAT mRNA between Full PPS and CTRL Full rats (**d**), whereas Full PPS rats showed a higher VGLUT1/VGAT ratio than CTRL Full rats (**e**). Juvenile Stress (**f**–**j**) and Puberty Stress (**k**–**o**) rats exhibited no changes in the expression of the genes examined here. (N: CTRL Full = 12–13, Full PPS = 12–15, CTRL Juvenile = 10, Juvenile Stress = 20, CTRL Puberty = 13–14, Puberty Stress = 14–16), *$P < 0.05$, ** $P < 0.01$ versus CTRL, results are the mean ± s.e.m.

transmission occurring during stress exposure. Importantly, increased glutamate efflux has been specifically reported in the rat CeA following exposure to acute stress.[63] Moreover, an increased expression of NR1 in the CeA has also been reported following alcohol consumption[64] and cocaine withdrawal,[65] two conditions that have been associated with increased anxiety and aggression.[66–69] Conversely, the reduced expression of GAD67—-an enzyme critically involved in the activity-dependent synthesis of GABA—that we detected in Full Peripubertal Stress rats, is consistent with the reduction in GAD67 reported in the brains of patients with neuropsychiatric disorders that typically present anxiety and/or social dysfunctions,[70] schizophrenia[71] and depression.[72] Importantly, in animals, the CeA has been shown to be particularly sensitive in displaying alterations in GAD67 expression levels following exposure to stress in early life,[73] whereas increased expression of GAD67 in limbic regions is typically found when stress is delivered at adulthood.[74,75] Evidence for a potential causal link with behavioral alterations comes from studies involving microinfusion of GABAergic antagonists into the rat amygdala that reported diminished social interactions.[76]

We also showed that the alterations in the expression of the genes examined in the CeA were not found in animals exposed only to the early or the late phase of the peripubertal stress protocol, that is, the Juvenile Stress or the Puberty Stress groups. These results should be interpreted with caution and certainly not as a proof that the shorter exposure to stressful events during these developmental periods is devoid of long-term consequences in the excitation/inhibition pathways. Previous work suggests that juvenile stress, when combined with emotional challenges in adulthood, leads to more pronounced behavioral[77,78] and neurobiological alterations.[79,80] In particular, region- and subunit-specific regulation of the GABA-A receptor expression profile within the limbic system has been found in

adult rats that have been stressed as juveniles but only if they are emotionally challenged in adulthood,[79] an effect that was particularly evident in the amygdala.[79,80] Therefore, exposure to juvenile and/or pubertal stress may provide an increased vulnerability to adult stress exposure and lead to latent alterations that can be observed when individuals are exposed to a second challenge. Indeed, this may be the case for our Full Peripubertal Stress protocol, such that latent changes induced by stress exposure during the juvenile period become effective upon further exposure to stress during the subsequent puberty phase. Note, however, that, although we found no evidence for the modulation of novel object reactivity or sociability with the shorter stress protocols, total aggressive behavior was mildly, although not significantly, increased in both groups. Interestingly, stress during juvenility resulted in a significant increase in the offensive upright type of aggressive behavior. Consistent with previous data in which stress was administered during different early developmental periods,[45,81,82] aggressive behavior appears to be particularly susceptible to long-term programming by early stress exposure. Moreover, we found evidence that could indicate increased anxiety-like behavior both in the Juvenile Stress and the Puberty Stress groups, as they showed decreased entries in the center of the arena and decreased distance traveled, respectively. These findings, concerning particularly the Juvenile Stress group, are in agreement with studies focusing on environmental stress during this period, and which similarly reported fewer crossings in an open field.[55,56,79]

Therefore, our study showed changes in the long-term programming of genes involved in excitatory and inhibitory neurotransmission in the CeA in the context of peripubertal stress-induced behavioral emotional and social alterations. Given the proposed role for the imbalance in the excitation/inhibition ratio in certain developmental psychopathologies involving social dysfunction, such as schizophrenia, our findings suggest that

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exposure to stress during peripuberty may enhance the vulnerability to neurodevelopmental perturbations elicited by genetic and/or other insults in the context of psychopathology.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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