

# Exposure to Stressors during Juvenility Disrupts Development-Related Alterations in the PSA-NCAM to NCAM Expression Ratio: Potential Relevance for Mood and Anxiety Disorders

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Childhood trauma is associated with higher rates of both mood and anxiety disorders in adulthood. The exposure of rats to stressors during juvenility has comparable effects, and was suggested as a model of induced predisposition for these disorders. The neural cell adhesion molecule (NCAM) and its polysialylated form PSA-NCAM are critically involved in neural development, activity-dependent synaptic plasticity, and learning processes. We examined the effects of exposure to stressors during juvenility on coping with stressors in adulthood and on NCAM and PSA-NCAM expression within the rat limbic system both soon after the exposure and in adulthood. Exposure to stressors during juvenility reduced novel-setting exploration and impaired two-way shuttle avoidance learning in adulthood. Among naive rats, a development-related decrease of about 50% was evident in the PSA-NCAM to NCAM expression ratio in the basolateral amygdala, in the CA1 and dentate gyrus regions of the hippocampus, and in the entorhinal cortex. In juvenile-stressed rats, we found no such decrease, but rather an increase in the polysialylation of NCAM (~50%), evident soon after the exposure to juvenile stress and also in adulthood. Our results suggest that exposure to stressors during juvenility alters the maturation of the limbic system, and potentially underlies the predisposition to exhibit stress-related symptoms in adulthood.

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## INTRODUCTION

Accumulating evidence indicates that chronic stress or continuous glucocorticoid elevation in adult rats affects the functioning of neural cell adhesion molecules (NCAMs), particularly NCAM and its polysialylated form PSA-NCAM (Sandi, 2004). NCAMs are membrane-bound glycoproteins of the immunoglobulin superfamily of adhesion molecules, which mediate cell–cell interactions; by interacting with cytoskeletal components, they can activate specific intracellular signaling pathways (Cremer *et al*, 1997). These molecules play a pivotal role in neural development and regeneration, and are strongly implicated in synaptic plasticity and memory formation processes (Schachner, 1997; Kiss *et al*, 2001; Sandi, 2004). NCAMs can be polysialylated on their extracellular domains by attachment

of long  $\alpha 2$ , 8-linked polysialic acid (PSA) homopolymer chains (Hoffman *et al*, 1982; Friedlander *et al*, 1985). This posttranslational modification, which is crucial to their morphoregulatory functions (Dey *et al*, 1999), weakens the adhesive properties of NCAM (Schachner, 1997) and is believed to inhibit NCAM-mediated interactions (Doherty and Walsh, 1991; Cremer *et al*, 1997; Schachner, 1997; Sandi *et al*, 2001). Thus, it was suggested that PSA-NCAM acts as a plasticity promoter by decreasing overall cell adhesion, thereby allowing structural remodeling to occur (Rutishauser and Landmesser, 1996), whereas NCAM acts as a stability promoter (Rønn *et al*, 2000). Normal brain development also involves temporal and spatial alterations in the expression of these NCAMs (Edelman, 1984; Rutishauser and Jessell, 1988; Rutishauser, 1989).

During early development, PSA-NCAM is widely expressed, but in the adult brain its expression is confined to a few restricted areas characterized by a high level of structural remodeling, such as the hippocampal formation (Rønn *et al*, 2000). The transient re-expression of PSA-NCAM was also associated with activity-dependent synaptic remodeling (Rønn *et al*, 1998, 2000). Throughout development, expression of the plasticity-promoting PSA-NCAM decreases,

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whereas the relative expression of the nonpolysialylated, stability-promoting NCAM increases (Rønn *et al*, 1998).

Developmentally regulated changes in PSA-NCAM expression influence fundamental morphogenetic processes, such as neuronal migration, neurite elongation, and synaptogenesis (Doherty and Walsh, 1991; Rutishauser and Landmesser, 1996) by modulating neuron–neuron (Nybroe and Bock, 1990), neuron–astrocyte (Keilhauer *et al*, 1985), and neuron–substratum (Covault, 1989) recognition and adhesion (Rutishauser, 1990). NCAM expression and its posttranslational polysialylation, which decreases the adhesive strength of NCAM, are critically involved in memory formation and activity-dependent synaptic remodeling processes (Doyle *et al*, 1992b; Lüthi *et al*, 1994; Rose, 1995; Arami *et al*, 1996; Becker *et al*, 1996; Foley *et al*, 2000; Welzl and Stork, 2003). Learning is also characterized by altered expression and glycosylation of NCAM (Doyle *et al*, 1992a; Rusakov *et al*, 1994; O'Connell *et al*, 1997; Tiunova *et al*, 1998; Sandi *et al*, 2004). Targeted disruption of the NCAM gene resulted in learning deficits (Cremer *et al*, 1994; Stork *et al*, 2000) accompanied by alterations in emotional/motivational behavior and by hyperresponsiveness of the serotonergic system (Stork *et al*, 1999, 2000).

A series of studies showed that chronic stress protocols known to produce cognitive and neural alterations (McEwen, 1999) markedly affected the expression of NCAMs in the hippocampus and other brain areas (Sandi, 2004). Chronic restraint stress (21 days  $\times$  6 h) diminished NCAM mRNA and protein expression in the hippocampus and other brain regions (Sandi *et al*, 2001; Venero *et al*, 2002); it did not alter the expression of NCAM-180 kDa isoform mRNA (Venero *et al*, 2002) but did specifically reduce the expression of NCAM-140 kDa isoform protein (Touyarot and Sandi, 2002). This reduction in NCAM-140 kDa expression levels was also observed following a social stress procedure, and seemed to be particularly pronounced in stressed rats exhibiting impaired water maze spatial learning as well as increased levels of corticosterone (CORT) (Touyarot *et al*, 2004).

Polysialylation of NCAM is also affected by stress. A chronic 3-week restraint stress protocol, which decreased cell proliferation in the hippocampus dentate gyrus (DG) (Sandi, 2004), also increased PSA-NCAM expression in the DG (Sandi *et al*, 2001) and in the CA3 section of the hippocampus (Nacher *et al*, 2004; Sandi, 2004), although downregulating PSA-NCAM expression in the amygdala complex (Cordero *et al*, 2005).

Though much is known about the effects of chronic stress on the functioning of NCAM in adult rats, relatively little is known about how these molecules are affected by early life stress (ELS) (Sandi, 2004). Recent evidence indicates that both prenatal and perinatal stress exposure causes profound downregulation of hippocampal and cortical NCAM expression, as well as long-lasting cognitive alterations in the adult offspring, with a parallel reduction in brain-derived neurotrophic factor (BDNF) and in the synaptic marker synaptophysin (Koo *et al*, 2003).

Stressful and traumatic experiences early in life predispose individuals to develop mood and anxiety disorders, and were shown to be associated with multiple endocrine and anatomical changes in the neuronal circuits that are critically involved in modulating stress responses,

emotional processing, and learning within the limbic system (Heim and Nemeroff, 2001; Heim *et al*, 2004; Nemeroff, 2004).

Most ELS rodent models focus on the perinatal to preweaning periods and involve some form of maternal deprivation or separation (for a review, see Sanchez *et al*, 2001). However, we focused on an alternative ELS-sensitive period in the rat ontogeny, namely the juvenile stage ( $\sim$ 28 days), the earlier phase of the adolescent/postweaning to prepubertal period (Avital and Richter-Levin, 2005; Tsooary and Richter-Levin, 2006; Avital *et al*, 2006; Tsooary *et al*, 2007). During the adolescent period (21–42 days), substantial maturational processes occur in the rat limbic system, including in the hippocampus and amygdala-based neurocircuits (for a review, see Spear, 2000).

During the juvenile period, the hypothalamic-pituitary-adrenal (HPA) axis response reaches its developmental asymptote (Vazquez, 1998); however this response lasts considerably longer than in adults (Vazquez, 1998; Romeo *et al*, 2004). Romeo *et al* (2004) suggested that this slower shutoff of the HPA axis during juvenility might derive from there being less centrally mediated feedback from various underdeveloped forebrain limbic regions at this age.

Exposure to stressors during juvenility was reported to produce more pronounced effects than exposure at earlier or later ages, affecting object exploration in adulthood (Einon and Morgan, 1977), fluid intake (McGivern *et al*, 1996), and adulthood social and nonsocial behaviors associated with dysregulation of endogenous opioid system development (Van den Berg *et al*, 1999a, b, c, 2000).

Several studies suggested that exposure to stressors during juvenility affects limbic system development, which may lead to enduring effects on coping with stressors in adulthood. Adult rats chronically exposed to variable stressors throughout juvenility had an enhanced acoustic startle response similar to patients with posttraumatic stress disorder (PTSD) (Maslova *et al*, 2002). Exposure to acute stressors during juvenility produces increased vulnerability to stressful events in adulthood, resulting in an augmented response to adverse experiences (Avital and Richter-Levin, 2005). In addition, a short-term juvenile exposure to variable stressors produced two types of impaired avoidance learning reminiscent of symptoms of both mood and anxiety disorders (Tsooary and Richter-Levin, 2006; Tsooary *et al*, 2007).

Exposure to the juvenile short-term variable stressor protocol transiently delayed body weight gain (Horovitz *et al*, 2007). In comparison with control (unexposed) rats, juvenile-stressed rats exhibited less body weight gain when examined soon after the exposure (at 34 days of age). However, later on during the maturation process (at 41, 48, 55, and 62 days of age), this difference was not evident. A two-way analysis of variance (ANOVA) of percent body weight gain for age and groups (control, juvenile-stressed) revealed a main effect for age ( $F(3,14) = 1642.61$ ;  $p < 0.01$ ), but not for groups ( $F(1,14) = 2.94$ ; NS) and not for the interaction between age and groups ( $F(3,14) = 1.09$ ; NS). Subsequent *t*-tests compared percent body weight gain between the groups at each of the noted ages and found a significant difference only at the age of 34 days ( $C = 170.81 \pm 2.23$ ;  $J = 162.73 \pm 0.93$ ;  $t(14) = 3.34$ ;  $p < 0.01$ ), indicating that though the stressor affected body weight gain

in the short run, in the long run juvenile-stressed rats continue to develop normally in terms of their body weight gain (Horovitz *et al*, 2007). This transient effect may be owing to the timing of the exposure to stressors, ie juvenility, close to their growth spurt at 4–5 weeks (Spear, 2000), which may have overshadowed any stress-related body weight reduction. Alternatively, the lack of a long-term effect on body weight may be due to the fact that stress-related reductions in body weight are prevalent following chronic stress procedures (eg Bekris *et al*, 2005; Cordero *et al*, 2005), but not following acute stress procedures (eg Mizoguchi *et al*, 2001; Retana-Marquez *et al*, 2003). Mizoguchi *et al* (2001) suggested that this difference might be associated with the differential effects these procedures have on HPA axis responses.

Furthermore, exposure to stressors during juvenility affected the HPA axis response to stressors in adulthood (Tssoory *et al*, 2004). A comparison of the baseline CORT levels ( $\mu\text{g/ml}$ ) of adult rats (60 days old) was undertaken between naive (N) rats and rats exposed to a brief predator scent stressor during juvenility (J, at 28 days of age) or during adulthood (A, at 59 days of age), or during both juvenility and adulthood (J + A, at 28 days of age and again at 59 days of age) (for a full description of the stress procedure and groups see Tssoory *et al*, 2007). An ANOVA indicated a significant main effect for these groups ( $F(3,30) = 18.36$ ;  $p < 0.05$ ). Scheffe *post hoc* tests revealed that compared to the baseline CORT levels of naive rats ( $N = 139.97 \pm 28.18$ ;  $n = 10$ ), only rats which were exposed to the stressor in adulthood alone exhibited significantly ( $p < 0.05$ ) increased baseline CORT levels ( $A = 263.11 \pm 42.28$ ;  $n = 8$ ), whereas rats which were exposed to the stressor only during juvenility ( $J = 16.76 \pm 2.42$ ;  $n = 8$ ) and those exposed to it during both juvenility and adulthood ( $J + A = 25.70 \pm 4.59$ ;  $n = 8$ ) exhibited significantly lower baseline CORT levels ( $p < 0.05$ ). The baseline CORT levels of J and J + A rats did not differ from each other. Taken together, with the substantial difference found between the baseline CORT levels of A and J + A rats, it was suggested that the juvenile exposure altered the consequences of the later, adulthood, exposure. Similar differences in HPA axis responses were recently reported among PTSD patients (Santa-Ana *et al*, 2006). Following a physical challenge (the cold pressor task), adult PTSD patients who experienced childhood trauma exhibited a blunted HPA axis response (ie significantly lower cortisol levels at baseline and throughout the 2 h following the challenge) compared to both control subjects and PTSD patients who were traumatized in adulthood. Control subjects and PTSD patients traumatized in adulthood did not differ in their HPA axis responses. The authors suggested that this might indicate that childhood trauma confers a greater impairment on HPA axis responses than does traumatization later in life (Santa-Ana *et al*, 2006).

Utilizing our 'juvenile stress' animal model (Tssoory and Richter-Levin, 2006), the present study sought to examine whether the 'juvenile stress' protocol that induces a predisposition for impaired coping with stressors in adulthood also affects NCAM and PSA-NCAM development-related alterations. To this end, we examined the effects of exposure to 'juvenile stress' on NCAM and PSA-NCAM expression both soon after the exposure and in adulthood, whereas

reconfirming our previous findings regarding altered emotional responses in adult 'juvenile-stressed' rats.

## MATERIALS AND METHODS

### Study Design

To evaluate the consequences of exposure to stressors during juvenility, on coping with stressors in adulthood, and to assess development-related changes in NCAM and PSA-NCAM expression, rats were randomly exposed to a short-term stress protocol during juvenility (juvenile-stressed) (27–29 days of age) or not exposed (naive). In each of these conditions, about a third of the rats were designated for behavioral assessment of coping with stressors in adulthood, to confirm our previous long-term behavioral effects findings. From the remaining two thirds, limbic brain tissues were collected for later immunoblotting. Collection took place either soon after the exposure, at the age of 33 days, or later in adulthood at the age of 9 weeks. Thus, limbic brain regions were harvested from the following four groups:

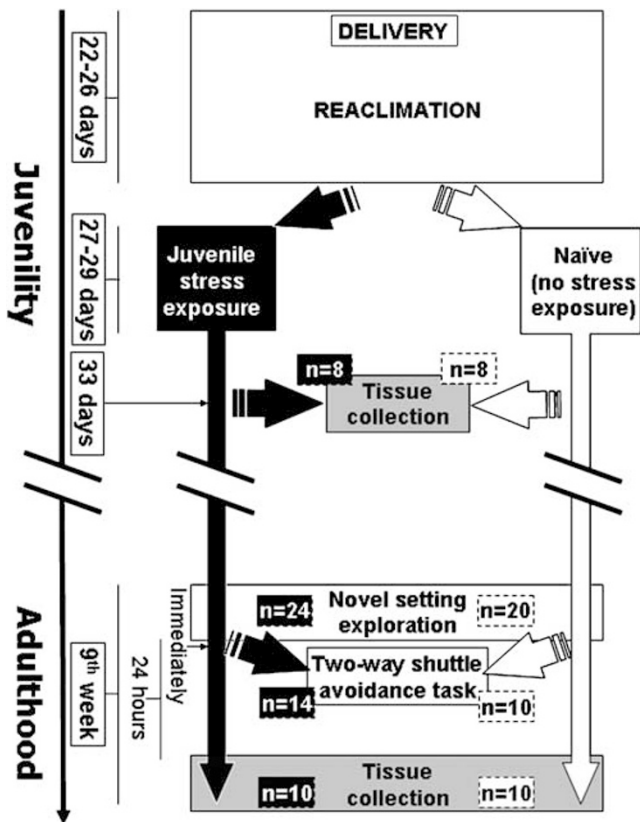
- (1) Juvenile-stressed rats (J-S): 33-day-old rats exposed to a short-term stress protocol during juvenility (27–29 days of age);  $n = 8$ .
- (2) Juvenile naive rats (J-N): 33-day-old naive rats, not exposed to adverse conditions;  $n = 8$ .
- (3) Adult juvenile-stressed rats (A-JS): 9-week-old rats previously exposed to the stress protocol during juvenility (27–29 days of age);  $n = 10$ .
- (4) Adult naive rats (A-N): 9-week-old naive rats, not exposed to adverse conditions;  $n = 10$ .

As depicted in Figure 1, to ascertain the long-term behavioral effects of exposure to stressors during juvenility, we verified its effect on novel-setting exploration in all the adult rats (A-N:  $n = 20$ ; A-JS:  $n = 24$ ). Following this test, about a half of all the adult rats (A-N:  $n = 10$ ; A-JS:  $n = 14$ ) were trained in the two-way shuttle avoidance task to confirm previous results regarding impaired learning under stressful conditions. No tissue was collected from these rats. Twenty-four hours following the exploratory assessment, limbic brain regions were collected from the adult rats, which were not trained in the avoidance task: the above-noted groups 3 (A-JS:  $n = 10$ ) and 4 (A-N:  $n = 10$ ).

### Animals

Sixty male Sprague–Dawley rats, 22 days old, weighing 35–49 g on delivery from Harlan Laboratories Jerusalem, were maintained for the entire duration of the experiment in the laboratory's vivarium. Vivarium conditions: lighting, 12 h light–dark cycle, lights on at 0700; temperature,  $22 \pm 2^\circ\text{C}$ ; cages, floor area of  $2653.5 \text{ cm}^2$  ( $610 \times 435 \text{ mm}$ ), height, 215 mm (Model # 2000P, TECNIPLAST S.p.a., Varese, Italy), 3–4 rats per cage, bedding of sterilized pine sawdust; Diet: *ad libitum* water and solid food pellets (Teklad Global Diet 2018S, Harlan Teklad Ltd, WI, USA).

All stress procedures and behavioral assessments were conducted in designated experiment rooms away from the vivarium, were approved by the institutional animal care



**Figure 1** Study design: Following weaning at the age of 21 days, 22-day-old male Sprague–Dawley rats were supplied by Harlan Laboratories (Jerusalem) and, after 5 days of acclimation to the vivarium, were randomly assigned to be exposed to stressors during juvenility (juvenile-stressed: black textboxes and arrows) or not exposed (naive: white textboxes and arrows). When the rats were 33-days old, limbic brain regions were harvested from some members of each group (J-S,  $n=8$ ; J-N,  $n=8$ ). To ascertain the long-term behavioral effects of exposure to stressors during juvenility, we verified its effect on novel-setting exploration by the remaining rats when they reached adulthood (9 weeks) (A-N,  $n=20$ ; A-JS,  $n=24$ ). Immediately following this test, about half of the adult rats (A-N,  $n=10$ ; A-JS,  $n=14$ ) were trained in the two-way shuttle avoidance task to ascertain impaired learning under stressful conditions. No tissue was collected from these rats, whereas from the other half, which were not trained in the shuttle avoidance task (A-JS,  $n=10$ ; A-N,  $n=10$ ), limbic brain regions were collected 24 hours after the exploration assessment.

committee, and adhered to the NIH Guide for the Care and Use of Laboratory Animals.

### ‘Juvenile Stress’ Procedure

Research indicates that variable stressor protocols elicit stronger stress responses than acute or repeated stressor protocols in adult rats (Garcia-Vallejo *et al*, 1998; Prieto *et al*, 2003; Simpkins and Devine, 2003), and that prepubertal male rats exhibit an enhanced stress response as compared with adults (Romeo *et al*, 2004). On the basis of these findings, we designed a juvenile short-term variable stressor protocol (Tsory and Richter-Levin, 2006; Tsory *et al*, 2007).

Rats in the juvenile stress group were exposed to a different stressor every day for 3 days (see below). Stress exposure took place during juvenility (ages 27–29 days) at

approximately midday (1200–1400) in designated experimental rooms (a different room each day) away from the vivarium. Each of the stressors was chosen for its well-documented effects on adult rats.

- Day 1 (aged 27 days) *Forced swim*: 10 min forced swim in an opaque circular water tank (diameter 0.5 m; height 0.5 m; water depth 0.4 m), water temperature  $22 \pm 2^\circ\text{C}$  (adapted from Avital *et al*, 2001). Similar forced swim protocols affected the stress-response mechanisms of adult rats (Hall *et al*, 2001).
- Day 2 (aged 28 days) *Elevated platform*: three 30 min trials; intertrial interval (ITI), 60 min in the home cage. Elevated platform,  $12 \times 12$  cm at a height of 70 cm above floor level, located in the middle of a small closet-like room (adapted from Maroun and Richter-Levin, 2003). Similar elevated platform protocols affected the stress-response mechanisms of adult rats (Degroot *et al*, 2004; Ebner *et al*, 2004).
- Day 3 (aged 29 days) *Foot-shock or restraint stressors*: rats were randomly subjected to a short foot-shock session or to 2 hours of restraint. This was carried out to minimize possible attenuation of stress-induced increase in anxiety owing to similarities between the electric foot shocks used in the juvenile stress protocol and those used later in adulthood in the two-way shuttle avoidance task. No differences were observed between rats exposed to the foot-shock stressor or the restraint stressor procedures in any of the measured indices.

### Foot Shock

A 3-min session of six unconditioned electric foot shocks (1 s, 0.8 mA); ITI: 29 s; apparatus: a small cube-like chamber ( $31 \times 31 \times 31$  cm) with a metal grid floor connected to a computer-controlled electrical shocker device (Solid State Shocker/Scrambler, Model no. 113–33, Lehigh Valley Electronics Ltd, Lehigh Valley, PA, USA). Similar foot shock procedures affected the stress-response mechanisms of adult rats (Li and Sawchenko, 1998; Passerin *et al*, 2000; Pezzone *et al*, 1993, 1992; Rassnick *et al*, 1998).

### Restraint

Rats were placed in a metal mesh-restraining box ( $11 \times 5 \times 4$  cm) that prevented forward-backward movement and limited side-to-side mobility, but did not discomfort the animal in any other way. Rats remained in the restraining box for 2 h at  $25^\circ\text{C}$  under dim illumination. Similar restraint procedures affected the stress-response mechanisms of adult rats (Pace *et al*, 2005; Martijena *et al*, 2002).

Protocols were applied in parallel to rats in the stress group, so as not to isolate any rat in its home cage. Upon completion of the each of the first two stress procedures, rats were returned to their home cage and were not handled until the next day. Following completion of the day 3 stress procedure, rats were returned to their home cage and were not handled again until either brain regions harvesting at the age of 33 days or behavioral assessments in adulthood at the age of 9 weeks, except for weekly cage maintenance.

## Behavioral Assessments in Adulthood

In adulthood, at the age of 9 weeks, the novel-setting exploratory behavior of rats and their learning under stressful conditions (ie during a two-way shuttle avoidance task) were assessed. This was carried out by a group of three experimenters who were aware of which group each rat belonged to, as they had to run the tests in semirandom order to avoid a 'group order effect'. However, data collection was performed by an automated, PC-controlled, system.

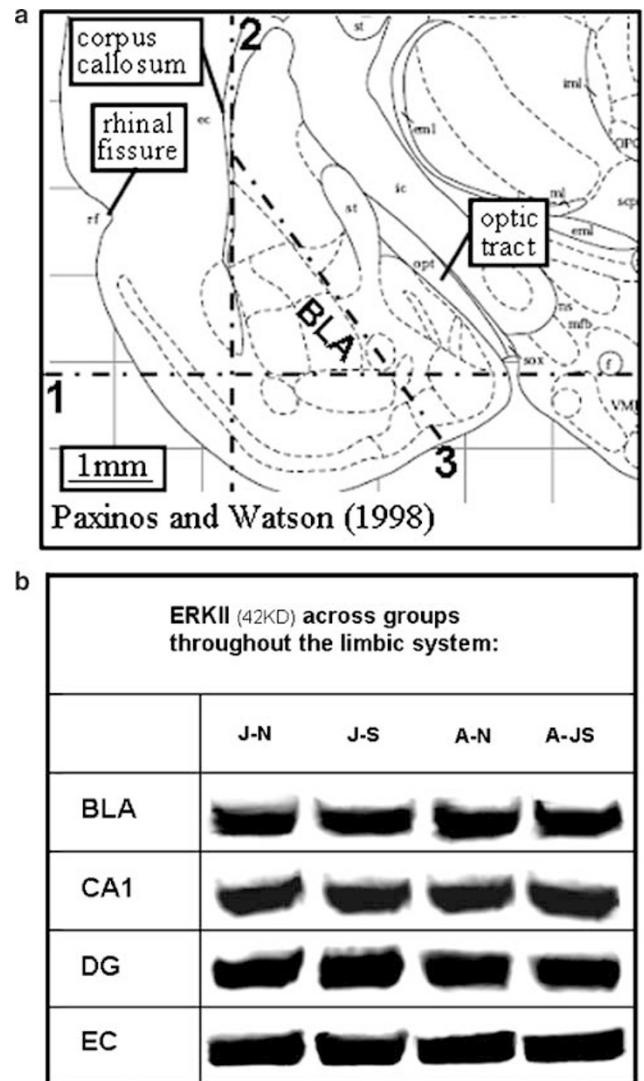
- **Novel-setting exploration.** Rats were placed in the two-way shuttle avoidance apparatus described below, although it was in an inoperative mode, and were allowed to explore both compartments for a total of 10 min. Crossingover between compartments provided an index of exploratory behavior. If a rat did not visit the adjacent compartment within 5 min, it was gently, manually directed through the door. The same was performed if the rat failed to cross back into the first compartment within another 5 min. An observing experimenter monitored the exploratory shuttles of the rats. Only voluntary shuttles were counted.
- **Two-way shuttle avoidance task.** Immediately following the exploratory behavior assessment, about a half of all adult rats (as noted above in 'Study Design' and in Figure 1) were trained in the two-way shuttle avoidance task in a single 100-trial session. **Apparatus:** The two-way shuttle avoidance box, placed in a dimly lit, ventilated, sound-attenuated cupboard, is a rectangular chamber (60 × 26 × 28 cm) divided by an opaque partition with a small passage (10 × 8 cm) connecting two equally sized, side-by-side, cube-shaped compartments. The metal grid floors of both compartments are weight sensitive, with microswitches transmitting information on the rat's location to a computer-controlled and automated data collection program. This program controls both conditioned stimulus (CS) presentations (a tone produced by loudspeakers located on the distal walls of the compartments) and unconditioned stimulus (US) electric shock deliveries (electrical shocker device: Solid State Shocker/Distributor, Model # 113-33, Coulbourn Instruments Inc., Lehigh Valley, PA, USA) as well as recording the rats' responses. **Procedure:** One session of 100 'trace conditioning' trials. CS: 10 s tone presentation; US: immediately following termination of the CS, an electric shock (0.5 mA) was delivered for a maximum of 10 s; ITI: 60 ± 12 s. Rats could produce one of three responses: (1) Avoidance: shuttling to the adjacent compartment upon hearing the CS-tone; thereafter, the tone stopped, an ITI commenced, and the rat avoided the electric shock. (2) Escape: shuttling to the adjacent compartment although the shock was on; the shock terminated and an ITI commenced. (3) Escape failure: failing to move to the adjacent compartment; the ITI commenced at the completion of the 10 s foot shock, so the rat was subjected to the full duration of the electric shock.

## Limbic Brain Regions Harvesting

Rats were decapitated by a guillotine (Stoelting, Wood Dale, IL, USA) and the following brain regions were collected

based on rat brain coordinates (Paxinos and Watson, 1998): basolateral amygdalae (BLAs), left and right separately (Figure 2a), the CA1 and DG subregions of the hippocampus, and the entorhinal cortex (EC).

Immediately following decapitation, the whole brain was removed from the skull and placed on an ice-cooled glass dish. First, the rostral hippocampus was dissected out and then the dorsal CA1 and DG sections were separated. Then a thick (~1 mm) coronal slice was cut ~6 mm anterior of the interaural plane (indicated by the rostral end of the cerebellum), and the BLAs were cut out as indicated in Figure 2a. This involved: (1) cutting the ventral part of the



**Figure 2** (a) BLA dissection: The dashed lines represent the dissection procedure employed on a thick (~1 mm) coronal slice cut ~6 mm rostral of interaural plane (indicated by the anterior 'end' of the cerebellum). (1) Cutting the ventral part of the slice just below the base of the optic tract; (2) cutting orthogonally to the first cut and in parallel to the corpus callosum about 2 mm medially to the rhinal fissure; (3) cutting out a small isosceles (~1.5 mm) right triangle. (b) Monitoring protein concentrations across groups and brain regions:  $\alpha$ -extracellular signal-regulated kinase type II ( $\alpha$ -ERKII) staining (1:1000, II:  $\alpha$ -Rabbit 1:10 000, cell-signaling; 12% acrylamide) across groups and brain regions indicating equal amounts of loaded protein.

slice just below the base of the optic tract; (2) cutting orthogonally to the first cut and in parallel to the corpus callosum about 2 mm medial to the rhinal fissure; (3) cutting out a small isosceles (~1.5 mm) right triangle. The sample from the EC (a small, ~1.5 mm, isosceles triangle) was then dissected from the posterior ventral part of the cerebral cortex.

The regions were immediately homogenized in an ice-cold glass/Teflon homogenizer (885502-0019; Kontes Glass Company, Vineland, NJ, USA) using 50 Teflon/glass mortar strokes in 300  $\mu$ l of ice-cold lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 0.8 M NaCl, 10% glycerol, pH 7.2–7.4), to which the following protease inhibitors were freshly added: 1 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1  $\mu$ g/ml leupeptine, 1  $\mu$ g/ml aprotinin, and 50 mM NaF. A total of 30  $\mu$ l of each lysate was saved for further protein concentration by Bradford analysis. 5  $\times$  protein Laemmli denaturation buffer (17.5 ml of 5  $\times$  concentrated stacking buffer, pH 6.8, 7.5 ml glycerol, 10% sodium dodecyl sulfate, 2.33 g bromophenol blue) was added to each remaining lysate, thoroughly mixed and denatured for 5 min at 95°C. The denatured proteins were stored at –80°C for further analysis.

### Immunoblotting

Protein concentration was monitored using Bradford assay, and equal amounts of loaded protein were verified using  $\alpha$ -extracellular signal-regulated kinase type II (ERKII) staining (1:1000, II:  $\alpha$ -Rabbit 1:10 000, cell-signaling; 12% acrylamide). No differences were observed between the groups in terms of ERKII concentrations in any of the examined regions (Figure 2b).

Individual samples from each region of each rat (100–120  $\mu$ g/ $\mu$ l of lysates) were loaded onto 8% sodium dodecyl sulfate-polyacrylamide gel gels. Following electrophoresis, the gels were transferred by wet transfer tanks to nitrocellulose membranes and stained against  $\alpha$ -NCAM (NCAM (H-94) Santa Cruz, sc8305-1:500;  $\alpha$ -Rabbit 1:7000, Chemicon) and  $\alpha$ -PSA-NCAM (PSA-NCAM: Chemicon MAB5324-1:1000,  $\alpha$ -mouse 1:10000, Chemicon). The membranes were ECL-stained by luminol and  $\beta$ -coumaric acid. For each lane, the mean band intensity was relatively assessed using the NIH Scion program for totalNCAM (based on all three major NCAM isoforms: 120, 140, and 180 kDa) and for totalPSA-NCAM (based on all three PSA-NCAM isoforms: 140, 180, and 220 kDa).

### Statistical Analysis

The results are expressed as mean  $\pm$  SEM. Differences between groups in terms of exploratory behaviors were determined by Student's *t*-tests. Differences between the responses of each group in the two-way shuttle avoidance task were determined using a two-way ANOVA for groups and for blocks of 10 trials (repeated measure analysis) followed by Student's *t*-tests per block. The effects of exposure to stressors during juvenility and development on totalNCAM and totalPSA-NCAM expression, indexed by blotting mean band intensity, were determined within each of the brain regions using a two-way ANOVA for 'juvenile

stress' and development followed by subsequent Student's *t*-tests with Bonferroni corrections.

## RESULTS

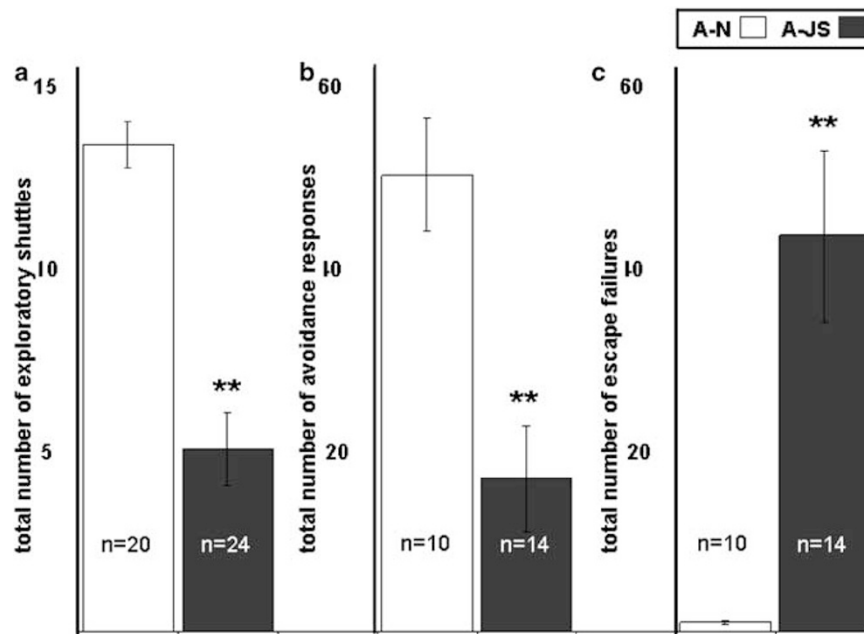
### The Effects of Exposure to Stressors during Juvenility on Adulthood Stress Responses

We have previously demonstrated that exposure to stressors during juvenility has long-lasting effects on rats' ability to cope with stressful situations in adulthood (Avital and Richter-Levin, 2005; Tsory and Richter-Levin, 2006; Tsory *et al*, 2007). The current study is aimed to assess the effects of exposure to stressors during juvenility on development-related changes in expression levels of NCAM and PSA-NCAM. Accordingly, we first verified the efficacy of the exposure to stressors during juvenility in the present study by examining its effects on adulthood coping responses by using stressful challenges, namely, novel-setting exploration and avoidance learning.

As in previous studies, exposure to stressors during juvenility significantly reduced adulthood exploratory behavior. Compared with adult naive rats (A-N, *n* = 20), adult juvenile-stressed rats (A-JS, *n* = 24) exhibited significantly less exploration of the novel setting, namely the shuttle box before avoidance learning ( $t(42) = 5.29$ ;  $p < 0.01$ ) (Figure 3a). Similarly, exposure to stressors during juvenility significantly impaired adulthood learning under stressful conditions, that is, it reduced the rates of avoidance responses and increased the rates of escape failures in the learning of the two-way shuttle avoidance task. Repeated measure analysis of avoidance shuttles per block of 10 trials revealed a significant main effect for groups ( $F(1,17) = 14.20$ ;  $p < 0.01$ ), for blocks ( $F(3,57) = 15.89$ ;  $p < 0.01$ ), and for their interaction ( $F(3,57) = 3.65$ ;  $p < 0.05$ ). Additional *t*-tests per block showed that adult juvenile-stressed rats (A-JS, *n* = 14) performed significantly fewer avoidance shuttles than naive adult rats (A-N, *n* = 10) in blocks 1, 3, and 5–10. Accordingly, the number of total avoidance shuttles by A-JS rats was significantly lower than that of naive adult rats ( $t(22) = 3.86$ ;  $p < 0.01$ ) (Figure 3b). No differences were found between these groups in terms of escape responses while learning the two-way shuttle avoidance task, but A-JS rats had higher rates of escape failure than naive adult rats. Repeated measure analysis for escape failures per block of 10 trials revealed a significant main effect for groups ( $F(1,17) = 10.19$ ;  $p < 0.01$ ) but not for blocks ( $F(3,51) = 0.99$ ; NS), nor for their interaction ( $F(3,51) = 0.85$ ; NS). Additional *t*-tests per block showed that A-JS rats (*n* = 14) exhibited significantly higher rates of escape failure than A-N rats (*n* = 10) in blocks 1–10. Accordingly, the number of total escape failure by A-JS rats was significantly higher than for A-N rats ( $t(22) = 4.59$ ;  $p < 0.001$ ) (Figure 3c).

### Alteration of TotalNCAM and TotalPSA-NCAM Development-Related Changes in Expression Following Exposure to Stressors during Juvenility

To evaluate the effects of exposure to stressors during juvenility on development-related changes in the expression of totalNCAM and totalPSA-NCAM, a two-way ANOVA was



**Figure 3** The effects of exposure to stressors during juvenility on novel-setting exploration and two-way shuttle avoidance learning in adulthood: exposure to stressors during juvenility significantly impaired coping with stressors in adulthood. Compared with adult naive rats (A-N), adult juvenile-stressed rats (A-JS) exhibited significantly fewer exploratory shuttles in a novel setting before avoidance learning (a); performed significantly fewer avoidance shuttles (b); and exhibited an increased rate of escape failures (c) whereas learning the two-way shuttle avoidance task. \*\*Significantly different from naive rats,  $p < 0.01$ .

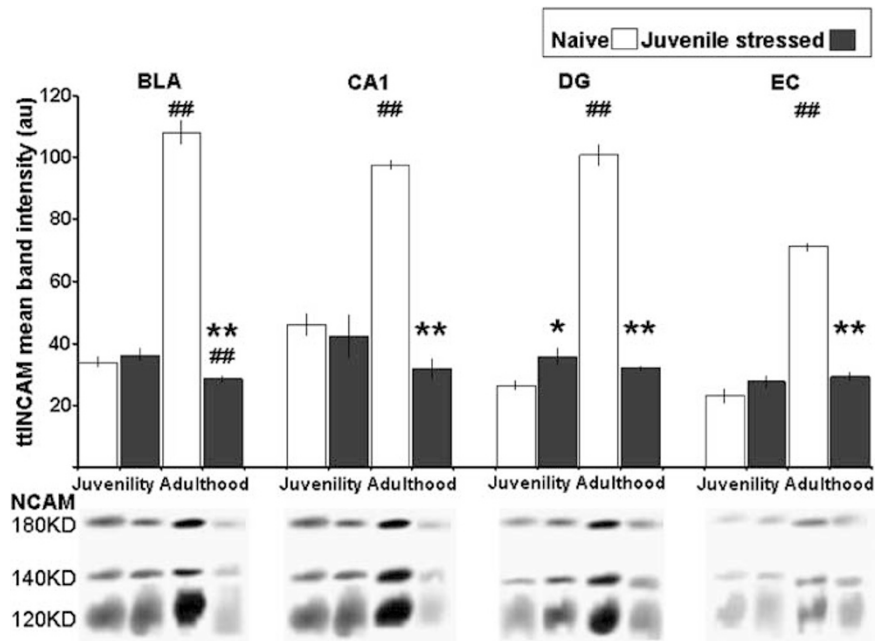
conducted on each of the monitored regions of the limbic system: the BLA (left and right pooled, although no laterality effect was evident), CA1, DG, and EC. The two-way ANOVAs compared juvenile-stressed rats ( $n = 18$  (J-S,  $n = 8 +$  A-JS,  $n = 10$ )) with naive rats ( $n = 18$  (J-N,  $n = 8 +$  A-N,  $n = 10$ )) to examine the effects of exposure to stressors during juvenility on totalNCAM and totalPSA-NCAM, and compared juvenile rats ( $n = 16$  (J-S,  $n = 8 +$  J-N,  $n = 8$ )) with adult rats ( $n = 20$  (A-JS,  $n = 10 +$  A-N,  $n = 10$ )) to examine the effects of development on these NCAMs.

#### Effects of Exposure to Stressors during Juvenility on Development-Related Alterations in the Expression Levels of TotalNCAM

Figure 4 depicts the effects of both exposure to stressors during juvenility and development on totalNCAM levels in each of the monitored regions of the limbic system. A two-way ANOVA for exposure to stressors during juvenility and for development indicated that in each of the monitored regions totalNCAM expression levels were significantly affected by juvenile stress exposure (BLA ( $F(1,32) = 208.69$ ;  $p < 0.01$ ); CA1 ( $F(1,32) = 75.08$ ;  $p < 0.01$ ); DG ( $F(1,32) = 131.21$ ;  $p < 0.01$ ); EC ( $F(1,32) = 107.04$ ;  $p < 0.01$ )) and by development (BLA ( $F(1,32) = 154.60$ ;  $p < 0.01$ ); CA1 ( $F(1,32) = 26.19$ ;  $p < 0.01$ ); DG ( $F(1,32) = 184.45$ ;  $p < 0.01$ ); EC ( $F(1,32) = 187.33$ ;  $p < 0.01$ )). The interaction between juvenile stress and development was also significant for totalNCAM in all the monitored limbic regions (BLA ( $F(1,32) = 234.12$ ;  $p < 0.01$ ); CA1 ( $F(1,32) = 59.43$ ;  $p < 0.01$ ); DG ( $F(1,32) = 226.94$ ;  $p < 0.01$ ); EC ( $F(1,32) = 162.60$ ;  $p < 0.01$ )).

*Examining the effect of exposure to juvenile stress on totalNCAM expression levels in each age group.* Subsequent *t*-tests with Bonferroni corrections were conducted to compare totalNCAM expression levels between juvenile stress-exposed and naive rats at juvenility and adulthood. These comparisons indicated that, during juvenility, totalNCAM levels did not differ significantly between juvenile-stressed rats (J-S;  $n = 8$ ) and naive rats (J-N,  $n = 8$ ) in the BLA ( $t(14) = 0.82$ ; NS), CA1 ( $t(14) = 0.49$ ; NS), and EC ( $t(14) = 1.39$ ; NS), whereas in the DG, there was a mild yet significant increase in the totalNCAM levels of J-S rats compared to J-N rats ( $t(14) = 2.81$ ;  $p < 0.05$ ). However, in adulthood, the expression levels of totalNCAM in adult juvenile-stressed rats (A-JS,  $n = 10$ ) appeared to be significantly lower than those of naive adult rats (A-N,  $n = 10$ ) in all examined regions (BLA ( $t(18) = 18.85$ ;  $p < 0.01$ ); CA1 ( $t(18) = 18.80$ ;  $p < 0.01$ ); DG ( $t(18) = 18.15$ ;  $p < 0.01$ ); EC ( $t(18) = 20.19$ ;  $p < 0.01$ )).

*Examining the effect of development on the totalNCAM expression levels among juvenile-stressed vs naive rats.* Additional *t*-tests with Bonferroni corrections compared juvenility and adulthood for rats exposed to stressors during juvenility and naive rats, and indicated that although among naive rats the totalNCAM levels significantly increased during maturation from juvenility into adulthood (BLA ( $t(16) = 16.98$ ;  $p < 0.01$ ); CA1 ( $t(16) = 13.28$ ;  $p < 0.01$ ); DG ( $t(16) = 18.43$ ;  $p < 0.01$ ); EC ( $t(16) = 18.07$ ;  $p < 0.01$ )), such development-related increases did not occur in rats exposed to stressors during juvenility in the CA1 ( $t(16) = 1.35$ ; NS), DG ( $t(16) = 1.27$ ; NS), and EC ( $t(16) = 0.64$ ; NS); whereas in the BLA a significant decrease was observed ( $t(16) = 3.02$ ;  $p < 0.05$ ).



**Figure 4** The effect of exposure to stressors during juvenility and the effect of development on totalNCAM levels across the limbic system. A two-way ANOVA indicated that in each of the monitored regions: the BLA, the CA1 and DG regions of the hippocampus, and the EC, totalNCAM levels were significantly affected by exposure to stressors during juvenility, by development, and by the interaction of exposure to stressors during juvenility with development. The insets depict representative bands of NCAM isoforms across groups (juvenile naive, J-N; juvenile-stressed, J-S; adult naive rats, A-N; and adult juvenile-stressed rats, A-J-S) and in the examined limbic regions: BLA, CA1, DG, and EC. At juvenility, totalNCAM levels did not differ significantly between J-S ( $n = 8$ ) and J-N ( $n = 8$ ) rats in the BLA, CA1, and EC, whereas, in the DG, there was a mild but significant increase in the totalNCAM levels of J-S rats compared to J-N rats. In adulthood, the totalNCAM levels of A-J-S rats ( $n = 10$ ) were significantly and substantially lower than those of A-N rats ( $n = 10$ ) in all examined limbic regions. \*\*significantly different from age-matched naive rats,  $p < 0.01$ . \*significantly different from age-matched naive rats,  $p < 0.05$ . Among naive rats, totalNCAM levels increased significantly during maturation from juvenility to adulthood in all examined limbic regions. However, such development-related increases did not occur among juvenile-stressed rats in the CA1, DG, and EC regions, whereas, in the BLA, a significant decrease was observed. ##significantly different from juvenile rats of the same group,  $p < 0.01$ .

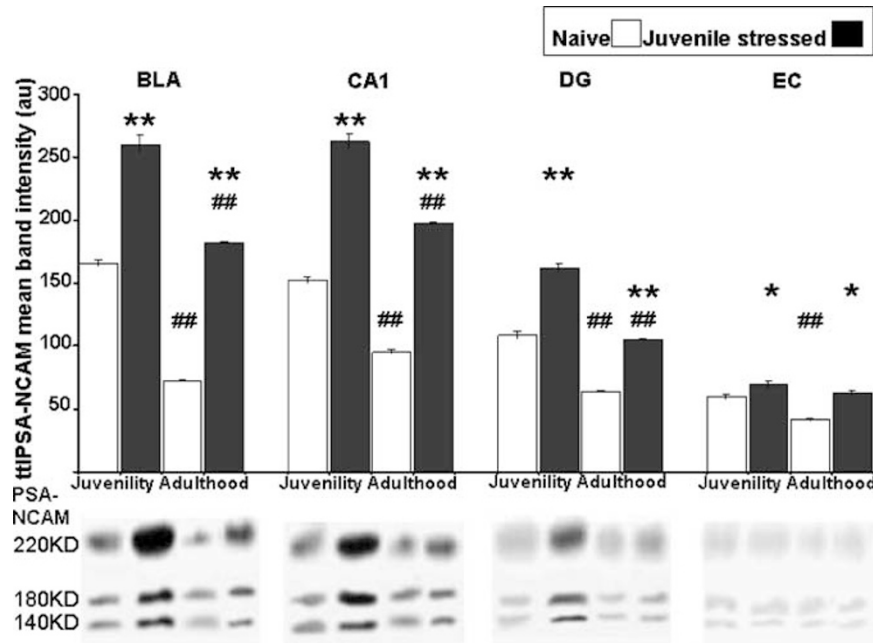
*Effects of exposure to stressors during juvenility on development-related alterations in the expression levels of totalPSA-NCAM.* Figure 5 depicts the effects of exposure to stressors during juvenility and the effects of development on totalPSA-NCAM levels in each of the monitored limbic regions. A two-way ANOVA for exposure to stressors during juvenility and for development indicated that in each of these regions totalPSA-NCAM expression levels were significantly affected by exposure to stressors during juvenility (BLA ( $F(1,32) = 887.23$ ;  $p < 0.01$ ); CA1 ( $F(1,32) = 1059.90$ ;  $p < 0.01$ ); DG ( $F(1,32) = 514.68$ ;  $p < 0.01$ ); EC ( $F(1,32) = 42.19$ ;  $p < 0.01$ )) and by development (BLA ( $F(1,32) = 617.40$ ;  $p < 0.01$ ); CA1 ( $F(1,32) = 352.73$ ;  $p < 0.01$ ); DG ( $F(1,32) = 595.31$ ;  $p < 0.01$ ); EC ( $F(1,32) = 28.83$ ;  $p < 0.01$ )). The interaction between juvenile stress and development was also significant for totalPSA-NCAM in the BLA ( $F(1,32) = 5.12$ ;  $p < 0.05$ ), DG ( $F(1,32) = 9.54$ ;  $p < 0.01$ ), and EC ( $F(1,32) = 6.80$ ;  $p < 0.05$ ), though not in the CA1 ( $F(1,32) = 1.54$ ; NS).

*Examining the effect of stress on totalPSA-NCAM expression levels in each age group.* Additional  $t$ -tests with Bonferroni corrections compared totalPSA-NCAM levels between juvenile stress-exposed and naive rats during juvenility and adulthood. These comparisons indicated that, during juvenility, totalPSA-NCAM levels were significantly higher in juvenile-stressed rats (J-S,  $n = 8$ ) than in naive rats (J-N,  $n = 8$ ) throughout the limbic system (BLA

( $t(14) = 12.60$ ;  $p < 0.01$ ); CA1 ( $t(14) = 16.45$ ;  $p < 0.01$ ); DG ( $t(14) = 12.46$ ;  $p < 0.01$ ); EC ( $t(14) = 2.60$ ;  $p < 0.05$ )). In adulthood, similarly increased totalPSA-NCAM levels were also evident in adult juvenile-stressed rats (A-JS,  $n = 10$ ) as compared with adult naive rats (A-N,  $n = 10$ ) throughout the limbic system (BLA ( $t(18) = 73.15$ ;  $p < 0.01$ ); CA1 ( $t(18) = 42.87$ ;  $p < 0.01$ ); DG ( $t(18) = 27.76$ ;  $p < 0.01$ ); EC ( $t(18) = 6.84$ ;  $p < 0.05$ )).

*Examining the effect of development on the totalPSA-NCAM expression levels among juvenile-stressed vs naive rats.* Further  $t$ -tests with Bonferroni corrections were conducted to compare totalPSA-NCAM levels between juvenility and adulthood in rats exposed to stressors during juvenility and in naive rats. These comparisons indicated that during maturation from juvenility into adulthood totalPSA-NCAM levels decreased significantly in naive rats (J-N,  $n = 8$  vs A-N,  $n = 10$ ) in the BLA ( $t(16) = 36.91$ ;  $p < 0.01$ ); CA1 ( $t(16) = 19.99$ ;  $p < 0.01$ ); DG ( $t(16) = 14.98$ ;  $p < 0.01$ ); and EC ( $t(16) = 7.17$ ;  $p < 0.01$ ), as well as in rats exposed to stressors during juvenility (J-S,  $n = 8$  vs A-JS,  $n = 10$ ) in the BLA ( $t(16) = 12.14$ ;  $p < 0.01$ ), CA1 ( $t(16) = 11.14$ ;  $p < 0.01$ ), and DG ( $t(16) = 19.55$ ;  $p < 0.01$ ), though not in the EC ( $t(16) = 1.66$ ; NS). However, as indicated by the previous  $t$ -test, despite the significant development-related decrease in totalPSA-NCAM levels, totalPSA-NCAM expression was still significantly higher in adult juvenile-stressed rats than in adult naive rats.





**Figure 5** The effect of exposure to stressors during juvenility and the effect of development on totalPSA-NCAM levels across the limbic system: a two-way ANOVA indicated that, in each of the monitored regions (the BLA, the CA1 and DG regions of the hippocampus, and the EC, totalPSA-NCAM levels were significantly affected by exposure to stressors during juvenility and by development. The interaction effect between exposure to stressors during juvenility and development was significant in the BLA, DG, and EC regions, but not in the CA1. The insets depict representative bands of PSA-NCAM isoforms across groups (juvenile naive, J-N; juvenile-stressed, J-S; adult naive rats, A-N; and adult juvenile-stressed rats, A-J-S) and in the examined limbic regions: BLA, CA1, DG, and EC. At juvenility, totalPSA-NCAM levels were significantly higher in J-S rats ( $n = 8$ ) than in J-N rats ( $n = 8$ ) throughout all four limbic regions: BLA, CA1, DG, and EC. Similar significant differences were evident in adulthood, with increased totalPSA-NCAM expression levels in A-JS rats ( $n = 10$ ) compared with A-N rats ( $n = 10$ ) in all examined limbic regions. \*\*significantly different from age-matched naive rats,  $p < 0.01$ . \*significantly different from age-matched naive rats,  $p < 0.05$ . Among naive rats, totalPSA-NCAM levels decreased significantly during maturation from juvenility to adulthood in all four limbic regions: BLA, CA1, DG, and EC. In juvenile-stressed rats, totalPSA-NCAM levels decreased significantly during maturation from juvenility to adulthood in the BLA, CA1, and DG regions, but not in the EC. ##significantly different from juvenile rats of the same group,  $p < 0.01$ .

### Effects of Exposure to Stressors during Juvenility on Development-Related Changes in the Relative Expression of NCAM and Its Polysialylated Form PSA-NCAM

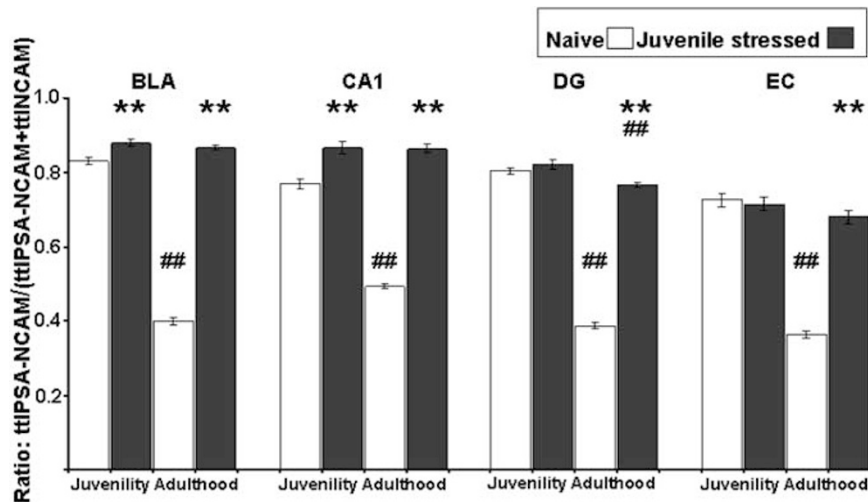
The developmental dynamics of NCAM and PSA-NCAM levels are opposite and complementary, with high levels of PSA-NCAM and relatively low levels of NCAM in early postnatal development, and the opposite in adulthood (Rønn *et al*, 1998). Chronic stress protocols in adult rats were also found to have opposite effects on the expression of NCAM and PSA-NCAM, decreasing the relative expression of NCAM, but increasing the relative expression of PSA-NCAM (Sandi, 2004).

We calculated the ratio of expression of totalPSA-NCAM to totalNCAM (totalPSA-NCAM/(totalNCAM + totalPSA-NCAM)) and examined stress-induced alterations to this ratio.

Figure 6 depicts the effects of exposure to stressors during juvenility and of development on the totalPSA-NCAM to totalNCAM expression ratio. As detailed below, a two-way ANOVA for exposure to stressors during juvenility and for development in each of the examined brain regions revealed a uniform pattern of effects on this ratio for exposure to stressors during juvenility, development, and for their interaction across all regions.

*Examining the effects of juvenile stress exposure and development by brain region.* Exposure to stressors during juvenility significantly affected the totalPSA-NCAM to totalNCAM expression ratio in each of the examined regions (BLA ( $F(1,32) = 857.19$ ;  $p < 0.01$ ); CA1 ( $F(1,32) = 367.70$ ;  $p < 0.01$ ); DG ( $F(1,32) = 589.94$ ;  $p < 0.01$ ); EC ( $F(1,32) = 87.09$ ;  $p < 0.01$ )). Similarly, a significant developmental effect was found in each region (BLA ( $F(1,32) = 637.24$ ;  $p < 0.01$ ); CA1 ( $F(1,32) = 131.41$ ;  $p < 0.01$ ); DG ( $F(1,32) = 842.90$ ;  $p < 0.01$ ); EC ( $F(1,32) = 147.29$ ;  $p < 0.01$ )), with significant interactions between exposure to stressors during juvenility and development in all examined regions (BLA ( $F(1,32) = 570.60$ ;  $p < 0.01$ ); CA1 ( $F(1,32) = 127.31$ ;  $p < 0.05$ ); DG ( $F(1,32) = 498.63$ ;  $p < 0.01$ ); EC ( $F(1,32) = 99.35$ ;  $p < 0.01$ )).

*Examining the effect of development on the totalPSA-NCAM to totalNCAM expression ratios among juvenile-stressed vs naive rats.* Additional *t*-tests with Bonferroni corrections compared the totalPSA-NCAM to totalNCAM expression ratios of juvenile stress-exposed and naive rats during juvenility and adulthood. These comparisons indicated that exposure to stressors during juvenility hindered the normal course of development, which manifests as a decrease in the totalPSA-NCAM to totalNCAM expression ratio from juvenility to adulthood.



**Figure 6** Effect of exposure to stressors during juvenility and effect of development on the totalPSA-NCAM to totalNCAM expression ratio across the limbic system: a two-way ANOVA for exposure to stressors during juvenility and development in each brain region indicated that the relative expression of totalPSA-NCAM to totalNCAM (totalPSA-NCAM/(totalPSA-NCAM + totalNCAM)) was affected by exposure to stressors during juvenility in a uniform manner across all tested limbic regions. A similar uniform pattern of effects was evident for development and for the interaction between exposure to stressors during juvenility and development. Comparisons of juvenile naive rats (J-N;  $n = 8$ ) with juvenile-stressed rats (J-S;  $n = 8$ ) revealed a mild yet significant stress-induced increase in the expression ratio of totalPSA-NCAM to totalNCAM soon after the exposure to stressors during juvenility in the BLA and in the CA1 region of the hippocampus, but not in the DG region of the hippocampus and EC. By contrast, in adulthood (adult naive (A-N);  $n = 10$ ; adult juvenile-stressed (A-JS);  $n = 10$ ) a significant and substantial juvenile stress-induced increase in the totalPSA-NCAM to totalNCAM expression ratio was evident in all four limbic regions: BLA, CA1, DG, and EC. \*\*Significantly different from age matched naive rats,  $p < 0.01$ . Exposure to stressors during juvenility disrupted the normative developmental decrease in the totalPSA-NCAM to totalNCAM expression ratio from juvenility to adulthood. A significant and substantial developmental decrease in the totalPSA-NCAM to totalNCAM expression ratio was evident only among naive rats (J-N,  $n = 8$ ; A-N,  $n = 10$ ) in all examined regions: BLA, CA1, DG, and EC. In contrast, among juvenile-stressed rats (J-S,  $n = 8$ ; A-JS,  $n = 10$ ), no differences in the totalPSA-NCAM to totalNCAM expression ratio were evident in the BLA, CA1 or EC, whereas the mild, but significant, decrease found only in the DG was incomparable with that found in naive rats. ##significantly different from juvenile rats of the same group,  $p < 0.01$ .

A significant developmental decrease in the totalPSA-NCAM to totalNCAM expression ratio in all limbic regions was evident only in naive rats (BLA ( $t(16) = 32.08$ ;  $p < 0.01$ ); CA1 ( $t(16) = 17.60$ ;  $p < 0.01$ ); DG ( $t(16) = 36.62$ ;  $p < 0.01$ ); EC ( $t(16) = 18.44$ ;  $p < 0.01$ )). However, in juvenile-stressed rats, a significant decrease in the totalPSA-NCAM to totalNCAM expression ratio was not evident in the BLA ( $t(16) = 1.05$ ; NS), CA1 ( $t(16) = 0.109$ ; NS), or EC ( $t(16) = 1.33$ ; NS). Although in the DG a mild yet significant decrease was found ( $t(16) = 4.56$ ;  $p < 0.01$ ), its magnitude was incomparable with that found in naive rats.

*Examining the effect of exposure to stressors during juvenility on the totalPSA-NCAM to totalNCAM expression ratios among juvenile vs adult rats.* Further *t*-tests with Bonferroni corrections compared the effect of exposure to stressors during juvenility on the totalPSA-NCAM to totalNCAM expression ratio as measured soon after the exposure during juvenility and in adulthood. A mild yet significant stress-induced increase in this ratio was found soon after exposure to stressors in the BLA ( $t(16) = 3.59$ ;  $p < 0.01$ ) and CA1 ( $t(16) = 4.39$ ;  $p < 0.01$ ) but not in the DG ( $t(16) = 1.22$ ; NS) or EC ( $t(16) = 0.41$ ; NS). However, in adulthood, a significant and substantial juvenile stress-induced increase in the totalPSA-NCAM to totalNCAM expression ratio was evident in all examined regions (BLA ( $t(16) = 40.63$ ;  $p < 0.01$ ); CA1 ( $t(16) = 28.37$ ;  $p < 0.01$ ); DG ( $t(16) = 37.26$ ;  $p < 0.01$ ); EC ( $t(16) = 15.08$ ;  $p < 0.01$ )).

## DISCUSSION

NCAMs mediate cell-cell interactions and are recognized as key players in activity-dependent synaptic remodeling, memory storage (Welzl and Stork, 2003), and developmental events (Dey *et al*, 1999). Exposure to stressors was found to affect the functioning of NCAMs in adult rats. These alterations were suggested to be associated with heightened anxiety, impaired cognitive and neural function, and compromised synaptic plasticity processes (Theodosios *et al*, 1999; Sandi *et al*, 2001, 2003; Sandi, 2004). The current study set out to characterize the relationship between the effects that exposure to stressors during juvenility imposes on emotional responses in adulthood, as manifested in altered behaviors under stressful conditions, and on development-related alterations in the expression of NCAM and its posttranslationally polysialylated form, PSA-NCAM. The current study also reconfirmed previously reported findings (Avital and Richter-Levin, 2005; Tsoory and Richter-Levin, 2006; Tsoory *et al*, 2007) that exposure to stressors during juvenility alters stress-coping responses in adulthood. Furthermore, these behavioral alterations were coupled with changes in the protein-expression levels of NCAM and PSA-NCAM, with these changes appearing shortly after exposure to stressors during juvenility and lasting into adulthood.

### Exposure to Stress during Juvenility Alters Emotional Responses in Adulthood

As previously demonstrated, exposure to stressors during juvenility results in altered emotional responses in adult-

hood, that is, a marked reduction in novel-setting exploration, impaired avoidance learning, and more escape failures in the two-way shuttle avoidance task (Tsoory and Richter-Levin, 2006; Tsoory *et al*, 2007) (Figure 3). Reduction in novel-setting exploration by rats following exposure to stressors is a commonly used and pharmacologically validated index for heightened anxiety, which is highly correlated with other accepted behavioral and physiological anxiety indices (Kalynchuk *et al*, 1997; Ramos and Mormede, 1998; Campbell *et al*, 2003; Gregus *et al*, 2005).

As learning and performance in the two-way shuttle avoidance task are dependent on the hippocampus (Schwegler *et al*, 1981; Becker *et al*, 1997) and the amygdalae (Savonenko *et al*, 2003), the impaired avoidance learning observed in adult juvenile-stressed rats may also indicate compromised emotional responses, possibly mediated by altered functioning of these neurocircuits. Poor two-way shuttle avoidance performance was observed following both negligible and high doses of injected CORT (Kademian *et al*, 2005). Selective breeding based on 'high/low-avoidance' performance was suggested to relate to differences in 'emotional' factors (state/trait anxiety) that influenced performance (Brush, 2003). For example, the 'high-avoidance' and 'low-avoidance' Hatano rat strains did not differ in locomotion activity (Ohta *et al*, 1999), but the 'low-avoidance' strain had a higher adrenocorticotropin (ACTH)-induced adrenal CORT release response than the 'high-avoidance' strain (Asai *et al*, 2004). The increased rates of escape failure in the shuttle box that we found among adult juvenilestressed rats (Figure 3c) may also imply an emotional disruption. Such increases in escape failures were suggested to correspond to learned helplessness, representing, in animals, depressive symptoms of nonresponsiveness (Pryce *et al*, 2005). Increased rates of escape failure follow exposure to inescapable/uncontrollable stressors (Russig *et al*, 2003; Rüedi-Bettschen *et al*, 2005); are more frequent among adult rats that were exposed to stressors during the perinatal period (Rüedi-Bettschen *et al*, 2005); and may be attenuated by antidepressants (Weiss and Kiltz, 1998; Chen *et al*, 2001; Vollmayr and Henn, 2001; Rüedi-Bettschen *et al*, 2004).

The behavioral effects of the current study support previous findings indicating that in the course of the neural development of the rat juvenility is a stress-sensitive period in which even a rather short-term period of exposure to stressors results in persistent behavioral disruptions. Exposure to stressors during juvenility augmented the effect of exposure to stressors in adulthood, reducing exploration in the open field whereas increasing startle responses and affecting water maze performance (Avital and Richter-Levin, 2005). Exposure to different short-term stressors during juvenility resulted in impaired coping responses when faced with stressors in adulthood, resembling both anxious and depressive symptoms (Tsoory *et al*, 2007). These 'juvenile stress' (27–29 days of age) effects were found to be stronger than the effects of exposure to the same stressors during mid-adolescence (33–35 days of age) (Tsoory and Richter-Levin, 2006).

*Exposure to stressors during juvenility alters development-related changes in totalNCAM expression.* In the current study, exposure to stressors during juvenility also

altered the expression levels of NCAM and PSA-NCAM throughout the monitored limbic brain regions shortly after the exposure and also in adulthood.

The expression of totalNCAM did not appear affected by juvenile exposure to stress shortly after that exposure, at the age of 33 days. However, in adulthood, at 9 weeks of age, decreased expression of totalNCAM was evident among juvenile-stressed rats (Figure 4).

During juvenility, NCAM expression levels appear low compared with adulthood. They rise in the course of neural development as depolysialylation of PSA-NCAM commences and NCAMs are expressed on the cell surface, forming cell-cell adhesions (Rønn *et al*, 1998). This normative developmental increase was evident in our study in the comparison of naive juvenile with naive adult rats in terms of totalNCAM expression levels (Figure 4). However, exposure to stressors during juvenility seems to alter the normative development-related increase in NCAM expression. The low levels of totalNCAM observed at 33 days of age did not increase significantly during the maturation process in juvenile-stressed rats as they did in controls. At the age of 9 weeks, adult juvenile-stressed rats exhibited totalNCAM levels that were significantly lower than those of the controls in all four brain regions: BLA, CA1, DG, and EC (Figure 4).

Several studies on adult rats have associated chronic stress protocols known to impair cognitive capacities and alter neural functioning with lower expression levels of various NCAM isoforms, mainly within the hippocampus (Sandi *et al*, 2001; Touyarot and Sandi, 2002; Touyarot *et al*, 2004; Venero *et al*, 2002). In a parallel vein, interference with hippocampal NCAM functioning by conditional ablation of the NCAM gene in adult mutant mice (NCAM<sup>ff</sup>+) yielded mimicry of the deleterious effect of stress exposure on both long-term potentiation (LTP) and long-term depression in the CA1, as well as on spatial orientation (Bukalo *et al*, 2004). Likewise, chronic variable stress protocols applied in the prenatal or postweaning period induced a profound downregulation in NCAM expression levels in the hippocampus and cortex, coupled with cognitive impairments and a reduction in the expression of the synaptic marker synaptophysin and BDNF (Koo *et al*, 2003).

Originally, NCAM-null mutant mice were reported to develop normally, with only minor anatomic aberrations in the olfactory bulbs and hippocampal mossy fiber system (Cremer *et al*, 1994). Later studies reported that, as adults, these NCAM-null mutants showed a reduction in thorny excrescences in CA3 pyramidal cells, as well as alterations in mossy fiber growth and fasciculation (Cremer *et al*, 1997; Cremer *et al*, 1998). As adults, the exploratory behavior of these null mutant mice, their spatial learning, fear conditioning, and LTP were inferior to those of the wild-type, and the mice were characterized by increased aggressiveness as well as alterations in emotional/motivational behavior and hyperresponsiveness of the serotonergic system (Muller *et al*, 1996; Stork *et al*, 1997, 1999, 2000). Interestingly, in these mutants, 'anxious' behaviors, but not learning, could be rescued through a transgenic manipulation causing reexpression of the NCAM-180 kDa isoform (Stork *et al*, 2000).

Several studies have indicated that glucocorticoid receptor activation may repress transcription and/or splicing of

NCAM (Colwell *et al*, 1992; Quandt *et al*, 1995; De Bosscher *et al*, 1997; Simpson and Morris, 2000; Datson *et al*, 2001; Karin and Chang, 2001; Takeuchi and Fukunaga, 2003; Sandi, 2004).

As after exposure to stressors, plasma CORT concentration levels in juvenile rats return to baseline levels at half the pace observed in adult rats (Romeo *et al*, 2004); the role of these glucocorticoids in the regulation of NCAM gene transcription should be considered. Adult juvenile stress rats exhibit an array of heightened anxiety indices (Avital and Richter-Levin, 2005; Tsory and Richter-Levin, 2006; Tsory *et al*, 2007), suggesting sensitized stress-response mechanisms which may include glucocorticoid receptor activation.

The fact that shortly after the exposure to the stressors during juvenility no significant decrease in totalNCAM expression was evident (Figure 4) may be due to a floor effect. That is, the normative low levels of NCAM at this age may be such that they cannot be downregulated further.

*Exposure to stressors during juvenility alters development-related changes in totalPSA-NCAM expression.* Exposure to stressors during juvenility altered PSA-NCAM levels in the BLA, CA1, DG, and EC. Increased totalPSA-NCAM expression was observed shortly after exposure to the stressors during juvenility as well as in adulthood (Figure 5). High levels of PSA-NCAM are expressed during early development and decrease as the animal matures (Doyle *et al*, 1992b; Rønn *et al*, 2000). In our study, this development-related decrease was evident when naive juvenile and adult rats were compared for their totalPSA-NCAM expression levels (Figure 5). A similar development-related decrease in totalPSA-NCAM expression was evident in juvenile-stressed rats, but because exposure to the stressors during juvenility significantly elevated PSA-NCAM expression, it was still significantly higher in adult juvenile-stressed rats than in naive adult rats. This may suggest that the stressors applied did not arrest this developmental process, but shifted its set point upward.

Several studies on adult rats have associated chronic stress exposure or repeated glucocorticoid administration-induced cognitive impairments with altered synaptic plasticity and increased levels of PSA-NCAM in different hippocampal subregions (for a review, see Sandi, 2004). However, acute restraint procedures, which failed to induce hippocampal structural alteration, also failed to increase polysialylation of NCAM (Pham *et al*, 2003). To the best of our knowledge, our study presents the first evidence of a persistent increase in expression levels of totalPSA-NCAM following a nonchronic stress procedure. These effects may be related to juvenile rats having an enhanced stress response (Romeo *et al*, 2004), which may have interacted with a limbic system that was underdeveloped and more susceptible to the effects of stress, and set into motion a PSA-NCAM counterreaction. The de-adhesive properties of PSA-NCAMs have been suggested to counteract the stress-induced excess glutamatergic input (Hoffman *et al*, 1997; Rutishauser, 1998; Sandi, 2004) and/or the reduction in BDNF that follows chronic stress protocols (Smith *et al*, 1995). As polysialylation of NCAM is critical for neurogenesis (Seki and Arai, 1993; Franceschini *et al*, 2001) and

for synaptic plasticity (Muller *et al*, 1996), increased PSA-NCAM expression levels were suggested to represent a compensatory response to the deleterious structural effects of stress (Sandi *et al*, 2001).

*Exposure to stressors during juvenility alters development-related changes in the dynamic equilibrium between totalNCAM and totalPSA-NCAM.* The equilibrium between the expression levels of PSA-NCAM and NCAM changes gradually from high levels of PSA-NCAM and low levels of NCAM in early postnatal development to the opposite in adulthood (Doyle *et al*, 1992b; Rønn *et al*, 1998; Rønn *et al*, 2000). These changes are regulated, on the one hand, by varying dynamics in the activity of ST8SIAIV/PST, a prominent polysialyltransferase (PST) in the postnatal brain, which binds the PSA-branched moieties to NCAM, and on the other hand, by a counteracting depolysialylation process which reduces expression of PSA-NCAM (Doyle *et al*, 1992b; Rønn *et al*, 2000).

Our data indicate that, soon after exposure to stressors during juvenility, totalPSA-NCAM levels increased (Figure 5), whereas totalNCAM levels were not affected (Figure 4). However, adult juvenile-stressed rats exhibited a relatively reduced level of totalNCAM (Figure 4), coupled with an increased level of totalPSA-NCAM, even though a significant development-related decrease in totalPSA-NCAM levels did occur with maturation, as in naive rats (Figure 5). This may imply that exposure to stressors during juvenility induced an enhanced and maybe persistent polysialylation action, possibly via continuous transcription of PST, producing increased levels of totalPSA-NCAM which, in the long run, may have overshadowed the effects of depolysialylation of totalPSA-NCAM, resulting in relatively lower levels of totalNCAM in adulthood. An immature equilibrium between these NCAMs may underlie faulty activity-dependent synaptic remodeling processes in the adult rat, rendering it susceptible to the effects of stress.

*Summary and concluding remarks.* Taken together, our data demonstrate altered emotional responses in adulthood following exposure to stressors during juvenility which were coupled with altered development-related changes in the expression of totalNCAM and its posttranslationally modified form totalPSA-NCAM. They strongly support the possibility of a role for altered functioning of these NCAMs, and possibly others, in generating experience-induced predispositions to stress-related psychopathologies.

It is noteworthy that the effects of development, exposure to stressors during juvenility, and their interaction on totalNCAM and totalPSA-NCAM expression appeared to be similar in all the examined limbic tissues, the BLA, the CA1 and DG subregions of the hippocampus, and the EC. These regions are well recognized for their pivotal role and interactive function in modulating emotional faculties as well as learning processes (Richter-Levin, 2004). Therefore, it would be interesting to evaluate the effects that exposure to stressors during juvenility may have on learning non-aversive tasks in adulthood and to correlate these effects with those it may impose on the expression of totalNCAM and totalPSA-NCAM. Furthermore, because, during the adolescent period, substantial maturation processes occur

in several brain mechanisms in different regions, including the prefrontal cortex (Spear, 2000), and considering the apparent nonspecificity of the effects regarding brain regions in the current study, it is tempting to speculate that exposure to stressors during juvenility may also affect development-related alterations in the expression of these and other NCAMs in other brain regions.

Modulations to the functioning of NCAMs are under investigation for their potential therapeutic effects in brain repair (Webb *et al*, 2001; Nguyen *et al*, 2003) and in neural and cognitive disorders (Berezin and Bock, 2004). Our 'juvenile stress' model, along with totalNCAM and totalPSA-NCAM expression assessments, may be utilized as an effective animal model for testing the antidepressant and/or anxiety-relieving competence of new treatments that modulate the regulation of NCAMs.

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