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# CHRONIC RESTRAINT STRESS DOWN-REGULATES AMYGDALOID EXPRESSION OF POLYSIALYLATED NEURAL CELL ADHESION MOLECULE

**M. I. CORDERO,[a,b]* J. J. RODRÍGUEZ,[a] H. A. DAVIES,[a] C. J. PEDDIE,[a] C. SANDI[b,c] AND M. G. STEWART[a]**

[a]*Department of Biological Sciences, The Open University, Walton Hall, Milton Keynes, MK7 6AA, UK*

[b]*Laboratory of Behavioral Genetics, Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland*

[c]*Department of Psychobiology, Universidad de Educación a Distancia, 28040 Madrid, Spain*

**Abstract—The amygdala is a brain area which plays a decisive role in fear and anxiety. Since exposure to chronic stress can induce profound effects in emotion and cognition, plasticity in specific amygdaloid nuclei in response to prior stress has been hypothesized to account for stress-induced emotional alterations. In order to identify amygdala nuclei which may be affected under chronic stress conditions we evaluated the effects of 21-days chronic restraint stress on the expression of a molecule implicated crucially in alterations in structural plasticity: the polysialylated neural cell adhesion molecule. We found that polysialylated neural cell adhesion molecule-immunoreactivity within the amygdala, present in somata and neuronal processes, has a regional gradient with the central medial and medial amygdaloid nuclei showing the highest levels. Our results demonstrate that chronic restraint stress induced an overall reduction in polysialylated neural cell adhesion molecule-immunoreactivity in the amygdaloid complex, mainly due to a significant decrease in the central medial amygdaloid and medial amygdaloid nuclei. Our data suggest that polysialylated neural cell adhesion molecule in these nuclei may play a prominent role in functional and structural remodeling induced by stress, being a potential mechanism for cognitive and emotional modulation. Furthermore, these finding provide the first clear evidence that life experiences can regulate the expression of polysialylated neural cell adhesion molecule in the amygdaloid complex.**


**Key words: hippocampus, chronic stress, synaptic plasticity, glucocorticoids, anxiety, fear.**

*Correspondence to: M. I. Cordero, Laboratory of Behavioral Genetics, Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland. Tel: +41-21-693-1661; fax: +41-21-693-1660.
E-mail address: Isabel.cordero@epfl.ch (M. I. Cordero).

*Abbreviations:* ANOVA, analysis of variance; BL, basolateral complex of the amygdala; Ce, central amygdaloid nucleus; CeC, central capsular amygdaloid nucleus; CeL, central lateral amygdaloid nucleus; CeM, central medial amygdaloid nucleus; CRS, chronic restraint stress; LaDL, lateral dorsolateral amygdaloid nucleus; ME, medial amygdaloid nucleus; OD, optical density; PB, phosphate buffer; PSA-NCAM, polysialylated neural cell adhesion molecule; PSA-NCAM-IR, polysialylated neural cell adhesion molecule immunoreactivity; SP, substance P; TBS, Tris-buffered saline; tPA, tissue plasminogen activator.

Exposure to chronic stress can induce profound effects in cognition and emotion. In particular, exposing rats for 21 days to stress or corticosterone treatments has been found consistently to impair spatial learning (Conrad et al., 1996; Venero et al., 2002; Sandi et al., 2003), but to potentiate fear conditioning (Conrad et al., 1999, 2004; Sandi et al., 2001; Cordero et al., 2003). Spatial learning deficits have been attributed to a myriad of structural (Magariños and McEwen, 1995; Magariños et al., 1997; Sousa et al., 2000; Sandi et al., 2003; Stewart et al., 2005), molecular (Magariños and McEwen, 1995; McEwen, 1999; Sapolsky, 2000; Molteni et al., 2001; Sandi, 2004) and neurophysiological (Pavlides et al., 2002; Alfarez et al., 2003) alterations that chronic stress impinges in the hippocampus. However, enhanced fear conditioning appears not to be dependent upon hippocampal alterations, since treatment with tianeptine, which prevents stress-induced dendritic atrophy in CA3 pyramidal cells, did not inhibit stress-induced facilitation of auditory or contextual fear conditioning (Conrad et al., 1999). Instead, stress-induced sensitization of the amygdala has been proposed to underlie chronic stress effects in fear conditioning.

However, information regarding the impact of repeated stress exposure at the level of the amygdala is scarce. Recently, exposure during 10 days to restraint stress was found to induce dendritic hypertrophy in the basolateral, but not the central, amygdala nuclei (Vyas et al., 2002, 2003). In order to identify amygdala nuclei that might be affected under chronic stress conditions leading to enhanced fear conditioning (Conrad et al., 1999; Sandi et al., 2001; Cordero et al., 2003), we aimed here to evaluate the effects of 21-days chronic restraint stress on the expression of a molecule crucially implicated on structural plasticity: the polysialylated neural cell adhesion molecule (PSA-NCAM). PSA-NCAM is an important post-translational modification of NCAM [a glycoprotein of the immunoglobulin superfamily that plays key roles in synaptic plasticity and memory formation (Schachner, 1997; Kiss and Muller, 2001)]. The addition of long chains of polysialic acid (PSA) to NCAM modifies the relative degree of overall membrane–membrane apposition between cells, a mechanism that is involved in neural plasticity (Rougon, 1993; Seki and Arai, 1993). Interestingly, PSA-NCAM is increased in the hippocampus 24–48 h after exposure to a 21-days chronic restraint procedure (Sandi et al., 2001; Pham et al., 2003). Recently, the presence of PSA-NCAM in different rat amygdala nuclei has been described (Nacher et al., 2002). However, no qualitative or quantitative analyses have been as yet performed on the effects of stress in the

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amygdala. Here we show that chronic stress results in marked alterations in the expression levels of this molecule in specific amygdala nuclei, providing the first evidence that life experiences can regulate the expression of PSA-NCAM in this brain area.

## EXPERIMENTAL PROCEDURES

### Animals

Male Wistar Hanover rats (Harlan Iberica, Barcelona, Spain), weighing 150–175 g on arrival, were housed in groups of two to three per cage, at a temperature of 22±2 °C, and 12-h light/dark cycle (lights on at 07:00 h). Animals had free access to food and water. Approximately 5 weeks after arrival, they were handled daily for 3 days before being weighed. Rats were then allocated to two groups (control and stress) with similar body weight average. On the 5th week after arrival (rats weighing around 280±20 g in their 13th week of life), the chronic restraint stress (CRS) procedure was initiated for those animals assigned to the stress group. Body weights were recorded every 7 days. All efforts were made to minimize the number and suffering of animals used. Animal care procedures were conducted in accordance with current national guidelines on the ethical use of animals set by the European community Council Directives (86/609/EEC).

### Chronic stress procedure

We followed a stress-induction protocol previously described in Sandi et al. (2001). Briefly, five rats were subjected to CRS for 21 days. The sessions consisted of 6 h/day (8:00–14:00 h) restraint of the rats in wire restrainers (Cibertec, Spain) secured at the head and tail ends with clips. During the restraint sessions, the rats were placed in a room adjacent to their colony room and every day, at the end of the stress session, they were returned to their home cages. During this period, control rats ($n$=6) were left undisturbed in their cages, except every 7th day when they were weighed for comparison of their body weights with those of stressed rats.

### Tissue preparation

Three days after the last restraint session, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.). The brains of these animals were fixed by aortic arch perfusion with 50 ml of 3.8% acrolein (Fluka, Spain) in a solution of 2% paraformaldehyde and 0.1 M phosphate buffer (PB), pH 7.4, followed by 250 ml of 2% paraformaldehyde. The brains were removed from the cranium and cut into 4–5 mm coronal slabs of tissue containing the entire rostrocaudal extent of the hippocampus. This tissue was then post-fixed for 30 min in 2% paraformaldehyde and sectioned at 40–50 μm on a VT1000, vibrating microtome (Leica, Milton Keynes, UK). Six coronal Vibratome hemisections per rat at a level −2.56 mm/−2.80 mm posterior to Bregma, according to the rat brain atlas of Paxinos and Watson (1998) were selected. To remove excess reactive aldehydes, sections were treated with 1% sodium borohydride in 0.1 M PB for 30 min. The sections were then rinsed with 0.1 M PB followed by 0.1 M Tris-buffered saline (TBS), pH 7.6.

### Immunohistochemistry

To optimize detection of PSA-NCAM containing cells and profiles the sections of all control and treated animals were processed using the avidin–biotin peroxidase complex (ABC) method (Hsu et al., 1981). For this procedure, vibrating microtome sections were first incubated for 30 min in 0.5% bovine serum albumin in TBS to minimize non-specific labeling. The tissue sections were then incubated overnight at room temperature in 0.1% bovine serum albumin in TBS containing 0.25% Triton X-100 and mouse monoclonal antibody against PSA-NCAM (Abcys SA, Paris, France) at a dilution of 1:250, followed by 24 h incubation at 4 °C. Sections were then washed and placed in (1) 1:200 dilution of biotinylated donkey anti-mouse immunoglobulin (IgM, Jackson Immunoresearch, West Grove, PA, USA) and (2) 1:200 dilution of biotin–avidin complex from the Elite kit (Vector Laboratories Ltd., Peterborough, UK) for 1 h each. All antisera dilutions were prepared in TBS, and the incubations were carried out at room temperature. The peroxidase reaction product was visualized by incubation in a solution containing 0.022% of 3,3′ diaminobenzidine (DAB, Aldrich, Gillingham, UK) and 0.003% $H_2O_2$ in TBS for 7 min. The sections were then permanently mounted on gelatin-coated glass slides and coverslipped with Entellan (Merck, Darmstadt, Germany) mounting medium. Specificity of PSA-NCAM antiserum was checked by incubating the sections with (i) normal serum instead of primary antibody, or (ii) normal serum instead of the secondary biotinylated antibody. No immunostaining was seen on any of these sections.

### Optical density (OD)

Polysialylated neural cell adhesion molecule immunoreactivity (PSA-NCAM-IR) was assessed by measuring OD using the software analySIS Pro (Software Imaging System), an image processing and analysis program connected to a Nikon Eclipse E800 Microscope with a 10× objective in. The OD of the neuropil PSA-NCAM-IR was evaluated in the following regions of the amygdaloid complex lateral nuclei dorsolateral (LaDL) and ventromedial (LaVM), basolateral nuclei, anterior (BLA) and posterior (BLP), central nuclei capsular (CeC), lateral (CeL), medial (CeM), and medial amygdaloid nucleus (ME; Fig. 1). The regions under study were delineated using a computer mouse and mean of OD and surface area was measured. The OD was calculated from a relative scale of intensity ranging from 0 to 255, with a measurement of 0 corresponding to non-specific binding levels and 255 corresponding to the densest area of labeling. Non-specific OD in sections was measured from the corpus callosum. Three measurements of mean density were taken and averaged, after background subtraction, from each amygdaloid nucleus in both the left and the right hemisphere of each slice. The results are shown as relative density (OD/area). All slides were coded prior to quantitative analysis, and the code was not broken until quantification was complete.

### Statistical analysis and figure preparation

The data were analyzed using SPSS for Windows v. 10.0.6 software. Results were expressed as mean±S.E.M. Student $t$-test analyses were carry out to examine differences per area in PSA-NCAM-IR between both groups. A one-way analysis of variance (ANOVA) was used to examine differences in PSA-NCAM-IR between the groups considering the different regions of the amygdaloid complex, and when appropriate Tukey's post hoc tests were used. A repeated measures ANOVA was used to analyze changes in body weight throughout the different weeks of stress. Significance was accepted at $P<0.05$. Figures were generated using Photoshop 7.0 (Adobe Inc.).

## RESULTS

Body weight changes were monitored at 7 (Control: 319.0+7.5; Stress: 291.5+3.4), 14 (Control: 344.7+8.2; Stress: 307.9+3.2), and 21 days (Control: 369.0+8.8; Stress: 321.1+2.8) from the starting of the stress-induction protocol. This chronic restraint procedure induced a significant reduction in body weight gain, as indicated by a