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Behavioral and neuroendocrine consequences of juvenile stress combined with adult immobilization in male rats

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Abstract

Exposure to stress during childhood and adolescence increases vulnerability to developing several psychopathologies in adulthood and alters the activity of the hypothalamic-pituitary-adrenal (HPA) axis, the prototypical stress system. Rodent models of juvenile stress appear to support this hypothesis because juvenile stress can result in reduced activity/exploration and enhanced anxiety, although results are not always consistent. Moreover, an in-depth characterization of changes in the HPA axis is lacking. In the present study, the long-lasting effects of juvenile stress on adult behavior and HPA function were evaluated in male rats. The juvenile stress consisted of a combination of stressors (cat odor, forced swim and footshock) during postnatal days 23-28. Juvenile stress reduced the maximum amplitude of the adrenocorticotropic hormone (ACTH) levels (reduced peak at lights off), without affecting the circadian corticosterone rhythm; but other aspects of the HPA function (negative glucocorticoid feedback, responsiveness to further stressors and brain gene expression of corticotrophin-releasing hormone and corticosteroid receptors) remained unaltered. The behavioral effects of juvenile stress itself at adulthood were modest (decreased activity in the circular corridor) with no evidence of enhanced anxiety. Imposition of an acute severe stressor (immobilization on boards, IMO) did not increase anxiety in control animals, as evaluated one week later in the elevated-plus maze (EPM), but it potentiated the acoustic startle response (ASR). However, acute IMO did enhance anxiety in the EPM, in juvenile stressed rats, thereby suggesting that juvenile stress sensitizes rats to the effects of additional stressors.

Keywords: Prepubertal stress, PTSD models, HPA axis, Anxiety, Vulnerability
1. Introduction

Adolescence is a transitional stage between childhood and adulthood that includes important changes in personality and cognition (see Casey et al., 2010 for a review). This period represents a critical phase in development during which the nervous system shows unique plasticity, maturation and rearrangement of major neurotransmitter pathways (Romeo 2003; Spear, 2000). In general, there are dramatic maturational changes in several brain areas important for cognition, emotion, motivation and reactivity to stress, including the prefrontal cortex and other limbic brain structures (see Ernst et al., 2009 and Spear, 2000 for a review). In rodents, “adolescence” broadly covers the entire postnatal period ranging from weaning (postnatal day, PND, 21) to adulthood (PND 60). The boundaries of puberty in rodents vary between males and females and between rats and mice (Schneider, 2013), but the period from PND 21 to PND 30-34 is considered prepubertal (Eiland and Romeo, 2013; Laviola et al., 2003). Although several groups during recent years have studied the long-term effects of several interventions between PND 21 and PND 60, the present study focused only on the prepubertal period because there are considerable developmental differences within this period (i.e., Foilb et al., 2011; Klein and Romeo, 2013).

In humans, exposure to stressful experiences during childhood and adolescence has a profound long-lasting impact and increases the risk of developing several psychopathologies later in life (Ehlert, 2013; Heim and Nemeroff, 2001; Teicher et al., 2003). In recent years, several animal models have been proposed to characterize the underlying mechanisms. To our knowledge, the first studies about the long-term effects of prepubertal stress during PND 21 to PND 32 were conducted in outbred rats by Maslova et al. (2002a, 2002b), using a combination
of unpredictable stressors. They found an increase in the acoustic startle response (ASR) but no changes in the elevated plus-maze (EPM) behavior.

Moreover, the McCormick laboratory characterized the long-term effects of adolescent social instability initiated during the prepubertal period (usually starting at PND 30) but extending approximately until PND 45. McCormick’s model induced anxiety-like behavior in adulthood, altered social behavior and induced a lasting impairment in performance on hippocampal-dependent learning and memory tasks (see McCormick, 2010 for a review). In addition, Richter-Levin’s group initiated another extensive series of studies (see Horovitz et al., 2012 for a review) using different combinations of stressors restricted to the juvenile period (i.e., an elevated platform, cat odor, forced swim, restraint or footshock) administered approximately from PND 27 to PND 29. In adult male rats, these models typically induced reduced exploration of novel environments and increased anxiety (e.g., Avital et al., 2006; Horovitz et al., 2014; Ilin and Richter-Levin, 2009; Jacobson-Pick et al., 2008; Jacobson-Pick and Richter-Levin, 2010, 2012; Tsoory et al., 2008; Tsoory and Richter-Levin, 2006). Finally, Sandi and colleagues have used another model of prepubertal stress that extends towards the peripubertal period. This procedure consists essentially of the unpredictable exposure to an elevated platform with bright light and/or to synthetic fox odor during 7 days between PND 28 and PND 42. The model induces increased anxiety and depressive-like behavior in adulthood, as well as decreased sociability and greatly enhanced aggression (Cordero et al., 2012, 2013; Márquez et al., 2013). Other similar models of prepubertal stress in rats and mice support the long-lasting increases in anxiety (Brydges et al., 2012; Giovanoli et al., 2013; Wilkin et al., 2012; Yee et al., 2011; Weathington et al., 2012). Thus, it is well recognized that stress during adolescence has enduring effects in adulthood (Green and McCormick, 2013; McCormick and Green, 2013).
In contrast to the attention paid to behavior, particularly anxiety-like behavior, most of the above models have not extensively characterized the long-term effects of prepubertal stress on the hypothalamic-pituitary-adrenal (HPA) axis, the key system in stress research. Most of these studies have only measured corticosterone response to stress at a single time point (Giovanoli et al., 2013; Maslova et al., 2002a; Toledo-Rodríguez and Sandi, 2007; Yee et al., 2011; Weathington et al., 2012), which is clearly insufficient considering the complexity of the HPA axis and the limited value of plasma corticosterone as an index of HPA responsiveness to stress (Armario, 2006). The paucity of data regarding the HPA axis is surprising, given that in humans childhood maltreatment (by itself or in combination with adult stressors) has been associated in adulthood with a dysregulation of basal and stress-induced activity of the HPA axis, although such results have not been always conclusive (see for reviews: Cooke and Weathington, 2014; Danese and McEwen, 2012; Heim and Nemeroff, 2001; Shea et al., 2004; Van Voornees and Scarpa, 2004).

Some of the long-term consequences of prepubertal stress may be latent, unmasked only after an additional insult in adulthood. This idea is in the framework of the “two-hit” proposal, initially developed by Bayer et al. (1999) and Maynard et al. (2001) in the field of schizophrenia, but that model can be translated to other mental illnesses. Essentially, this conceptualization assumes that some psychopathologies are neurodevelopmental in origin and require some environmental and/or genetic “hits”. The “first hit” (genetic background and/or early environmental insult, such as stress, viral infection or exposure to drugs) primes or sensitizes the CNS to the “second hit” (another early or adult environmental insult). Thus, the “first hit” makes the organism more vulnerable to the effects of the “second hit” that precipitates the disease. This idea is similar to the “cumulative stress” hypothesis, which proposes that acute and chronic stressful experiences in adulthood accumulate over early-life
adversity, compromising the ability of the organism to manage stress (McEwen, 1998; Taylor, 2010). Alternatively, the “match-mismatch” models predict that after a juvenile stress experience, individuals will show improvements in coping behavior and adaptability to further exposures to stress in adulthood (Claessens et al., 2011; Oitzl et al., 2010; Schmidt, 2011).

Given all of the above, the aims of the present study were three-fold: (i) to characterize, in our conditions, the long-term effects of juvenile stress (restricted to this very limited period of life) on activity and anxiety; (ii) to characterize the long-term consequences of juvenile stress on the peripheral and central functioning of the HPA; and (iii) to test the “two-hit” hypothesis, studying how prior juvenile stress experience can alter the long-term consequences of a single exposure to a severe stressor such as immobilization on boards (IMO). This stressor has been extensively characterized in our laboratory (Belda et al., 2008b) and has been determined to induce changes in activity and anxiety that can last for some days (Belda et al., 2008a), as well as more protracted impairment of spatial memory in the Morris water maze (Andero et al., 2012) and fear extinction (Andero et al., 2011).

2. Methods

2.1 Animals and general procedures

We used 76 male Sprague–Dawley rats, obtained from the breeding center of the Universitat Autònoma de Barcelona (derived from Harlan Interfauna Ibérica, Barcelona, Spain). They were obtained from 13 different mothers; after birth, each litter was culled to 10 pups (sex ratio after culling: 0.65 ± 0.10 males versus females). Pups were weaned at PND 21 and housed in groups of 4 males. After that, they were maintained in opaque polypropylene wire-topped
cages with solid bottoms (27.5 x 52.5 x 14.5 cm; Type “1000 cm2”, Panlab S.L.U., Barcelona, Spain) containing sawdust bedding (Ultrasorb, Panlab, S.L.U., Barcelona, Spain). At PND 45, they were housed in pairs, and they remained in these housing conditions until the end of the study. Animals were maintained in standard temperature conditions (21 ± 1 ºC) and in a 12-h light/12-h dark schedule (lights on at 8:00 h). Food (SAFE-diet A04, Panlab S.L.U., Barcelona, Spain) and water were available ad libitum. The rats of the same home cage had the same treatment. Except for the juvenile stress (see below), all behavioral manipulations were performed in the morning. The experiments were conducted using two different cohorts, the first corresponding to experiments 1 and 2, which were carried out simultaneously, and the second corresponding to experiment 3. The experimental protocol was approved by the Committee of Ethics of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, followed the principles of laboratory animal care and was conducted in accordance with the European Communities Council Directives (86/609/EEC).

Animals were handled on 4 days for approximately 2 min a day. During each handling period, each rat was removed from its cage, placed on the tabletop, had its neck and back gently stroked by the experimenter and was gently wrapped with the cloth that would be used for the tail nick. On the last day of handling, animals were exposed to the tail-nick procedure to allow habituation to the procedure (experiments 1 and 2). The tail nick consisted of gently wrapping the animal with a cloth, making a 2-mm incision at the end of one of the tail veins and then massaging the tail while collecting, within 2 min, 300 µl of blood into ice-cold EDTA capillary tubes (Sarsted, Granollers, Spain). This procedure is extensively used because very low resting levels of hormones are obtained under appropriate conditions (Belda et al., 2004; Vahl et al., 2005). The two cage-mates were sampled simultaneously (two experimenters took samples at the same time, and a third gently held the two rats).
2.2. Experimental designs

A summary of the experimental designs can be seen in Figure 1.

Experiment 1. The purpose of this study was to evaluate in adults the long-term effects of juvenile stress on: (i) the circadian rhythm of HPA hormones; (ii) the integrity of the negative glucocorticoid feedback mechanisms, and (iii) the tonic functioning of the HPA axis at the brain level. Twenty rats were used for this experiment (control: n = 10, and juvenile stress: n = 10).

After handling, at PND 61, blood samples were taken from rats under basal conditions at 9:00, 15:00, 19:00, 23:00 and again at 9:00 h on the next day. After 7 days of resting, corticosterone was injected (10 mg/kg, sc, dissolved in 0.4 ml of sesame oil) at 13:00 h, and the rats were sampled under basal conditions at 15:00, 19:00 and again at 9:00 h on the next day. After 7 additional days of rest, animals were perfused in basal conditions, and the adrenals and brain were extracted for further analysis. Central functioning of the HPA axis was evaluated by in situ hybridization for: (i) glucocorticoid receptor (GR) expression in the medial parvocellular dorsal division of the paraventricular nucleus of the hypothalamus (PVNmpd), the dorsal hippocampal formation (dentate gyrus: DG, CA1, CA3) and the prefrontal cortex (cingulate cortex area 1: Cg1, infralimbic: IL, prelimbic: PrL), (ii) mineralocorticoid receptor (MR) expression in the hippocampal formation (DG, CA1, CA2, CA3) and (iii) corticotrophin releasing hormone (CRF) expression in the PVNmpd, the central amygdala (CeA) and the lateral dorsal and ventral subdivisions of the bed nucleus of the stria terminalis (BNSTdl, BNSTvl). The precise coordinates are indicated in section 2.6.

Experiment 2. The purpose of this study was to characterize in adults the behavioral consequences of juvenile stress in tests of activity and anxiety as well as the HPA
responsiveness to stressors. Twenty rats were used (control: n = 10, and juvenile stress: n = 10). At PND 68-69, rats were exposed to the circular corridor for 15 min, and blood samples were taken immediately after the end of the test, in an adjacent room. Two days later, animals were exposed to the EPM for 5 min and returned to the vivarium, and their blood was sampled in an adjacent room 10 min after the end of the test. The following day, rats underwent the dark-light test, and the ASR the day after that. Eleven days later, all animals were exposed to 120 min of IMO and sampled just before IMO (basal conditions), at 30 and 120 min from the start of IMO and 60 and 180 min after the end of IMO (R60 and R180 min). Immediately after IMO, the subjects were returned to the vivarium until sampling.

Experiment 3. The objective of the study was to analyze the behavioral effects of the superimposition at adulthood of a severe stressor (IMO) in animals that had been previously exposed to juvenile stress. Thirty-six rats maintained as described were used for this experiment (control: n = 18, and juvenile stress: n = 18). At adulthood (PND 68), each group of rats was further divided into two groups regarding IMO exposure (NO IMO: n = 8-10, IMO: n = 8-10). Thus, four groups were obtained (control-NO IMO, control-IMO, juvenile stress-NO IMO and juvenile stress-IMO). The duration of IMO was 120 min, and the procedure was performed on two days (one day, one of the rats from the cage was immobilized; the next, the other rat). No HPA response study was conducted after IMO because this response was already studied in experiment 2, and no differences were found. After 7 days under undisturbed conditions other than cleaning maintenance, animals were exposed to the EPM. In the EPM testing, one rat from the home cage was exposed one day, and its cage-mate was exposed the next day. On the third day, all rats were exposed to the dark-light test, and the ASR testing started on the following day. ASR measurements lasted for 3 days so that all rats could be tested.
2.3. Juvenile stress

The juvenile stress lasted six consecutive days, starting at PND 23. For juvenile stress, a variable repeated model was chosen to minimize adaptation. Juvenile stressed rats were exposed to one different stressor daily, in the following order: footshock, forced swim, cat odor, footshock, forced swim and cat odor. These stressors are routinely used in the field (i.e. Rabasa et al. 2011, 2013; Wright et al. 2013). Animals were weighed immediately before the first session and 24 h after the last session to evaluate the overall impact of the procedure. Stressor exposure always started at 14:00 h, and it was performed in a room different from those used for any of the behavioral and endocrine studies. After the end of the juvenile stress, the rats remained undisturbed, except for cleaning maintenance, until adulthood. Control rats always remained undisturbed in the vivarium.

Rats were shocked in individual clear Plexiglas boxes (12 x 6 x 13.1 cm) with a metal removable grid floor of 13 stainless steel rods arranged in 9 channels, 0.15 cm diameter and spaced 0.75 cm center to center connected to a shoker that delivered scrambled AC current (Cibertec SA, Madrid, Spain). Animals were allowed to explore the chamber for 3 min and then received shocks (1.5 mA, 3 s of duration, ITI 60 s) for 30 min. After the last shock, animals remained in the chamber for 1 min. The four rats of the same home cage were tested simultaneously. The chambers were carefully cleaned between rats with an ethanol solution in tap water (5% v/v).

For forced swim exposure, animals were placed in transparent cylindrical plastic tanks (height 40 cm, internal diameter 19 cm) containing water (36 °C) to a level of 15 cm. Four identical tanks were used, and the animals of the same home-cage were separated by black screens and exposed simultaneously. Water was always changed for each rat. Animals were exposed to 2
sessions in the same day, each of 10 min duration, 1 h apart. After each session, animals were carefully dried and returned to their home cages.

For the cat-odor exposure test, rats were introduced into individual Plexiglas chambers painted black (25 x 25 x 30 cm) and remained there for 15 min. Inside the chamber was a piece of a collar (2.5 cm long) previously worn for 3 weeks by a male non-castrated cat of “European short-hair” origin (European IQHsdCpb:CATS(SPF) from Isoquimen SL, Sant Feliu de Codines, Spain). The procedure to obtain the collars was performed in the Isoquimen animal facility; immediately afterward, each collar was separately sealed in airtight plastic containers and frozen at -80 ºC until use. Before using the collars, they were maintained at room temperature for 1 h. The collars were always handled with plastic gloves. Each piece of the collar was only used once.

2.4. Adult behavioral procedures

In adulthood several tests were used primarily measuring activity and anxiety-like behaviours. All of the adult behavioral testing was conducted in a black-painted room, and the experimenter remained in an adjacent room. The apparatuses were cleaned between rats with an ethanol solution in tap water (5% v/v). Each animal of a given home-cage was tested individually, and its cage-mate was tested the following day, except for the dark-light test, in which the two animals were tested simultaneously in separated chambers.

The circular corridor (Nadal et al., 2005) was used to evaluate activity in a novel environment that has been related to exploration/novelty-seeking measures (Dellu et al., 1996). The apparatus consisted of a white circular arena (80 cm diameter x 34 cm high) with a cylinder
inside (52 × 34 cm), forming a corridor 14 cm wide. Thus, the structure of the test avoided the exploration of the center of the apparatus. Each corridor had 4 photobeams placed above the surface of the floor. A dim light provided an intensity of 6 lux. The rat was placed inside the corridor and could move around a circular track between the walls. The duration of the session was 15 min. Activity (ambulation) was scored as the number of photobeams interrupted. Defecations were also scored.

The EPM was used to measure anxiety-like behavior (Pellow and File, 1986). The apparatus consisted of 4 white wooden arms (Formica) at right angles to each other, connected to a central square (10 cm²) to form the shape of a plus sign and elevated 50 cm above the floor. Each arm was 46 cm long and 10 cm wide. Two of the opposite arms had high walls (enclosed arms, 43 cm high), whereas the other two were the open arms, with a 0.7-cm ridge to provide an additional grip. The illumination was provided from two white bulbs (25 W) placed in each of the open arms at 164 cm from the floor of the EPM (in the open arms: 10 lux, in the closed arms: 1 lux). The apparatus was surrounded by black screens. The rat was placed in the center, facing a closed arm. The duration of the session was 5 min. Behavior was videotaped from the top with a camera (Sony SSC-M388 CE, BW) situated 150 cm above the center of the apparatus. A video tracking system (Smart version 2.5.10, Panlab SLU, Barcelona, Spain) was used to measure distance travelled into open and closed arms. Time spent in open and closed arms, latency to the first open arm entry, number of entries in each type of arm, time spent in grooming behavior, number of head-dippings and time in risk-assessment behavior in the center of the maze were measured with a stopwatch. A subject was considered to enter in a given arm when all paws were inside. Head-dipping was defined as protruding the head over the ledge of an open arm and down towards the floor while being in the center or in an enclosed arm (protected) or while being in an open arm (unprotected). Risk assessment was
defined by the “stretched attend posture”, when the rat in the center stretches toward the open arms and retracts to its original position. Defecations were also measured.

The dark-light test (Ramos et al., 2003) was used to measure anxiety-like behavior. The apparatus was made of wood covered with Formica and consisted of two compartments. One was white (30 × 30 × 30 cm), and the other was black (30 × 30 × 30 cm) and covered with a roof. The compartments were separated by a wall and connected by a small opening of 9.5 × 8 cm. The white compartment was illuminated by a white 25-W bulb located 164 cm above the center of this compartment (8.7 lux). Each rat was individually placed in the center of the black compartment, facing the small opening. The procedure lasted for 10 min. Behavior was videotaped from the top, as mentioned, and the video tracking system was used to measure the number of transitions between the two compartments (with all four paws), activity in the white compartment and time spent with all four paws in the white compartment. Defecation, latency to enter the white compartment, attempts to enter the white compartment and rearings were also measured by hand. An attempted entry was defined as when the animal did not enter the white compartment with all four paws but instead returned to the dark compartment (this pattern includes the “stretched attend posture”, when the rat stretches forward and retracts to its original position). A decrease in the transitions between compartments, as well as a decrease in time or activity in the white compartment, is considered to reflect anxiety (Ramos et al., 2003).

The ASR was also measured because it is modulated by a variety of experimental changes in the perceptual or emotional state of the organism, and can be enhanced by conditioned and unconditioned aversive events, making it useful for the study of anxiety-related behaviors (Koch, 1999). To test the ASR, a commercially available system was used (Cibertec SA, Madrid,
Spain). The system comprised one sound-attenuating chamber, equipped with a Plexiglas animal enclosure (16.5 cm x 30 cm with an adjustable lid). The chamber was illuminated with a dim light (10 W) and ventilated by a small electric fan that provided background white noise (55 dB) throughout the test. Broad and tone pulses were presented by a speaker positioned 27 cm directly above the animal enclosure. A piezoelectric accelerometer affixed to the animal enclosure frame was used to detect and to transduce motion resulting from the animals’ responses. Tone-pulse parameters were controlled by a computer, using a software package and an interface assembly that digitized, rectified and recorded stabilimeter readings. Animals were placed in the Plexiglas enclosure for 5 min of acclimation, and then a pulse (startling tone of 40 ms, 110 dB) was presented alone. The number of trials was 60 in experiment 1 and 30 in experiment 2. The interval between each pair of trials was programmed following a variable time schedule, with an average duration of 30 s (range 25—35 s). The startle response during the 200 ms following the onset of each startle stimulus was recorded and stored on a computer. The measures taken were peak of the startle response, latency of the first response, latency to the peak of the response and area under the curve of the startle response.

The immobilization (IMO) stress was conducted by gently restraining the animal in a prone position and taping its four limbs to metal mounts attached to a wooden board. This procedure was originally described by Kvetnansky and Mikulaj (1970), and it has been used in several labs (Nostramo et al., 2012; Nostramo et al., 2013; Ondrejcakova et al., 2010; Tillinger et al., 2013). In our group, the procedure has been adapted to minimize the movement of the animal and therefore the probability to induce inflammation in the paws. Thus, head movements were restricted with plastic pieces (7 x 6 cm) placed on each side of the head, and the body was secured to the board by means of a piece of plastic cloth (10 cm wide) attached with Velcro® that surrounded the entire trunk. This procedure has been extensively used in our
group in rats (i.e., Andero et al., 2012; Belda et al., 2008a; Belda et al., 2012; Daviu et al., 2012, Daviu et al., 2014; Gagliano et al., 2008; Muñoz-Abellán et al., 2008; Muñoz-Abellán et al., 2001) and mice (Amador-Arjona et al., 2010; Andero et al., 2011).

2.5. Biochemical analysis

Plasma adrenocorticotropic hormone (ACTH) and corticosterone levels were determined by double-antibody radioimmunoassay (RIA) to study the effects on the peripheral HPA axis. ACTH RIA used $^{125}$I-ACTH (PerkinElmer Life Science, Boston, USA) as the tracer, rat synthetic ACTH 1–39 (Sigma, Barcelona, Spain) as the standard and an antibody raised against rat ACTH (rb7), kindly provided by Dr. W.C. Engeland (Department of Surgery, University of Minnesota, Minneapolis, USA). The characteristics of the antibody have been described previously (Engeland et al., 1989), and we followed a non-equilibrium procedure. The first antibody was added on day 1, and $^{125}$I-ACTH was added on day 2. After one additional 18-h incubation period, the second antibody was added. Corticosterone RIA used $^{125}$I-corticosterone–carboxymethyloxime–tyrosine–methylester (Laboratorios Leti, Barcelona, Spain) as the tracer, synthetic corticosterone (Sigma, Barcelona, Spain) as the standard, and an antibody raised in rabbits against corticosterone–carboxymethyloxime-BSA, which were kindly provided by Dr. G. Makara (Institute of Experimental Medicine, Budapest, Hungary). The characteristics of the antibody and the basic RIA procedure have been described previously (Zelena et al., 2003). Plasma glucose levels were measured by the glucose-oxidase method using a commercial kit (Glucose RTU, Biomerieux, Barcelona, Spain) to study medulloadrenal activation (Bialik et al., 1989). All samples to be statistically compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 6% for ACTH, 9% for corticosterone and
2.8% for glucose. The sensitivity of the assays was 33 pg/ml for ACTH, 1 ng/ml for corticosterone and 5 mg/dl for glucose.

### 2.6. In situ hybridization

Rats were anesthetized with isoflurane and perfused with saline solution (4°C) for 2 min and with 4% paraformaldehyde and 3.8% borax (4°C) for 8-10 min. Then, brains were removed, post-fixed overnight at 4°C and cryoprotected [0.2 M NaCl, 43 mM potassium phosphate (KPBS) containing 30% sucrose] for 48 h at 4°C. Next, each brain was frozen on dry ice, and four series of 20-μm sections were obtained and stored at −20°C in an anti-freeze solution (30% ethylene glycol, 20% glycerol in 0.25 mM phosphate buffer at pH 7.3).

Serial coronal sections (20 µm thick) through the medial prefrontal cortex (Bregma 2.20 to 3.20), the BNST (Bregma -0.26 to -0.80), the PVN (Bregma -1.80 to -2.30), CeA (Bregma -1.60 to -2.30) and the hippocampal formation (Bregma -3.14 to -4.16) were obtained from the frozen brains with a cryostat (Frigocut 2800; Leica, Nussloch, Germany) and mounted onto Superfrost Plus slides (Thermo scientific; Menzel-Gläser, Braunschweig), which were then maintained at −80 °C until the day of analysis. See Supplementary Figure 1 to see the size of the areas analyzed.

The CRF probe was generated from a pGEM-4Z plasmid containing an EcoRI fragment (1.2 kb) of rat cDNA (Dr. K. Mayo, Northwestern University, Evanston, IL, USA), linearized with HindIII. The GR probe was transcribed from a 500-bp rat cDNA fragment that encodes the N-terminal region of the rat liver GR (courtesy of Dr. K.R. Yamamoto and Dr. R. Miesfeld, Department of Biochemistry, University of Arizona, Tucson, AZ, USA). This fragment was subcloned from a 2.8-
kb fragment and transfected into the pGEM3 plasmid (courtesy of Dr. M.C. Bohn, Department of Pediatrics, Northwestern University Medical School, Chicago, IL, USA). It was linearized with BamHI. MR probe was generated from a pBS SK+ plasmid containing an EcoRI/HindIII fragment (550 bp) from a 3′ coding region and 3′ untranslated region of rat MR cDNA (courtesy of Dr. K.R. Yamamoto and Dr. R. Miesfeld, Department of Biochemistry, University of Arizona, Tucson, AZ, USA). It was linearized with EcoRI. Radioactive antisense cRNA copies were generated using a transcription kit (Roche, Germany) in the presence of [α-35S]-UTP (specific activity >1000 Ci/mmol, Perkin Elmer, Spain). The cRNAs were purified with mini Quick Spin RNA Columns (Roche, Spain) and stored at −20 °C.

The protocol used was adapted from Simmons et al. (1989). All solutions were pretreated with DEPC and sterilized before use. Sections were post-fixed in 4% PFA + Borax, rinsed in KPBS, digested with 0.01 mg/ml proteinase K (Roche Diagnostic, Germany), rinsed in DEPC-treated water and 0.1 M triethanolamine pH 8.0 (TEA; Sigma) and acetylated in 0.25% acetic anhydride in 0.01 M TEA, washed in 2×SCC, dehydrated through graded concentrations of ethanol and air-dried. Thereafter, 100 μl of hybridization solution (50% formamide, NaCl 0.3 M, Tris–HCl 10 mM pH 8.0, EDTA 1 mM pH 8.0, 1×Denhardt’s solution, 10% dextran sulfate, yeast tRNA 500 g/ml and 10 mM DTT) containing 1×10^6 dpm of the labeled probe was spotted onto each slide and sealed with a coverslip. After a 16–18 h incubation in a humid chamber at 60 °C, the slides were washed in descending concentrations of SSC containing 1 mM DTT (Sigma, Spain), including one wash at 60 °C, digested with RNase A (0.02 mg/ml, Roche, Spain), dehydrated through a series of ethanol solutions and air-dried. The slides were then exposed to autoradiography film (XAR-5 Kodak Biomax MR, Kodak, Spain) for between 13 h and 6 days for CRF mRNA, from 36-48 h for MR mRNA and from 36-48 h for GR mRNA. All the slides to be compared were exposed in the same cassette.
Densitometric analyses were performed on the autoradiography films. The mRNA levels were semiquantitatively determined in both hemispheres in 3-5 sections per brain area and per animal. The sections to be analyzed were photographed under a 10x-4x microscope objective (Eclipse E400, Nikon, Japan) with a DXM1200 digital camera (Nikon, Japan). The illumination conditions, exposure time, sensitivity to light and resolution conditions were kept constant across all the photographs to be compared. Photomicrographs were subsequently quantified in a blind manner with Scion Image software (W. Rasband, NIH, USA; available on the web at http://rsb.info.nih.gov/nih-image or http://www.scioncorp.com). (The program uses an 8-bit scale for grey values, with 0 indicating absolutely white and 255 absolutely black). As indicated in the Supplementary Figure 1f, for the hippocampal formation, several measures were taken for each slide and hemisphere in the different areas (CA1, CA2, CA3 and DG). The final values for each area and animal were the averages of all the measures obtained. For each brain area, a threshold for gray value was selected, and the pixels with values under these thresholds were not further considered in the analysis. Thresholds were chosen in a way that remained the specific signal and was removed most of the background. Once the optimal threshold was determined, it was kept constant across all the subjects to be compared. For each slide, background measurements were taken and subtracted from the specific signal. For the CRF background, measures were taken in areas without specific signal, adjacent to the nuclei of interest. For GR and MR, background was measured in the film area immediately adjacent to the brain slices. We have observed only small differences between background measures taken from sections and from the film. Measures were obtained in arbitrary units (square pixels × mean grey value). In all cases, the intensity of the signal was within the linear range as evaluated by comparison with $^{14}$C microscales (GE Healthcare, UK). An example of negative controls using sense riboprobes is seen in Supplementary Figure 2.
2.7. Statistical analysis

The SPSS (*Statistical program for the Social Sciences*, version 15) program was used to analyze behavioral, endocrine and histological data. Normality of the data was studied by means of Kolmogorov-Smirnov tests. If normality was achieved, parametric tests were performed: Student’s t-test or repeated-measures ANOVAs (general linear model: GLM), as indicated later. If normality was not achieved, nonparametric ANOVAs were used (Kruskal-Wallis followed by Mann-Whitney U tests). If homogeneity of variance was not achieved, data were log-transformed. If log-transformations were not useful (because of some data with zero values) a generalized linear model (GzLM) was used (McCulloch et al., 2008).

3. Results

3.1. Experiment 1

Because experiments 1 and 2 were conducted in parallel, body-weight data are reported together. Control and juvenile stress groups did not differ at weaning (control: 56.5 ± 1.3 g vs. juvenile stress: 55.8 ± 1.1 g, NS), but at the end of the juvenile stress period, body weight gain was lower in the juvenile stress group than in control rats (control: 43.7 ± 1.0 g vs. juvenile stress: 38.2 ± 0.9 g, t (38) = 3.98, P < 0.01). On the last day of handling in adulthood, those differences had vanished (juvenile stress: 334.0 ± 4.0 g vs. control: 340.9 ± 5.0 g, NS). When adults, the animals showed the expected circadian rhythms in plasma ACTH and corticosterone (Figure 2). From the inspection of the Figure it became evident that the effect was restricted at night and a priori contrast was made, and the results showed that juvenile
stress decreased basal ACTH levels at 19.00 h \([t(18) = 2.12, p < 0.05]\) without affecting corticosterone levels.

The effects of corticosterone injection to suppress ACTH secretion (Figure 3) were analyzed by repeated-measures ANOVA with juvenile stress as the between-subjects factor and two within-subjects factors (corticosterone injection, two levels: basal and suppression, and time, three levels: 15:00, 19:00 and 9:00 h). Whereas the effect of juvenile stress was not statistically significant, there were significant effects due to the suppression produced by corticosterone injection \([F (1, 36) = 95.80, p < 0.001]\) and time of day \([time: F (2, 36) = 50.98, p < 0.01]\). The degree of suppression was dependent on time of day \([corticosterone \times time: F (2, 36) = 38.58, p < 0.001]\). The decomposition of the interaction showed that the suppression induced by corticosterone injection was maintained at the three blood sampling times, being maximal in the first two samples \([15:00 \text{ h: } F (1, 18) = 49.72, p < 0.01; \text{ 19:00 h: } F (1, 18) = 69.87, p < 0.01; \text{ 09:00 h: } F (1, 18) = 39.47, p < 0.01]\). All other interactions \([juvenile \text{ stress } \times \text{ time}, \text{ juvenile stress } \times \text{ corticosterone}, \text{ juvenile stress } \times \text{ corticosterone } \times \text{ time}]\) were not statistically significant.

Absolute adrenal weight was lower in the rats subjected to juvenile stress (control: 75 ± 2 mg vs. juvenile stress: 67 ± 3 mg, \(t(18) = 2.21, p < 0.05\)), but this difference disappeared when the relative adrenal weight was calculated (control: 14.5 ± 0.54 vs. juvenile stress: 13.5 ± 0.50 mg of adrenal weight/100 g of body weight, NS). No significant group effects were observed with regard to the parameters related to the central functioning of the HPA axis (Table 1, Figures 4-6): (i) in the GR expression in the PVNmpd, hippocampal formation and prefrontal cortex, (ii) in the MR expression in the hippocampal formation or (iii) in the CRF expression in the PVNmpd, CeA and BNSTdl/BNSTvl.
3.2. Experiment 2

The effect of juvenile stress on adult activity in the circular corridor (Figure 7) was analyzed with a repeated-measure ANOVA, with one between-subjects factor (juvenile stress, two levels) and one within-subjects factor (time: 3 levels, blocks of 5 min). The analysis revealed that activity decreased across time \([F (2, 36) = 26.89, p < 0.001]\), that juvenile stress decreased this activity \([F (1, 18) = 5.73, p < 0.05]\) and that the reduction was detected in all the sessions \([\text{juvenile stress } \times \text{time: NS}].\) In the other behavioral tests (EPM, dark and light, and ASR), no statistically significant effects of juvenile stress were observed (Table 2). The HPA response to the circular corridor and the EPM was also unaffected by the treatment (Table 2).

The effects of juvenile stress on the HPA response to IMO were evaluated by a repeated-measures ANOVA with one between-subjects factor (juvenile stress) and one within-subjects factor (time, 5 levels, corresponding to the blood sampling times). The analysis showed the expected effect of time (Figure 8) in that IMO increased ACTH and corticosterone levels with regard to basal levels \([\text{time: } F (4, 72) = 146.22, p < 0.001; F (4, 68) = 107.0, p < 0.001,\) respectively], and they were still elevated 3 h after the end of the stressor. However, juvenile stress did not affect the HPA response to IMO. This lack of effect of juvenile stress on endocrine reactivity to adult stress was corroborated by glucose levels in response to IMO (see Supplementary Table 1).

3.3. Experiment 3

As in the previous experiments, animals did not show differences in body weight at weaning (control: \(70.5 \pm 1.3\) g vs. juvenile stress: \(68.7 \pm 1.4\) g, NS), but at the end of the juvenile stress,
body weight gain was decreased in juvenile stress rats (control: 43.3 ± 0.9 g vs. juvenile stress: 39.4 ± 1.0 g, t (34) = 2.90, P < 0.01). At adulthood, these differences had vanished (control: 396.5 ± 3.8 g vs. juvenile stress: 390.5 ± 5.1 g, NS).

Neither juvenile stress nor IMO by themselves exerted anxiogenic effects in the EPM (Figure 9). Because the homogeneity of variances was not achieved for the percent of time spent in open arms with regard to time in open + closed arms (Figure 9A), and log-transformations were not useful (data included some zero values), a GzLM was performed in this case. Statistical analysis indicated that juvenile stress was NS, but IMO and juvenile stress x IMO were statistically significant [Χ²(1) = 6.07, p < 0.05; Χ²(1) = 4.17, p < 0.05, respectively]. The decomposition of the interaction between juvenile stress and IMO showed that adult IMO decreased the percentage of time spent in open arms only in animals subjected to juvenile stress (p < 0.01), suggestive of an anxiogenic-like effect. The same results were found with regard to the raw time spent in open arms [Figure 9B, juvenile stress: NS, IMO: Χ²(1) = 5.13, p < 0.05, juvenile stress x IMO: Χ²(1) = 3.85, p = 0.05]. Because normality was not achieved for activity in the open arms of the EPM (Figure 9C), a nonparametric Kruskal-Wallis test was performed (between-subject factor “group” with four levels), and it reported statistically significant differences [Χ²(3) = 8.98, p < 0.05]. In juvenile stress animals, IMO superimposed at adulthood decreased the activity in open arms [U: 10.0, p < 0.05]. The indexes of unspecific motor activity such as the distance traveled in the closed arms (Figure 9D) were not affected (NS), and no between-groups differences were observed in the other measures analyzed in the EPM (data not shown). Moreover, ANOVA analyses revealed that the behavioral parameters recorded in the dark-light test were not affected by the treatments [juvenile stress, IMO, juvenile stress x IMO: all NS, see Table 3].
Regarding the ASR, it must be considered that animals with higher body weight exerted more force against the platform and may therefore present higher amplitude responses. Thus, usually the indexes are adjusted by body weight (Servatius et al., 2005), or a covariance analysis is performed, using body weight as a covariate (Young and Cook, 2004). In the present experiment, as expected, IMO animals tended to have lower weights (control: 485.1 ± 7.8 g vs. IMO: 467.5 ± 6.5 g, t (34) = 1.73, p = 0.093). An ANOVA was performed to analyze differences in the peak of the amplitude of the ASR with body weight as the covariate, two between-subject factors (juvenile stress, two levels, and IMO, two levels) and one within-subject factor (time, 3 blocks of 10 trials). This analysis (see Figure 10) revealed a statistically significant effect of body weight [F (1, 31) = 7.67, P < 0.01] and IMO [F (1, 31) = 7.73, P < 0.01]. All the other factors and interactions were not statistically significant (time, juvenile stress, juvenile stress x IMO, time x juvenile stress, time x IMO, time x juvenile stress x IMO). Thus, IMO increased the amplitude of the ASR, whereas juvenile stress is ineffective by itself and it is not able to potentiate the effects of IMO at adulthood. Similar results were obtained with the area under the curve of the response instead of the peak of the amplitude of the response (data not shown). Latency of response was not affected by the treatments (data not shown).

4. Discussion

The major findings of the present study are that juvenile stress exerts only minor long-term effects on behavior and HPA function in adulthood. However, juvenile stress did potentiate the long-term effects of adult exposure to a severe stressor (IMO) on anxiety measured in the EPM, giving some support to the “two-hit” hypothesis. It therefore appears that even if exposure to stress only during the prepubertal period is unable to exert major long-lasting
effects on reactivity to stress, attention should be paid to latent effects that can be unmasked under appropriate conditions.

Although our model of juvenile stress was able to reduce body weight gain, which is characteristic of stressors of intermediate to high intensity (Armario et al., 1990; Martí et al., 1994), this effect vanished at adulthood. Our model of juvenile stress by itself decreased adult exploration of novel environments, consistent with previous data using other stress models (i.e. Avital et al., 2006; Tsoory and Richter-Levin, 2006, Tsoory et al., 2007, 2008). However, no long-term effects were detected with classical measures of anxiety (EPM, dark-light or ASR tests). The reduction of activity/exploration in novel environments has been interpreted as reflective of anhedonic-like behavior (Fukushiro et al., 2012). The lack of anxiogenic effects of the present model contrasts with other previous reports (Avital et al., 2006; Bazak et al., 2009; Ilin and Richter-Levin, 2009; Jacobson-Pick and Richter-Levin, 2012; Jacobson-Pick et al., 2008; Tsoory et al., 2007, 2010). Although this might suggest that our procedure is of lower intensity than previous protocols, this is difficult to demonstrate and not supported by the observed reduction of body weight gain. It is more likely that the discrepancies are due to the use of animals differing in susceptibility to stress (see Armario and Nadal, 2013, for a review).

Surprisingly, juvenile stress had a small impact on the HPA axis at adulthood. The only observed change was a flattened circadian rhythm of ACTH, with lower than normal peak levels at lights off. This altered ACTH maximum amplitude was not associated with an altered corticosterone rhythm. It is well known that the amplitude of the circadian rhythm of corticosterone is higher than that of ACTH (i.e., Dallman et al., 1978). This is because the circadian rhythm of the HPA axis is subject to mechanisms of amplification in the cascade of changes and that this amplification occurs between the hypothalamus and the adrenal cortex,
including the pituitary. The amplification between the pituitary (ACTH) and the adrenal cortex (corticosterone) is mediated by the neural innervation of the adrenal by the splanchnic nerve (i.e., Ottenweller et al., 1978).

Most studies in humans characterize the integrity of negative glucocorticoid feedback using the classical dexamethasone-suppression test under basal conditions. We therefore studied negative feedback mechanisms under basal conditions. Moreover, we used corticosterone instead of dexamethasone because the former is the natural glucocorticoid in rats and the latter is excluded from the brain in rats (Meijer et al., 1998; Mason et al., 2008). We did not observe any effect of juvenile stress on the integrity of negative glucocorticoid feedback. These results are compatible with the lack of differences in the expression of GR and MR receptors in brain areas known to be critical for negative glucocorticoid feedback (e.g., Mizoguchi et al., 2003): the medial prefrontal cortex, the hippocampal formation and the PVN. Finally, we did not observe any change in CRF gene expression either in the PVNmpd or in other brain areas where prominent CRF gene expression has been detected (Kovacs 2013): the CeA, the BNSTdl and BNSTvl. Finally, the response of HPA hormones to various stressors differing in duration and intensity (circular corridor, EPM and IMO) was not modified by prior exposure to juvenile stress. Therefore, no evidence for an altered regulation of the HPA axis and the brain CRF systems was obtained in rats subjected to juvenile stress.

To our knowledge, there is only one previous report on the long-term effects of exposure to stress during the post-weaning period for 4 weeks on central components of the HPA axis and the CRF system. Isgor et al. (2004) showed that post-weaning stress caused reduced expression of GR in the DG and CA1 regions of the hippocampal formation associated with a higher corticosterone responsiveness to stressors when assessed either just after the chronic.
stress protocol or after 3 weeks of delay, although the enhanced corticosterone responsiveness was modest at the later time. Although the post-weaning chronic stress protocol was much more prolonged in Igor et al. (2004), our results are compatible with these data. In fact, only a few reports have studied peripheral HPA hormones, and those studies were restricted to plasma corticosterone. In general, resting corticosterone levels and their responsiveness to additional stressors showed null or small effects (Giavanoli et al., 2013; Ilin and Richter-Levin, 2009; Jacobson-Pick et al., 2012; Maslova et al., 2002a; Toledo-Rodríguez and Sandi, 2007, 2011; Yee et al., 2011). Interestingly, there is some evidence for a stronger effect of stress during adolescence (prepubertal or posterior) on adult HPA reactivity to stress in females than in males (Barha et al., 2011; Bourke and Neigh, 2011; Jacobson-Pick et al., 2012; Pohl et al., 2007; Weathington et al., 2012), although these gender differences have not been found consistently (Mathews et al. 2008; McCormick et al., 2005, 2008; Toledo-Rodriguez and Sandi, 2007; Wright et al., 2008). The particular type of stressor may be one of the factors to account for those inconsistencies.

In the present study, juvenile stress by itself did not induce anxiogenic effects in adulthood as evaluated by the EPM, nor did it alter the ASR. This contrasts with previous reports of increased anxiety (Giovanoli et al., 2013; Jacobson-Pick et al., 2008; Maslova et al., 2002a, 2002b; Tsoory et al., 2007; Yee et al., 2011), although negative or inconsistent results have also been reported (Avital and Richter-Levin, 2005; Bourke and Neigh, 2011; Jacobson-Pick and Richter-Levin, 2010; McCormick et al., 2007; Peleg-Raibstein and Feldon, 2011; Pohl et al., 2007; Toledo-Rodríguez and Sandi, 2007). This is likely related to a differential susceptibility to stress among the different strains of rats and mice used (e.g., Maslova et al., 2002a, 2002b).

However, other factors might be relevant. For instance, adolescence social defeat stress could
specifically induce long-term changes in anxiety when tests were conducted in the context of prior social defeat but not in other contexts (Vidal et al., 2011).

Exposure to IMO elicited a marked and prolonged activation of the HPA axis, confirming the severity of this stressor (i.e., García et al., 2000; Márquez et al., 2002; Martí et al., 2001). Juvenile stress did not affect either the HPA or hyperglycemic responses to IMO. Because stress-induced increases in plasma glucose are an index of the activation of the adrenal medulla and particularly of plasma adrenaline (Martí and Armario, 1998), the present finding argues against an effect of juvenile stress on the activation of the sympathetic-medulloadrenal axis. Despite this severity, IMO by itself had no long-term (7 days post-stress) anxiogenic effects in the EPM. Previous data from our laboratory indicate that the anxiogenic effects of IMO in this test are transitory and vanish over days (Belda et al., 2004a, 2008a). In contrast, this stressor did induce long-term effects in the ASR, potentiating the amplitude of the reflex. Although the long-term effects of a single exposure to severe stressors on the ASR are not always consistent (see Armario et al., 2008 for a review), the observed increase is in accordance with some previous studies using other stress models of presumably high severity (e.g., Cohen et al., 2005; Khan and Liberzon, 2004; Servatius et al., 1995).

Interestingly, prior juvenile stress potentiated the negative consequences of a superimposed stressor on anxiety-like behavior in the EPM. In contrast, such a potentiation was not detected with the ASR, suggesting that the latter test does not measure the same component of anxiety as the EPM (Yilmazer-Hanke et al., 2004). The present results agree with other studies indicating that juvenile stress increases the response of adult rats to further stressors that by themselves were ineffective in stress-naïve rats (Avital and Richter-Levin, 2005; Horovitz et al.,
2014; Maggio and Segal, 2011), although this potentiation is not always found (Bazak et al., 2009; Tsoory et al., 2007).

The primary difference between the present result and certain previous works is that, in our case, the type of stressors experienced by the animal at the juvenile period was different from the adult stressor, which may affect the interpretation of the data. In addition, in the present work, we studied how juvenile stress was affecting the “long-term” (and not the “short-term”) effects of the adult stressor. The fact that IMO exposure unmasked the latent effect of juvenile stress is within the theoretical framework of the “two-hit” hypothesis (see Introduction).

Another possible hypothesis to account for the long-term effects of early-life stress is the “match-mismatch” hypothesis. This hypothesis, originally developed by Gluckman et al. (2007), states that early-life stress increases coping behavior and adaptability to further exposures to stress during adulthood and that it is the “match” or “mismatch” between the early and adult environment that makes the early stress “adaptative” or “detrimental” (Claessens et al., 2011; Oitzl et al., 2010; Schmidt, 2011). Although this is a very attractive idea in the field of juvenile stress, which is supported by some experimental data (Buwalda et al., 2013; Overmier and Murison, 1991), it does not seem to explain the present results. Interestingly, the laboratory of Richter-Levin has also demonstrated that juvenile stress impairs active avoidance learning in adulthood (Ilin and Richter-Levin, 2009; Tsoory and Richter-Levin, 2006; Tsoory et al., 2008, 2010), which is by itself a stressful procedure, thus supporting sensitization to further stressors.

In conclusion, the present results demonstrated only minor effects of juvenile stress on the peripheral and central functioning of the HPA axis. However, whereas only minor behavioral consequences were noted in juvenile stress rats, this procedure potentiated the anxiogenic
effects of an adult stressor, supporting the “two-hit” hypothesis. The behavioral “sensitization”
induced by previous exposure to juvenile stress has important implications in that early-life
stressful experiences may have long-term latent effects only unmasked after adult exposure to
severe stressors.
Acknowledgments

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Captions for Figures

Figure 1. Summary of the experimental methods (see text for details). ASR: acoustic startle response, EPM: elevated-plus maze, IMO: immobilization, PND: postnatal day.

Figure 2. Basal plasma levels of ACTH (pg/ml) and corticosterone (μg/ml) during the day in adulthood, for control (CTR) rats and rats exposed to juvenile stress (JS). Means and SEM are represented; + p < 0.05 vs. control in the same hour.

Figure 3. Plasma levels of ACTH (pg/ml) in adulthood, for control (CTR) rats and rats exposed to juvenile stress (JS). Basal levels (baseline) were taken one week before the suppression. To study the suppression, corticosterone was injected at 13:00 h, and the rats were sampled in basal conditions at 15:00, 19:00 and again at 9:00 h on the next day. Means and SEM are represented; ** p < 0.001 vs. baseline.

Figure 4. Representative autoradiographs of corticotrophin-releasing factor (CRF) mRNA in the paraventricular nucleus of the hypothalamus (PVN), central amygdala (CeA), bed nucleus of the stria terminalis lateral dorsal and ventral (BNSTdl and BNSTvl), for control (CTR) rats and rats exposed to juvenile stress (JS).

Figure 5. Representative autoradiographs of glucocorticoid receptor (GR) mRNA in the cingulate cortex area 1 (Cg1), infralimbic cortex (IL), prelimbic cortex (PrL), paraventricular nucleus of the hypothalamus (PVN), and hippocampal formation (dentate gyrus or DG, CA1 and CA3) for control (CTR) rats and rats exposed to juvenile stress (JS).
Figure 6. Representative autoradiographs of mineralocorticoid receptor (MR) mRNA in the hippocampal formation (dentate gyrus or DG, CA1, CA2 and CA3) for control (CTR) rats and rats exposed to juvenile stress (JS).

Figure 7. Numbers of ambulations in a circular corridor in adulthood, in blocks of 5 min, for control (CTR) rats and rats exposed to juvenile stress (JS). Means and SEM are represented; * p < 0.05 vs. control in the same block.

Figure 8. ACTH (pg/ml) and corticosterone (µg/ml) responses to immobilization (IMO) stress in adulthood, for control (CTR) rats and rats exposed to juvenile stress (JS). Rats were sampled before IMO (0), at 30 min after the start of IMO, at the end of 120 min of IMO (120), and 60 and 180 min after the end of IMO (R60, R180). Means and SEM are represented; ** p < 0.01, *** p < 0.001 vs. pre-IMO (0).

Figure 9. Behavioral measures in the elevated plus-maze (EPM) in adulthood, for control (CTR) rats and rats exposed to juvenile stress (JS). In adulthood, groups were undisturbed before the EPM (NO IMO) or exposed to immobilization (IMO) stress one week before testing. Means and SEM are represented; + p < 0.05, ++ p < 0.01 vs. JS-NO IMO.

Figure 10. Acoustic startle response (ASR) in mV, in adulthood, for control (CTR) rats and rats exposed to juvenile stress (JS). In adulthood, groups were undisturbed before the ASR test (NO IMO) or exposed to immobilization (IMO) stress 9-11 days before testing. Means and SEM are represented; ## p < 0.01 vs. NO IMO.
References


Maggio, N., Segal, M., 2011. Persistent changes in ability to express long-term potentiation/depression in the rat hippocampus after juvenile/adult stress. Biol. Psychiatry. 69, 748-753.


Figure 1
Figure 2

BASAL CIRCADIAN RHYTHM

A

ACTH (pg/ml)

B

Corticosterone (µg/dl)

Dark period

CTR
JS

0900 1500 1900 2300 0900

0 25 50 75 100

25 50 100

0 5 10 15 20 25

0900 1500 1900 2300 0900

Dark period

Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
CIRCULAR CORRIDOR

![Graph showing ambulations over blocks of 5 min for different conditions.]

Figure 7
Figure 8
Figure 9
Figure 10

ACOUSTIC STARTLE RESPONSE

Blocks of 10 Trials

ASR (mV)

0 750 1500 2250 3000

1 2 3

CTR-NO IMO
CTR-IMO
JS-NO IMO
JS-IMO
Table 1. Expression (mRNA) of glucocorticoid (GR) and mineralocorticoid (MR) receptors and CRF in several brain regions in control (CTR) animals or exposed to juvenile stress (JS). BNSTld and BNSTlv: lateral dorsal and ventral bed nucleus of the stria terminalis, CeA: central amygdala, Cg1: cingulate cortex area 1, IBI and OBI: inner and outer blade of the dentate gyrus, IL: infralimbic cortex, PrL: prelimbic cortex, PVNmpd: medial parvocellular dorsal division of the paraventricular nucleus of the hypothalamus.

<table>
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<th>PREFRONTAL CORTEX</th>
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<th>PVN</th>
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<td>mRNA CRF</td>
<td>mRNA CRF</td>
<td>mRNA GR</td>
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<td>Cg1</td>
<td>CTR</td>
<td>554±65</td>
<td>430±54</td>
<td>739±81</td>
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<td></td>
<td>JS</td>
<td>555±62</td>
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<td>IBI</td>
<td>CTR</td>
<td>583±48</td>
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<tr>
<td></td>
<td>JS</td>
<td>530±25</td>
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</table>

The values are expressed in arbitrary units as mean ± SEM. (n=10 per group). No statistical significant differences between the groups were observed.
Table 2. Representative behavioral measures in the elevated-plus maze (EPM), dark-light (DL) and acoustic startle response (ASR) tests, and plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone (CORT) after exposure to the circular corridor and to the EPM for control (CTR) and juvenile stress (JS) rats.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>CTR</th>
<th>JS</th>
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<tr>
<td>Time in the open arm (s) EPM</td>
<td>35.1 ± 9.4</td>
<td>31.6 ± 10.8</td>
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<td>Open arm entries (Nº) EPM</td>
<td>2.9 ± 0.8</td>
<td>2.2 ± 0.8</td>
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<td>Closed arm entries (Nº) EPM</td>
<td>9.3 ± 1.0</td>
<td>7.7 ± 1.1</td>
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<td>Entries into white compartment (Nº) DL</td>
<td>7.3 ± 1.3</td>
<td>7.7 ± 1.3</td>
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<td>Latency to enter the white compartment (s) DL</td>
<td>191 ± 67</td>
<td>173 ± 65</td>
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<td>Activity into the white compartment (cm) DL</td>
<td>444 ± 83</td>
<td>496 ± 90</td>
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<tr>
<td>Peak of the startle response (mV) ASR</td>
<td>2164 ± 320</td>
<td>1876 ± 187</td>
</tr>
<tr>
<td>Plasma levels of ACTH after circular corridor (pg/ml)</td>
<td>69.4 ± 9.8</td>
<td>99.5 ± 16.3</td>
</tr>
<tr>
<td>Plasma levels of ACTH after EPM (pg/ml)</td>
<td>65.4 ± 12.2</td>
<td>56.7 ± 8</td>
</tr>
<tr>
<td>Plasma levels of CORT after circular corridor (µg/ml)</td>
<td>3.39 ± 1.00</td>
<td>6.44 ± 1.43</td>
</tr>
<tr>
<td>Plasma levels of CORT after EPM (µg/ml)</td>
<td>7.76 ± 1.59</td>
<td>10.17 ± 2.14</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. (n=10 per group). No statistical significant differences between the groups were observed.
Table 3. Representative behavioral measures in the dark-light test in control (CTR) rats or exposed to juvenile stress (JS) and/or to an adult immobilization stress (IMO).

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>NO-IMO</th>
<th>IMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Stress Treatment</td>
<td>CTR</td>
<td>JS</td>
</tr>
<tr>
<td>Juvenile Stress Treatment</td>
<td>CTR</td>
<td>JS</td>
</tr>
<tr>
<td>Entries into white compartment (N°)</td>
<td>6.1 ± 0.9</td>
<td>5.0 ± 0.9</td>
</tr>
<tr>
<td>Latency to enter the white compartment (s)</td>
<td>215 ± 61</td>
<td>323 ± 88</td>
</tr>
<tr>
<td>Activity into the white compartment (cm)</td>
<td>368 ± 57</td>
<td>333 ± 115</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. (n=8-10 per group). No statistical significant differences between the groups were observed.
HIGHLIGHTS

- Juvenile stress in rats decreased exploration in adulthood without changing anxiety
- Juvenile stress altered the circadian rhythm of ACTH in adulthood
- Immobilization in adulthood increased the ASR without changing EPM
- Juvenile stress potentiated the anxiogenic effects of immobilization in the EPM