Title: *The bed nucleus of stria terminalis and the amygdala as targets of antenatal glucocorticoids: implications for fear and anxiety responses*

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Disclosure/conflict of interest: The authors have no conflicts of interest to declare.

Acknowledgments: This study was supported by a grant from the Institute for the Study of Affective Neuroscience (ISAN). AJR is supported by a Fundação para a Ciência e Tecnologia (FCT) grant.

ABSTRACT

Rationale: Several human and experimental studies have shown that early life adverse events can shape physical and mental health in adulthood. Stress or elevated levels of glucocorticoids (GCs) during critical periods of development seem to contribute for the appearance of neurospyschiatric conditions such as anxiety and depression, albeit the underlying mechanisms remain to be fully elucidated.

Objectives: The aim of the present study was to determine the long-term effect of prenatal erxposure to dexamethasone- DEX (synthetic GC widely used in clinics) in fear and anxious behavior and identify the neurochemical, morphological and molecular correlates.

Results: Prenatal exposure to DEX triggers a hyperanxious phenotype and altered fear behavior in adulthood. These behavioral traits were correlated with increased volume of the bed nucleus of the stria terminalis (BNST), particularly the anteromedial subivision which presented increased dendritic length; in parallel, we found an increased expression of synapsin and NCAM in the BNST of these animals. Remarkably, DEX effects were opposite in the amygdala, as this region presented reduced volume due to significant dendritic atrophy. Albeit no differences were found in dopamine and its metabolite levels in the BNST, this neurotransmitter was substantially reduced in the amygdala, which also presented an up-regulation of dopamine receptor 2.

Conclusions: Altogether our results show that *in utero* DEX exposure can modulate anxiety and fear behavior in parallel with significant morphological, neurochemical and molecular changes; importantly, GCs seem to differentially affect distinct brain regions involved in this type of behaviors.

KEYWORDS: anxiety; fear; amygdala; BNST; stereology; neurodevelopment; corticosteroids

INTRODUCTION

Since the initial evidence on its benefit in the prevention of respiratory distress syndrome in preterm infants (Liggins and Howie, 1972), antenatal glucocorticoids (GC) have gained wide clinical use in the enhancement of fetal lung maturation in pregnancies at risk of preterm delivery (Crane et al., 2003; Crowley, 1995; NIH, 1995). In fact, accumulating evidence shows a substantial decrease in neonatal morbidity and mortality, which strongly supported the recommendations for its clinical use (Hofmeyr, 2009; NIH, 1995). Additionally, antenatal GCs have been use to treat other conditions such as congenital adrenal hyperplasia (Speiser et al., 2010).

GCs act predominantly through intracellular receptors which control the transcription of GC-responsive target genes (Matthews, 2001); Sousa et al., 2008). While endogenous GCs (corticosterone in rats and cortisone in humans), at basal levels, display higher affinity to the mineralocorticoid receptors (MR), leaving glucocorticoid receptors (GR) receptors largely unoccupied (De Kloet et al., 1998), synthetic GCs such as dexamethasone (DEX) can readily cross the placenta and bind almost exclusively to GR (Reul et al., 1987). GRs can also be activated when endogenous GC secretion is elevated and this chronic activation is noxious for both the periphery and the brain (Sapolsky, 1999). One relevant finding is that despite both being efficacious in preventing respiratory distress syndrome (Crowley, 2000), synthetic GCs effects seem to be more deleterious than endogenous GCs (Oliveira et al., 2006).

While the use of antenatal GCs triggers long-term gains in several clinical conditions, evidence is also revealing the detrimental effects of antenatal exposure to these hormones; in fact, it is now established a correlation between GC exposure in early life with several metabolic (Barker, 1995; Lindsay et al., 1996) and emotional and mood disorders in both animal models and humans in adulthood (Mesquita et al., 2009; Weinstock, 2001). These so-called “programming effects” of GCs may result from endocrine, morphological, neurochemical and molecular changes in specific brain regions; as examples, we have shown that antenatal exposure to DEX significantly changes the mesolimbic circuit (Leao et al., 2007) while others have shown deleterious effects in the prefrontal cortex (Diaz Heijtz et al., 2010), amygdala and hypothalamus (Nagano et al., 2008). Importantly, previous studies from our lab have shown that antenatal exposure to GCs induces a hyperemotional phenotype in

adulthood (Oliveira et al., 2006). Given the established the role of the amygdala and bed nucleus of stria terminalis (BNST) in fear and anxiety (Davis, 1992a, b, 1998, 2006), herein we decided to perform an extensive morphological and molecular analysis of these brain regions in adult animals exposed to antenatal DEX and to search for correlations with fear and anxiety behaviors.

MATERIALS AND METHODS

Animals and Treatments

Experiments were conducted in accordance with European Union regulations (Directive 86/609/EEC) and National Institutes of Health guidelines on animal care and experimentation.

Fourteen pregnant Wistar rats (Charles River Laboratories, Barcelona, Spain) were individually housed under standard laboratory conditions (room temperature 22ºC; 12/12h light/dark cycle; food and water *ad libitum*). Subcutaneous injections of dexamethasone (DEX, 1 mg/kg; N=7) or vehicle (CONT, 1 mL/kg; N=7) were administered on embryonic (ED) 18 and 19 of pregnancy, as previously described (Oliveira et al., 2006; Oliveira et al., 2011). Weaning was carried out at postnatal day 21; male offspring was randomly distributed in groups of two animals per cage according to treatment. Ten males from 4 to 5 different litters of each treatment were tested for behavior at 3 months age.

Elevated plus maze

The elevated plus maze test, used to assess the degree of anxiousness, was performed on a black polypropylene plus-shaped platform elevated 72.4 cm above the floor (ENV-560; MedAssociates Inc, St Albans, VT, USA). The maze consisted of two open arms (50.8 X 10.2 cm) and two closed arms (50.8 X 10.2 X 40.6 cm). Animals were tested over a period of 5 minutes, under bright white light. The number of entries into each of the arms and the time spent therein were recorded using a system of infrared photobeams. Data were processed to obtain the ratio of time spent in the open arms versus total time and the number of entries into each arm of the maze.

Acoustic startle as a function of stimulus intensity

Acoustic startle reflex was measured in a startle response system (SR-LAB, San Diego Instruments, San Diego, CA, USA). The apparatus consisted of a non-restrictive Plexiglas cylinder (inner diameter 8.8 cm, length 22.2 cm), mounted on a Plexiglas platform and placed in a ventilated, sound-attenuated chamber. A piezoelectric element detected cylinder

movements. Background white noise (intensity 63 dB) was used to minimize the impact of external acoustic stimuli.

Animals were habituated to the apparatus (5 minutes daily) for 2 days before testing. In the trial day, following 5 minutes acclimatization to the chamber, rats were presented 5 baseline startle stimuli (50 ms pulse of white noise at 120 dB) at a 30 s inter-stimulus interval, in order to become familiarized with the startle stimuli. Then, 60 startle stimuli were randomly presented (50 ms duration and variable intensity between 70 and 120 dB at 10 dB increments). Startle magnitude was assessed at 1 ms intervals, during the 200 ms period following stimulus. Chambers were cleaned between tests (70% ethanol and water) in order to remove olfactory cues.

Prepulse inhibition

The test lasted 20 minutes, following a 5 minutes acclimatization period. A white background noise (70 dB) was provided. Following five introductory 120 dB startle trials (noise lasting 40 ms), a total of 35 test trials were pseudo-randomly delivered as follows: a) five trials with background noise only, b) 10 startle trials of 120 dB and c) five prepulses of each of four different intensities preceding a startle trial. Prepulse intensitites of 2, 4, 8 and 16 dB above the background noise level lasted 20 ms and preceded the 120 dB startle presentation in 100 ms. Intertrial intervals ranged from 10 to 20 seconds. The average startle response (AVG) was assessed in the 100 ms period following the onset of the startle stimulus presentation. The mean AVG for the ten 120dB startle trials was employed in the formula used to assess the percentage of reduction in AVG compared to startle trials alone: % PPI = 100 X [1-(AVG at prepulse plus startle trial)/(AVG at startle trial)].

Fear-potentiated startle

A stainless steel grid was adapted to the floor of the testing chamber, through which an electric current could be passed under software control. Following a 5 minutes acclimatization period, 20 light-shock pairings were presented at 30 seconds intervals. The

shock (0.6 mA) was presented during the last 500 ms of the 5 s light pulse (3 watt incandescent light bulb). Following conditioning, animals were returned to their home cages.

On the testing day, the steel grid used during conditioning, was maintained. After 5 minutes acclimatization, 20 baseline startle stimuli were presented. Startles were measured. Then, animals were randomly presented 10 startle stimuli (intensity 120 dB, duration 50 ms), five of them during the last 50 ms of the delivery of a 5 s luminous conditioned stimulus (CS).

Histological procedures

Following behavioral assessment, 8 male animals (derived from 4 to 5 different litters) of each group were placed under deep pentobarbital anesthesia and transcardially perfused with either 4% paraformaldehyde solution (N=4) or 0.9% saline (N=4).

Brains from the former set of subjects were embedded in glycolmethacrylate (Tecnovit 7100; Heraeus Kulzer, Werheim, Germany) and 30 µm coronal sections were obtained by microtome (Cerqueira et al., 2005). These were placed on a gelatinized slide, stained with Giemsa, mounted with Entellan (Merck, Darmstadt, Germany) and coverslipped. Shrinkage factor was calculated according to previous studies (Madeira et al., 1990).

Brains from animals perfused with 0.9% saline were processed for Golgi-Cox staining as previously described (Gibb and Kolb, 1998); details are given in supplementary material.

Regional boundaries

We analyzed stereological parameters in three regions of the amygdaloid complex, namely the basolateral anterior (BLa), lateral (La) and central (CeA), as previously outlined (Paxinos and Watson, 2005). CeA and La nuclei were differentiated using prior definitions (De Omlos et al., 2004) and the BLa division was distinguished from surrounding areas on the basis of cell size and staining intensity (De Omlos et al., 2004; Krettek and Price, 1978). In accordance with a recent revision of anatomical and projection studies of the BNST (Dong et al., 2001), three divisions were considered, the anteromedial (BNSTam), anterolateral (BNSTal) and

posterior (BNSTp). In order to avoid observer bias and to permit comparison between groups, the same observer drew all regional boundaries.

Stereological procedures

Volume and cell number estimations were obtained using StereoInvestigator® software (MicroBrightField, Williston, VT, USA) and a motorized microscope (Axioplan 2, Carl Zeiss, Hamburg, Germany) attached to a camera (DXC-390, Sony Corporation, Tokyo, Japan). The Cavalieri´s principle (Gundersen et al., 1988) was applied to evaluate the volume of each region. Average cell numbers were estimated using the optical fractionator method (West et al., 1991). Coefficients of error were computed according to previously published formulas for cell numbers (Gundersen et al., 1999) and volume estimates (Gundersen and Jensen, 1987). Detailed methods are provided in supplementary material.

Dendritic tree analysis

BLa pyramidal-like, CeA multipolar and BNSTam bipolar neurons were chosen as described elsewhere (McDonald, 1982a, b, 1983). For each selected neuron, all branches of the dendritic tree were reconstructed at 600x magnification using a motorized microscope (Axioplan 2, Carl Zeiss), with oil-immersion objectives, and attached to a camera (DXC-390, Sony Corporation) and Neurolucida software (MicroBrightField). A 3-D analysis of the reconstructed neurons was performed using NeuroExplorer software (MicroBrightField). Dendritic branches were sampled in order to estimate spine density; spines in the selected segments were classified in thin, wide, ramified and mushroom categories (Harris et al., 1992). Thin spines were considered immature, while the other spine types were classified as mature. A total of 20 neurons/group/area were drawn (total 120 neurons).

Brain catecholamines

A different set of males was sacrificed by decapitation (n=6 animals derived from 3 different litters per group). After brain snap freezing, macrodissection of the amygdala and bed

nucleus of stria terminalis was rapidly performed under a stereomicroscope (Model SZX7, Olympus America Inc., Center Valley, PA, USA). Whole brains were placed upside down and, using delicate forceps (Dumont #7 forceps, Fine Science Tools USA Inc., Foster City, CA, USA), the areas of interest were dissected according to stereological coordinates (Paxinos and Watson, 2005). Samples were frozen in liquid nitrogen (overnight at -20ºC) after adding percloric acid 0.2M; then, samples were briefly sonicated, centrifuged and 50 µl aliquots of the supernatant injected on a high performance liquid chromatography (HPLC) combined with electrochemical detection system. A mobile phase of 0.7 M aqueous potassium phosphate (monobasic) (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/l) and Na-EDTA (40 mg/l) was used.

Levels of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA) were determined using a Gilson instrument (Gilson Inc., Middleton, WI, USA), fitted with an analytical column (Supelco Supelcosil LC-18 3 M; 7.5 cm x 4.6 mm; flow rate: 1.0–1.5 ml/min; Supelco, Bellefonte, PA, USA). A standard curve was then obtained and data presented as concentration (nanogram per milligram of tissue protein).

Molecular correlates

In order to establish molecular correlates, the mRNA expression of dopamine D1 and D2 receptors (Drd1 and Drd2, respectively) was assessed in the BNST and amygdala. Moreover, we evaluated the expression of several synaptic plasticity-related genes, including synapsin (Syn), brain-derived neurotrophic factor (BDNF) and neural cell adhesion molecule (NCAM). The amygdala and bed nucleus of stria terminalis of an additional set of animals (N=8 animals derived from 4 different litters/group) were isolated as described above. For Real-Time PCR analysis, total RNA was isolated from frozen areas using Trizol (Invitrogen) and DNase treated (Fermentas), according to manufacturer. Two µg of RNA were converted into cDNA using the iSCRIPT kit (Biorad). RT-PCR was performed using SyberGreen (Qiagen) and the Biorad q-PCR CFX96 apparatus. HPRT was used as a housekeeping gene. We used relative quantification to determine the fold change difference between control and DEX animals,

using the ΔΔCT method as described before (Pfaffl, 2001). Primer sequences available in Supplementary Material.

Statistical analysis

Results are presented as mean ± SEM. A repeated measures test was used to analyze data from acoustic startle, prepulse inhibition and fear potentiated startle; Greenhouse-Geissser and Huynh-Feldt´s corrections were applied for acoustic and fear potentiated startle data, respectively. The comparison of means between groups was performed using the Student's t-test for the remaining variables. Statistical significance was considered for $P<0.05$.

RESULTS

Brief antenatal exposure to DEX triggers hyperanxiety and impairs fear conditioning

Animals exposed to either DEX 1mg/kg or vehicle (controls) at embryonic day 18 and 19 were tested at 3 months of age. Antenatal exposure to DEX resulted in a significant reduction on the ratio of time spent in open arm when compared to controls (t=3.636, P=0.004; Figure 1A); these results confirm our previous findings (Oliveira et al., 2006). Furthermore, while the number of open arm entries was also decreased following DEX exposure (t=2.424, P=0.026; Figure 1B), there were no significant differences between groups in the number of closed arm entries (t=0.340, P=0.738), thus showing that the increased anxiety behavior should not be attributed to locomotory differences.

In the acoustic startle test, startle amplitudes for DEX exposed subjects increased more rapidly as a function of stimulus intensity when compared to controls ($F_{(3,47)}$=4.036, P=0.016; Table 1). When comparing responsiveness to individual noise intensities between groups, DEX exposed rats displayed significantly increased startle amplitudes at 70 (t=5.121, P<0.001), 80 (t=4.252, P=0.001), 90 (t=3.537, P=0.006), 100 (t=3.045, P=0.012) and 110 dB (t=2.452, P=0.025).

Interestingly, the acoustic startle response to inhibitory prepulses was not affected by exposure to antenatal DEX, as shown by the analysis of treatment x prepulse intensity interaction ($F_{(3,54)}$=3.858, P=0.994; Figure 2). Moreover, comparison between groups failed to reveal significant differences at any prepulse intensity. The fact that all groups had similar percentages of reduction in the average of startle response, supports the absence of sensorimotor deficits.

Startle amplitude varied as a function of the treatment X stimulus interaction in the fear potentiated startle ($F_{(1,18)}$=8.379; P=0.01; Table 2). While controls displayed enhanced startle amplitude after being presented a conditioned stimulus startle, DEX exposure resulted in an

impaired fear conditioning in the acoustic startle, as shown by the significantly decreased ratio of conditioned stimulus/basal startle responsiveness in the DEX group (t=-2.602, P=0.018; Figure 3).

Antenatal-DEX exposure promotes dendritic remodeling in the BNST and in the amygdala

To further understand the behavioral findings, we have performed a detailed morphological characterization of the BNST and amygdala brain regions, known to be involved in fear and anxiety, respectively (Walker and Davis, 1997).

Volumetric determinations reveal that antenatal exposure to DEX resulted in an increase in BNST total volume (t=3.841, P=0.009; data not shown). This effect was largely due to increased volumes in anteromedial division of the BNST of DEX-exposed animals (t=5.887, P=.001; Figure 4A); conversely, the volumes of BNSTal and BNSTp were not influenced by such antenatal exposure (t=-0.484, P=.646 and t=0.718, P=.500, respectively). DEX exposure during pregnancy did not affect the total number of cells in the BNST (t=1.595, P=0.162) nor in its divisions (BNSTam: t=1.475, P=0.191; BNSTal: t=-1.506, P=0.183; BNSTpost: t=0.946, P=0.381; Figure 4B).

The volumetric increase in the BNST was further scrutinized through a 3-D morphological analysis of dendritic arborizations of neurons in the BNSTam; the neurons in this brain region were previously described as cells with ovoid soma, moderate polarized dendritic branching, with sparse spines (McDonald, 1983). Data revealed that antenatal exposure to DEX resulted in a significant increase of total dendritic length when compared to controls (t=4.498, P<0.001; Table 3). Spine densities were not affected by antenatal DEX treatment (t=-1.779, P=0.085), even when a separate analysis of mature (t=-1.825, P=.076) and immature forms (t=-0.534, P=.0596) was performed.

In contrast to the BNST, antenatal exposure to DEX resulted in a decreased volume of BLa and CeA divisions of the amygdala (BLa t=-3.564, P=0.02; CeA t=-3.072, P=0.027) but not in the La division (t=-0.561, P=0.605) (Figure 4C). No effect was observed on the estimated number of cells in the BLa, CeA and La divisions of the amygdala (t=1.457, P=0.196; t=-1.383, P=.216; t=-0.725, P=0.496, respectively; Figure 4D).

The analysis of the dendritic arborizations of pyramidal-like neurons in the BLa region of the amygdala, which represent the predominant cell-type in the area (70%; type I) (McDonald, 1982b; Sah et al., 2003), revealed a reduction in total and basal dendritic lengths following DEX treatment (t=-2.471, P=0.018 and t=-3.252, P=0.002, respectively; Table 3); however, apical dendritic length was not affected (t=-0.832, P=0.411) by antenatal DEX-exposure. The density of spines in these neurons was not affected by antenatal exposure to DEX (t=0.165, P=0.870), even when assessing mature and immature spines separately (t=-0.122, P=0.903 and t=0.598, P=0.555, respectively).

Treatment also affected the CeA predominant cell type, previously called medium spiny neurons (by comparison with neurons in the striatum) (Sah et al., 2003). These neurons have an ovoid or fusiform soma and three to five nonspiny primary dendrites from which moderately spiny, sparsely branching secondary and tertiary dendrites arise. These neurons in the DEX progeny displayed a significant dendritic atrophy when compared to controls (t=-3,491, P=0.002; Table 3). No effect was found on the densities of total, mature and immature spines (t=0.435, P=0.666, t=0.023, P=0.982 and t=1.332, P=0.191, respectively).

Neurochemical and molecular correlates

To complement the behavioral and morphometric analysis, we decided to measure dopamine levels and its metabolites. Antenatal DEX exposure had no effect on the concentration of dopamine in the BNST, nor its turnover (dopamine t=0.026 P=0.980; dopamine turnover t=0.291, P=0.777; Figure 5A-B).

In contrast, the neurochemical analysis in the amygdala revealed a decrease in dopamine concentration following antenatal exposure to DEX (t=5.006, P=0.001; Figure 5C). Moreover, dopamine turnover was significantly increased in these subjects (t=-4.405, P=0.001; Figure 5D).

At a molecular level, and given the impact of antenatal DEX-exposure on dendritic arborization, we assessed the expression of the synaptic plasticity-related genes in the BNST and amygdala, namely a synaptic gene (synapsin (Syn)), a neurotrophin (brain-derived neurotrophic factor (BDNF)) and a cell adhesion molecule (neural cell adhesion molecule

(NCAM)), all known to be involved in synaptic/dendritic plasticy. Moreover, as the dopaminergic innervation of the BLa is considered to facilitate amygdala dependent functions (Asan, 1997), and since a hypodopaminergic status was previously associated with DEX-exposure (Leao et al., 2007; Rodrigues et al., 2010), the expression of dopamine D1 and D2 receptors (Drd1 and Drd2, respectively) was also evaluated.

In the BNST, DEX exposed animals displayed increased expression of synapsin (t=-2.418, P=0.030) and NCAM (t=-2.032, P=0.045) (Table 4). No significant differences between groups were found in the expression of the remaining genes analysed (Drd1 t=-0.663, P=0.514; Drd2 t=-0.881, P=0.386; BDNF t=-0.214, P=0.832).

In the amygdala, Drd2 mRNA levels were significantly increased in the DEX exposed subjects (t=-3392, P=0.008), while Drd1 were not affected (t=0.370, P=0.717) (Table 4). The expression levels of the synaptic plasticity-related genes, namely synapsin, BDNF and NCAM were not significantly different between groups (t=0.546, P=0.594, t=-1.457, P=0.167 and t=-0.376, P=0.713, respectively).

DISCUSSION

In utero and early postnatal environment, namely the GC milieu, is crucial for neurodevelopment not only through its activational effects, but also through important programming effects. Increasing evidence suggests that early life exposure to GC triggers undesired metabolic, cardiovascular, neuroendocrine and behavioral phenotypes in adulthood (Mesquita et al., 2009). Both synthetic (such as DEX) and naturally occurring GCs are able to exert a programming effect in the brain; however, it is important to remember that their effects can be quite distinct (Oliveira et al., 2006) as endogenous GCs preferentially bind to MR, whereas DEX binds almost exclusive to GR (Reul et al., 1987), the receptors that mediate most of the detrimental effects of GCs. Although it has been argued that synthetic GCs have a limited capacity to reach the brain in mice (De Kloet et al., 1975), its deleterious effects in the central nervous system are undeniable (Cerqueira et al., 2005; Cerqueira et al., 2007; Leao et al., 2007; McArthur et al., 2005; Yu et al., 2010), specially in rats, and may arise from the use of higher dosages which can indeed pass the barriers and activate brain GC receptors (Miller et al., 1992; Reul et al., 1987). Moreover, in the context of the present study it is important to highlight the fact that the exposure to DEX has occurred during a period in which the brain barriers are still immature (Saunders et al., 2000)."

The data herein presented extends our previous observations that antenatal exposure to DEX results in anxiety-like behavior in adult animals (Oliveira et al., 2006). In fact, while we confirmed a hyperanxious phenotype in the elevated plus maze, we also found an increased acoustic startle response (ASR) in DEX-exposed rats. The startle, a fast protective response from the organism to a sudden and intense stimulus (Koch, 1999), is considered an indicator of anxiety-like behavior. The ASR results from the activation of a trisynaptic circuit, where auditory inputs from several brainstem nuclei (dorsal cochlear nucleus, cochlear root nucleus, ventral cochlear nucleus, lateral superior olive and ventrolateral tegmental nucleus) are conveyed to a main sensorimotor interface, the caudal pontine reticular nucleus, which then projects to spinal motor neurons to trigger the motor response (Koch, 1999; Lee et al., 1996). Importantly, it was demonstrated that a direct projection from the BNST to the caudal pontine reticular nucleus, but also an indirect one via the CeA, are responsible for the modulatory response of stressors upon the ASR (Davis et al., 1997).

The BNST is one of the relay stations that conveys inputs from stress-sensitive areas of the cortex and limbic system both to the HPA axis but also to brain stem nuclei implicated in several emotional behaviors (Herman and Cullinan, 1997). Here, we show a hypertrophy of the BNST, due to the enlargement of its anteromedial region. Interestingly, this region presented alterations in the expression levels of NCAM, a molecule important for neuronal plasticity (Bisaz and Sandi, 2010; Nacher et al., 2002). Augmented levels of synapsin, a protein involved in the regulation of neurotransmitter release (Rosahl et al., 1995), also suggest increased synaptic activity of this brain region in DEX-exposed animals, which is in line with the increased dendritic arborization of these animals. Such plastic changes are paralleled with an increased startle response, thus supporting the idea of an overactivation of this brain subregion. This increased activity of the BNSTam is also in accordance with our previous observation of a hyperresponsive HPA axis in these subjects (Oliveira et al., 2006). Importantly, the activational increase of circulating corticosteroids might further promote the activation of the BNST as it is known that these hormones are implicated in the neuroanatomical changes observed in this brain region (Pego et al., 2010).

Several projections to the caudal pontine reticular nucleus are considered responsible for the increase in startle amplitude induced by fear conditioning to a previous neutral stimulus. These include projections from the CeA, either direct or via mesencephalic reticular formation and deep layers of superior colliculus, but also indirect projections from the medial amygdala via ventromedial hypothalamus and ventral periaqueductal gray (Davis, 2006). Previous data supports that while baseline startle amplitudes are not affected by interference with these connections, the response to fear conditioning is. Interestingly, we here show that antenatal DEX exposure impairs fear conditioning in adulthood, which is consistent with deficits in amygdalar function and memory consolidation for emotionally arousing experiences. These results, together with previous data showing that early life stress (neonatal isolation) resulted in impairment of context-induced fear conditioning in adult male rats (Kosten et al., 2006), confirms that fear conditioning can be modulated by adverse early life events. The role of different divisions of the amygdala in fear behavior, in particular the CeA and BLa, has been extensively studied. Several forms of CeA lesions have been correlated with disruption of fear-potentiated startle (Campeau and Davis, 1995; Hitchcock and Davis, 1987; Walker and Davis, 1997). Electrolytic lesions of the CeA

completely blocked the expression of fear-potentiated startle in rats (Kim and Davis, 1993). Conversely, inactivation of BNST did not disrupt fear-potentiated startle (Gewirtz et al., 1998; Walker and Davis, 1997); however, more recently, it has been suggested that BNSTlat tonically inhibits fear-potentiated startle, probably through projections to the CeA (Meloni et al., 2006). Thus, the present observations of decreased volumes in the CeA and BLa in DEX-exposed rats are likely to be implicated in the changes in fear conditioning displayed by these animals; once again, the volumetric decreases in these amygdalar divisions result largely from dendritic atrophy-which fits previous observations of the effects of chronic unpredictable stress in the BLa (but not the CeA) (Vyas et al., 2003; Vyas et al., 2002). It is relevant to stress at this point that others have shown that a single course of betamethasone at postnatal day one failed to affect volumes of cerebral cortex, corpus callosum, hippocampus, dentate gyrus or amygdala (Yossuck et al., 2006), however, several technical differences may explain this discrepancy. The time of the exposure and the nature of insults seems to be critical issues to consider, as several windows of vulnerability to the programming effects of different stimuli seem to occur. Indeed, stress effects in neuronal morphology of specific brain regions are not necessarily equal across life and depend on the type of stress. While stress effects in the BNST morphology seem more similar, since different types of stress induce a general hypertrophy of dendrites in the neurons of this brain region (Pego et al., 2008; Vyas et al., 2003), the effects of stress in the amygdala are less concordant to our present observation of dendritic atrophy. Whereas chronic juvenile stress leads to a general hypertrophy of amygdalar dendrites (Eiland et al., 2011), others failed to find any significant change in dendritic structure after stress exposure in adulthood (Pego et al., 2008) and others have shown remarkably divergent changes in amygdalar neurons following different types of stress (Vyas et al., 2003; Vyas et al., 2002).

Remarkably, such morphological effects in the amygdala were accompanied by changes in dopamine but not other cathecolamines levels. Dopamine, arising mainly form the VTA, plays a facilitative role in the function of the amygdala (Asan, 1997). *In vitro* studies also suggest that the excitability of amygdalar, namely BLa, neurons is modulated by dopamine (Kroner et al., 2005). In addition, behavioral data showed that VTA lesion results in blocked fear-potentiated startle (Borowski and Kokkinidis, 1996). At least in part, the facilitation of affective behaviors by DA may be explained by actions at the cellular level on BLa neurons. It

has been suggested that DA receptor activation could simultaneously facilitate the BLa output in response to strong inputs that cause spike firing, while suppressing weaker inputs via activation of GABAergic interneurons (Kroner et al., 2005). Thus, the present observation of a hypodopaminergic status in the amygdala seems to be of relevance to the deficits in fear memory. In fact, it confirms observations in models of dopamine deficient mice (Fadok et al., 2010), in which the restoration of dopamine in the BLa was shown to be required for the formation of fear-related memory (Fadok et al., 2010). Dopamine levels in the amygdala are also determinant for sensorimotor gating, as it was shown that NAcc and amygdalar infusions of this neurotransmitter significantly impair PPI (Swerdlow et al., 1992); surprisingly, we did not find any differences in PPI in DEX-exposed animals, which is also in accordance with another previous study (Hauser et al., 2006).

Herein, the decreased amygdalar levels of dopamine are associated with increased expression of D2 receptor mRNA in DEX exposed subjects; such up-regulation could be seen as a compensation mechanism. Remarkably, no differences were found in D1 receptor expression, suggesting that D2 receptor has a prominent role in fear behavior. Indeed, while D1 receptors may participate in recognition of danger, D2 receptors seem to have a role in setting up adaptive responses to adverse stimuli (de la Mora et al., 2010). Moreover, D2 (but not D1) antagonists injection in the BLa impairs fear-potentiated startle probably due to reduced dopaminergic tone (de Oliveira et al., 2011), which is in agreement with the amygdalar hypodopaminergic status seen in DEX-exposed animals. These findings are consistent with recent data showing that D2 receptor pathway connecting the VTA and BLa modulates conditioned fear (de Oliveira et al., 2011).

The present findings unravel the impact of antenatal exposure to DEX in the brain regions implicated in fear and anxiety behaviors. We show that subjects exposed to GCs during neurodevelopment present marked neuroanatomical, neurochemical and molecular programming changes in the BNST and amygdala, and are more vulnerable to anxiety and fear pathology in adulthood. Thus, our findings further support the concerns raised on the potential deleterious effects of antenatal exposure to synthetic corticosteroids (Weinstock et al., 2005; Talge et al., 2007; Mesquita et al., 2009) and call for the need of a parsimonious use of these drugs.

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TABLES

Noise (dB)	Controls	DEX
70 dB	30,51 ± 3,03	**97,50 ± 15,07 ***
80 dB	32,50 ± 4,72	**291,17 ± 86,56 ***
90 dB	145,19 ± 21,39	**561,26 ± 115,66 ***
100 dB	1203,54 ± 168,88	**3126,46 ± 608,44 ***
110 dB	4645,30 ± 461,93	**6221,83 ± 447,09 ***
120 dB	6358,69 ± 257,17	7101,62 ± 519,47

Table 1 – Acoustic startle data. Startle amplitude (arbitrary units) in response to acoustic stimulus. Data presented as mean ± SEM. * different from controls, P<.05.

	Controls	DEX
Startle	5290.89 ± 658.66	5252.08 ± 716.75
CS + Startle	6229.04 ± 425.83	**4554.13 ± 577.61***

Table 2 – Fear-potentiated acoustic startle data. Startle amplitude (arbitrary units) in response to acoustic stimulus with or without previous conditioned stimulus (CS). Data presented as mean ± SEM. * different from controls, P<.05.

	Controls	DEX
BNSTam (bipolar)		
Dendritic length (µm)	395 ± 17	**511 ± 19***
Density of spines (n/µm)	0.58 ± 0.042	0.49 ± 0.027
Mature	0.47 ± 0.035	0.39 ± 0.026
Immature	0.10 ± 0.015	0.09 ± 0.016
BLa (pyramidal)		
Dendritic length (µm)	1969 ± 128	**1506 ± 137***
Basal dendrites	1088 ± 87	**721 ± 72***
Apical dendrite	881 ± 80	785 ± 83
Density of spines (n/µm)	0.95 ± 0.046	0.95 ± 0.027
Mature spines	0.71 ± 0.037	0.70 ± 0.026
Immature spines	0.24 ± 0.022	0.26 ± 0.012
CeA		
Dendritic length (µm)	818 ± 38	**670 ± 19***
Density of spines (n/µm)	0.45 ± 0.043	0.47 ± 0.037
Mature	0.35 ± 0.039	0.36 ± 0.032
Immature	0.09 ± 0.009	0.12 ± 0.015

Table 3 – Dendritic length and spine density of bipolar neurons of anteromedial region of the bed nucleus of stria terminalis (BNSTam), pyramidal-like neurons of basolateral amygdaloid nucleus (BLa) and multipolar neurons of the central nucleus of the amygdala (CeA) and Data presented as mean ± SEM. * different from controls, P<.05.

Treatment	Drd1	Drd2	Syn	BDNF	NCAM
BNST					
Controls	0.950 ± 0.12	1.070 ± 0.12	0.918 ± 0.05	1.127 ± 0.14	0.987 ± 0.04
DEX	1.229 ± 0.21	1.226 ± 0.13	**1.200 ± 0.11***	1.196 ± 0.27	**1.318 ± 0.25***
Amygdala					
Controls	1.066 ± 0.13	0.927 ± 0.07	1.167 ± 0.19	0.920 ± 0.04	1.043 ± 0.10
DEX	0.990 ± 0.16	**2.494 ± 0.62***	1.060 ± 0.04	1.102 ± 0.12	1.115 ± 0.17

Table 4 - Gene expression analysis (measured by real-time PCR) in amygdala and bed nucleus of stria terminalis (BNST). The mRNA expression levels are presented as the fold change increase in relation to the respective control. Data presented as mean ± SEM. * different from controls, P<.05. Dopamine D1 receptor (Drd1), dopamine D2 receptor (Drd2), synapsin (Syn), brain-derived neurotrophic factor (BDNF) and neural cell adhesion molecule (NCAM).

LEGENDS TO FIGURES

Figure 1 – Elevated plus maze data. (A) Ratio of time spent in open arm over total time. (B) Number of open and closed arm entries. Data presented as mean ± SEM. * different from controls, P<.05.

Figure 2 – Prepulse inhibition test. Percentages of reduction in average startle response compared with startle trials at prepulse intensities of 2, 4, 8 and 16 dB above background noise level.

Figure 3 – Fear potentiated acoustic startle data. Data presented as the average of ratios between conditioned stimulus/basal startle responsiveness ± SEM. * different from controls, P<.05.

Figure 4 – (A) Estimated volumes of anterolateral (BNSTal), anteromedial (BNSTam) and posterior (BNSTpost) divisions of the bed nucleus of stria terminalis. Data presented in mm^3, as mean ± SEM. (B) Estimated total number of cells in the anterolateral (BNSTal), anteromedial (BNSTam) and posterior (BNSTpost) divisions of the bed nucleus of stria terminalis. (C) Estimated volumes of BLa, CeA and La divisions of the amygdala. Data presented in mm^3, as mean ± SEM. (D) Estimated total number of cells in the BLa, CeA and La divisions of the amygdala. Data presented as mean ± SEM; * different from controls, P<.05

Figure 5 – Neurochemical analysis by HPLC of the bed nucleus of stria terminalis (BNST) (A-B) and the amygdala (C-D). (A, C) Concentration of each neurotransmitter (ng/mg of protein) is presented. 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA). (B, D) Dopamine turnover assessed by the ratio between dopamine metabolites and dopamine concentrations. Data presented as mean ± SEM.