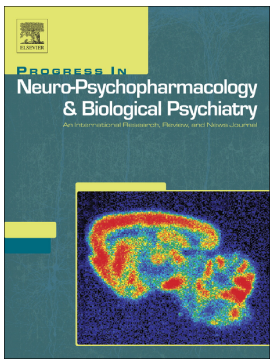

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Long-Term Impact of Chronic Variable Stress in Adolescence versus Adulthood

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ABSTRACT:

Adolescence is a period of active development of stress regulatory neurocircuitry. As a consequence, mechanisms that control the responses to stress are not fully matured during this developmental period, which may result in vulnerability to chronic stress. We hypothesized that adolescent chronic stress would have negative consequences on stress adaptation later in life. Male Wistar rats (PND40) were subjected to chronic variable stress (CVS) for 2 weeks, with 2 daily stressors randomly presented and overnight social stressors twice a week. After five weeks, animals were evaluated during adulthood, using the elevated plus maze (EPM) and the forced swim test (FST). The hypothalamic-pituitary adrenal (HPA) axis response to a 30-min restraint was also assessed. Results are compared to those of adult rats tested 5 weeks following CVS cessation. Our results demonstrate that the long-term effects of CVS are specific to the age of application of the stress regime. We show how behavior and HPA axis response as well as hypothalamic paraventricular nucleus activation can differ with age, resulting in differential behavioral adaptations for animals stressed in adolescence and dysregulation of the HPA axis in the animals stressed in adulthood, These data underscore the importance of the adolescent period in determining resilience of the HPA axis and programming behavioral responses later in life.

KEY WORDS

Adolescent, Stress, chronic, corticosterone, ACTH, HPA axis, PVN

INTRODUCTION:

Adolescence is a period of physiological, behavioral and neurobiological transition that prepares the animal for adult life (Spear, 2000). Prefrontal cortical circuits are among the last to attain adult configuration. Indeed, late adolescence is a time of active synaptic pruning of prefrontal-amygdala connections (Cressman et al., 2010), alterations in the expression of prefrontal neurotransmitters and receptors, and increased myelination of key corticolimbic pathways (reviewed in (Andersen, 2003). In humans, the adolescence period is the major period of onset for stress-related diseases, including depression and anxiety disorders (Andersen and Teicher, 2008), and stress during adolescence has been frequently associated to the first episode of numerous psychiatric disorders (Goodyer, 2000; Patel et al., 2007). Likewise, it has been reported that individuals with previous history of depression in adolescence have a higher risk to suffer depressive episodes in adulthood (Aalto-Setälä et al., 2002; Lewinsohn et al., 2000). Together, these finding suggest that adolescence may be a critical period for development of emotional regulatory processes.

Chronic stress and accompanying alterations in neuroendocrine (hypothalamo-pituitary-adrenocortical (HPA) axis) function are vulnerability factors contributing to the development of neuropsychiatric disorders (reviewed in (de Kloet et al., 2005). Traumatic experiences during adolescent development of the brain can be associated with psychopathologies such as depression as well as altered neuroendocrine function in adulthood (Andersen and Teicher, 2008; Oitzl et al., 2010). Susceptibility to stress and trauma during this period may be linked to disproportionate HPA reactivity. The magnitude and duration of the hypothalamic pituitary-adrenocortical (HPA) axis response to stress is dramatically higher in adolescence compared to pre-pubertal and adult rats, likely due to reduced negative feedback regulation during this

period (Romeo, 2010). Several authors have hypothesized that adolescents are more sensitive to stress relative to adults, given that this exacerbated HPA response, in combination with the active development of the brain, would define a conducive environment for the damaging effects of glucocorticoids (Jankord et al., 2011; Romeo, 2010; Romeo and McEwen, 2006).

Prior studies in humans and animals have tried to delineate how stress during adolescence affects the development of the brain, physiology and behavior. While many of these studies have focused on the immediate effects of stress at this age (Eiland et al., 2012; Jankord et al., 2011; Romeo, 2013), some authors have also explored the long-term, lasting effects of chronic stress on subsequent behavior and stress reactivity (Bourke and Neigh, 2011; Chaby et al., 2015, 2013; Isgor et al., 2004; McCormick et al., 2005; McCormick and Green, 2013; Weintraub et al., 2010; Wulsin et al., 2016; Yohn and Blendy, 2017). Experiments performed in female rats exposed to adolescent chronic stress and tested as adults indicate HPA hyporeactivity and enhanced immobility in the forced swim test, whereas no HPA or behavioral effects are observed immediately after the stress exposure (Wulsin et al., 2016). This contrast between the two time points evaluated suggests that chronic stress during adolescence may have unique long-term 'incubation' effects, consistent with differential stress reactivity in adulthood. Nevertheless, the question whether these changes were specific to the age of the stress, or if they were merely protracted effects of the stress protocol regardless the age, remains unresolved.

In the present study we demonstrate that the long-term effects of a history of CVS are specific to the age of application of the stress regimen. Our data indicate that chronic stress in adolescence produces behavioral changes later in life, both in anxiety-related behavior and passive coping, whereas HPA axis reactivity was unaffected. In contrast, HPA axis stress responses were potentiated in animals receiving chronic stress in adulthood and testing 5 weeks later, whereas

behavioral responses were unaffected. The data support the hypothesis that adolescent stress has long-term effects on emotional behaviors, which is distinct from stress exposure in adulthood.

MATERIALS AND METHODS:

Experimental Animals: Wistar male rats were bred in-house, weaned at postnatal day (PND) 21 and housed 3/cage (45cm x 30cm x 18cm) under a 12h light/12h dark cycle (lights on at 8:00 am) at constant temperature (22ºC) with free access to food and water. Animals from at least 5 different litters were used for each experiment, with no more than 2 animals per litter in the same experimental group. Two cohorts of animals derived from different litters were used to carry out the experiments. All tests were performed in an isolated behavioral room during the light cycle, between 09:00 AM and 4:00 PM. All procedures and care performed in the animals were approved by National Department of Animal Care and Health (SENASA, Argentina) and were in accordance with the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications Eight Edition, 2011) and complied with the regulations of the Guide for Care and Use of Laboratory Animals of Argentinean National Research Council, 1996. Best efforts were made to reduce the number of animals used and to minimize their suffering.

Chronic Variable Stress: The stress paradigm was adapted from the originally proposed by Herman (Herman et al., 1995) and its variant on (Ziegler et al., 1999) , that was inspired by the model of (Katz et al., 1981). Briefly, 2 daily stressors (AM between 9:00-12:00 and PM between 2:00-6:00) were applied across a 2 week-period, delivered in an unpredictable manner, with no stressors were repeated within the same day. The stressors used were: hypoxia (30 min with 8%

O2 and 92 %N,); rotation (orbital shaker 100 rpm, 1 h), cold exposure (8-10°C, 1 h); lithium chloride injection: (i.p. 1.5 M LiCl, 2% vol/body weight in saline); foot shock (animals were placed in a conditioning chamber and were immediately subjected to an electric shock of 1mA, 1s); space restriction (animals were placed for 2 hours in a metabolic box (12 x 20 cm) with metal wire mesh floors and walls) and novel environment (animals were placed for 5 minutes in a circular transparent open field of 30 cm diameter). This last stressor was repeated 3 times, the first time the animal was only exposed to the open field, while the following times a different plastic object was placed on the ground to promote novelty on the situation. In addition, twice a week, an overnight stressor (social isolation or overcrowding (6 per cage) was applied. During the 2 weeks of CVS, control animals were maintained under standard animal facility rearing conditions (i.e., housed 3/cage).

Experiment 1: Long-term effect of CVS during adolescence. Male rats were submitted to 2 weeks of CVS starting at PND 40. The age of onset was based on previous results showing that the immediate effects of adolescent chronic variable stress are more prominent in the late adolescence period compared to early adolescence (Jankord et al., 2011). Animals then remained undisturbed until PND 90 when the effects of CVS on behavior and the HPA response to an acute challenge were assessed.

Experiment 2: Long term effects of CVS in adults rats. A different cohort of adult male rats was submitted to 2 weeks of CVS using the same sequence of stressors as Experiment 1, starting at PND 60, to later assess the same endpoints following the same post-stress incubation time. To maintain the same time interval from stress cessation to testing, behavioral assessments were started on PND 110.

Both experiments included their own age-matched controls of unstressed animals. See Figure 1 A for experimental timeline.

Exploration behavior: Exploratory behavior was tested in the elevated plus maze (EPM). The apparatus consists of a plus shaped maze of Plexiglas with two opposite open arms and two opposite closed arms (all 7.5 cm wide), placed at a height of 50 cm from the floor. Each animal was placed in the central square of the maze to begin the test. All entries to both arms were recorded for 5 min. An entry was considered when the two front paws and more of than half of the animal body were inside an arm. The animals were evaluated between 12:00 and 4:00 PM. The EPM was carefully cleaned after each test with a 70% ethanol solution. Manual scoring was later performed by individuals blind to experimental grouping. The original purpose of this behavioral procedure was to evaluate anxiety-like behavior. However, under our experimental conditions the open arm times were too small to allow clear assessment of changes using this typical index for anxiety-related behavior (<30% of the time, see (Handley and McBlane, 1993). Indeed, most of the behavior displayed consisted of transitions between the dark arms. Therefore, we report only the number of total arm entries as a measure of general exploration in a novel environment as a way to assess anxiety-like behavior.

HPA response to an acute stressor: Corticosterone and adrenocorticotropic hormone (ACTH) responses to an acute restraint challenge conducted 5 days after testing in the EPM. Rats were restrained for 30 min in plastic cylindrical restrainers, with blood samples taken by tail nick at the beginning of the stressor (time 0, baseline, less than 3 minutes after restraint onset), at right before the end of the stress challenge (30 minutes) and twice after the animals were returned to their home cages (60 m and 120 min) (Vahl et al., 2005). Initial samples were obtained by clipping the distal tip of the tail and collecting ~300 ul of blood into an EDTA containing tube.

Subsequent samples were obtained by gently removing the clot from the distal tail to recommence bleeding. After collection blood was centrifuged at 1500 rpm, for 15 minutes at 4° C and then plasma samples were extracted and kept at -20° C until hormones level determination. All samples were collected before noon in order to precede the diurnal rise in corticosterone secretion seen late in the light phase.

Corticosterone and ACTH detection: ACTH was measured using a commercial kit (IMMULITE® 1000 ACTH, solid-phase, two-site sequential chemiluminescent immuno- metric assay from DPC Diagnostic Products Corporation, Los Angeles, CA, USA). Corticosterone concentration was determined by radioimmunoassay (ImmuChem Double Antibody Corticosterone I125 RIA (ICN Biomedicals, Costa Mesa, CA) according to the specifications of the manufacturer.

Adaptive behavior in forced swimming test: Five days after restraint testing, animals received a forced swim test (FST) between 12:00 and 4:00 PM. Rats were individually placed in a Plexiglas cylinder (20 cm diameter, 80 cm high). The water temperature was 29 +/-2° C. The tanks were filled at a level that prevented the animals from touching the bottom. The performance was recorded on video for later scoring of behavior (blinded observers). The method of scoring was sampling the behavior of the animal every 5 seconds to determine which of the following behaviors the animal was doing: swimming, climbing, diving or immobility; i.e. float without movement greater than necessary to keep the float and stability. The test lasted ten minutes and results were expressed as the percentage of behavioral observations over the total number of samples (6 in 1 minute, 120 in 10 minutes).

Immunohistochemistry: 24 h after the FST, rats were euthanized and brains were processed for the determination of Fos-like immunoreactivity (Fos-LI) (polyclonal antibody Santa Cruz k-25,

sc 253). This antibody detects Fos, but also FosB, Fra-1 and Fra-2 (Davern and Head, 2007; Lohmeier et al., 2002). Briefly, animals were anesthetized, between 09:00 and 12:00, with an i.p. injection with 30 % chloral hydrate and transcardially perfused with 100 ml of a blood-washing solution consisting of 0.8% sucrose, 0.8% NaCl, and 0.4% glucose, followed by 4% paraformaldehyde in 0.2 M borate buffer, pH 7.4 (Riedel-de Haenn, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany). Brains were postfixed in the paraformaldehyde solution overnight at 4°C, and then removed and placed in 30% sucrose until they sank. The brains were cut in the coronal plane at 30µm with a freezing microtome. Alternate sections were collected in a well containing a cryoprotectant solution to be stored at -20ºC until immunostaining (0.1% Polyvinyl-pyrrolidone (Sigma), 30% Sucrose (Fisher), 30% ethylene glycol (Fisher), 0.01% Sodium azide (Fisher) in PBS 0.1M ph7.4. For the detection of Fos-LI immunoreactivity, after rinsing the cryoprotectant solution 6x5min PBS 0.1M, endogenous peroxidase activity was quenched by incubating 20 min in 3% H2O2 in PBS 0.1 M. After that, unspecific binding was blocked in a mix of 4% normal goat serum, 0.4% triton X-100 and 0.2 % bovine serum albumin in PBS 0.1 M for 2h. Immediately after, sections were incubated overnight with the primary antibody mixed in blocking solution (1:3000) at room temperature. After rinsing, the slices were incubated for 1h in goat anti-rabbit IgG (Vector Labs) diluted 1:400 of each component in blocking solution. After washing, the tissue was incubated with the avidin-biotin horseradish peroxidase complex (Vectastain ABC elite Kit, Vector Laboratories), 1:400 solution in PBS 0.1M during 1h. Chromagen reactions were performed by incubating tissue in a solution of 0.02% 3,30-diaminobenzidine (Sigma), 1% of a 2% aqueous solution of nickel sulfate and 0.04% of H2O2 (30%) in PBS 0.1 M peroxide for 5 min. All the steps were performed at room temperature. Tissue was rinsed 3x5min in PBS 0.1M between each step. Sections were

mounted on gelatinized slides, allowed to dry, dehydrated with xylene and cover slipped with DPX mountant (Sigma-Aldrich). Sections from 4-5 brains per experimental group were processed (to avoid a possible cage effect, animals to be included in the study were randomly selected from different cages). For analysis, we counted 3 sections covering the anterior, medial and posterior portions of the paraventricular nucleus of the hypothalamus (PVN) from equivalent coordinates, as calculated from bregma (using coordinates from rat brain atlas (Paxinos and Watson, 2007) see schematic in figure 1B.

Statistical analysis: All data were analyzed using STATISTICA 7.0 (Statsoft, Inc.,Tulsa, USA) and Prism 7 (GraphPad Software, La Jolla California USA). For the analysis of the results from EPM, a t-test analysis was performed with a level of significance $p < 0.05$. HPA axis response over time was analyzed by repeated measurements ANOVA. Variation factors were "stress" and "time", with their respective levels and a significance of $p < 0.05$. In the cases where significant differences were found, a Tukey pot-hoc test was performed. Area under the curve was analyzed by t-test. For FST, t-test and repeated measurements ANOVA were used to evaluate both the total value of each behavior, as well as the temporal progression of them during the test.

RESULTS:

Experiment 1:

We first tested animals receiving adolescent CVS for behavioral and HPA axis reactivity in adulthood, 5 weeks after stress cessation. The rats stressed during adolescence showed a reduction in the number of total entries in the EPM compared with control animals ($t_{(16)=}2.793$, $p=0.013$ (Fig. 2A). Five days later, these animals HPA responses were determined following

acute restraint. There was no effect of adolescent stress on HPA responses to a 30-min restraint, with no main effect of stress or time by stress interaction for either ACTH, $F(1,18)=0.1796$, $P=0.67$; $F(1,54)=0.16$, $P=0.92$ or corticosterone $F(1,18)=1.41$, $P=0.24$; $F(1,54)= 0.33$, $p=0.8$ (Fig. 3A and 3B). In addition, there was no difference in the integrated stress response (as measured by area under the curve) for either ACTH ($t_{(18)}=0.50$, $p= 0.62$) and corticosterone ($t_{(18)}= 1.75$, $p=0.097$) (Table1). The index of adrenal sensitivity was calculated as the concentration of corticosterone during its peak of secretion (30 min) over the logarithm of the ACTH concentration during at the same time. This index gives an idea of how responsive the adrenal cortex is to the effects of ACTH (Engeland et al., 1981). Again, there were no difference in adrenal response index for the animals that were stressed during adolescence ($t_{(18)}= 0.38$, $p=0.71$) (Table 1).

We then tested behavioral adaptation following exposure to the FST. Rats with a previous history of adolescent stress showed a significant increase in time spent immobile compared with non-stressed subjects ($t(17)=2.454$, $p=0.025$) (Fig 2B). The time course analysis showed no interaction for any particular time-point during the test ($F(1,153)= 1.29$, $p=0.24$), corroborating the main effect between groups ($F(1.17)=5.11$, $p=0.037$) (Fig 2C). Nevertheless, it is important to point out that the total difference is mainly due to the difference between the groups during the second half of the time of the test, a phase in which the swimming behavior was also reduced (not shown) in the CVS group, indicating that the effect was in fact a behavioral adaptation after struggling equally during the initial phase (not shown) and not an initial response to the aversive situation.

To analyze prolonged neuronal activation in the PVN 24 h after challenging the animals with FST, Fos-LI, a broad indicator of long-term changes in neuronal transcription activation after a

stimulus (Sharp et al., 1991), was quantified. The number of cells expressing Fos-LI in the paraventricular neurons was reduced in rats with a previous history of stress of adolescent stress ($t(7)=3.91$, $p=0.0058$), suggesting an increased inhibitory tone in the central limb of the HPA axis (Fig 4 A,B), despite equivalent peripheral responses.

Experiment 2:

We then evaluated the long-term effects of CVS in adult rats, in order to determine whether the effects of observed following adolescent CVS are unique from those seen after similar stress exposure and rest interval in adulthood. We administered an identical CVS exposure protocol in a group of adult rats, starting at PND 60, and assessed the same variables after an identical post-stress incubation time as that used to test adolescents. Thus, PND 60-74 rats were submitted to CVS, with behavioral, HPA testing commencing 5 weeks later, controlling for CVS-testing interval (Fig. 1). In contrast to adolescent stress, CVS exposure in adults did not affect EPM exploration, with equivalent numbers of entries observed in both CVS and control animals ($t(20)=0.57$, $p=0.58$) (Fig 2D).

Also, in contrast to adolescents-CVS rats (experiment 1), the adult-stressed animals showed significant differences in the HPA response to 30-min restraint. The repeated measures ANOVA showed no main effect for ACTH ($F(1,19)=3.82$, $p=0.07$) but a significant interaction STRESS x TIME ($F(1, 57)=3.2$, $p=0.03$). Post hoc Tukey's tests showed that ACTH was significantly higher in CVS-exposed rats 60-min after the onset of the acute stressor ($p<0.05$) (Fig. 3C). The integrated values under the secretion curve were also higher for the animals stressed during adulthood $t(19)=2.6$, $p=0.018$ (Table 1). Similarly, there was a significant main effect of stress

(F(1,19)= 4.98, p=0.04) on corticosterone secretion, and a significant stress by time interaction (F(1,57)=4.23, p=0.009). The post hoc Tukey's test showed greater corticosterone secretion 30-min after the onset of the restraining (p< 0.05) (fig 3D). However, group differences in the area under the curve did not reach the criterion for statistical significance (t(19)=1.985, p=0.062). Interestingly, when evaluating the adrenal sensitivity at the 30-min time point, we observed that the CVS animals have a higher index compared with control rats (t(19)=3.23, p=0.004) (Table 1), suggesting that the adrenal gland is more sensitive to the stimulation by ACTH in animals that were previously stressed.

Unlike animals exposed to CVS in adolescence (experiment 1), animals that were subjected to CVS during adulthood showed no differences in FST immobility duration relative to controls (t(19)=0.2165, p=0.83). Similarly, there were no group differences with respect to immobility as a function of time F(1,162)= 1.18, p=0.31 (Fig 2 E,F).

Finally, animals stressed in adulthood did not show differences in Fos-LI in the PVN (t(6)=0.39, p=0.71), suggesting that the hyper-response of the HPA axis after an acute stressor in animals stressed in adulthood does not correlate with prolonged activation of the neurons in this area, as determined 24 h later.

DISCUSSION

The findings of this study provide strong evidence that CVS during adolescence causes long-term behavioral changes that are not recapitulated by a similar stress protocol in adulthood. Conversely, chronic stress in adulthood causes lasting alterations in HPA axis reactivity that are not observed following adolescent exposure to stress. The data are consistent with marked stress plasticity during both periods of life.

Our results suggest that adolescent stress causes lasting behavioral changes that are consistent with reorganization of mechanisms regulating emotional reactivity and possibly, stress-related pathologies, given the changes observed in both the EPM and FST tests. These data are roughly consistent with prior work in another rat strain (Bourke and Neigh, 2011), indicating that the effects of adolescent stress are reproducible and likely to reflect a developmental feature of the rat brain. While the mechanisms underlying the susceptibility of the adolescent brain to chronic stress remain to be determined, it is likely that lasting effects are related to interference with known developmental processes (e.g., synaptic pruning) occurring in corticolimbic connections across this period (e.g., (Cressman et al., 2010).

Prior studies observed enhanced passive coping (immobility) following CVS (Wulsin et al, 2016) in females (males not tested) or a combination of social stressors that includes restraint (Bourke and Neigh, 2011) in both males and females. Interestingly, social isolation stress (Hong et al., 2012) evoked this effect in females, but not males. It is notable in this regard that prior studies and recent work from our group suggest that female rats may be more sensitive than males to isolation (unpublished data), possibly contributing to the noted sex difference.

Remarkably, the response of the HPA axis to an acute stressor was not affected, indicating a resilient phenotype after the chronic aversive experience. These data are intriguing, given previous studies demonstrating increased HPA axis reactivity immediately after cessation of the stress protocol (Jankord et al., 2011; Wulsin et al., 2016). The lack of an altered HPA axis response is accompanied by reduced Fos-LI immune-reactivity in PVN of adolescent CVS rats, indicating that the long-term activation of these neurons after a stressor was reduced by a previous history of CVS. This could be explained by a greater capacity for plasticity in HPA regulatory circuits in the adolescent brain, especially considering that HPA axis feedback

mechanisms are still under development during this time window (Romeo, 2010). Consequently, animals may have the opportunity to modulate the development of circuits involved in the control of the axis, allowing more efficient HPA axis control in the context of stressor exposure. Together, these observations suggest that prior stress reduces central HPA drive, perhaps related to enhanced pituitary reactivity to hypophysiotrophic hormones (i.c., corticotropin releasing hormone).

It is notable that CVS causes hypersecretion of HPA hormones in response to acute stressor at several ages (Franco et al., 2016; Jankord et al., 2011; Ulrich-Lai et al., 2006; Wulsin et al., 2016). In general, this effect has been studied immediately after the application of the stress model, although some authors have assessed longer lasting effects. For example, (Bourke and Neigh, 2011) studied the effect of chronic stress using a combination of restraint and social stressors during adolescence, indicating no changes in the corticosterone response to an acute stress in males later in adulthood, whereas hypersecretion was observed in females. Prior studies in our group (Wulsin et al., 2016), using CVS and a similar experimental design to our study in terms of age and time of recovery, also reported a decrease in the HPA axis stress response in adult females following adolescent stress. However, there was no effect of adolescent stress on HPA stress reactivity in adulthood in males (Cotella et al., 2017).

The results for adult CVS rats stand in striking contrast to those seen in adolescence. The lack of behavioral effects of adult CVS suggests that unlike adolescents, adults do not effective 'incubate' the experience of prior CVS into lasting behavioral change. These data are consistent with prior studies showing substantial recovery of adult rats from deleterious effects of chronic stress. For example, a previous study from our group demonstrates that standard effects of CVS on FST immobility and weight loss are no longer observed 3 weeks post chronic restraint (Smith

et al., 2017). These results suggest that adults display resilience to behavioral change, suggesting that stress-related reorganization of stress response mechanisms are less pronounced in adulthood. The results highlight the uniqueness of the adolescent period to long-term programing of the HPA axis.

Also in contrast to animals stressed in adolescence, those stressed in adulthood showed hyper-activity of the HPA and increased adrenal sensitivity after acute stressor exposure, without an accompanying change in PVN Fos-LI. In combination, the data suggest that the mechanism for the exacerbated HPA response might be due to peripheral regulation at the level of the pituitary or adrenal rather than increased PVN activation. These results are in line with (Ulrich-Lai et al., 2006) showing adrenal gland hyperplasia and hypertrophy in adult rats following two weeks of CVS. Nevertheless, we cannot discard short-term facilitation at the PVN that could have been detected at a shorter time point after the stressor, as showed in (Bhatnagar and Dallman, 1998; Sterrenburg et al., 2011) using Fos as a marker. The fact that animals stressed in adulthood did not exhibit behavioral changes suggests that the behavior and HPA axis responsivity are dissociable along this time-course, and that plasticity of the HPA axis is more pronounced following adult stress exposure. Given the link between glucocorticoids and brain aging (Sapolsky, 1999), long-lasting stress hyper-reactivity in adulthood may be disadvantageous to coping with neuronal insults over time.

Classically, the number of arm entries in the EPM is used as a general indicator of the locomotor activity of animals (Weiss et al., 1998). In this matter, other authors have reported a decrease in the locomotor activity assessed in open field test in rats that were submitted to an adolescent variable stress protocol for one week, then evaluated at PND 60 (Luo et al., 2014). However, other researchers showed that the repeated isolation stress for 1 hour over 15 days in early

adolescence caused a slight increase in locomotion in animals when they were evaluated 3 weeks and a half later (McCormick et al., 2008). These conflicting reports may be due to differences in the stress models used and the ages of application of stress and evaluation.

Altogether, our results show how previous experience affects the later responses to stress, and how the age at which a stressful experience takes place determines different types of adaptations in the responses. It is likely that the experience of stress contributes to individuality and subjectivity of stress responses (McCarty et al., 1988; Ursin and Eriksen, 2004), and that programming in adolescence may play a major role in how the individual will then react to new stressors. Whether the developmental changes incurred by adolescent stress are advantageous or deleterious to individual coping will likely depend on the context of subsequent stressful events. For example, a withdrawal response (exemplified in reduced exploratory behavior or passive coping) may be of use during periods of environmental adversity or illness, but less so during social interactions. Conversely, limited glucocorticoid response may be advantageous during conditions of energy repletion, less so when added resources need to be mobilized. Thus, developmental changes engendered by chronic stress may be adaptive in nature in some contexts but not others, and may contribute to disease states that are precipitated or complicated by stress exposure.

Overall, our data underscore the importance of the adolescent period in programming behavioral and HPA axis reactivity later in life. Uncovering mechanisms underlying this plasticity will be critical for defining strategies to promote stress resilience and/or intervene in stress-related diseased states.

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AUTHOR CONTRIBUTIONS:

EMC and ASG postulated the hypothesis, designed experiments, performed stress protocol and behavioral testing, and obtained tissue samples. G.F collaborated to discuss plan and execute experiments. CH processed blood samples for hormones determination. MGP and JPH supervised the project. EMC, CC and PL processed brain tissue for immuhistochemistry. EMC processed, analyzed and graphed the data. EMC and ASC collaborated on preliminary draft. EMC, JPH and MGP reviewed and edited last version of manuscript.

CONFLICTS OF INTEREST:

The authors declare that this study was conducted in the absence of any financial or commercial relationships that could be construed as a potential conflict of interest.

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Figure1: Experimental design: Experiment 1: Rats were exposed to CVS during 2 weeks during late adolescence (PND40-54) and allowed to recover 5 weeks before being tested in the elevated plus maze (EPM) (PND90). 5 days later the HPA response after 30 min restraint was evaluated. 5 days after, they were tested in the forced swim test (FST) and 24 h after euthanized to obtain fixed brains. Age matched control animals were kept in normal facility rearing conditions. Experiment 2: The same design was applied in adult animals starting at PND 60 and the recovery time and delay between the different evaluations was the same in experiment 1. Age matched control animals were kept in normal facility rearing conditions. B: Representative images from the coordinates analyzed for Fos-like immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) obtained from the Paxinos and Watson rat brain atlas.

Figure 2: Upper panel: Animals that were subjected to adolescent CVS (adol CVS) during 2 weeks and evaluated after 5 weeks of recovery, and their age-matched controls. Lower panel: Animals that were subjected to adult CVS during 2 weeks and evaluated after 5 week of recovery, and their age-matched controls. A, D: Total number of entries in the elevated plus maze after 5 min exploration. B, E: Total percent of immobility in the forced swim test after 10 min exposure. C, F: Percent of immobility over time during the exposure to the forced swim test. Data are presented as mean $\pm$ s.e.m. *: significant result $p<0.05$.

Figure 3: A: Plasma ACTH concentration (pg/ml) and B: Plasma corticosterone concentration (ng/ml) after 30 min restraint and in the animals subjected to chronic variable stress during adolescence (adol CVS) and their age-matched controls after 5 weeks of recovery. C: Plasma ACTH concentration (pg/ml) and D: Plasma corticosterone concentration (ng/ml) after 30 min restraint and in the animals subjected to chronic variable stress as adults and their age-matched

controls after 5 weeks of recovery. The grey panel represents the time animals were restrained. Data are presented as mean ± s.e.m. *: Significant result $p<0.05$.

Figure 4: Fos-Li immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) counts as positive nuclei/0.01mm^2 in the animals subjected to CVS during adolescence (adol CVS) and their age- matched controls (A) and subjected to CVS as adults and their age-matched controls (C). Data are presented as mean ± s.e.m. *: significant result $p<0.05$.

B and D: Representative photomicrographs of the immunoreactivity in the PVN in each experimental group.

Table1: Results of the integration under the response curve of ACTH and corticosterone and adrenal sensitivity index in the animals stressed during adolescence (Experiment 1) or adulthood (Experimen2) and their respective aged matched controls evaluated 5weeks after the endo of CVS. *: significant result $p<0.05$.

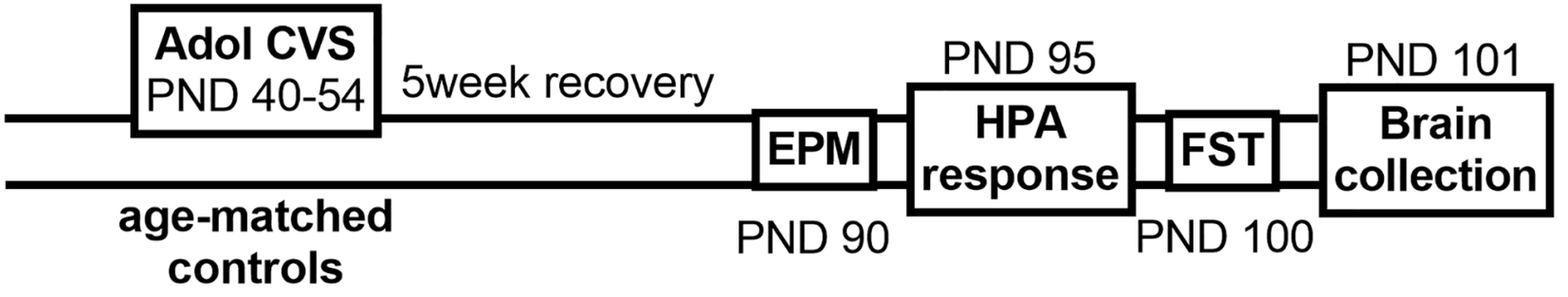
	Variable	Group	Mean ± sem
Experiment 1	ACTH AUC (arbitrary units)	Control	12201 ± 1911
		Adol CVS	13327 ± 1177
	Cort AUC (arbitrary units)	Control	26019 ± 2638
		Adult CVS	36250 ± 5227
	Peak Adrenal sensitivity [Cort]/log[ACTH]	Control	58.3 ± 12.47
		Adult CVS	64.2 ± 9.08
Experiment 2	ACTH AUC (arbitrary units) *	Control	17348 ± 1886
		Adol CVS	28814 ± 4140
	Cort AUC (arbitrary units)	Control	46428 ± 6231
		Adult CVS	62406 ± 4918
	Peak Adrenal sensitivity [Cort]/log[ACTH] *	Control	83.16 ± 18.27
		Adult CVS	153.3 ± 10.6

Highlights

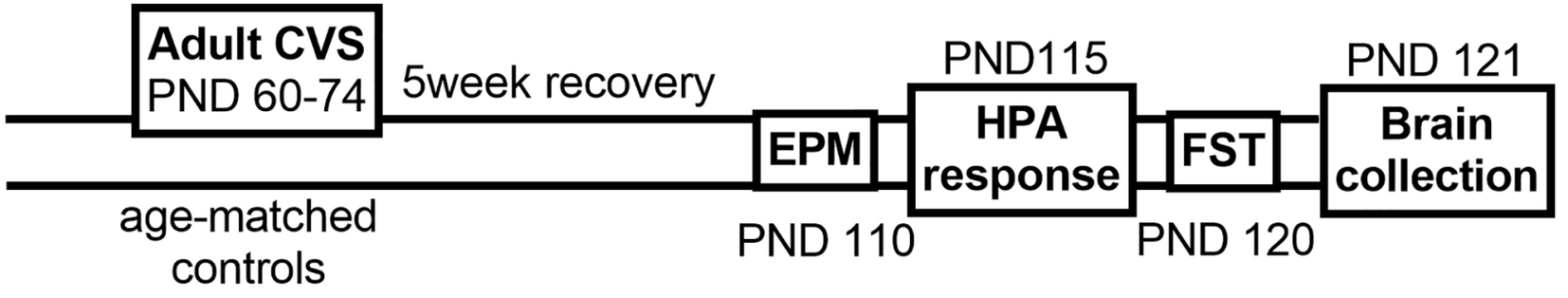
- Long-term effects of chronic variable stress are age-specific
- Adolescent stress affected behaviors tested, but had not effect on the HPA response
- Adult stress caused protracted HPA hyper response, but no had no behavioral effects

A

Experiment 1



Experiment 2



B

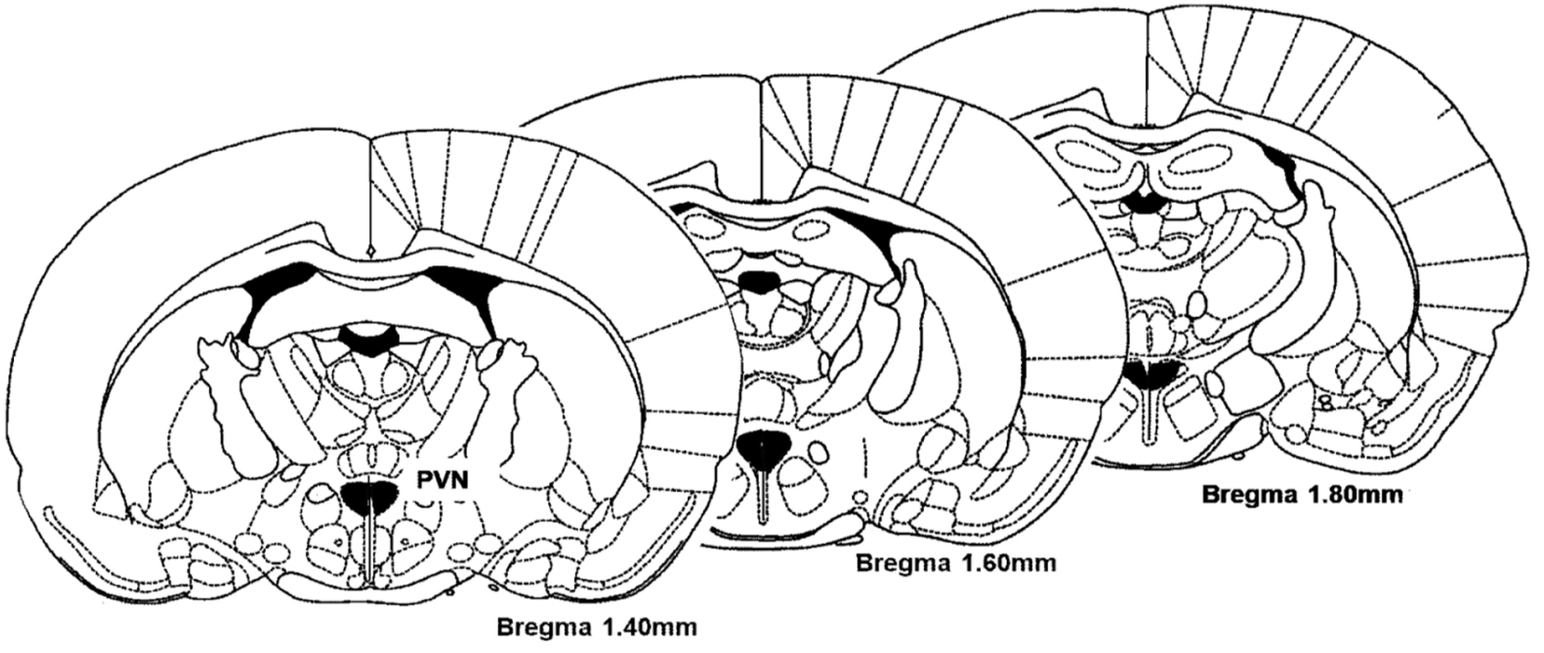


Figure 1

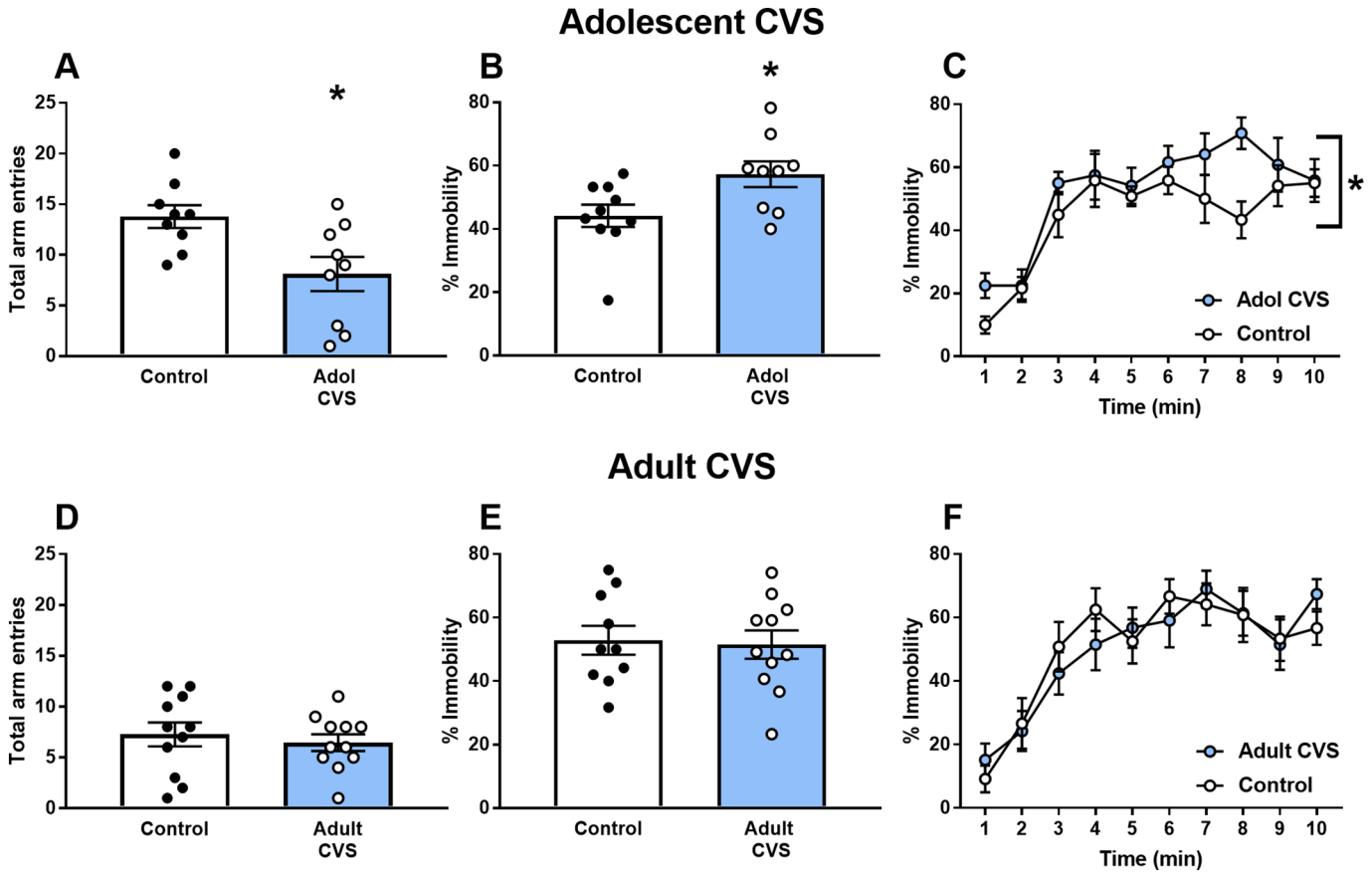

Adolescent CVS
A
Total arm entries
25
20
15
10
5
0
*
Control
Adol CVS
B
% Immobility
80
60
40
20
0
*
Control
Adol CVS
C
% Immobility
80
60
40
20
0
*
Adol CVS
Control
1 2 3 4 5 6 7 8 9 10
Time (min)
Adult CVS
D
Total arm entries
25
20
15
10
5
0
Control
Adult CVS
E
% Immobility
80
60
40
20
0
Control
Adult CVS
F
% Immobility
80
60
40
20
0
Adult CVS
Control
1 2 3 4 5 6 7 8 9 10
Time (min)

Figure 2

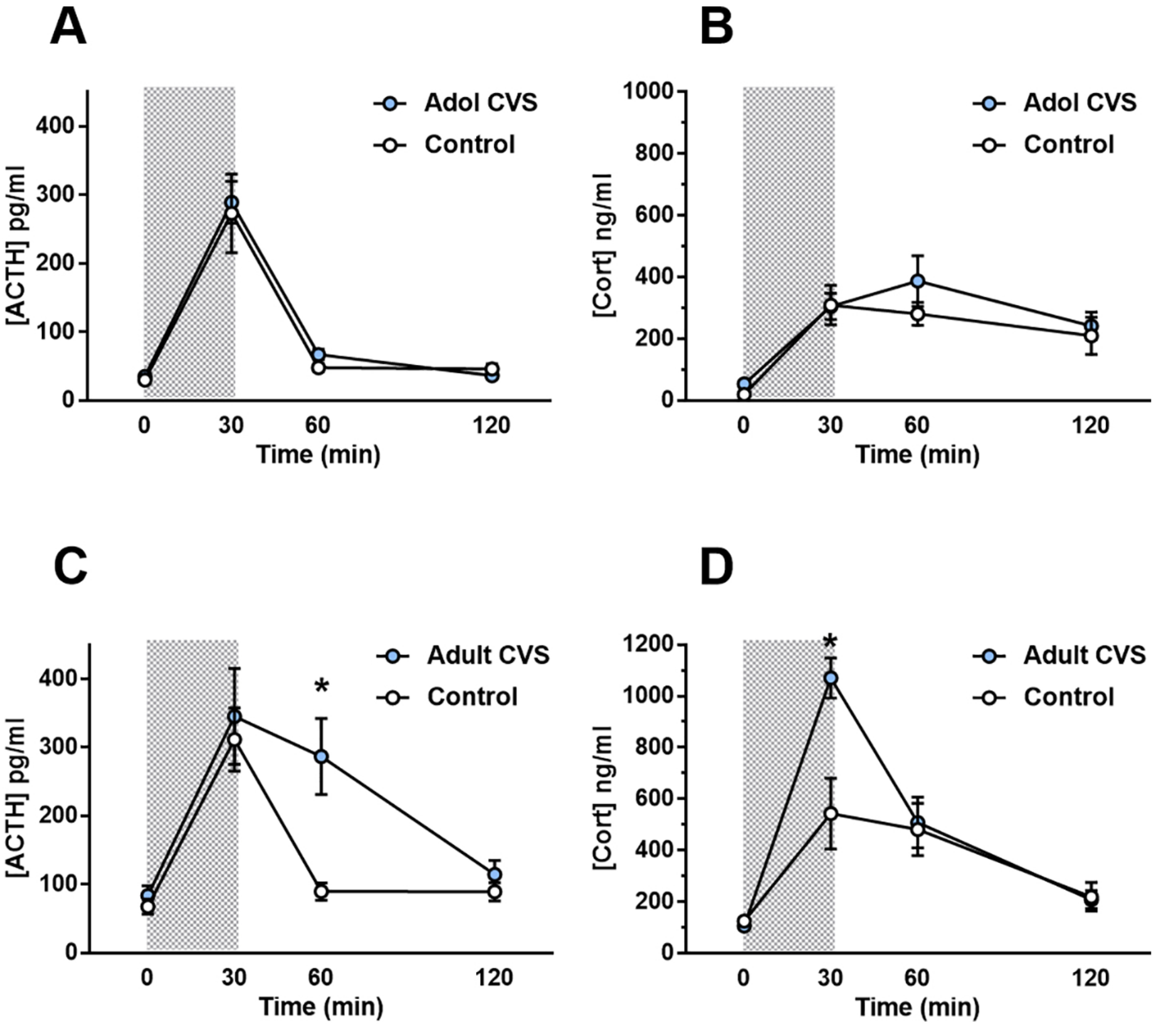

A
Adol CVS
Control
[ACTH] pg/ml
0
100
200
300
400
0
30
60
120
Time (min)
B
Adol CVS
Control
[Cort] ng/ml
0
200
400
600
800
1000
0
30
60
120
Time (min)
C
Adult CVS
Control
*
[ACTH] pg/ml
0
100
200
300
400
0
30
60
120
Time (min)
D
Adult CVS
Control
*
[Cort] ng/ml
0
200
400
600
800
1000
1200
0
30
60
120
Time (min)

Figure 3

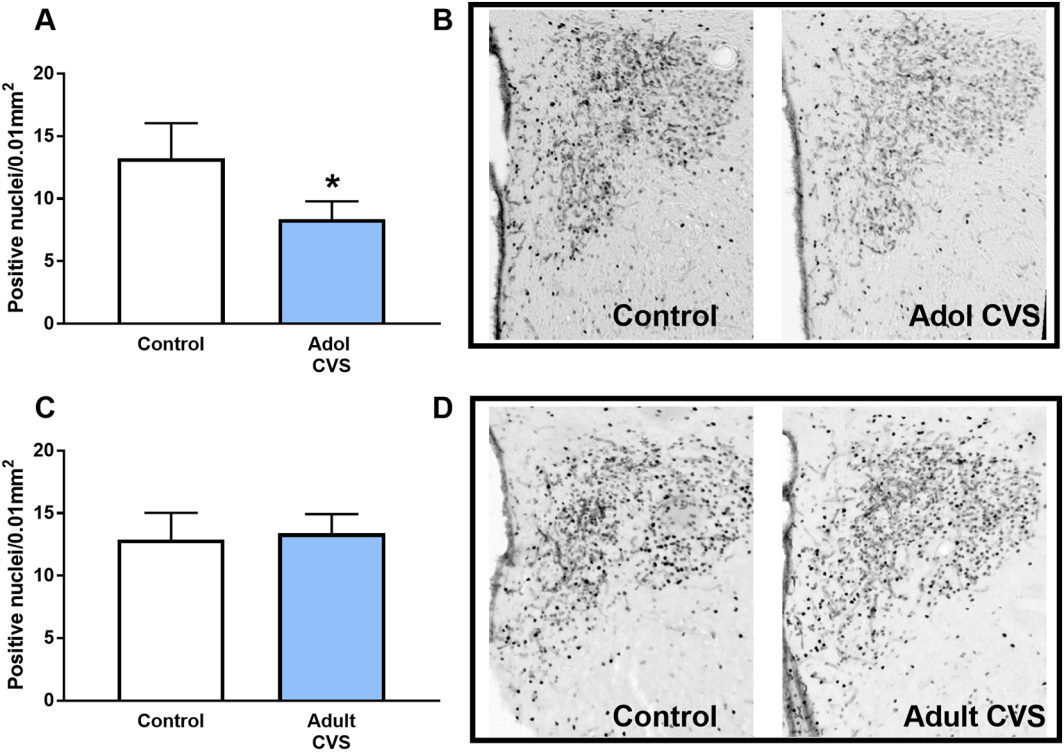

A
Positive nuclei/0.01mm2
20
15
10
5
0
*
Control
Adol CVS
B
Control
Adol CVS
C
Positive nuclei/0.01mm2
20
15
10
5
0
Control
Adult CVS
D
Control
Adult CVS

Figure 4