Published in final edited form as:

Neuroscience. 2010 September 29; 170(1): 138–148. doi:10.1016/j.neuroscience.2010.06.052.

Inescapable but not escapable stress leads to increased struggling behavior and basolateral amygdala *c-fos* gene expression in response to subsequent novel stress challenge

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Abstract

Control over an aversive experience can greatly impact the organism's response to subsequent stressors. We compared the effects of escapable (ES) and yoked inescapable (IS) electric tail shocks on the hypothalamic-pituitary-adrenal (HPA) axis hormonal (corticosterone and ACTH), neural (*c-fos* mRNA) and behavioral (struggling) response to subsequent restraint. We found that although the HPA axis response during restraint of both previously stressed groups were higher than stress-naïve rats and not different from each other, lack of control over the tailshock experience led to an increase in restraint-induced struggling behavior of the IS rats compared to both stress-naïve and ES rats. Additionally, *c-fos* expression in the basolateral amygdala was increased selectively in the IS group, and relative *c-fos* mRNA expression in the basolateral amygdala positively correlated with struggling behavior. Restraint-induced *c-fos* expression in the medial prefrontal cortex, a brain area critical for mediating some of the differential neurochemical and behavioral effects of ES and IS, was surprisingly similar in both ES and IS groups, lower than that of stress-naïve rats, and did not correlate with struggling behavior. Our findings indicate that basolateral amygdala activity may be connected with the differential effects of ES and IS on subsequent behavioral responses to restraint, without contributing to the concurrent HPA axis hormone response.

Keywords

facilitation; PFC; amygdala; helplessness; anxiety; sensitization

Introduction

Control over a previous stressful experience is an important factor in determining an organism's neural and behavioral response to future stressful events. Rats exposed to a single session of escapable (ES) or yoked inescapable (IS) tail shocks show striking behavioral differences when subsequently challenged with experiences such as a shuttle box (Amat et al., 2006), social interaction (Short and Maier, 1993), social exploration (Christianson et al., 2009) or conditioned fear stimuli (Baratta et al., 2007). Moreover, there is evidence for differential neural activity and subsequent neurochemical alterations elicited by ES and IS, some of which have been demonstrated to contribute to the different behavioral outcomes (Weiss et al., 1980, Weiss and Simson, 1986, Maier et al., 1995). On the other hand, both the ES and IS session elicit strong and indistinguishable HPA axis hormone secretion profiles (Maier et al., 1986). Thus, the subsequent differential neurochemical and behavioral outcomes cannot be attributed to different levels of HPA axis activity present during ES and IS. One of the well characterized consequences of IS, however, is sensitized HPA axis hormone secretion upon subsequent challenge with a variety of physical and psychological stressors (Johnson et al., 2002, Johnson et al., 2003, O'Connor et al., 2004). IS can also enhance the behavioral struggling responses to subsequent novel stressors (Grissom et al., 2008), and is also known to produce a sensitized neural response (increased *c-fos* mRNA) in the hypothalamic paraventricular nucleus (PVN) to subsequent novel stress challenge (O'Connor et al., 2004). To date, there is no report of the HPA axis activity present in ES rats during subsequent exposure to a novel stressor. Thus, it is unknown whether the emergence of a sensitized HPA axis response to novel stressors observed after IS is an outcome that depends on the lack of stressor controllability during the initial tailshock session. This study, therefore, addressed two related questions: 1) Do ES and IS produce differential sensitization of HPA axis and neural responses to a novel stressor challenge? 2) Are some of the differential behavioral responses to subsequent stressful situations observed after ES and IS correlated with differential HPA axis activity, and are brain regions known to underlie HPA and behavioral responses to stress differentially activated during novel stress as a result of prior ES or IS?

For this investigation we examined the hormonal, neural and behavioral (struggling) response of ES, IS, and stress-naïve rats to subsequent challenge with restraint. Restraint is characterized as a processive, or psychological stressor (Herman and Cullinan, 1997), and the neuroendocrine (Bhatnagar and Dallman, 1998, Bhatnagar and Vining, 2003, Girotti et al., 2006, Weinberg et al., 2009, Weinberg et al., 2010) and behavioral (Grissom et al., 2008) responses to restraint are highly sensitive to previous stress experience. To assess neural activity we measured the relative mRNA expression levels of the immediate early gene *c-fos* (Morgan and Curran, 1989) that were induced by 30 min of restraint challenge. We focused on two brain regions of interest, the amygdala and the medial prefrontal cortex. Amygdala activity can influence stress-related behaviors, such as struggling. For instance, rats bred for faster seizure development upon amygdala stimulation struggle to a greater extent during a stressor challenge than rats bred for slower seizure development (Anisman et al., 1997, McIntyre et al., 1999, Merali et al., 2001). Medial prefrontal cortex (mPFC) activation may also be related to possible controllability-based differences present during restraint challenge. Medial PFC activity during ES is necessary to prevent some of the subsequent detrimental neurochemical and behavioral effects that are evident in yoked IS rats (Amat et al., 2005, Amat et al., 2006, Amat et al., 2008, Christianson et al., 2009). Ventral mPFC neural activity is also necessary during behavioral testing in order to see a differential conditioned fear response between rats previously exposed to ES or IS (Baratta et al., 2008).

We supposed that if ES and IS rats exhibit different behavioral responses to the subsequent restraint experience then there might also be a difference in their HPA axis hormonal response. However, we found that although the behavioral response of IS rats to restraint was markedly different from that of stress naïve and ES animals, both stress groups showed a comparably sensitized corticosterone response. Interestingly, those behavioral differences were strongly related to basolateral amygdala, but not mPFC neural activity.

Materials and Methods

Subjects

36 male Sprague-Dawley rats (Harlan Laboratories, Madison, WI, USA, weight approximately 275 g at time of experiment), divided across two cohorts (N=24 and 12) were allowed a one-week acclimation period after arrival to the animal facilities at the University of Colorado before experimentation. Rats were housed in groups of four in polycarbonate cages with wood shavings. Food (Purina Rat Chow; Ralston Purina, St. Louis, MO, USA) and tap water were provided *ad libitum*. The colony room lights were maintained on a 12-h light/dark cycle, with lights on at 07:00 h. Procedures for ethical treatment of animals conformed to the guidelines found in the “Guide for the Care and Use of Laboratory Animals,” DHHS Publication No. (NIH) 80-23, revised 1996 ed. and were approved by the University of Colorado Institutional Animal Care and Use Committee.

Wheel-turn escape/yoked IS procedure

Rats from both cohorts were equally divided into nonshocked home cage (HC), escapable stress (ES), and inescapable stress (IS) treatment groups. Cagemates all received the same treatment. Animals were transported from the colony room to the experimental location at the same time, and HC rats were placed in a quiet area of a non-adjacent room. ES and IS-designated rats were placed in Plexiglas boxes (14×11×17 cm) with a wheel mounted in the front and a Plexiglas rod extending from the back. The rat's tail was taped to the Plexiglas rod and affixed with copper electrodes. Rats received shocks in yoked pairs (ES and IS). The treatment consisted of 100 trials with an average intertrial interval of 60 s. Shocks began simultaneously for both rats in a pair and terminated for both whenever the ES rat met a response criterion. Initially, the shock was terminated by a quarter turn of the wheel. The response requirement was increased by one quarter turn when a successful response was completed in less than 5 s. Responses made within 5-15 s resulted in a decrease of one criterion level; that is, at the half-wheel turn requirement a response of 20 sec would lower the criterion to ¼ turn level. For successful responses, subsequent latencies under 5 s increased the requirement by 50% up to a maximum of four full turns. If the requirement was not reached in less than 30 s, the shock was terminated and the requirement reduced to a single quarter turn. This procedure was used to ensure that the ES rats learned an operant response. Shock intensity was 1.0 mA for the first 35 min, 1.3 mA for the second 35 min and 1.6 mA through completion of the 100th trial, to maintain good escape responding. Shortly after shock procedure, rats were replaced in fresh cages with previous cagemates and returned to the colony room.

Restraint procedure

Approximately 48 h after shock, rats from all groups, including HC, were placed in restraint tubes for 30 min. Cagemates all received restraint at the same time, and behavior in restraint tubes was video recorded. Restraint consisted of taking rats from the home cage and placing them in adjustable length tubes (15.5 ± 2.5 cm long and 6.3 cm diameter Plexiglas tubes with air holes in the front, top and back). This stressor is considered to be primarily psychological in nature because it does not produce pain or direct physical insult (Herman and Cullinan, 1997). Restraint was administered in a separate room adjacent to the home

cage room. All behavioral manipulations were performed between 08:00 and 13:00. This time of day was chosen because it is near to the circadian trough of HPA hormone secretion, when variability between baseline HPA-axis activity is minimal.

Rats were killed immediately after 30 min via decapitation. Brains were collected, flash-frozen in an isopentane bath maintained between -30°C and -20°C and stored at -80°C . Trunk blood was taken in EDTA coated tubes, kept on wet ice and centrifuged at 5000g for 10 minutes within 45 min after collection. Plasma was then rapidly frozen and stored at -80°C until hormone assay.

Corticosterone ELISA and ACTH radioimmunoassay

Measurement of plasma corticosterone was conducted on 20 μL of plasma with an enzyme immunoassay kit (Assay Design, Ann Arbor, MI) according to manufacturer's instructions. Plasma concentrations of ACTH were determined by radioimmunoassay as described previously (Girotti et al., 2006). Sensitivity for the CORT assay was $130\text{ ng } 100\text{ mL}^{-1}$. The intra-assay coefficient of variability (CV) for the CORT assay was approximately 4%. The detection limit for the ACTH assay was 15 pg mL^{-1} for a 50 μL sample, the intra-assay CVs were between approximately 1 and 3%. ACTH inter-assay CVs in our laboratory are less than 10%.

Behavioral analysis

Behavior during restraint (struggling/strong mobility, light mobility, immobility) was defined, acquired, and analyzed largely as described in Grissom et al. (Grissom et al., 2008). Briefly, video recordings of all animals during the entire 30 min restraint were analyzed using the mobility parameter in Noldus Ethovision 3.1, which detects an animal as a field of pixels and calculates percent mobility by analyzing the difference in field size and shape between samples. Struggling was analyzed as strong mobility, corresponding to $>5\%$ pixel change, and included robust rolling and forelimb movement. Light mobility, corresponding to 2-5% pixel change, included smaller movements of the head, grooming, and subthreshold struggling behaviors. Immobility, corresponding to $<2\%$ pixel change, measured movements no larger than those generated by the animal breathing. 5 min periods were binned across the 30 min restraint yielding seconds of activity across six-5 min bins. Behavioral analyses were automated, and researchers blind to experimental conditions performed program setup.

In situ hybridization and autoradiographic image analysis

Brain sections (14 μm) were cut on a cryostat (Leica Microsystems, Bannockburn, IL, USA, model 1850) through the extent of prefrontal cortex (3.2 mm anterior to bregma) and amygdala (2.3 – 3.6 mm posterior to bregma), based on a brain atlas (Paxinos G, 1998), and thaw-mounted onto poly-L-lysine-coated slides and stored at -80°C . *In situ* hybridization using a 35S-radiolabelled riboprobe for *c-fos* mRNA was performed as described previously (Girotti et al., 2006). Semi-quantitative analyses of autoradiographs were performed on digitized images from X-ray films (Scion Image) as described (Campeau and Watson, 1997) with the following specifications: For prefrontal cortex, a 25×25 pixel square was centered within the dorsomedial prefrontal cortex (dmPFC), ventromedial prefrontal cortex (vmPFC), or ventral orbital prefrontal cortex (VO). A 30-pixel diameter circle was centered over the medial amygdala (MeA), the combined basolateral / lateral amygdala (BLA), or the central nucleus (CeA) of the amygdala, using the optic tract as a reference point for medial amygdala, and the external capsule as a reference point for lateral amygdala. Average integrated density was expressed as average percent difference from restrained stress naïve rats (HC).

Double *in situ* hybridization

A subset of prefrontal cortex sections across all three treatment groups were subject to further phenotypic characterization at the level of the prefrontal cortex using double *in situ* hybridization. Tissue sections adjacent to those used in the single-label *c-fos* expression study were analyzed using double *in situ* hybridization. Sections were hybridized with 35S-labeled GAD65/67 anti-sense mRNA and non-radiolabeled digoxigenin (Dig)-labeled *c-fos* anti-sense mRNA probes. Double-labeled slides were processed similarly as [35S] probe-only slides (detailed in Girotti et al., (2006)) with the following additions (based on Day et al., (1999)): For the *in vitro* non-radioactive transcription reaction, 2 μ L of a diluted DIG-uridine triphosphate (UTP) mix (1 part DIG-UTP (Roche, Indianapolis) to 3 parts cold UTP (Fisher Scientific, Pittsburgh)) was utilized in place of [35S]-UTP. Full-length transcription products were separated from unincorporated nucleotides using sephadex G50-50 column chromatography. The following day, after the high stringency 0.01 $\times$ standard saline citrate (SSC) wash, slides were washed 3 $\times$ in 0.1M phosphate buffered saline (PBS), pH 7.4, blocked in 0.1M PBS pH 7.4 with 0.5% Triton-X 100 and 0.25% carrageenan lamda, and incubated overnight at room temperature in primary antibody buffer (1:20,000 anti-DIG conjugated to alkaline phosphatase (Roche) in blocking buffer). The following day, sections were washed 3 $\times$ in 0.1M PBS, 3 $\times$ in 0.1M tris-buffered saline (TBS) pH 7.4, and 1 $\times$ in an alkaline substrate buffer (ASB; 0.1M TBS pH 9.5). For the color reaction, slides were placed in a light-sealed container containing ASB with 5% polyvinyl alcohol (Sigma, St. Louis, MO), 0.025% levamisole (Sigma), 0.45% 4-nitro blue tetrazolium chloride (NBT, Roche) and 0.35% 5-bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt (BCIP, Roche). The color reaction took 4 hours (based on microscope examination), and slides were washed in water, incubated in 0.1M glycine and 0.5% Triton-X 100 for 10 minutes at room temperature, and washed in water. Sections were fixed using 2.5% glutaraldehyde for 1 hour, washed in water, and air-dried. The radioactive GAD65/67 probes were detected by coating slides in Ilford KD-5 emulsion and incubating them in light-tight boxes at 4°C for 5 days. Slides were developed in pre-chilled Kodak D-19 developer to minimize background signal, dehydrated in ascending ethanol washes, delipidized with xylenes, and coverslipped.

Identification of single and double-labeled cells in the mPFC

For each slide, encompassing four to six brain sections, photomicrographs were generated for ROIs using a Zeiss Axio Scope microscope (20 $\times$ objective) and AxioVision imaging software's multidimensional acquisition function. Images of the dmPFC and vmPFC were captured, using dorsoventral positioning from the ventral orbital area as a place reference, totaling approximately 15-25 multichannel images per brain area. For each section, bright- and darkfield images were captured in a single plane of focus. Converted files were recoded for blind analysis, and were imported as Adobe Photoshop (CS3) layers. Independent, translucent layers atop of the bright- or darkfield layers were used to outline GAD and *c-fos* positive cells. GAD positive cells were determined in the darkfield layer, and included clusters of approximately 10 or greater neighboring silver grains. Blue-purple precipitate from the *c-fos* riboprobe formed filled oval shapes which we identified as *c-fos*-positive cells using brightfield microscopy. There was low background signal in both channels. Positive cells were painted in translucent colors approximating the visible grain/color boundaries. Comparison of outline layers alone revealed the number of *c-fos*, GAD, and *c-fos*-GAD-double-labeled cells.

Statistics, graphing, and exclusions from data analysis

One-way ANOVAs were performed when comparing gene expression within a given brain region and when comparing HPA-axis hormone levels. Plasma ACTH and CORT values were log[10] transformed for analysis. For behavioral analyses, a mixed-model repeated measures ANOVA was performed (time-bin $\times$ group) for the three designations of behavior

(immobility, light mobility, and strong mobility), with post-hoc tests of between group differences across time bin determined using a Fisher's least significant differences test. Post-hoc tests of between-groups effects within each time bin were performed using Bonferroni correction. When testing pair wise group differences in hormone levels and gene expression, Fisher's least significant difference post-hoc tests were performed in cases of significant F-test. A Pearson's coefficient was calculated for correlation analysis between struggling behavior and hormone levels / *c-fos* mRNA expression. A small number of plasma and histological samples were excluded from analysis due to assay problems. Additionally, 4 (of 12) videos of restrained stress naïve rats were damaged and unable to be properly analyzed.

Results

Effects of previous stress experience on the restraint-induced HPA-axis response

Previous tailshock led to higher plasma CORT responses to restraint than the stress-naïve group. There was a significant effect of previous experience on CORT ($F_{1,32} = 4.02$, $P < 0.05$). Specifically, both IS and ES rats had greater CORT secretion than did stress naïve rats (Fig 1). The pattern of ACTH levels only partially matched that of CORT. IS rats but not ES rats had higher plasma ACTH than the stress naïve rats, but overall there was no statistical effect of previous experience on ACTH ($F_{1,30} = 2.49$, $P = 0.1$). Plasma ACTH levels typically peak earlier than corticosterone levels, and differences in ACTH levels between treatment groups may be less pronounced at this relatively late time-point after stress onset.

Effects of previous stress experience on the behavioral response to restraint

Previous IS led to very different behavioral responses to subsequent restraint than did either previous ES or no previous stress. Whereas both ES and HC rats showed no differences in mobility, either across the 30 min, or within any of six 5 min blocks, IS rats showed consistently higher mobility, and lower immobility (separately measured) than either of the other two groups across time bins, and overall (Fig 2). The three groups did not significantly differ in strong mobility over the first 10 mins, or in immobility for the first 5 mins, but thereafter group differences generally increased and persisted for the remainder of the 30 mins. MANOVA (Pillai's Trace) revealed a significant effect of Group: ($F_{1,29} = 9.46$, $P < 0.01$), Time bin: ($F_{5,25} = 2.78$, $P < 0.05$), and Time bin $\times$ Group: ($F_{10, 52} = 3.582$, $P < 0.01$).

Effects of previous stress experience on restraint-induced *c-fos* mRNA expression in the prefrontal cortex

Semi-quantitative analyses of autoradiograms indicate that both IS and ES rats challenged with restraint responded with similar overall *c-fos* mRNA expression in the mPFC (both dorsal and ventral aspects). However, while there was virtually no difference in the response of ES to IS rats, both groups showed less *c-fos* mRNA induction in the dmPFC and vmPFC than a restrained, stress naïve group (HC, Fig 3). Thus, there was a significant effect of previous experience on restraint-induced *c-fos* induction in the dmPFC: ($F_{1,33} = 5.746$, $P < 0.01$), and vmPFC: ($F_{1,33} = 12.01$, $P < 0.001$). There was no significant difference in overall *c-fos* expression in the VO between groups (data not shown). In parallel with the semi-quantitative autoradiographic results based on *c-fos* mRNA radiolabeled *in situ* hybridization, non-radioactive labeling of *c-fos* mRNA revealed that IS and ES rats had fewer total *c-fos* mRNA-expressing cells in the mPFC than did HC rats (Fig 4). There was a group effect on the number of *c-fos*-positive cells in the dmPFC: ($F_{2,9} = 10.38$, $P < 0.01$), with a trend for the same in the vmPFC: ($F_{2,9} = 3.219$, $P = 0.09$). As IS and ES groups showed comparable total numbers of *c-fos*-positive cells in the mPFC, we further examined whether the *c-fos*-positive cells were of different neuronal subtype. To do so, we examined the number of GABAergic interneurons concurrently labeled with with the *c-fos* probe. We

found that the numbers of *gad65/67*-positive neurons were similar across groups and that both previously stressed groups had significantly fewer restraint-induced *c-fos*-positive GABAergic cells in the mPFC than stress naive rats – data paralleling these groups' relatively fewer total *c-fos*-positive cells. ANOVAs for *c-fos*-positive GABAergic cells in the mPFC were as follows: dmPFC: ($F_{2,9} = 17.059$, $P < 0.01$); and vmPFC: ($F_{2,9} = 6.346$, $P < 0.05$).

Effects of previous stress experience on restraint-induced *c-fos* mRNA expression in the amygdala

IS rats responded to restraint with significantly greater *c-fos* expression in the basolateral amygdala than either ES or HC rats (Fig 5). There was a significant group difference in *c-fos* expression in the BLA: ($F_{1,29} = 4.80$, $P < 0.05$). MeA *c-fos* expression followed the same pattern as the BLA, but there was only a trend for significant group differences in this brain area: ($F_{1,29} = 2.54$, $P = 0.1$). There was no significant group difference in the CeA.

Correlation of HPA-axis hormone secretion / amygdala *c-fos* induction to struggling behavior

Regression analyses revealed that struggling / strong mobility was positively correlated with ACTH ($r = 0.44$, $P < 0.05$), CORT ($r = 0.41$, $P < 0.05$) and BLA *c-fos* expression ($r = 0.65$, $P < 0.001$) (Fig 6). Although none of the data points included in the BLA *c-fos* mRNA by struggling behavior correlational analysis were considered outliers, we analyzed the correlation in the absence of the data point highest on both *c-fos* expression and struggling time (included on Fig 6). With this point removed, the correlation was 0.41 , $P < 0.05$. There was not a significant correlation between *c-fos* expression and strong mobility in other amygdala brain areas. Additionally, there were no significant correlations between mPFC *c-fos* expression and HPA-axis responses, or struggling behaviors.

While generally rats with lower ACTH and CORT levels struggled less than rats with higher plasma HPA-axis hormone levels, there were several notable exceptions: one rat, for example, had high plasma CORT levels (48 $\mu\text{g/dL}$) but showed only minimal strong mobility. However, this rat did not remain immobile for the duration of restraint however – its overall light mobility levels were in the top 10% of all animals. Conversely, a rat with only average circulating CORT levels (25 $\mu\text{g/dL}$) showed the greatest extent of strong mobility. It is also important to point out that although the total strong mobility measure reflected behavior across the full extent of the restraint session, plasma hormone levels at 30 min may not necessarily reflect ACTH or CORT values earlier in the restraint session, when the majority of strong mobility occurred. Thus while we describe an overall significant positive correlation between ACTH / CORT and struggling behavior, there are notable exceptions and caveats to this pattern which may warrant further study.

Discussion

The present study evaluated the effect of previous stress exposure and the behavioral controllability of that experience on hormonal, behavioral, and neuronal responses of animals to a subsequent novel stressor. We found that rats that previously experienced tailshock, whether under controllable (ES) or uncontrollable (IS) conditions, responded to a subsequent novel stressor with an increased HPA-axis response over stress-naïve animals. A previous study found that rats also responded to shock re-exposure 24 h after ES or IS with a sensitized response (Maier et al., 1986). Although we found no difference between the HPA-axis response of the two previously stressed groups, we found a striking difference in their behavioral responses to the stress challenge. Whereas ES rats behaved nearly identically to

the tailshock-naïve rats, IS rats struggled substantially more during restraint challenge than did either of the other groups.

The degree of struggling during restraint has previously been found to correspond in general with HPA-axis activity patterns. For instance, repeated restraint results in habituated plasma HPA-axis hormones, as well as habituated struggling behavior and exposure to a previous stressor can facilitate both the HPA-axis (Bhatnagar and Dallman, 1998, Bhatnagar and Vining, 2003, Weinberg et al., 2009) and struggling responses (Grissom et al., 2008) to restraint. Although in this study we found an overall positive correlation between struggling and HPA-axis hormone levels, that correlation was due primarily to the differential responses of the IS and stress naïve rats. Whereas the IS group struggled more than the stress naïve group, consistent with that group's elevated HPA-axis response to the stressor, the ES group struggled to the same extent as the stress naïve group, and demonstrated the same temporal shifts in struggling behavior. Thus, on our behavioral index of stress sensitization, ES animals were unaffected. In contrast, the IS group showed very little reduction in struggling behavior over the restraint session, consistent with behavioral stress sensitization (Grissom et al., 2008). The pattern of ACTH data, while not significant, may suggest a greater degree of HPA-axis response sensitization in the IS group. However, corticosterone levels indicate otherwise. Therefore we conclude that while acute struggling behavior is correlated to HPA-axis activity, lack of perceived control over previous stress experience accounts for the correlation between struggling behavior and the HPA-axis response of rats challenged with restraint.

IS rats not only displayed increased struggling behavior in response to subsequent restraint, but also had greater *c-fos* mRNA expression in the BLA than ES and stress naïve rats. While the present study cannot causatively link increased struggling behavior in IS rats with their increased amygdala activity, regression analysis indicates that BLA activity was positively correlated with struggling behavior. These findings further support the documented relationship between amygdala activity and struggling behavior (Anisman et al., 1997, McIntyre et al., 1999, Merali et al., 2001), and moreover suggest that previous exposure to an uncontrollable stress experience may result in sensitized amygdala responsivity to subsequent stressors. Various lines of evidence suggest that the BLA plays an important role in anxiety states. For instance, activation of the BLA (Sajdyk and Shekhar, 1997, Spiga et al., 2006) can increase anxiety-like behaviors on a social interaction test, whereas inhibition of that brain area can decrease anxiety-like behaviors (Gonzalez et al., 1996, Sajdyk and Shekhar, 1997). In addition, fMRI-based findings in humans suggest that the degree of amygdala activation in response to negative valence images correlates with social anxiety severity in subjects with generalized social anxiety disorder (Shah et al., 2009). Struggling behavior during restraint may reflect a state of anxiety (Grissom et al., 2008), and thus one possibility is that the increase in BLA activity in inescapably stressed rats is reflective of increased anxiety and is directly related to the behavioral changes we observed in these animals. It should be noted, however, that if the ES and IS rats differed in their relative anxiety during the restraint challenge, that this difference was not reflected in their relative magnitude of HPA axis response. This observation is consistent with several others studies that found a dissociation between relative HPA axis activity and inferred anxiety state (Wilson et al., 2004, Agrati et al., 2008, Gagliano et al., 2008, Munoz-Abellan et al., 2008, Pego et al., 2008). Thus, the behavioral sensitization in IS animals exposed to novel restraint may reflect increased anxiety induced by uncontrollable stress, which could be related to altered basolateral amygdala activity.

Given the important role that the mPFC plays in the initiation of some of the differential behavioral consequences of ES and IS (Amat et al., 2005, Baratta et al., 2007), we examined relative *c-fos* gene expression in the mPFC of rats during restraint challenge. Both IS and ES

rats had reduced regional *c-fos* mRNA expression in both dorsal and ventral aspects of the mPFC compared with the tailshock stress naïve group, and comparable *c-fos* gene expression to one another. Thus, in this study it appears that previous exposure to stress, but not stress controllability, affected the mPFC response to a subsequent stressor. Using a colorimetric *in situ* hybridization procedure, we also found that the relative number of *c-fos*-positive cells in the mPFC of restrained ES and IS rats was lower than stress naïve rats. Although our study shows that relative *c-fos* mRNA expression was similar in ES and IS rats during restraint challenge, this does not preclude the possibility that different subpopulations of mPFC neurons were activated in ES and IS rats. If such was the case, the different profiles appear to not be due to differences in the relative proportion of GABAergic neurons that were activated. Alternatively, there may have been a difference between ES and IS rats in the activity of certain excitatory projection neurons, such as those that project to the basolateral amygdala. Baratta and colleagues observed that although ES and IS elicited similar levels of total Fos positive cells in the prelimbic cortex, a higher percentage of those cells in ES rats compared to IS rats projected to serotonergic neurons of the dorsal raphe (Baratta et al., 2009). This differential mPFC Fos expression profile was also present when both ES and IS rats were subsequently (re-)exposed a week later to IS.

Interestingly, *c-fos* mRNA expression in the mPFC was lowest in the two groups (ES and IS rats) with the highest HPA-axis response to restraint. Although we did not find a significant correlation between mPFC and HPA-axis activity, other studies have. For example, Kern and colleagues (Kern et al., 2008) found that metabolic activity in the mPFC of humans challenged with a psychosocial stressor was negatively correlated with cortisol secretion. In addition to less overall *c-fos* mRNA expression, we observed fewer *c-fos*-positive GABAergic cells in the dmPFC and vmPFC of restraint-challenged ES and IS rats compared to restraint-challenged stress naïve rats. The local circuit GABAergic interneurons of the mPFC synapse on glutamatergic pyramidal projection neurons and regulate PFC output (Fuster, 1997). The decreased number of *c-fos*-positive GABAergic cells possibly reflects decreased activity of those cells in ES and IS rats. We recently demonstrated that increased activation of the mPFC (microinfusion into the mPFC of a GABA-A receptor antagonist) suppressed the HPA-axis response to restraint (Weinberg et al., 2010). Moreover, Radley et al. (Radley et al., 2009) have demonstrated the presence of an indirect inhibitory influence of the prelimbic cortex on HPA axis activity. Perhaps related, here we find relatively less mPFC GABAergic activity in groups having the greatest HPA-axis response to restraint.

Stress exposure and perceived controllability of stressful experiences strongly contribute to the mental and physical well being of an individual (Weiss and Simson, 1986, Chrousos and Gold, 1992, Kyrou et al., 2006). We have found that ES, while lacking many of the subsequent adverse consequences produced by IS, led to a sensitized HPA axis response to novel stress, as well as altered mPFC neural activity. However, only the IS rats displayed a greater degree of struggling behavior and increased *c-fos* mRNA in the basolateral amygdala in response to challenge with novel stress. Thus, the consequences of stressor controllability may pertain more to subsequent control of stress-related behaviors than to stress hormone responses.

Acknowledgments

This work was supported by NIH grant MH75968. Our thanks to Aadra Bhatt and Dr. Heidi Day for their technical assistance with performing double *in situ* hybridization.

Abbreviations

ACTH adrenocorticotrophic hormone

ANOVA	analysis of variance
BCIP	5-bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt
BLA	basolateral / lateral amygdala
CeA	central nucleus of the amygdala
C-fos	cellular V-fos FBJ murine osteosarcoma viral oncogene homolog
CV	coefficient of variability
DIG	digoxigenin
dmPFC	dorsomedial prefrontal cortex
ES	escapable stress
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
HC	homecage control
HPA-axis	hypothalamic-pituitary-adrenal axis
IS	inescapable stress
MANOVA	multivariate ANOVA
MeA	medial amygdala
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
NBT	4-nitro blue tetrazolium chloride
PBS	phosphate-buffered saline
ROI	region of interest
SSC	standard saline citrate
TBS	tris-buffered saline
UTP	uridine triphosphate
vmPFC	ventromedial prefrontal cortex
VO	ventral orbital prefrontal cortex

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Fig 1.

Effect of previous stress experience on the restraint-induced HPA-axis response. Groups were challenged with 30 min restraint 48 h after no stress (HC), escapable stress (ES), or inescapable stress (IS), and average plasma ACTH and CORT secretion were compared. ACTH measures appear in grey bars, and correspond to the left y-axis, and CORT measures appear in black and correspond to the right y-axis. #, significantly different than HC ($P < 0.05$). Error bars represent standard error of the mean.

Fig 2.

Effect of previous stress experience on the behavioral response to restraint. Groups were challenged with 30 min restraint 48 h after no stress (HC), escapable stress (ES), or inescapable stress (IS), and behaviors made in the restraint tube were recorded. Panels A-C reflect differences between groups in immobility, light mobility, and strong mobility, respectively, across 5 min time bins. Panels D-F reflect group differences in overall time spent immobile, lightly mobile, and strongly mobile across the 30 min experience. #, significantly different than HC ($P < 0.05$). *, significantly different than ES ($P < 0.05$). Error bars represent standard error of the mean.

Fig 3.

Effect of previous stress experience on restraint-induced *c-fos* expression in the mPFC. Groups were challenged with 30 min restraint 48 h after no stress (HC), escapable stress (ES), or inescapable stress (IS), and *c-fos* induction in response to 30 min restraint was determined. Panel A shows overall expression levels (integrated density) of groups. ##, significantly different than HC ($P < 0.01$). Error bars represent standard error of the mean. Panel B shows a section at the level of the prefrontal cortex, adapted from Paxinos and Watson (Paxinos G, 1998), in which areas marked in black reflect dorsal, and areas marked in grey reflect ventral medial prefrontal cortex. Panel C shows representative autoradiograms of *c-fos* mRNA expression across the different groups.

Fig 4.

Effect of previous stress experience on distribution of *c-fos*-positive cells in the mPFC. Data is reported for the dorsal and ventral medial prefrontal cortex. Panel A, average number of *c-fos*-positive cells. Panel B, average number of *GAD65/67*-positive cells. Panel C: Number of *GAD65/67* cells double-labeled for *c-fos*. Panel D, representative brightfield and darkfield images, showing *c-fos*-positive neurons as black round bodies in brightfield (marked as + in the labeling box) and *GAD65/67*-positive neurons as clusters of silver grains in darkfield (marked as circles in the labeling box). #, ##, significantly different than homecage ($P < 0.05$, $P < 0.01$, respectively). Error bars represent standard error of the mean.

Fig 5.

Effect of previous stress experience on restraint-induced *c-fos* expression in the amygdala. Groups were challenged with 30 min restraint 48 h after no stress (HC), escapable stress (ES), or inescapable stress (IS), and *c-fos* induction in response to 30 min restraint was determined. Panel A shows overall expression levels (integrated density) of groups. #, significantly different than HC ($p < 0.05$). **, significantly different than ES ($p < 0.01$). Error bars represent standard error of the mean. Panel B shows a section at the level of the amygdala, adapted from Paxinos and Watson (Paxinos G, 1998), in which areas marked in black reflect medial amygdala (MeA), light gray basolateral / lateral amygdala (BLA), and dark gray central nucleus (CeA) of the amygdala. Panel C shows representative autoradiograms of *c-fos* mRNA expression across the different groups.

Fig 6.

Correlation of HPA-axis hormone levels / basolateral amygdala *c-fos* expression to struggling behavior. Groups were challenged with 30 min restraint 48 h after no stress (HC), escapable stress (ES), or inescapable stress (IS). Panel A reflect the correlation between ACTH / CORT and time spent strongly mobile. Unfilled dots are used to represent ACTH, and filled dots represent CORT. The lower x-axis charts plasma ACTH, and the upper x-axis CORT levels. ACTH vs. strong mobility: $r = 0.44$, $P < 0.05$. CORT vs. strong mobility: $r = 0.41$, $P < 0.05$. Panel B reflects the correlation between basolateral amygdala (BLA) *c-fos* mRNA expression and time spent strongly mobile. Basolateral amygdala *c-fos* expression vs. strong mobility: $r = 0.65$, $P < 0.001$.