HABITUATION TO REPEATED RESTRAINT STRESS IS ASSOCIATED WITH LACK OF STRESS-INDUCED c-fos EXPRESSION IN PRIMARY SENSORY PROCESSING AREAS OF THE RAT BRAIN

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Abstract—Rats repeatedly exposed to restraint show a reduced hypothalamic–pituitary–adrenal axis response upon restraint re-exposure. This hypothalamic–pituitary–adrenal axis response habituation to restraint does not generalize to other novel stressors and is associated with a decrease in stress-induced c-fos expression in a number of stress-reactive brain regions. We examined whether habituation to repeated restraint is also associated with adaptation of immediate early gene expression in brain regions that process and relay primary sensory information. These brain regions may not be expected to show gene expression adaptation to repeated restraint because of their necessary role in experience discrimination. Rats were divided into a repeated restraint group (five 1-hour daily restraint sessions) and an unstressed group (restraint naïve). On the sixth day rats from each group were either killed with no additional stress experience or at 15, 30 or 60 min during restraint. Immediate early gene expression (corticotropin-releasing hormone heteronuclear RNA, c-fos mRNA, zif268 mRNA) was determined by in situ hybridization. A reduction in stress-induced hypothalamic–pituitary–adrenal axis hormone secretion (plasma corticosterone and adrenocorticotropic hormone) and immediate early gene expression levels in the paraventricular nucleus of the hypothalamus, the lateral septum and the orbital cortex was observed in repeated restraint as compared with restraint naïve animals. This reduction was already evident at 15 min of restraint. Unexpectedly, we also found in repeated restraint rats a reduction in restraint-induced c-fos expression in primary sensory-processing brain areas (primary somatosensory cortex, and ventroposterior medial and dorsolateral geniculate nuclei of thalamus). The overall levels of hippocampal mineralocorticoid receptor heteronuclear RNA or glucocorticoid receptor mRNA were not decreased by repeated restraint, as may occur in response to severe chronic stress. We propose that repeated restraint leads to a systems-level adaptation whereby re-exposure to restraint elicits a rapid inhibitory modulation of primary sensory processing (i.e. sensory gating), thereby producing a widespread attenuation of the neural response to restraint.

Key words: in situ hybridization, HPA axis, immediate early genes.

Experiences that are characterized as stressful elicit a complex array of behavioral and physiological changes believed to contribute to optimal coping of the organism with the situation. These responses include the release of glucocorticoid hormones from the adrenal cortex, controlled by the hypothalamus–pituitary–adrenal system (HPA axis). Glucocorticoids produce a wide range of regulatory physiological effects such as the promotion of glycogen, muscle, and fat catabolism in order to mobilize energy required for the immediate adaptive responses to stress (Munck et al., 1984). An important feature of many real-life stressors is their recurrent or persistent nature; under these circumstances, chronic elevation of glucocorticoid levels can exacerbate a number of pathologies including immune disorders, neurodegeneration and major depression (McEwen and Stellar, 1993; Gold and Chrousos, 2002). Often repeated stress leads to a decrease in the HPA axis response (stress habituation), protecting the organism from the potentially damaging effects of hypercorticosteroidism (Armario et al., 2004). The mechanisms involved in the adaptation to daily stress, however, are poorly understood. Glucocorticoid negative feedback participates in the depression of the HPA response (Cole et al., 2000) and of corticotropin-releasing hormone (CRH) gene expression (Pinnock and Herbert, 2001) during habituation to restraint stress. However, decreased HPA axis activity appears not to be the result of a generalized diminished responsivity of the axis, but rather, of changes in the neural activity of afferents involved in its control. This is evidenced by the fact that habituation is stimulus specific. Consequently, prior habituation to one stressor (homotypic stimulus) does not reduce the HPA response to a different stressor (heterotypic stimulus) (Armario et al., 1988; Hashimoto et al., 1988; Hauger et al., 1988; Terrazino et al., 1995; Akana and Dallman, 1997; Ma et al., 1999; Pace et al., 2001; Fernandes et al., 2002). Thus, some degree of altered central neural control appears responsible for habituation of HPA responses to stress.

To assess whether stress response habituation is associated with a reduction in the stress-induced neural ac-
tivation present in various brain regions, a number of studies have examined immediate early gene expression. These studies have looked at habituation to a variety of stressors (e.g. prone restraint, supine restraint, immobilization, noise, social defeat) with a special focus on response adaptation of the HPA axis and of c-fos mRNA/protein in primary stress-responsive areas in the forebrain and brain stem (Lachuer et al., 1994; Melia et al., 1994; Umemoto et al., 1994a; Watanabe et al., 1994; Chen and Herbert, 1995; Umemoto et al., 1997; Bonaz and Rivest, 1998; Martinez et al., 1998; Stamp and Herbert, 1999; Campeau et al., 2002). With some exceptions, probably due to use of more intense stressors (Makino et al., 1995; Umemoto et al., 1997), the majority of studies report a robust habituation of the HPA axis hormone response to repeated stress. The stress-induced expression of c-fos also reliably decreases with repeated stress exposure in the paraventricular nucleus of the hypothalamus (PVN) and in other stress-responsive cortical (e.g. medial prefrontal cortex), limbic (e.g. bed nucleus of the stria terminalis, various hypothalamic subregions and amygdala) and brainstem (e.g. locus ceruleus, raphe nuclei, central gray, nucleus of the solitary tract) structures. Two anatomical exceptions that have been highlighted are the orbital frontal cortex and the lateral septum (LS). Campeau and co-workers (2002) observed a sensitization rather than habituation of the c-fos mRNA response in the orbital frontal cortex to repeated noise stress. Also, a lack of habituation of Fos protein in the LS to repeated supine restraint has been noted, although the same group did see habituation of this brain region in subsequent studies (Chen and Herbert, 1995; Chung et al., 1999; Stamp and Herbert, 1999). Due to the habituation of stress-induced c-fos expression reported in many other brain regions, a failure of habituation in the orbital frontal cortex and LS may reflect a special role of these structures in the development and/or maintenance of habituation.

While a wealth of information has been provided from these previous studies, generalizations about the specific brain regions affected and underlying mechanisms of stress adaptation are lacking, partly due to the different stress protocols used and variety of brain regions examined. In order to further examine the extent to which habituation of an HPA axis response to repeated stress is associated with changes in central neural activity, we have studied habituation to repeated restraint (six daily 1 h sessions). Exposing rats to repeated restraint is a widely used model of psychological stress adaptation (Glavin et al., 1994). We have chosen to study habituation to a psychological stressor since understanding the adaptation processes to this type of stress may be especially informative to the neurobiology underlying stress-related disorders (Chrousos and Gold, 1992). For this study we have examined the expression of two different immediate early genes, c-fos and zif268. Both genes are rapidly induced in the rat brain by a wide range of experiences, including those characterized as stressors (Herdegen and Leah, 1998). The specific expression patterns of these two genes may differ, however, and their direct comparison may provide a more comprehensive reflection of neural activity (Cullinan et al., 1995; Kaplan et al., 1996). We have examined gene expression at the level of mRNA rather than protein because of the increased temporal resolution provided by the more rapid induction and transient nature of these mRNA levels. This has allowed us to examine the acute response time-course in order to see if habituation is expressed early or later during the response to restraint challenge. Finally, we have selected different brain regions to examine that may represent different stages in the neural processing of the restraint experience. As examined in previous studies, we have used the HPA axis response as an indicator of the activity of a functional stress response system—thus we have measured plasma corticosterone and adrenocorticotropic hormone (ACTH), and observed concurrent stress activation of the c-fos and zif268 genes in the PVN. Although other studies have shown that the majority of Fos induction in the PVN with restraint is localized to CRH neurons (Dayas et al., 1999), we have also examined CRH heteronuclear RNA (hnRNA) as a direct measure of activity within this cell population. In addition, in order to examine the extent of habituation throughout the brain we have selected two brain regions that have previously been reported to be stress reactive, but, as discussed above, do not necessarily show habituation of immediate early gene response to repeated homotypic stress (LS and orbital frontal cortex) (Chen and Herbert, 1995; Campeau et al., 2002). Most importantly, a unique aspect of this study is the examination of the immediate early gene response within primary sensory pathways. We have specifically examined the region of primary somatosensory cortex and associated thalamic relay nucleus that receives sensory input from the vibrissae. We expect considerable stimulation of the whiskers as they are deflected by the walls of the restrainer in rats challenged with restraint for the first and sixth time. Our assumption is that neural activity in primary sensory systems precedes central processing of the stressfulness of the experience, and therefore may not exhibit immediate early gene response habituation. As a final measure, we examined mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) gene expression in the hippocampus. This measure may provide an indicator of the severity of our repeated stress paradigm since other studies have found that chronic stress can lead to a downregulation of hippocampal mineralocorticoid and/or GR expression (Sapolsky et al., 1984; Makino et al., 1995; Lopez et al., 1998; Paskitt et al., 2000).

**EXPERIMENTAL PROCEDURES**

**Animals**

Male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN, USA) were allowed a two week acclimation period after arrival to the animal facilities at the University of Colorado before experimental use (10 weeks old, weight range 270–290 g at experimental onset). Animals were housed two per polycarbonate tub with wood shavings, and were allowed food (Purina Rat Chow; Ralston Purina, St. Louis, MO, USA) and tap water ad libitum. The colony room lights were regulated 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. All efforts were made to minimize the number of animals used and their suffering.

**Restraint procedure**

Restraint involved taking rats from the home cage and placing them in adjustable length (15.5±2.5 cm long and 6.3 cm diameter) Plexiglass 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 took place in a separate room adjacent to the home cage room.

**Stress exposure paradigm**

Rats (N=48) were divided into two groups, naïve and repeated restraint animals. Restraint naïve animals (N) were left untouched in their home cages until the test day. Repeated restraint animals (RR) were placed into restrainers for 1 h (between 09:30 h and 11:30 h) for five consecutive days prior to the test day. On the sixth day (test day) animals from each group were either killed by decapitation without being exposed to the restrainer or they were placed in restrainers and killed at different durations of restraint (15, 30 and 60 min). Each group (N0, N15, N30, N60 and RR0, RR15, RR30, RR60) was composed of six animals. The brains were collected, flash-frozen in a dry ice butanol bath maintained between −40 °C and −30 °C and stored at −80 °C.

On the test day, all animals received an i.p. injection of saline 1 h prior to restraint or kill (no stress group). The injection was included as a control procedure for future drug studies that will examine the influence of various pharmacological agents on the expression of habituation.

**In situ hybridization histochemistry**

Ten micrometer sections were cut on a cryostat (Leica model 1850) through the extent of the orbital frontal cortex (approximately 4.20 mm anterior to bregma, Paxinos and Watson, 1998), rostral and caudal septum (approximately 0.20 anterior and 0.26 mm posterior to bregma, respectively), PVN (approximately 1.80 mm posterior to bregma) and dorsal hippocampus (approximately 3.14 mm posterior to bregma), thaw-mounted onto poly-L-lysine-coated slides and stored at −80 °C.

In situ hybridization for MR hnRNA and GR mRNA was performed as previously described (Paskitti et al., 2000). In situ hybridization for CRH hnRNA, c-fos mRNA and zif268 mRNA was performed as previously described (Ginsberg et al., 2003) with the following modifications. Slides were fixed in buffered 4% paraformaldehyde for 15 min at room temperature, washed in 2× standard saline citrate solution (SSC, 1 X NaCl, 0.015 M sodium citrate), acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride, pH 8, for 10 min, rinsed in double distilled water, dehydrated through graded ethanol solutions and air dried. For the generation of the probes, plasmids containing a fragment of c-fos cDNA (courtesy of Dr. T. Curran, St. Jude Children’s Research Hospital, Memphis), or zif268 cDNA (courtesy of Dr. J. Milbrandt, Washington University School of Medicine, St Louis) or a portion of the CRH intron (kindly provided by Dr. R. Thompson, University of Michigan) were used. [35S]-labeled complementary RNA probes were generated using standard in vitro transcription reagents (Promega, Madison, WI, USA). Briefly, 0.5–1 µg of linearized plasmid were incubated at 37 °C for 90 min in transcription buffer (Promega) in the presence of 100 µCi [35S]UTP, 75 µCi [3H]UTP, 40 µM each of ATP and GTP, 10 mM DTT, 40 units of RNase inhibitor, and 20 units of T7 RNA polymerase. Following purification through a G50/50 Sephadex column, the probe was applied to slides at 1.5–2×10⁶ cpm per slide in 65 µl hybridization buffer containing 50% formamide, 10% dextran sulfate, 2X SSC, 50 mM phosphate-buffered saline, pH 7.4, 1X Denhardt’s solution and 0.1 mg/ml yeast tRNA. Hybridization was performed in a 50% formamide humidified atmosphere at 55 °C for 14–16 h. The following day, sections were treated with RNase A, 200 µg/ml at 37 °C (1 h), washed in decreasing concentrations of SSC at room temperature and finally in 0.1X SSC at 70 °C for 1 h. Dehydrated sections were exposed to X-ray film for 1–2 weeks.

**Image analysis**

Semi-quantitative analyses were performed on digitized images from X-ray films (NIH image) as described (Campeau and Watson, 1997). The regions of interest (ROIs) were determined on the autoradiographic images using the Paxinos and Watson (1998) atlas for guidance. The ROIs were first drawn on images with positive signals then saved and positioned on control brain.
images according to identifiable landmarks. Background areas were chosen in the white matter or in a cell-poor area close to the ROIs. Signal pixels of the brain area of interest were defined as being 3.5 SDs above the mean of the set background. The number of pixels and the average pixel values above the set background were then computed and multiplied, giving an integrated densitometric measure of arbitrary units (integrated gray level).

Corticosterone radioimmunoassay
Measurement of plasma corticosterone was conducted with radioimmunoassay procedures as previously described (Spencer et al., 1998) except for the use of a different antiserum (B3-163, Endocrine Sciences, Calabasas Hills, CA, USA). The detection limit for this assay, based on a sample volume of 20 μl, was 0.5 μg/100 ml. The intra-assay coefficient of variability was 13%.

ACTH radioimmunoassay
Blood samples for the ACTH assay were collected into EDTA-coated tubes and stored at −80 °C. Plasma concentrations of ACTH were determined by radioimmunoassay procedures described in (Nicholson et al., 1984) using antiserum (rabbit antibody Rb7) courtesy of Dr. Bill Engeland, University of Minnesota. The detection limit for this assay was 15 pg/ml for a 50 μl sample and the intra-assay coefficient of variability was 11%.

Statistical analysis
Data were analyzed with separate two-way analysis of variance (ANOVA) tests for the between groups factors, Prior Restraint Experience and Restraint Duration, in a 2×4 factorial design using a Statistical Analysis System (SAS) package for UNIX. In cases where there were significant effects on the Prior Restraint Experience factor or significant interactions, post hoc tests (Fisher’s least significant difference test) were performed to reveal the time points of significant difference between treatment groups and the results are indicated on the data figures. An α level of P<0.05 was used to determine statistical significance. Data presented represent group mean±S.E.M.

RESULTS
Effect of acute and repeated restraint on HPA axis hormone secretion
Fig. 1 shows that the paradigm of repeated restraint used in this study allowed for a substantial HPA axis response habituation. Both corticosterone and ACTH levels showed a distinct stress response with peaks at 30 min in N animals. In repeatedly restrained (RR) animals the hormone responses were attenuated, and this attenuation was especially pronounced at the latter post-restraint onset timepoints (30 and 60 min). Thus, there was an overall effect of Prior Restraint Experience on corticosterone [F(1,40)=11.7, P<0.05] as well as an overall effect of Restraint Duration on both corticosterone [F(3,40)=16.3, P<0.001] and ACTH levels [F(3,40)=4.1, P<0.05]. There was also a significant interaction between both factors in the case of corticosterone levels [F(3,40)=3.44, P<0.05].

Effect of repeated restraint on overall c-fos expression in the rat brain
Visual examination of the autoradiograms for c-fos mRNA in situ hybridization indicated that the adaptation of the neuroendocrine response to restraint re-exposure in RR animals was mirrored by lower c-fos gene induction in

Fig. 2. Reduced c-fos mRNA response to restraint in RR rats is apparent at all rostral–caudal forebrain levels sampled. Top row: diagrammatic representations from the Paxinos and Watson (1998) atlas of brain sections at the levels of frontal cortex, septum nuclei, PVN and VPM. Reprinted from Paxinos G. and Watson C. (1998), The rat brain in stereotaxic coordinates, 4th ed., with permission from Elsevier. Shaded areas represent the ROIs analyzed in this study (gray areas: orbital cortex, LS, PVN and lateral geniculate; striped areas: barrel field and VPM). The ROIs were determined on the autoradiographic images using the Paxinos and Watson (1998) atlas for guidance. Middle and bottom rows: autoradiogram images of c-fos mRNA (in situ hybridization) in brain sections corresponding to the above atlas diagrams, of N unstressed rats (N0) and of N and RR rats at 15 min of restraint (N15 and RR15 respectively). Induction of c-fos expression is evident by 15 min in N animals and a widespread reduction in c-fos mRNA levels is observed in RR animals.
many forebrain structures. Fig. 2 shows sample autoradiograms for N and RR rats after 15 min of restraint and illustrates the specific brain regions that were quantified by densitometry.

**Effect of acute and repeated restraint on gene expression in PVN**

In the PVN (Fig. 3), short-term changes in CRH primary transcript (hnRNA) represent recent changes in transcription rate that are not otherwise readily discernable in the large pool of CRH mRNA (Herman et al., 1992). CRH hnRNA rapidly accumulated with a peak of expression at 15 min and quickly returned to basal levels in acutely stressed rats (Fig. 3A). RR animals, on the other hand, showed a nearly complete lack of transcriptional activation, at all time points [Prior Restraint Experience $F(1,40)=4.81$, $P<0.05$; Restraint Duration $F(3,40)=10.8$, $P<0.001$; Interaction $F(3,40)=3.66$, $P<0.05$].

Similarly, c-fos and zif268 mRNAs (Fig. 3B and 3C) were increased by stress exposure with peaks at 15 min in N animals but not in RR animals [c-fos: Prior Restraint Experience $F(1,38)=15.3$, $P<0.001$; Restraint Duration $F(3,38)=6.3$, $P<0.01$; Interaction $F(3,38)=4.67$, $P<0.01$; zif268: Prior Restraint Experience $F(1,34)=17.2$, $P<0.01$; Restraint Duration $F(3,34)=2.75$, $P=0.05$; Interaction not significant]. Interestingly, in N rats our time course showed a much more sustained induction of zif268 mRNA than c-fos mRNA in the PVN.

**Effect of acute and repeated restraint on gene expression in LS**

In the LS (Fig. 4), we observed a spatial dissociation between c-fos and zif268 expression. c-fos mRNA was prominent in more rostral sections (corresponding to the rostral–caudal level where the head of the anterior commissure lies below the lateral ventricles) but it was not so evident in more caudal sections where the anterior commissure starts to cross the midline. Conversely, zif268 expression was clearly evident in the caudal, but much less so in the rostral areas (see Fig. 4, panel E). For this reason, we analyzed separately the rostral and the caudal LS sections. Despite the difference in overall signal intensity, c-fos expression had similar treatment-dependent patterns in both rostral and caudal septal regions (Fig. 4A and B). In restraint naive animals a clear induction, with a peak at 30 min, was observed, while RR animals had a substantially flattened response to restraint [c-fos-rostral: Prior Restraint Experience $F(1,34)=18.6$, $P<0.001$; Restraint Duration $F(3,34)=9.11$, $P<0.001$; Interaction $F(3,34)=3.94$, $P<0.05$; c-fos-caudal: Prior Restraint Experience $F(1,32)=15.6$, $P<0.001$; Restraint Duration and Interaction not significant]. In the caudal regions of the LS there was also a significantly lower basal c-fos mRNA level present in RR animals than in naive controls (Fisher LSD, $P<0.05$).
Zif268 expression was nearly absent in the rostral sections and there was no significant effect of stress, regardless of prior restraint experience (Fig. 4C). In caudal LS sections, zif268 mRNA expression was more prominent, even at constitutive levels in unstressed animals. A three- to four-fold increase of zif268 mRNA at
15 and 30 min of acute stress was detected (significantly different from 0 min by Fisher’s LSD, $P<0.05$), but the overall effect of acute stress did not reach significance. The zif268 mRNA stress-dependent increase was less pronounced in RR rats at the 30 min time-point, but the overall Prior Restraint Experience ANOVA factor was not significant.

Effect of acute and repeated restraint on gene expression in the orbital cortex

In the orbital cortex (Fig. 5) there was a significant c-fos mRNA induction by restraint in restraint naive animals, with a peak at 15 min. In RR animals there was a lack of induction for all restraint durations on the test day [Prior Restraint Experience $F(1,40)=7.09$, $P<0.05$; Restraint Duration $F(3,40)=3.66$, $P<0.05$; Interaction not significant].

Zif268 expression in the orbital cortex did not differ significantly between N or RR rats, however, there was an overall effect of Restraint Duration due to elevated zif268 mRNA levels in both groups after 60 min of restraint [$F(1,40)=5.68$, $P<0.05$].

Effect of acute and repeated restraint on gene expression in primary somatosensory cortex and thalamic relay regions

In order to establish whether habituation of immediate early gene induction to restraint can be observed within primary sensory neural pathways, we analyzed gene expression in the region of primary somatosensory cortex that relays tactile information from the whiskers (barrel fields). Expression of both c-fos and zif268 was especially evident in cortical layers IV and VI. Layer IV receives direct innervation from the thalamic relay (ventroposteriomedial [VPM] thalamic nucleus) of the vibrissae sensory afferents. Layer VI pyramidal cells project back to the thalamus. In both layers, we observed a marked c-fos response to restraint which was absent in RR animals [Fig. 6A and B] [layer IV: Prior Restraint Experience $F(1,39)=21.82$, $P<0.0001$; Restraint Duration $F(3,39)=4.67$, $P<0.01$; Interaction $F(3,39)=3.05$, $P<0.05$; layer VI: Prior Restraint Experience $F(1,39)=22.62$, $P<0.0001$; Restraint Duration $F(3,39)=4.18$, $P<0.05$; Interaction $F(3,39)=4.84$, $P<0.01$]. Zif268 expression also showed a marked difference between N and RR rats (Fig. 6C and D), although the overall effect of restraint exposure in naive animals did not reach significance [layer IV: Prior Restraint Experience $F(1,32)=10.19$, $P<0.01$; layer VI: Prior Restraint Experience $F(1,32)=11.50$, $P<0.01$]. At the peak of the response, the fold increase over the corresponding time 0 in acutely stressed rats was larger for c-fos mRNA than for zif268 mRNA, probably reflecting a higher constitutive expression of zif268 in the cortical areas. Taken together, the data indicate that immediate early gene adaptation was present in the primary somatosensory cortex and was evident within 15 min after restraint onset.

To determine whether the adaptation of the immediate early gene response was present at an earlier stage in the whisker-to-barrel sensory pathway, we analyzed gene expression in the corresponding thalamic relay nucleus, the VPM. Moreover, in order to determine if adaptation could be found across different sensory modalities we measured c-fos and zif268 mRNA in the dorsolateral geniculate nucleus, which relays visual information from the retina to the primary visual cortex. As shown in Fig. 7, while zif268 expression was not appreciably affected by restraint on the test day, c-fos expression was significantly increased by restraint in the N compared with the RR rats in both thalamic nuclei. The overall restraint duration factor in acutely stressed animals, however, did not reach significance due to a rather large within group variability at each time point [c-fos-VPM: Prior Restraint Experience $F(1,40)=4.56$, $P<0.05$; c-fos dorsolateral geniculate: Prior Restraint Experience $F(1,40)=5.01$, $P<0.05$].
Effects of acute and repeated restraint on MR and GR expression in the hippocampus

As is evident from Fig. 8, there was no overall difference in corticosteroid receptor expression between N and RR rats during the sixth restraint experience. RR rats had lower mean GR mRNA basal levels (time 0) compared with N rats, however, this difference disappeared within 15 min of restraint. There was also a small but significant decline in overall MR hnRNA levels across the 60 min of restraint [MR-CA1, Restraint Duration $F(3,40)=3.39, P=0.03$; MR-DG, Restraint Duration $F(3,40)=4.5, P<0.01$].

DISCUSSION

In this study we have shown that repeated intermittent exposure to restraint stress produced not only habituation of the HPA axis response but also a widespread depression in stress-induced c-fos expression in the brain. The decreased c-fos induction was observed in typical stress-responsive brain regions (PVN, orbital frontal cortex, and LS), but unexpectedly was also evident in somatosensory cortex and primary sensory relay nuclei. Moreover, habituation of the c-fos response to repeated restraint was expressed within 15 min of re-exposure to the stimulus. These data suggest that widespread neural habituation to repeated restraint may result from a resetting of the excitation threshold of sensory pathways to the specific stimuli.

Habituation of HPA hormone and PVN immediate early gene response

In our study, both systemic corticosterone and ACTH levels in response to restraint were lower after repeated exposure than in naïve controls. Repeated restraint induced an increase in ACTH secretion within the first 15 min of stimulation, but the levels returned more rapidly to baseline than in N animals. In the PVN, initial exposure to restraint...
produced a rapid increase in CRH gene primary transcript levels (CRH hnRNA) with a very distinct peak of induction around 15 min after acute restraint stress onset. A similarly rapid and transient increase of CRH hnRNA has been previously reported to occur in response to hypertonic saline and ether stress (Kovacs and Sawchenko, 1996; Ma and Aguilera, 1999). The peak of early CRH hnRNA induction observed in this study was completely absent in RR animals, despite the early ACTH response evident in these animals. While habituation of CRH hnRNA induction has been reported previously in paradigms of repeated restraint stress, those studies did not examine CRH hnRNA expression at times less than 1 h after stress onset (Ma et al., 1997; Ma and Lightman, 1998).

The lack of stress-induced CRH gene expression after repeated restraint could be a result of repeated restraint leading to a specific repression of the CRH gene. Chronic glucocorticoid elevation could produce such an effect. Glucocorticoids have been implicated in the functional regulation of the CRH gene and suppression of CRH hnRNA and mRNA expression by glucocorticoids has been shown (Itoi et al., 1998; Ma and Aguilera, 1999; Ginsberg et al., 2003). Moreover, Pinnock and Herbert (2001) have hypothesized that CRH gene adaptation during habituation to repeated stress requires elevated corticosterone levels. At the molecular level, studies conducted in the pituitary cell line AT20 indicate that the GR can mediate a cis-repression of the CRH gene by binding to a negative regulatory element in the promoter (negative GREs, (Malkoski et al., 1997; Malkoski and Dorin, 1999)). Paradoxically, however, while chronic glucocorticoid treatment of rats leads to suppressed basal and stress-induced CRH mRNA in the PVN (Beyer et al., 1988; Lightman and Young, 1989), severe chronic stress has been found to increase CRH mRNA (Umemoto et al., 1994b; Makino et al., 1995).
An alternative explanation for habituation of stress-induced CRH gene induction could be altered presynaptic input to the CRH neuron after repeated restraint. Changes in presynaptic inputs to the CRH neurons could involve either decreased excitatory afferent activity or an activation of inhibitory GABAergic projections (Herman et al., 2004). Such a situation would also explain the complete lack of induction in the PVN of the other two genes examined, c-fos and zif268, in RR animals. The time course of c-fos mRNA induction with novel restraint followed an established pattern (Imaki et al., 1992, 1995; Cullinan et al., 1995) with a peak around 30 min of restraint. In repeatedly stressed animals, decreased levels of c-fos mRNA (Melia et al., 1994; Umemoto et al., 1994a, 1997) and c-Fos immunoreactivity (Viau and Sawchenko, 2002) after homotypic stress challenge have also been observed; however, the response time-course of the c-fos gene during re-exposure to the stressor had never been previously reported. Here we show that the lack of c-fos induction was already evident by 15 min of restraint re-exposure. The same pattern was observed for zif268 gene expression.

Zif268 is another immediate early gene with a widespread neural distribution and it has also been used as a marker of neuronal activation because of its rapid induction in acute stress paradigms (Worley et al., 1991; Rosen et al., 1992; Cullinan et al., 1995). However, only a limited number of studies have looked at the pattern of zif268 expression during repeated stress (Melia et al., 1994; Umemoto et al., 1994a, 1997; Watanabe et al., 1994). In agreement with our data, Melia et al. (1994) and Watanabe et al. (1994) report a decrease in stress-induced zif268 expression in the PVN following repeated restraint. Interestingly, Umemoto and colleagues (1994a, 1997) found that both repeated immobilization stress and chronic glucocorticoid treatment suppressed stress-induced c-fos, but not zif268 gene expression in the PVN. They concluded that the suppression of c-fos expression was due to a direct cumulative effect of chronically elevated endoge-
nous or exogenous glucocorticoids, and that the zif268 gene was resistant to this direct glucocorticoid effect. Those studies, however, may not be directly comparable to our study since the stress regimen (repeated immobilization) used in those studies did not produce habitation of plasma corticosterone responses. The fact that we see both a decreased c-fos mRNA and zif268 mRNA in the PVN after repeated restraint suggests that this gene response habitation is not due to a cumulative effect of glucocorticoids acting directly on the PVN. In contrast to the studies by Umemoto and colleagues (1994a, 1997), our repeated restraint regimen does not appear to produce chronic HPA axis hyperactivity. In past studies using this regimen and this strain of rat we did not see adrenal hypertrophy or thymus involution (Dhabhar et al., 1997). Several studies indicate that chronic stress exposure regimens can decrease hippocampal GR and/or MR mRNA or ligand binding levels (Sapolsky et al., 1984; Herman and Watson, 1995; Makino et al., 1995; Gomez et al., 1996; Lopez et al., 1998). In this study there was no overall downregulation of hippocampal MR and GR gene expression, although there appeared to be lower resting levels of hippocampal GR mRNA present in RR rats compared with N rats. This difference, however, was not present after 15 min of restraint challenge. Previous studies also indicate that acute stress induces rapid down-regulation of MR gene transcription in hippocampal subfields (Herman and Watson, 1995; Paskitti et al., 2000). The current data lend some support to these findings, as MR hnRNA was slightly, but significantly decreased by one hour of restraint in both N and RR groups. Responses of the MR gene to stress are critically dependent on glucocorticoids (Herman and Watson, 1994); thus, the fact that a decrease in MR hnRNA was present in both groups, despite very different corticosterone profiles, suggests that inhibition of MR may be related to low-level secretion of glucocorticoids, perhaps acting via hippocampal MR.

**Habitation of c-fos expression in LS, orbital cortex and primary sensory pathways**

If HPA response habitation reflects decreased neural excitation of the PVN then one would expect to also see decreased neural activity in some other brain regions believed to directly or indirectly control PVN activation during acute stress. Indeed, visual inspection of our autoradiograms (see Fig. 2) suggested to us that there was widespread habitation of the c-fos mRNA response throughout the forebrain. We quantified two brain regions, LS and orbital frontal cortex, that have previously been reported to not exhibit habitation of c-fos responses to repeated audiogenic stress and supine restraint, respectively (Chen and Herbert, 1995; Campeau et al., 2002). In contrast to those two other reports, in this study there was a clear habitation of the c-fos mRNA response in RR animals, perhaps indicating differential adaptation evident within these brain regions depending on the type of stressor utilized. In the case of repeated restraint it appears that activation of these brain areas was not required for the expression of habitation to restraint stress. It should also be noted that a procedural aspect of our study that may have led to response patterns different from other stress habitation studies is that all of our animals were given a saline injection 1 h prior to restraint challenge. We included this control injection in order to provide for a characterization of habitation patterns that would be directly comparable to future studies in which the effects of drug pretreatment on response habitation could be studied. Consistent with our previous studies, we found that this control injection did not interfere with the expression of HPA axis response habitation (Cole et al., 2000), and the corticosterone and ACTH levels in no-restraint control animals one hour after injection were well within the baseline range for non-stressed animals (Sapolsky, 1992).

Other studies report habitation of immediate early gene responses to repeated stress in a number of additional brain regions believed to be involved in the control of HPA axis activity. For example, habitation of c-fos mRNA or protein expression has been reported in prefrontal cortex, hippocampus, septum, bed nucleus of the stria terminalis, amygdala, various hypothalamic subnuclei, locus ceruleus, central gray, raphe nuclei, and nucleus of the solitary tract (Melia et al., 1994; Umemoto et al., 1994a; Watanabe et al., 1994; Chen and Herbert, 1995; Bonaz and Rivest, 1998; Chung et al., 1999; Stamp and Herbert, 1999; Campeau et al., 2002). To test the prospect that habitation of c-fos expression to repeated restraint was manifest throughout the brain, we examined a region of primary sensory cortex. We wanted to determine if the neural activity of this area, which would be expected to receive comparable stimulation during initial and re-exposure to restraint, would show signs of habitation. We focused on the barrel field portion of primary somatosensory cortex that receives sensory input from the vibrissae through medullary and thalamic relays. Induction of both c-fos and zif268 proteins in the barrel field by whisker stimulation has been well characterized (Bisler et al., 2002). As expected, in rats restrained for the first time, increases in c-fos and zif268 mRNAs were observed in this region, likely representing the deflection of the whiskers by the walls of the restrainer. Surprisingly, in animals with prior exposure to the restrainer we observed a very distinct decrease in both c-fos and zif268 mRNA in the barrel field cortex. Even more surprising, was a clear habitation of the c-fos response in the major thalamic relay center to the barrel field, namely the VPM. Finally, the pattern of c-fos habitation was not restricted to the vibrissae sensory pathway, but it was also observed in the dorsolateral geniculate nucleus that relays visual information from the retina to the primary visual cortex. Thus, habitation to restraint stress was associated with a generalized decrease in c-fos expression that was evident in primary sensory thalamic relays of multiple sensory pathways. Interestingly, repeated audiogenic stress has also been reported to produce habitation of c-fos mRNA responses within primary auditory processing centers at the thalamic (medial geniculate) and cortical level (primary auditory cortex) (Campeau et al., 2002). However, in the case of audiogenic stress, the habituated c-fos mRNA response was apparently restricted to the audi-
tory modality as there was no habituation of c-fos induction within the lateral geniculate nucleus.

**Comparison of c-fos and zif268 mRNA expression patterns**

It has been noted before that the temporal and phenotypic expression patterns in the brain can vary between different immediate early genes (Cullinan et al., 1995; Kaplan et al., 1996). Interestingly, in this study we see a range of temporal, phenotypic and treatment-dependent incongruence between c-fos and zif268 expression patterns. Within the PVN, both genes had very low constitutive expression in unstressed animals, and both genes were rapidly induced (within 15 min) by novel restraint. However, the duration of peak expression was sustained (at least one hour) for zif268 mRNA compared with c-fos (approaching basal levels by one hour). As widely observed in other studies, c-fos gene constitutive expression was also very low in other brain regions of the unstressed animals, including LS, thalamus and cortex. In contrast, the zif268 gene exhibited relatively high constitutive expression in these other brain regions. Nevertheless, in some of these brain regions (LS and somatosensory cortex) there was a further induction of zif268 expression by acute restraint. The lack of a significant induction in the remaining brain areas could be due to a masking of small increases by the high constitutive zif268 mRNA expression. In the somatosensory cortex, as in the PVN, the increased zif268 expression after restraint was more prolonged than the c-fos induction, and may be due to its long-lived transcriptional steady state (Kaplan et al., 1996). Interestingly, in the LS we observed a different distribution of c-fos and zif268 mRNA signals: in the rostral LS there was a marked c-fos acute induction but very little zif268 expression, whereas in more caudal regions the pattern was reversed (Fig. 4). This phenomenon could be due to separate LS cell populations responding with increased c-fos or zif268 expression after restraint, or to a differential stimulation threshold or sensitivity to different signaling pathways of the two genes within the same population of LS cells. Importantly, in both rostral and caudal LS, the detectable c-fos expression showed induction with acute stress and much less induction in repeated stress. In contrast, zif268 gene changes were less dramatic, and while its expression appeared to increase in the caudal LS with acute stress, the overall difference between treatment groups did not reach statistical significance. Finally, we saw some dissociation between these two genes in their response to treatment. In all brain regions examined, c-fos expression was significantly increased with novel restraint, and the response was attenuated or absent in RR rats. In contrast, there were some brain regions in which we did not detect a significant effect of acute or repeated restraint on zif268 mRNA levels (rostral LS, VPM thalamus and dorsolateral geniculate). In the orbital frontal cortex there was a small but significant delayed increase in zif268 mRNA after restraint, regardless of prior restraint experience.

**Proposed model of habituation**

A great deal of literature supports using the induction of c-fos as a relatively faithful marker of increased neuronal activity [(Morgan and Curran, 1989), for review see (Hoffman and Lyo, 2002)]. However, the changes in intracellular activity that lead to c-fos induction are not directly coupled to membrane depolarization and the resulting relationship between c-fos expression and neuronal firing appears to be complex (Labiner et al., 1993). On the premise that increased c-fos expression reflects some level of increased cellular activity, one interpretation of the present data is that habituation to repeated restraint is a result of a widespread reduction in neuronal activity throughout the brain. This could be a result of a pervasive cellular change leading to an intrinsic decrease in overall level of neuronal activity. There is also the possibility that the relationship between neuronal activity and immediate early gene expression has been altered (e.g. uncoupling) as a result of repeated restraint and that only the immediate early gene response has been suppressed. For example, long-term expression of transcriptional negative regulators could lead to lowered immediate early gene expression during repeated restraint. Potential negative regulators of c-fos have been found in members of the Fos family [for review see (McClung et al., 2004)]. Other mechanisms for c-fos transcriptional regulation involving mRNA stability, which have been studied in cell lines (Peng et al., 1998), have not been documented in whole organisms but may be interesting to explore.

The greater context of experimental findings, however, makes the prospect of a widespread cellular/molecular downregulation with repeated restraint unlikely. If repeated restraint produces a tonic widespread neuronal depression then one would not expect there to be a normal neural and hormonal response when the rat is challenged with a novel stimulus. However, such normal responding to novel stimuli for both the HPA axis (Pace et al., 2001) and immediate early gene response is well documented (Melia et al., 1994; Watanabe et al., 1994; Bhatnagar and Dallman, 1994; Ma et al., 1999). Consequently, an alternate hypothesis is that habituation to restraint is fundamentally a result of a systems level adaptation. This seems especially likely in the case of restraint since the stressfulness associated with the experience depends on higher level processing. Restraint does not produce direct physical disruption (e.g. metabolic disturbance) to the organism, so one would not expect that the experience produces a direct pervasive impact on neurons throughout the brain. Rather, the association of stressfulness with this experience would seem to depend on systems-level processes, such as multimodal sensory integration, situation-specific discrimination and recall of related past experience and associated outcomes. It is surprising, however, that if this habituation is a result of a systems-level process that it then has such a widespread neural impact. One might expect that the habituated responses would only be manifest in neural signaling that occurs subsequent in time and space to the neural processing necessary for interpreting the situation as being stressful. Instead, we see that there seems to be a suppression of neural activity in primary sensory pathways. Presumably activity in these pathways is at least momentarily normal on the test day, since the stimulus specificit
of habituation implies that the animal is able to discriminate the restraint experience from others. However, our data suggest that within a very short period of time after restraint onset, activity in sensory pathways becomes attenuated. A rapid decrease of neural responses to sensory information could be a very effective means to produce multi-system habituation throughout the brain. This widespread neural habituation could be an example of altered sensory gating, perhaps implemented at the thalamic level (McCormick and Bal, 1994). Thus, we propose a model by which stressor integrative brain region(s) in the context of repeated homotypic stress exposure can rapidly decrease neural responses to primary sensory stimuli. This altered sensory gating would thereby produce attenuated responses (habituation) to homotypic stress challenge (Fig. 9). An implication of this model is that the habituation phenomenon will not be restricted to stress responses, but will include all other responses to the experience that are dependent on central neural systems.

REFERENCES


Fig. 9. Diagrammatic model for restraint habituation in the rat. The experience of restraint produces a variety of multi-modal sensory information, which is relayed to primary sensory cortex through thalamic relay nuclei. Efferents from this region projecting to associative cortical areas transfer the primary sensory information to central cortical and subcortical integrative areas. From here, projections innervating effector control centers (motor, autonomic, neurosecretory) will direct autonomic and behavioral outputs. After repeated exposure to restraint it is possible that very rapidly during re-experiencing of restraint there is a systems-level recognition of the situation and a subsequent active inhibition of sensory processing acting directly on thalamic relays and/or primary sensory cortex (indicated by the dashed arrows), thereby efficiently attenuating the overall neural response to restraint.


