Repeated Social Defeat Selectively Increases ΔFosB Expression and Histone H3 Acetylation in the Infralimbic Medial Prefrontal Cortex

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Exposure to social stress has been linked to the development and maintenance of mood-related psychopathology; however, the underlying neurobiological changes remain uncertain. In this study, we examined numbers of ΔFosB-immunoreactive cells in the forebrains of rats subjected to 12 episodes of social defeat. This was achieved using the social conflict model whereby animals are introduced into the home cage of older males (“residents”) trained to attack and defeat all such “intruders”; importantly, controls were treated identically except that the resident was absent. Our results indicated that the only region in which ΔFosB-positive cells were found in significantly higher numbers in intruders than in controls was the infralimbic medial prefrontal cortex (mPFC). This same effect was not apparent using another psychological stressor, noise stress. Cells of the infralimbic mPFC also displayed evidence of chromatin remodeling. We found that exposure to repeated episodes of social defeat increased numbers of cells immunoreactive for histone H3 acetylation, but not for histone H3 phosphoacetylation, in the infralimbic mPFC. Collectively, these findings highlight the importance of the infralimbic mPFC in responding to social stress—a finding that provides insight into the possible neurobiological alterations associated with stress-induced psychiatric illness.

Keywords: chronic stress, infralimbic cortex, layer II/III, (medial) prefrontal cortex, social conflict

Introduction

Social defeat has been consistently implicated in both the induction and the exacerbation of mood-related psychopathology (Bjorkqvist 2001; Huhman 2006). Because of this, there have been a number of preclinical studies aimed at elucidating the underlying neuronal circuitry. Most such studies have utilized the expression of immediate early genes, particularly c-fos, to assess the defeat-induced pattern of neuronal activation (Kollack-Walker et al. 1997; Martinez et al. 1998; Chung et al. 1999; Kollack-Walker et al. 1999; Martinez et al. 2002). While this approach has been very useful for identifying circuits activated by a single social defeat, it is not well suited to mapping neuronal activation resulting from repeated or chronic defeat. This is because of the declining ability of neurons to continue to express c-fos in the face of repeated activation (Martinez et al. 1998; McClung et al. 2004). This limitation is unfortunate as repeated or chronic stress is a more frequent antecedent to the development of psychopathology than a single episode (Rygula et al. 2005; Huhman 2006). There has been recent interest in ΔFosB as an alternative marker of neuronal activation in response to chronic events. In contrast with other known Fos family members, ΔFosB, a truncated splice variant of FosB, progressively accumulates in repeatedly activated neurons and persists there for several weeks (Chen et al. 1997; McClung et al. 2004; Perrotti et al. 2004). Accordingly, in the present study, we assessed the effect of repeated episodes of social defeat on the expression of FosB-like immunoreactivity in limbic forebrain regions associated with the control of mood (Di Chiara et al. 1999; Scheggi et al. 2002; Boyce and Finlay 2005). It is important to recognize that the results from these social defeat studies cannot be generalized to females as defeat can only be reliably elicited in males. Nevertheless, as several other studies have indicated that different forms of stress elicit distinct patterns of neuronal activation in the forebrain (Gullinan et al. 1995; Abraham and Kovacs 2000; Sawchenko et al. 2000; Dayas et al. 2001), we considered it prudent to examine the ΔFosB response to another form of psychological stress, noise stress. These studies failed to produce any effect of repeated noise stress on ΔFosB expression in the forebrain. Intriguingly, this work revealed only one region in which there was a significant increase in numbers of FosB-positive neurons after exposure to repeated social defeat, the infralimbic medial prefrontal cortex (mPFC). In subsequent work, FosB-positive cells in this region were further characterized as to their neurotransmitter phenotype. Additionally, because of recent evidence suggesting that neuronal activation can also induce chromatin remodeling (Miller et al. 2008; Ching and Liem 2009; Sweatt 2009), we examined cells in this region for evidence of acetylation, or phosphoacetylation, of histone H3.

Materials and Methods

Animals

Adult Sprague-Dawley male rats (350–450 g; ~70 days old, n = 8–11 per group) were obtained from the University of Newcastle Central Animal House for use in this study. Animals were maintained in temperature-controlled holding rooms (21 ± 1 °C) for the duration of the study, with food and water provided ad libitum. The rooms were held under a 12-h light:dark cycle (lights on at 1400 h), and all experimental procedures were conducted between 0800 and 1200 h. This study was approved by the University of Newcastle Animal Care and Ethics Committee and was performed in accordance with the New South Wales Animals Research Act and the Australian Code of Practice for the use of animals for scientific purposes. All animals were housed in groups of 4 prior to the commencement of the experiment but individually housed thereafter (with the exception of nonhandled controls).

Experimental Design

Subjects were exposed to either noise (NS+) or social defeat (SC+) stress, a total of 12 times over a 16-day period. Two types of controls
were used: sham stress controls (NS- and SC-) that were exposed to exactly the same procedures as the stressed animals except that the stressor was not applied and home cage controls (HCCs) that were simply left in their home cage for the duration of the experiment. An additional group was run where animals were exposed to a single social defeat and sacrificed 24 h later in order to confirm that ΔFosB was not expressed at this time.

Social Defeat
The social defeat procedure used is adapted with modifications from Miczek (1979). Briefly, subjects ("intruders") were exposed to the attacks of a dominant male (the "resident") in the resident's home cage. Residents (Sprague-Dawley males, 500–800 g) were at least 4 months old and had been co-housed in a large multi-compartment acrylic cage, with a ligated female for at least 6 weeks prior to the commencement of the experiment. Prior to the study commencing, all residents were screened to ensure that they would reliably attack intruders with a latency of less than 1 min. Fifteen minutes prior to each defeat taking place, females were removed from the resident home cage.

Intruders were removed from their home cage and placed directly into a resident home cage. After the first attack by the resident, and subsequent expression of submissive postures (lying on the back or side with no sign of resistance while being inspected by the resident) by the intruder, a wire mesh barrier was inserted into the cage to separate the 2 animals. This ensured that the animals had auditory, visual, and olfactory contact with one another but prevented further physical contact. The intruder remained in the resident’s home cage for a total of 30 min, including both interaction and separation time. To allow a proper distinction between the effects of exposure to the resident cage (novelty, handling, olfactory) and the effects of social stress, a group of sham stress control animals (SC- ) were exposed to an empty resident cage for 30 min. Exposures were time-locked to the corresponding social defeat group. As such, these animals differ from the intruders in just one respect: Only intruders were subjected to social defeat.

Noise Stress
Subjects were exposed to white noise at 105 dB for half an hour at a time. Exposure occurred within a soundproof box that the animal’s home cage was placed into. Sham stress control animals (NS-) were removed from their holding room and their home cage placed into the sound chamber but not exposed to any sound.

Immunohistochemistry
Twenty-four hours after the last treatment, animals were deeply anesthetized with sodium pentobarbital and then perfused transcardially with 2% sodium nitrate solution, followed by 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). Critically, this time of sacrifice was chosen expressly to ensure that ΔFosB would be the only FosB isoform present as it has been previously shown that all other Fos family proteins have degraded prior to this time (McClung et al. 2004; Perrotti et al. 2004; Berton et al. 2007; Nikulina et al. 2008). Additionally, evidence suggests that this same fixation time will reveal maximum expression of acetylated and phosphoacetylated histone H3 proteins (Renthal and Nestler 2009). Stable changes in histone acetylation have been found to persist for at least 2 weeks after cessation of a repeated stimulus (Renthal et al. 2007; Tsankova et al. 2007; Covington et al. 2009; Renthal and Nestler 2009). After the brains were removed, they were postfixed for 17 h in the same fixative solution. After fixation, brains were transferred to a 12.5% sucrose solution in 0.1 M phosphate-buffered saline for cryopreservation. Serial coronal sections (40 μm) were cut using a freezing microtome (Leica 2000R) and processed for FosB, acetylated histone H3, or phosphoacetylated histone H3 immunoreactivity.

For ΔFosB, we used a rabbit polyclonal antibody that had been raised against an N-terminal region of FosB and recognizes both FosB and ΔFosB (SC-48, dilution 1:500; Santa Cruz Biotechnology). Labeling for acetylated histone H3 [Ac(Lys9)-Ac(Lys 14)-H3] and phosphoacetylated histone H3 [P(Ser 10)-Ac(Lys 14)-H3] was performed with rabbit polyclonal antibodies (06-599, 1,500, and 07-081, 1:250, respectively, both from Upstate). To our knowledge, the antibody for acetylated histone H3 has not been used previously for immunohistochemistry. However, the antibody has been validated for use in western blot procedures both by the manufacturer and in previous studies (Steffan et al. 2001; Kumar et al. 2005; Renthal et al. 2007; Covington et al. 2009; Perisic et al. 2010) and in all instances produces a single band at ~17 kDa. We additionally performed a preabsorption test using the peptide for acetylated H3 to confirm that the antibody is specific for this protein. We observed no labeling in tissue treated with preabsorbed antibody (see Fig. 1 below). The histone H3 phosphoacetylation antibody used in this study has been previously validated for use in immunohistochemistry in a recent series of studies (Chandramohan et al. 2007; Reul and Chandramohan 2007; Chandramohan et al. 2008).

A 1-in-4 series of forebrain was processed batch wise for ΔFosB, or a 1-in-8 series of mPFC was processed for acetylated and phosphoacetylated H3, using the following immunoperoxidase technique.

Free-floating sections were first treated with 1% H2O2 in 0.1 M phosphate buffer and then incubated for 30 min in 5% normal horse serum in 0.1 M phosphate buffer. Sections were incubated overnight at 4 °C in 1% normal horse serum, 0.1% bovine serum albumin, 0.3% Triton X-100, and primary antibody. Sections were washed and incubated in the secondary antibody biotinylated goat anti-rabbit (BA1000, 1:500; Vector Laboratories) for 1 h, washed, and incubated in a 1:1000 dilution of Extravidin (Sigma-Aldrich) for 2 h. Peroxidase activity was visualized using a reaction with 3,3′-diaminobenzidine. The reaction was terminated when an optimal contrast between specific cellular labeling and nonspecific background was reached. Sections were then mounted onto chrome-alum-subbed slides, dehydrated in a series of alcohols (70%, 95%, 100%, absolute), cleared in xylene, and coverslipped using Permount (Fisher Scientific).

Analysis
Slides were coded, and an experimenter blind to the code counted immunoreactive cells for each region of interest. Only those forebrain regions associated with the control of mood or regulation of the stress...
response were examined for the presence of FosB-positive cells (Di Chiara et al. 1999; Weiss et al. 2001; Scheggi et al. 2002). No significant labeling was found in the paraventricular nucleus of the hypothalamus; the bed nucleus of the stria terminalis; or the medial, central, or basolateral amygdala. However, significant numbers of FosB-positive cells were present in prefrontal cortex, nucleus accumbens (NAC), and lateral septum. Therefore, more precise quantification was carried out in these regions, specifically the prelimbic and infralimbic cortices of the mPFC (5.0–2.50 and 3.72–2.44 mm from bregma, respectively), the core and shell of the NAC (2.82–0.90 mm from bregma), and the ventral lateral septum (VLS) (2.04 to –0.36 mm from bregma). Regions were defined according to Paxinos and Watson (2005). For each region of interest, immunoreactive cells were counted bilaterally at 320-μm intervals over a number of rostrocaudal levels, their boundaries being determined by cytoarchitectural features mapped in adjacent double-labeled ΔFosB and Nissl-stained sections (Gerfen 2003). Acetyl-H3- and phosphoacetyl-H3-positive cells were counted in mPFC only. Positive cells were counted bilaterally at 320-μm intervals. Analysis was performed using Metamorph Imaging System software (Universal Imaging Corp.; Volpicelli-Daley and Levey 2003).

Double-Label Immunofluorescence
Immunohistofluorescence was used to double label ΔFosB (SC-48, 1:100; Santa Cruz Biotechnology) and several other proteins. These included neuron-specific nuclear protein NeuN (MAB377, 1:100; Chemicon), parvalbumin (MAB1572, 1:5000; Chemicon), calbindin (AB1778, 1:100; Chemicon), and calretinin (AB5054, 1:1000; Chemicon). Immunohistofluorescence was also used to double label acetylated histone H3 (06-599, 1:100; Chemicon) and neuron-specific nuclear protein NeuN (MAB377, 1:100; Chemicon). The proteins were visualized using AlexaFluor-labeled secondary antibodies (488 or 594, 1:400; Molecular Probes). Localization of protein expression was performed using an Olympus BX51 microscope fitted with an Olympus DP71 camera and U-RFL-T burner connected to a personal computer running DP-BSW software.

Figure 2. Left: Photomicrograph of HCC infralimbic cortex labeled with ΔFosB. Center: Photomicrograph of infralimbic cortex from an animal exposed to a single social defeat and sacrificed 24 h later and labeled with ΔFosB. Right: Photomicrograph of infralimbic cortex from an animal exposed to 12 social defeats and sacrificed 24 h after the final episode and labeled with ΔFosB. Scale bars = 50 μm.

Results
Stress Induction of ΔFosB Protein in Brain
As several previous studies have demonstrated that ΔFosB is only apparent after repeated stimulation, we were interested in confirming that ΔFosB expression was low after a single social defeat episode. Consistent with previous reports, we found no evidence of ΔFosB in tissue collected 24 h after a single social defeat (see Fig. 2 below).

Each of the regions examined are represented in Fig. 3, below. While immunoreactive cells were counted bilaterally, there were no significant differences between the left and the right sides of any brain region counted. A one-way ANOVA was used to test differences between the stress groups in each brain region counted. In the infralimbic (IL) mPFC, there was a significant effect of group, F4,38 = 21.54, P < 0.001. Tukey’s post hoc comparisons revealed that the repeated social defeat stress group has significantly higher ΔFosB counts than the sham social defeat group (P < 0.001), the noise stress group (P < 0.001), the sham noise stress group (P < 0.001), and the HCCs (P < 0.001). The sham stress group was also significantly different to the HCCs (P = 0.045).

In the prelimbic (PrL) mPFC, a one-way ANOVA suggested that there was a significant effect of group, F4,38 = 4.21, P = 0.006. Tukey’s post hoc comparisons revealed significant differences

Data Analysis
Raw counts of positive immunoreactive cells were analyzed using analysis of variance (ANOVA), followed by post hoc testing. In all figures and tables, data are expressed as the mean ± standard error (SEM). The data were considered to reach statistical significance where P < 0.05.
between the repeated social defeat group and both the HCCs (\(P = 0.01\)) and the sham noise stress condition (\(P = 0.047\)).

In the shell of the NAc, there was a significant effect of group, \(F_{3,38} = 4.64, P = 0.004\). Tukey’s post hoc comparisons showed significant differences between the repeated social defeat condition and both the HCCs (\(P = 0.009\)) and the sham noise stress group (\(P = 0.014\)).

In the core of the NAc, a one-way ANOVA revealed a significant main effect of group, \(F_{3,38} = 5.26, P = 0.002\). Tukey’s post hoc testing revealed significant differences between the repeated social defeat group and the noise stress (\(P = 0.013\)), sham noise stress (\(P = 0.019\)), and HCC (\(P = 0.008\)) groups.

In the VLS, a one-way ANOVA was not significant overall (\(P = 0.16\)). Tukey’s post hoc analysis revealed a significant difference between the repeated noise stress condition and the HCCs only (\(P = 0.017\)) (see Table 1 below).

Overall, relative to HCCs, numbers of \(\Delta FosB\)-positive cells were significantly elevated in just one of the regions counted in the forebrains of animals subjected to sham social defeat (SC-), the IL mPFC, and 4 of the regions counted in the forebrains of animals subjected to repeated social defeat (SC+), the Prl mPFC, the IL mPFC, the NAc core, and the NAc shell. However, relative to the SC- group, only the increase observed in the IL mPFC of SC+ animals was statistically significant (see Fig. 4 below).

Relative to HCC animals, numbers of \(\Delta FosB\)-positive cells were not significantly elevated in any of the forebrain regions counted in animals subjected to sham noise stress (NS-), but they were significantly elevated in one of the regions counted in the forebrains of animals subjected to repeated noise stress (NS+) that being the VLS. Importantly, however, this increase in the VLS of NS+ animals was not statistically significant relative to NS- animals.

**Cellular Specificity of \(\Delta FosB\) Induction by Chronic Stress in the Infralimbic Cortex**

\(\Delta FosB\)-positive cells observed in the infralimbic cortex of animals subjected to social defeat were further characterized. Tissue immunolabelled for \(\Delta FosB\) was also subjected to a Nissl stain. This showed that \(\Delta FosB\)-positive cells were densest in cortical layers II and III (Fig. 5). Double-label immunofluorescence showed that almost all of these cells were also immunopositive for Neun, a marker of mature neurons (Fig. 6; Magavi and Macklis 2008). However, no \(\Delta FosB\)-positive cells were immunoreactive for calbindin-D28k, parvalbumin, or calretinin, all of which are established markers of γ-Aminobutyric acid (GABAergic) neurons (Kawaguchi and Kubota 1997). As the cortex contains primarily GABAergic interneurons and glutamatergic pyramidal projection neurons, this suggests that the \(\Delta FosB\)-positive cells were of the latter type (Fig. 7).

**Stress Induction of Changes in Histone Acetylation Status in mPFC**

We used immunohistochemistry to quantify changes to histone H3 acetylation status in the IL and Prl of animals exposed to repeated social defeat. While there was a significant difference in levels of acetylated histone H3 [Ac(Lys9)-Ac(Lys14)-H3] immunoreactivity between repeated social defeat and sham social defeat controls in the IL (\(P = 0.02\); Fig. 8), we found no difference between the 2 groups for phosphoacetylated histone H3 [P(Ser10)-Ac(Lys14)-H3] immunoreactivity. We found no differences between repeated social defeat and sham social defeat controls in levels of acetylated [Ac(Lys9)-Ac(Lys14)-H3] or phosphoacetylated [P(Ser10)-Ac(Lys14)-H3] histone H3 immunoreactivity in the Prl (see Table 2). Further analysis of the acetylated histone H3 (Lys9-Lys14) response in the IL using dual-label immunofluorescence showed partial colocalization with Neun, suggesting that this change occurred in both neurons and glial cells (Fig. 6).

**Discussion**

In this study, it was found that, although repeated noise stress was without effect, repeated social defeat led to a significant increase in numbers of \(\Delta FosB\)-immunoreactive neurons in just one forebrain area, the infralimbic mPFC. Because a single social defeat does not elicit detectable \(\Delta FosB\)-immunolabelling in the forebrain, this result is assumed to reflect the cumulative impact of multiple defeat episodes over the preceding 16 days, an interpretation consistent with reports that this protein progressively accumulates in the nuclei of repeatedly activated neurons (McClung et al. 2004; Nikulina et al. 2008; Renthal et al. 2008).

The current findings are somewhat surprising as there have been 2 previous studies suggesting that repeated stress (both social defeat and restraint stress) increases \(\Delta FosB\) expression in multiple forebrain regions (Perrotti et al. 2004; Nikulina et al. 2008). However, it is critical to note that, in both these previous studies, the reported increases were relative to levels observed in control animals that were, at most, subjected to brief handling by the experimenters. In contrast, in the current study, the critical controls were animals that were treated identically to the stressed animals, except for application of the stressor itself. For example, sham social defeat controls were, in addition to being handled by the experimenters, also placed in the temporarily vacated home cage of an aggressive conspecific for 30 min. Such a cage would obviously constitute a highly novel environment, being rich in spatial and olfactory stimuli. Previous work suggests that this should elicit neuronal activation well beyond that produced by handling alone (Hosokawa and Chiba 2005; Cavalcante et al. 2006; Nugent et al. 2009), and indeed, we found that \(\Delta FosB\)-positive cell numbers in the IL and Prl mPFC of the sham social defeat controls were significantly higher than that in HCCs (see Table 1). It is our view then that, particularly when assessing the impact of complex stressors, the most appropriate control

**Table 1**

<table>
<thead>
<tr>
<th>Region</th>
<th>SC+</th>
<th>NS+</th>
<th>HCC</th>
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</thead>
<tbody>
<tr>
<td>IL mPFC</td>
<td>110</td>
<td>65</td>
<td>23</td>
</tr>
<tr>
<td>Prl mPFC</td>
<td>156</td>
<td>117</td>
<td>54</td>
</tr>
<tr>
<td>NAc shell</td>
<td>191</td>
<td>143</td>
<td>140</td>
</tr>
<tr>
<td>NAc core</td>
<td>312</td>
<td>189</td>
<td>169</td>
</tr>
<tr>
<td>VLS</td>
<td>115</td>
<td>95</td>
<td>50</td>
</tr>
</tbody>
</table>

Note: Number of \(\Delta FosB\)-positive cells in forebrain regions of rats exposed to repeated social defeat (SC+) or noise stress (NS+), matched sham stress control groups (SC- and NS-), and nonhandled HCCs. Data are expressed as mean ± standard error (SEM) (n = 8–11 animals per group).

\*Significantly different from nonhandled HCC.

\*Significantly different from matched sham stress control group.

\*P < 0.05.
Figure 4. Quantification of ΔFosB induction in the infralimbic mPFC of animals exposed to 12 days of repeated social defeat. Levels of ΔFosB immunoreactivity were assessed by immunohistochemistry in (A) animals subjected to repeated social defeat and (B) animals exposed to sham social defeat. (C) Line graph showing the distribution of ΔFosB-positive cells across the rostrocaudal axis of the infralimbic cortex of animals exposed to chronic social stress (●), sham stress controls (■), and nonhandled HCCs (▲). Inset: Bar graph displaying mean totals of ΔFosB-positive cells in the infralimbic mPFC of animals exposed to chronic social stress (SC+), chronic sham social stress (SC-), and nonhandled HCCs. Data are expressed as mean ± standard error (SEM). *P < 0.05 = mdline (layer II). Scale bars = 50 μm.

Figure 5. Layer-specific ΔFosB labeling in the infralimbic cortex. (A) Light photomicrograph showing laminar distribution of cresyl violet-stained cells in the infralimbic cortex using cortical layer demarcation adapted from Gabbott et al. (2005). Photomicrographs demonstrating layer-specific labeling of ΔFosB with cresyl violet. (B) Animal exposed to chronic social defeat. Inset: High-power photomicrograph with arrows indicating cells double labeled with both cresyl violet and FosB in layer II/III. (C) Animal exposed to chronic sham social defeat. Inset: Examples of cells from layer II/III. Scale bars = 100 μm (A), 50 μm (B and C), and 10 μm (insets).
group is one differing from their experimental counterparts only in the absence of the critical stress element, which, in the case of the social defeat paradigm, is a aggressive social interaction culminating in defeat.

It is particularly interesting that in the present study: 1) Although repeated social defeat elicited a significant increase in $\Delta FosB$ expression, repeated noise stress did not and 2) this change occurred only in the infralimbic mPFC. One possibility is that social defeat is simply a more intense stressor than noise stress. This is consistent with the considerable body of evidence showing that the infralimbic mPFC plays a key role in regulating the activity of the subcortical stress response system (Diorio et al. 1993; Figueiredo et al. 2003; Spencer et al. 2004; Radley et al. 2006). However, one problematic aspect of this explanation is that we would expect levels of $\Delta FosB$ to be higher in the noise stress group than in the sham social defeat group, a group only exposed to an empty social conflict arena, when in fact they are statistically equivalent. Alternatively, the results could be taken to suggest that the infralimbic mPFC is uniquely responsive to social forms of stress. Certainly, this

Figure 6. The upper series of 3 panels shows a representative fluorescent photomicrograph displaying colocalization of FosB with NeuN, a marker of mature neurons, in the infralimbic cortex. Left: $\Delta$FosB only. Center: NeuN only. Right: Merged. Arrows indicate double-labeled cells. The lower series of 3 panels shows a representative fluorescent photomicrograph demonstrating incomplete colocalization of acetyl-H3-positive cells with NeuN, a marker of mature neurons. Left: Acetyl-H3 only. Center: NeuN only. Right: Merged. Arrows indicate acetyl-H3-positive cells that are not neurons. Scale bars = 50 $\mu$m.

Figure 7. Cellular specificity of $\Delta FosB$ induction in the infralimbic mPFC following repeated social defeat. Representative fluorescent photomicrographs show no colocalization of FosB with parvalbumin (A–C), calretinin (D–F), or calbindin (G–I), markers of GABAergic interneurons. Scale bars = 50 $\mu$m.
HCCs. Data are expressed as mean ± standard error (SEM). *Significantly different from nonhandled HCC. aSignificantly different from matched sham stress control group. bSignificantly different from chronic sham social stress controls (SC−), and nonhandled HCCs. Data are expressed as mean ± standard error (SEM). *P < 0.05.

Table 2

| Total acetylated H3- and phosphoacetylated H3-positive cell counts by condition and region |
|----------------------------------|-----------------|-----------------|-----------------|
|                                  | SC−             | SC+             | HCC             |
| Acetylated histone H3            |                 |                 |                 |
| IL mPFC                          | 4186 (231)      | 5896 (476)      | 4476 (449)      |
| PrL mPFC                         | 8096 (1045)     | 9619 (868)      | 11,900 (464)    |
| Phosphoacetylated histone H3     |                 |                 |                 |
| IL mPFC                          | 3270 (1060)     | 3817 (1154)     | 1460 (460)      |
| PrL mPFC                         | 4354 (640)      | 4453 (613)      | 2495 (640)      |

Note: Number of acetylated histone H3-immunoreactive cells in the mPFCs of rats exposed to repeated social defeat (SC+), matched sham stress control group (SC−), and nonhandled HCCs. Data are expressed as mean ± standard error (SEM) (n = 8-11 animals per group).

*Significantly different from matched sham stress control group.

**Significantly different from nonhandled HCC.

**P < 0.05.

Figure 8. Quantification of acetyl-H3 induction in the infralimbic mPFC of animals exposed to repeated social defeat. Line graph showing the rostrocaudal distribution of acetyl-H3-positive cells in the infralimbic cortex of animals exposed to chronic social defeat (SC+), chronic sham social stress controls (SC−), and nonhandled HCCs. Inset: Bar graph displaying mean totals of acetyl-H3-positive cells in the infralimbic cortex of animals exposed to chronic social defeat (SC+), chronic sham social stress (SC−), and nonhandled HCCs. Data are expressed as mean ± standard error (SEM). *P < 0.05.

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Hypothesis is consistent with evidence indicating that the mPFC is involved in modulating social interactions (Ovtcharoff and Braun 2001; Shah and Treit 2003; Kiyokawa et al. 2007; Leussis et al. 2008). Experimentally, teasing out whether the infralimbic mPFC is uniquely responsive to social stressors will certainly be a challenge. One promising avenue may be to examine a wider range of social (social isolation) and nonsocial stressors (predation, shock) that vary in their inherent intensity.

It is generally agreed that neurons located within the mPFC are either glutamatergic pyramidal neurons or GABAergic interneurons with regard to phenotype (Gabott et al. 1997). Moreover, it is generally accepted that neurons containing the calcium-binding proteins calretinin, calbindin-D28k, or parvalbumin also contain GABA (Kawaguchi and Kubota 1997; Gritti et al. 2003; Molyneaux et al. 2007). Consequently, our finding that none of the FosB-positive cells were immunoreactive for any of these 3 calcium-binding proteins suggests that these cells are likely to be glutamatergic pyramidal projection neurons. Notably, most of these presumptive glutamatergic pyramidal neurons were located in cortical layer II/III. Previous studies have shown that the pyramidal neurons of layer II/III are stress sensitive, with prolonged exposure to stress leading to extensive and reversible dendritic remodeling (Radley et al. 2006; Radley, Rocher et al. 2008; Radley, Williams et al. 2008; Watakabe 2009). Furthermore, while some of these neurons project to subcortical regions including the basolateral amygdala, lateral hypothalamus, and NAc, it is thought that the majority of pyramidal neurons in layer II/III project to other cortical areas, including orbital, insular, and entorhinal cortices (Vertes 2004; Gabbott et al. 2005; Hoover and Vertes 2007).

As ΔFosB is a transcription factor, increased numbers of FosB-positive cells in the infralimbic mPFC has been interpreted as a change in the functional capacity of the neurons in this region (McClung et al. 2004; Perrotti et al. 2008; Wallace et al. 2008). Recent studies indicate that another way in which transcriptional modification of neurons can be achieved is via chromatin remodeling (Bilang-Bleuel et al. 2005; Tsankova et al. 2006; Tsankova et al. 2007; Jiang et al. 2008). One way in which this can occur is through the posttranslational modification of core histone proteins at their N-terminus, increasing the accessibility of DNA to binding proteins (Kouzarides 2007; Renthal et al. 2007). Thus, it has been demonstrated in several studies that the acetylation [Ac(Lys9)-Ac(Lys 14)] and phosphoacetylation [P(Ser 10)-Ac(Lys 14)] of histone H3, events associated with the activation and maintenance of transcription, can be modified in several brain regions by exposure to stress (Bilang-Bleuel et al. 2005; Chandramohan et al. 2007; Reul and Chandramohan 2007; Chandramohan et al. 2008; Fuchikami et al. 2009; Sweatt 2009). It was against this background that we examined cells in the infralimbic mPFC for immunoreactivity against acetylated or phosphoacetylated histone H3 after exposure to repeated social defeat. This revealed that, although there was no change in numbers of immunoreactive cells for phosphoacetylated histone H3, there was a significant increase in acetylated histone H3 immunoreactivity. Ideally, we would have wished to demonstrate increases in acetylated histone H3 colocalized to cells that were FosB positive. However, this was not possible as all commercially available antibodies for these proteins are currently raised in the same species, making it impossible to...
achieve reliable double labeling. Nevertheless, to our knowledge, it still stands that this is the first provision of evidence that stress-induced chromatin remodeling occurs in the prefrontal cortex. We additionally examined cells in the adjacent prelimbic mPFC for immunoreactive cells for acetylated and phospho-acetylated histone H3. Interestingly, in line with the null result found in this region for ΔFosB, we also found no significant differences in chromatin remodeling between groups.

One of the major functions of the mesocorticolimbic system, which includes the mPFC, is to prevent excessive behavioral and physiological responses to stress (Sullivan 2004). Both electrolytic lesions and injections of dopamine receptor antagonists into the mPFC have previously been shown to exaggerate chronic stress-induced neuroendocrine responses (Gerrits et al. 2003; Sullivan and Dufresne 2006). Our results extend this body of work by elucidating specific neurobiological alterations that occur following exposure to chronic stress within the mPFC. From a clinical standpoint, these results are in keeping with functional neuroimaging studies in humans, which report increased metabolic activity in the subgenual cingulate cortex (the closest analogous region to the rat infralimbic cortex) in patients with depression (Gotlib et al. 2005; Beauregard et al. 2006; Drevets et al. 2008), a condition that may be precipitated by exposure to chronic psychosocial stress (Bjorkqvist 2001).

In conclusion, this study confirms that repeated social defeat leads to an increase in ΔFosB expression in the forebrain of the rat. In contrast to the sole previous report, we demonstrated that this is restricted to a single area of the forebrain, the infralimbic mPFC. In particular, this increase in FosB-like immunoreactivity was localized to glutamatergic pyramidal neurons, largely in layer II/III in that region. Moreover, evidence was provided of histone modifications occurring in this same region after repeated exposure to social defeat, this being the first time any evidence of this functionally important change is occurring in prefrontal cortex. While these results are interesting, it is important to note that these experiments were carried out using male rats only, and at this point, the extent to which they generalize the female brain is unknown. Importantly, however, it has been shown that exposure to a nonsocial form of chronic stress increases the number of ΔFosB-positive nuclei in the mPFC of female rats, suggesting that the mPFC is similarly responsive to chronic stress in females (Gerrits et al. 2006).

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References


