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Structural and functional alterations to rat medial prefrontal cortex following chronic restraint stress and recovery

Deena S. Goldwater^{*}, Constantine Pavlides[^], Richard G. Hunter[^], Erik B. Bloss^{*}, Patrick R. Hof^{*,+,#}, Bruce S. McEwen[^], and John H. Morrison^{*,+}

^{*}Fishberg Department of Neuroscience and Kastor Neurobiology of Aging Laboratories, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1065, New York, NY 10029

⁺Department of Geriatrics and Adult Development, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1065, New York, NY 10029

[#]Computational Neurobiology and Imaging Center, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1065, New York, NY 10029

[^]Laboratory of Neuroendocrinology, Rockefeller University, New York, NY 10021, USA

Abstract

Chronic stress has been shown in animal models to result in altered dendritic morphology of pyramidal neurons of the medial prefrontal cortex (mPFC). It has been hypothesized that the stress-induced dendritic retractions and spine loss lead to disrupted connectivity that results in stress-induced functional impairment of mPFC. While these alterations were initially viewed as a neurodegenerative event, it has recently been established that stress induced dendritic alterations are reversible if animals are given time to recover from chronic stress. However, whether spine growth accompanies dendritic extension remains to be demonstrated. It is also not known if recovery-phase dendritic extension allows for re-establishment of functional capacity. The goal of this study, therefore, was to characterize the structural and functional effects of chronic stress and recovery on the infralimbic (IL) region of the rat mPFC. We compared neuronal morphology of layer V IL pyramidal neurons from animals subjected to 21 days of chronic restraint stress (CRS) to those that experienced CRS followed by a 21 day recovery period. Layer V pyramidal cell functional capacity was assessed by intra-IL long-term potentiation (LTP) both in the absence and presence of SKF38393, a dopamine receptor partial agonist and a known PFC LTP modulator. We found that stress-induced IL apical dendritic retraction and spine loss co-occur with receptor-mediated impairments to catecholaminergic facilitation of synaptic plasticity. We also found that while post-stress recovery did not reverse distal dendritic retraction, it did result in over-extension of proximal dendritic neuroarchitecture and spine growth as well as a full reversal of CRS-induced impairments to catecholaminergic-mediated synaptic plasticity. Our results support the hypothesis that disease-related PFC dysfunction is a consequence of network disruption secondary to altered structural and functional plasticity and that circuitry reestablishment may underlie elements of recovery. Accordingly, we believe that pharmacological treatments targeted at preventing dendritic retraction

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Corresponding Author: Dr. John H. Morrison Department of Neuroscience, Box 1065, Mount Sinai School of Medicine One Gustave L. Levy Place New York, NY 10029 Phone: 212-659-5985 Fax: 212-849-2510 John.Morrison@mssm.edu.

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and spine loss or encouraging circuitry reestablishment and stabilization may be advantageous in the prevention and treatment of mood and anxiety disorders.

Keywords

infralimbic; dendritic morphology; dendritic spines; dopamine; long-term potentiation

Introduction

Exposure to chronic stress is a well-established risk factor for mood and anxiety disorders (Mazure, 1995), yet the mechanism by which chronic stress acts as a precipitating agent is incompletely understood. It has been suggested that chronic stress results in neuronal network disruption of susceptible brain regions, via altered structural and functional plasticity, which ultimately leads to disease-related cortical dysfunction (Castrén, 2005). The prefrontal cortex (PFC) is a neocortical region directly involved in high-order executive processes, including integration of cognitive information and emotional states and modulation of subcortical systems (Diorio et al., 1993, Groenewegen and Uylings, 2000, Uylings et al., 2003). In rodent models, chronic stress results in neuronal morphological alterations in the medial PFC (mPFC) as evidenced by retraction of the apical dendritic branches of layers II/III pyramidal cells (Cook and Wellman, 2004, Radley et al., 2004, Liston et al., 2006) as well as spine loss on those same neurons (Radley et al., 2008). Initially thought to be a degenerative event, this dendritic retraction of layers II/III neurons is a reversible phenomenon, with apical dendrites re-extending to original lengths following a post-stress rest period (Radley et al., 2005).

Evidence also exists for stress-induced impairment of functional plasticity in the rodent PFC. For example, Cerqueira et al. (2007) demonstrated that chronic stress inhibits the maintenance of *in vivo* hippocampus-to-mPFC LTP. Functions mediated by the PFC are heavily dependent upon tightly regulated release of dopamine (DA) from neurons of the mesocortical projection system. Via D1 and D2 receptors (D1R and D2R), DA works in the PFC to modulate postsynaptic neuronal responses to glutamatergic input (Goldman-Rakic et al., 2000). Establishment of both *in vivo* and *in vitro* long-term potentiation (LTP) in mPFC is influenced by D1 receptor stimulation (Huang et al., 2004). Although alterations of DA release are evident following chronic stress (Di Chiara et al., 1999, Mizoguchi et al., 2000, Murphy et al., 2003), whether there exists a postsynaptic component to such stress-induced impairment of DAergic PFC modulation remains to be investigated. Furthermore, whether functional capacity returns to baseline following a recovery period also remains unknown.

In contrast to the mostly corticocortical projections of layers II/III, the majority of layer V mPFC pyramidal neurons project to and regulate subcortical output systems (Gabbott et al., 2005). However, chronic stress consistently results in dysregulation of mPFC layer V subcortical targets such as the hypothalamus and amygdala (Beyer et al., 2000, Shelton, 2000, Shin et al., 2005, Yao et al., 2008), suggesting a stress-induced impairment of mPFC top-down regulation. However, the effects of chronic restraint stress (CRS) on layer V mPFC neurons are unknown. The goal of this study was therefore to investigate the as yet uncharacterized structural and functional consequences of chronic stress and recovery on layer V pyramidal neurons of the infralimbic (IL) cortex of the rat mPFC. Specifically, we assessed the effects of CRS on IL layer V apical dendritic architecture as well as the ability of CRS to affect D1 receptor modulation of *in vitro* mPFC LTP induction and maintenance.

Experimental procedures

Animals

Given the different nature of tissue processing necessary for each component of this study, a different set of animals was used for each experiment. Male Sprague-Dawley rats (Charles River, Wilmington, MA), weighing between 150 and 200 g at the start of the experiments, were double- (morphology) or triple- (LTP/receptor assays) housed and each cage of animals was randomly assigned to a Stress, Rest, or Control group. Animals were housed at Rockefeller University Laboratory Animal Research Center in a normal 12:12 hours light dark cycle (8 A.M. — 8 P.M.) with ad libitum access to food and water and one week to adjust to the housing environment prior to stress protocol onset. Chronic restraint stress (CRS) entailed restraint in a wire-mesh restrainer for 21 consecutive days, 6 hours/day (10 A.M. — 4 P.M.), during the rats' normal low-activity period. Stress was staggered such that all animals were sacrificed at 14 weeks of age in order to prevent age-related differences at time of sacrifice. Therefore, Rest animals began stress at 8 weeks of age for 21 days and remained undisturbed in their home cages for an additional 21 days. The Stress group began CRS at 11 weeks of age for 21 days and was sacrificed 40 hours after their final stress exposure to minimize the effects of acute stress. Control animals remained undisturbed in their home cages for the full 44-day period except for weekly weighing and regular husbandry.

Morphology

Euthanasia

Eighteen animals, with 6 animals per Stress, Rest and Control groups, were used in the morphology study. As in Radley et al. (2004,2005), animals were deeply anesthetized with an i.p. injection of a 30% chloral hydrate solution and perfused transcardially with 1% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4) for 1 min (40 ml/min) then 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4) and 0.125 % glutaraldehyde for 12 min (40 ml/min). Brains were carefully dissected from the skull and postfixed in the paraformaldehyde/glutaraldehyde solution for 6 hours until placement in PBS for short-term storage. The mPFC of each brain (1.5-3.5 mm rostral from Bregma; (Paxinos and Watson, 1997)) was then serial sectioned in the coronal axis at thicknesses of 250 μ m (for cell loading) and 50 μ m (for immunohistochemistry) on a Vibratome (Leica, Bannockburn, IL).

Analysis of mPFC apical dendritic morphology

The cell loading and analysis procedure was identical to that described in Radley et al. (2004,2008). Each 250 μ m-thick mPFC slice was immersed in 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO), a fluorescent staining solution, to visualize pyramidal cell nuclei and aid in layer differentiation within the IL. The sections were then mounted on nitrocellulose filter paper and submerged in PBS. Sections were visualized with a water immersion 5X lens under fluorescent light with a UV filter to localize pyramidal cells within the larger in IL for intracellular loading. The Paxinos and Watson (1997) atlas was used as a reference for each slice and IL regional boundaries were identified using the midpoint of genu of the corpus callosum for the dorsal limit and the ventral extent of the lateral ventricle as the ventral limit (Paxinos and Watson, 1997). Moreover, the anterior-posterior limits of the IL were determined to be 500 μ m caudal to the initiation of the genu of the corpus callosum and 250 μ m rostral to obvious intrahemispheric corpus callosum communication. Identified neuronal targets were then visualized at 20X and loaded with iontophoretic injections of 5% Lucifer Yellow (LY; Molecular Probes, Eugene, OR), using a DC current of 5-10 nA for 5-10 min or until distal processes were fully visualized and no further loading was observed. Sections were then mounted and coverslipped using PermaFluor aqueous mounting medium (Thermo

Electron, Boston, MA). The edges of the coverslip were sealed with hard polish to prevent mounting medium leakage.

An average of 3.5 neurons from the IL region of each animal were traced using Neurolucida software (MBF Bioscience, Williston, VT). As in Radley et al. (2004), LY-loaded neurons were visualized using a Zeiss Axiophot 2 microscope (Oberkochen, Germany) equipped with a Zeiss 40X 1.4 numerical aperture (N.A.) lens with a motorized stage and video camera to send live images to the computer for tracing. Layer V neurons included in the analysis needed to meet the following criteria: 1) the soma was within IL layer V; 2) the dendritic tree was completely filled with LY as well as entirely traceable; 3) neuronal primary, secondary, and tertiary branches were intact; 4) at least one apical dendritic branch reached the pial surface (Fig. 1A-C).

For spine imaging, two-dimensional (2-D) maximal projections of reconstructed layer V neurons were used to identify apical dendritic branches within layers V, II/III, and I, at 30-120 μm , 150-280 μm , and 300-480 μm from the soma, respectively. Similar to Radley et al. (2008), dendritic branches were identified in the 2-D reconstruction through a systematic random procedure and then imaged in three dimensions on a Zeiss LSM510 confocal microscope fitted with Zeiss 100X 1.4 N.A. oil-immersion objective with standard filter sets and a standard (1 Airy disk) pinhole. LY was excited by an Ar/Kr laser at 453 nm with laser attenuation set at 8. Settings for gain and offset were optimized for each dendritic segment sampled to maximize the image and minimize background. Confocal stacks through the dendritic branch were obtained with a z-step of 0.1 μm at a resolution of 512 $\times$ 512 pixels with a digital zoom of 5. All confocal stacks included at least 1 μm above and below the target dendritic segment. Three dendritic segments per layer per neuron (i.e., 9 dendritic segments/neuron) were captured with a total of 12 neurons per group. Z-stacks were deconvolved using AutoDeblur (version 8.0.2.; Autoquant, Troy, NY). As in Radley et al. (2008), the Rayburst automated algorithm (Rodriguez et al., 2008) was used to reconstruct digitally each deconvolved confocal image stack in three dimensions in order to obtain spine density (Fig. 1D,E).

Statistical analyses

All sampling occurred with investigator blind to group. One-way analysis of variance (ANOVA) was used to compare total dendritic length between the three groups with an α level of 0.05 for statistical significance. Sholl analysis of dendritic length within successive 30 μm radial increments from the soma was analyzed with two-way repeated measures ANOVA (treatment group $\times$ distance from soma) with post hoc Bonferroni pairwise comparisons to discriminate effects of treatment, with $\alpha = 0.05$ considered significant. Data are presented as mean $\pm$ SEM.

Spine density was analyzed using two-way repeated measures ANOVA (treatment group $\times$ layer) with post hoc Bonferroni pairwise comparisons to identify layer and treatment effects. A significance level of $\alpha = 0.05$ was considered significant for all tests. Data are presented as mean $\pm$ SEM.

LTP

Animal sacrifice and tissue preparation

Rats were deeply anesthetized in a carbon dioxide chamber. Brains were rapidly removed and submerged for one minute in 4°C artificial cerebrospinal fluid (ACSF). The composition of ACSF was as follows: 10 mM D-glucose, 124 mM NaCl, 1.25 mM NaH_2PO_4 , 26 mM NaHCO_3 , 5 mM KCl, 2.4 mM CaCl_2 , and 1.3 mM MgSO_4 . Coronal slices (350 μm -thick) of the PFC (1.5-3.5 mm rostral from Bregma) were cut on a Vibratome while immersed in 4°C

ACSF and transferred to a holding chamber filled room temperature ACSF and constantly bubbled with O₂/CO₂ (95/5% mixture). Slices remained undisturbed in the holding chamber for at least one hour before being placed into an interface chamber where they were perfused with 32°C ACSF (1 ml/min) that was constantly bubbled with the oxygen mixture. Slices placed in the interface chamber again remained undisturbed for at least 20 min to temperature-adjust before recording began.

Recordings

The LTP protocol was adapted from Huang et al. (2004). Field excitatory postsynaptic potentials (fEPSP) recordings were obtained using a glass pipette filled with 2 M NaCl (1–3 MΩ) placed on IL layer V. Stimuli (50–200 μA; 100 μs pulse duration) were given using a unipolar electrode placed on IL layer II. An input/output curve ranging from minimum to maximum responses was performed on each slice and stimuli strength was adjusted such that synaptic response size was 50% of maximum. Test-stimulus field potentials were obtained once every minute. High frequency stimulation (HFS) protocol consisted of 3 trains of 300 Hz tetanus (100 μs pulse width; 0.5 s train duration) given with a 3 min inter-train interval.

Pharmacological manipulations

A D1 receptor agonist, SKF38393 (3 μM, Sigma-Aldrich, St. Louis, MO), was infused at a rate of 0.1 ml/min for 30 min total (20 min before tetanization and 10 min during the HFS protocol) such that final concentration at the slice was 0.3 μM. As a control, a final slice concentration of 0.3 μM SKF38393 was infused for 30 min without HFS to determine baseline drug effect. Infusion of 100 μM SCH23390 (Sigma-Aldrich, St. Louis, MO), a D1R antagonist, such that the final concentration at the slice was 10 μM, 5 min before a 30 min infusion of 0.3 μM SKF38393 with HFS was used as a second control. Each pharmacological manipulation was used only once per animal.

Data analysis

Amplitude averages of fEPSP responses following tetanization were obtained for every 10 min post-HFS. Averages across the three groups were analyzed using two-way repeated measure ANOVA (treatment group X time). Post hoc Bonferroni comparisons, with $\alpha = 0.05$ considered significant, was used to further discriminate treatment effects. If any seizure activity was seen, the slice was eliminated from the analysis. Data are presented as mean $\pm$ SEM.

Receptor Assays

Animal sacrifice and tissue preparation

Animals were deeply anesthetized in a CO₂ chamber. Brains were then rapidly removed, fresh-frozen on dry ice, and stored at –70°C until further processing. Coronal mPFC sections (20 μm; 1.5–3.5 mm rostral from Bregma) were obtained on a cryostat and stored at –70 °C until binding assays.

Binding assays

D1 receptor ligand binding was performed as described previously (Davidoff and Benes, 1998). Coronal slices from Stress, Rest, and Control groups were incubated for 10 min at room temperature in assay buffer (pH 7.4) containing 0.05 M Tris-HCl, 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 μM pargyline, 0.001% ascorbic acid, and 300 nM mianserin. Sections were then incubated in the same buffer with 10 nM [³H]SCH23390 (Sigma-Aldrich, St. Louis, MO) at room temperature for 45 min. Finally, sections were washed 2 × 10 min in 0.05 M Tris buffer, pH 7.4 followed by a quick rinse in ice-cold ddH₂O. Incubation of sections for basal binding identification was carried out in an identical manner except for the addition

of the competitive inhibitor SKF38393 (10 μ M, Sigma-Aldrich, St. Louis, MO) to the buffer containing 10 nM [3 H]SCH23390. Sections were allowed to dry overnight at room temperature before being exposed to Kodak BioMax MS film (Sigma-Aldrich, St. Louis, MO) for 12 weeks. Film was developed according to company specifications.

Data acquisition and analysis

Prior to slicing, the IL region was punched unilaterally, alternating left vs. right sided punch per animal, in the event that ligand binding did not allow for proper IL region visualization. Optical density (OD) measurements, therefore, were obtained unilaterally, in three sections per animal, rostral to caudal. OD was determined using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Baseline OD was calculated by subtracting the optical density of basal-binding sections from that of experimental sections and averages were obtained per animal. Statistical significance was determined using one-way ANOVA with post hoc Newman-Keuls multiple comparisons test significant; data are presented as percent of Control with mean $\pm$ SEM.

Results

Rats gain weight more slowly during three weeks of chronic restraint stress

Corticosterone (CORT) release habituates over the course of chronic restraint stress paradigms, resulting in similar baseline CORT levels between groups (Watanabe et al., 1992, Magariños and McEwen, 1995, Conrad et al., 1999). For this reason, measurements of weight gain are used as a marker of cumulative stress. Amount of weight gain was determined in three-week blocks over the six-week restraint stress protocol. There were no interactions between sets of animals, therefore weight data was collapsed across animal sets. As expected, two-way repeated measures ANOVA ($N = 22$ animals per group) revealed significant effects of treatment ($F_{(2,63)} = 3.6$; $p = 0.03$) and week number ($F_{(1,63)} = 1,465$; $p < 0.0001$), as well as a significant interaction between the two ($F_{(2,63)} = 110.9$; $p < 0.0001$; data not shown). Bonferroni pairwise comparisons further revealed that Rest and Stress animals gained weight slower than the other groups during their respective three weeks of chronic restraint (weeks 1-3: Control vs. Rest: $t = 5.21$, $p < 0.001$; Stress vs. Rest: $t = 4.9$, $p < 0.01$. weeks 3-6: Control vs. Stress: $t = 4.28$, $p < 0.001$; Rest vs. Stress: $t = 5.43$, $p < 0.001$). At the end of the experiment, body weights were as follows: Control rats = 461.3 ± 15 g, Stress = 403.2 ± 5 g, and Rest = 413.2 ± 21 g.

A post-stress rest period promotes significant IL layer V pyramidal cell proximal dendritic extension but does not reverse stress-induced distal retraction

We investigated whether IL layer V pyramidal neurons retained plasticity following CRS. Similar to Radley et al. (2005), we found a trend toward a CRS effect on total apical dendritic length (one-way ANOVA, $N = 13$, $F_{(2,36)} = 2.64$; $p = 0.08$) (Fig. 2A-C,D). Two-way repeated measures ANOVA on data gathered in the Sholl analysis revealed a trend toward an effect of treatment ($F_{(2,540)} = 2.6$; $p = 0.09$), a highly significant effect of distance from the soma ($F_{(15,540)} = 46.42$; $p < 0.0001$), and an interaction between distance and treatment ($F_{(30,540)} = 2.16$; $p = 0.01$) (Fig. 2E). Although stress-induced alterations to total dendritic length never reached statistical significance, Bonferroni post hoc comparisons on the more sensitive Sholl analysis revealed significant stress- and rest-induced morphological alterations in specific 60 μ m segments: CRS resulted in dendritic retraction at 240 μ m (Control vs. Stress: $t = 2.81$; $p < 0.05$) from the soma, while proximal dendritic extension was seen in the Rest group at 120 μ m (Control vs. Rest: $t = 3.05$; $p < 0.05$; Stress vs. Rest: $t = 3.83$; $p < 0.01$) from the soma, accounting for the significant interaction seen in the two-way repeated measure ANOVA.

Basal dendritic length was not assessed in these neurons because, in order to obtain a fully loaded apical dendritic tree, the soma of loaded neurons was necessarily very deep within the tissue. As such, we did not feel that the total basal dendritic tree could be reliably traced owing to the radial distribution of the basal branches around the soma. Another study focused specifically on basal architecture will be needed to obtain these data.

IL layer V pyramidal neuron spine density remains constant despite behaviorally induced dendritic retraction and extension

LY-loaded neurons were then subjected to spine density analysis as previously described by Radley et al. (2008). Spine density averages per layer per neuron were compared across groups (Fig. 3). Two-way repeated measures ANOVA identified significant differences only between cortical layers ($F_{(2,64)} = 129.3$; $p < 0.0001$) with no significant differences between treatment groups ($F_{(2,64)} = 1.60$; $p = 0.22$) or an interaction between spine densities per treatment group and cortical layer ($F_{(4,64)} = 1.32$; $p = 0.27$) with average spine density of layer I = 2.0 ± 0.7 spines/ μm , layers II/III = 3.8 ± 0.12 spines/ μm , and layer V = 2.6 ± 0.09 spines/ μm (Fig. 4A).

Note that the consistency of layer-specific spine density in the setting of significant dendritic plasticity suggests an active process of spine retraction and growth. These results therefore confirm that stress-induced retraction is accompanied by spine loss and show for the first time that spine growth co-occurs with rest-promoted dendritic extension.

Stress-induced impairment of DAergic LTP modulation is restored following recovery period

To address the impact of CRS and Rest on DAergic modulation of rat PFC activity, fEPSP responses were analyzed as 10-min averages that were compared across treatment groups. Input/output curves performed on each slice yielded no significant differences in experimental stimulation intensity between groups (one-way ANOVA: $F_{(2,44)} = 1.91$, $p = 0.16$, data not shown). In the absence of D1 receptor agonist infusion, LTP was induced and maintained in each group 50 minutes post-tetanzation (Control: $N = 6$, $145 \pm 11\%$ of baseline; Stress: $N = 7$, $177 \pm 35\%$ of baseline; Rest: $N = 4$, $129 \pm 9\%$ of baseline), yet no differences overall were found between groups (two-way repeated measure ANOVA of all ten minute averages: interaction: $F_{(12,84)} = 0.87$, $p = 0.58$; treatment group: $F_{(2,84)} = 0.02$, $p = 0.98$; time: $F_{(6,84)} = 9.51$, $p < 0.0001$) (Fig. 5A,B). On the other hand, infusion of $0.3 \mu\text{M}$ SKF38393, a D1 receptor agonist, dramatically enhanced the neuronal excitability following HFS in Control and Rest but not Stress (two-way repeated measure ANOVA with $N = 4$ (Control), $N = 7$ (Stress) and $N = 3$ (Rest): interaction: $F_{(12,66)} = 3.39$, $p = 0.0007$; treatment group: $F_{(2,66)} = 4.95$, $p = 0.03$; time: $F_{(6,66)} = 11.35$, $p < 0.0001$) with Bonferroni posttests revealing significant differences between Control and Rest vs. Stress at 30, 40, and 50 min post-tetanzation (30 min: Control = $246 \pm 23\%$ baseline, Rest = $232 \pm 41\%$ baseline, Stress = $151 \pm 15\%$ baseline; Control vs. Stress: $t = 2.84$, $p < 0.05$; 40 min: Control = $261 \pm 24\%$ baseline, Rest = $274 \pm 55\%$ baseline, Stress = $147 \pm 18\%$ baseline; Control vs. Stress: $t = 3.38$, $p < 0.001$; Stress vs. Rest: $t = 3.37$, $p < 0.001$; 50 min: Control = $289 \pm 52\%$ baseline, Rest = $294 \pm 64\%$ baseline, Stress = $150 \pm 25\%$ baseline; Control vs. Stress: $t = 4.09$, $p < 0.0001$; Stress vs. Rest: $t = 3.86$, $p < 0.001$) (Fig. 5C,D). Two controls, addition of D1 receptor antagonist SCH23390 ($10 \mu\text{M}$) in combination with SKF38393, as well as a 30 min infusion of SKF38393 in the absence of HFS, inhibited the development of D1R-dependent LTP enhancement (Control at 50 min post-tetanzation: SCH23390 with SKF38393 = $109 \pm 26\%$ baseline, $N = 4$; SKF38393 with no HFS = $134 \pm 17\%$ of baseline, $N = 4$, data not shown). The DA-modulated enhancement of neuronal excitability following HFS seen here potentially stems from the effect of D1 receptors on receptor dynamics, as D1 receptor activation upregulates alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor insertion into the perisynaptic space and HFS induces movement of AMPARs into the synapse (Sun et al., 2005), allowing for increased ion flux during each post-HFS depolarization.

Rest period restores D1R density to pre-stress levels

Given that D1R-mediated synaptic plasticity is compromised by CRS, we investigated whether D1R density is altered by treatment. IL slices from Control, Stress and Rest (Fig. 6A-C, respectively) were subjected D1R ligand binding. Using [^3H]SCH23390 as the D1R ligand, we found significant differences between groups (one-way ANOVA with $N = 8$ for all groups: $F_{(2,23)} = 6.82$; $p = 0.005$) with post hoc Neuman-Keuls tests revealing a $13 \pm 1.8\%$ stress-induced decrease in IL D1R density (Control vs. Stress: $q = 4.80$; $p < 0.01$), which was completely reversed in Rest (Stress vs. Rest: $q = 4.17$; $p < 0.01$) (Fig. 6D). Therefore, stress-induced inhibition of D1R-modulated synaptic plasticity may be partially explained by the stress-induced decrease in D1R availability, which itself may be a consequence of stress-induced apical dendritic retraction of layer V pyramidal neurons (Lambe et al., 2007, Liu and Aghajanian, 2008).

Discussion

We found that the apical dendrites of layer V IL pyramidal neurons undergo behaviorally-induced neuroarchitectural changes in response to both stress and recovery. Specifically, CRS induced significant distal dendritic retraction while Rest promoted proximal dendritic over-extension, yet did not reverse CRS-mediated distal retraction. Moreover, layer-specific spine density remained constant despite dendritic architectural change, implying that total spine number decreased with retraction and increased with extension. We also show that D1R-mediated modulation of synaptic plasticity is significantly impaired by chronic stress, yet fully restored by a post-stress rest period, and that this loss of D1R activity at least partially results from stress-induced downregulation of D1R availability.

Following 21 days of CRS, we found the apical dendrites of IL layer V pyramidal neurons undergo significant retraction within cortical layers II/III $240\ \mu\text{m}$ from the soma and show that spine density remains constant in this setting of dendritic retraction. While these data are the first to describe IL layer V neuronal retraction and spine loss in response to CRS, they differ subtly from previous work done in layers II/III of the anterior cingulate cortex (ACg), PL, and IL regions of the mPFC, which show significant total apical dendritic length decrease as well as spine density decrease in response to CRS. (Cook and Wellman, 2004, Radley et al., 2004, Liston et al., 2006, Radley et al., 2008). Likewise, using Golgi analysis methods, Liu and Aghajanian (2008) recently demonstrated that a milder stressor, 30 minutes of restraint stress per day for seven consecutive days, results in apical dendritic retraction of the most distal regions of layer V pyramidal neurons in ACg and PL. While these results suggest that pyramidal neurons within all regions and layers of the mPFC are morphologically susceptible to CRS, specific differences in retraction pattern and spine loss likely result from a combination of differences in stress exposure, intra-mPFC regional susceptibility, and individual study methodology. Furthermore, there is initial data suggesting that dendritic plasticity of individual neurons differs depending on the projection target of each cell (Shansky et al., 2009). Therefore, isolating specific IL layer V circuits versus random sampling of the layer V IL pyramidal cell population may identify distinct mPFC-projection populations (i.e. mPFC to amygdala projection neurons) that have an increased vulnerability to or enhanced protection from the effects of CRS.

We also present the first evidence that layer V mPFC neurons possess the potential for significant post-stress rest-promoted dendritic remodeling. We demonstrate proximal apical dendritic extension beyond that of Control lengths $120\ \mu\text{m}$ from the soma, within the cortical layer V-III junction. Moreover, an increase in total apical dendritic spine number accompanies this dendritic extension as spine density is maintained at Control levels. Interestingly, we did not find CRS-mediated retraction to be reversed in Rest, as there was not a significant difference between Stress and Rest at $240\ \mu\text{m}$. However, dendritic length in Rest and Control groups did

not differ, either, suggesting not only that the retraction process does not continue during recovery, but some re-extension likely occurred given the lack of difference between Rest and Control. Similar to the subtle regional differences observed with respect to dendritic retraction, the finding of proximal dendritic over-extension appears to be a distinctive characteristic of layer V IL mPFC neurons, as neither the recovered ACg and PL neurons of layers II/III nor hippocampal CA3 neurons undergo this dramatic plasticity event (Conrad et al., 1999, Sousa et al., 2000, Radley et al., 2005).

Although the mechanism of dendritic remodeling has yet to be fully elucidated, hippocampal studies have demonstrated that interruption of glucocorticoid production (Magariños and McEwen, 1995), and inhibition of the glucocorticoid receptor (Liu and Aghajanian, 2008) and *N*-methyl-D-aspartic acid (NMDA) receptors (Magariños and McEwen, 1995) prevent CRS-induced retraction. The mechanism of Rest-promoted dendritic extension has yet to be investigated. Perhaps the withdrawal of the retraction-inducing stimuli in concert with additional presynaptic stimuli encourages dendritic extension. Interestingly, the mPFC layer V-III junction is unique in that it receives dense DA projections from the ventral tegmental area (VTA) (Tzschentke, 2000), and possesses correspondingly high concentrations of D1R located on postsynaptic spines and dendrites of apical branches of pyramidal cells (Vincent et al., 1993, Bergson et al., 1995). While the effects of DA on dendritic morphology in adult animal models are unknown, *in vivo* and *in vitro* experiments have shown embryological neocortical dendritic extension and retraction processes to be influenced by DA activation of D1Rs (Reinoso et al., 1996, Jones et al., 2000, Song et al., 2002). Given that chronic stress is known to dysregulate mPFC catecholaminergic neurotransmission (Di Chiara et al., 1999, Mizoguchi et al., 2000, Murphy et al., 2003), perhaps behavioral CRS- and Rest prime these layer-specific apical branches for neuroarchitectural change. Undoubtedly, future work to elucidate mechanisms of rest-promoted dendritic extension will yield a deeper understanding of stress-related disease processes and the potential for recovery.

In addition to neuronal structure, there is growing evidence of stress-induced impairment to monoaminergic modulation of layer V neuronal function. The mesocortical circuitry as described above is a crucial modulator of both *in vivo* and *in vitro* LTP in rat mPFC (Gurden et al., 2000, Huang et al., 2004, Jay et al., 2004). We show here that CRS inhibits D1R-mediated LTP modulation, occurring in the setting of a stress-induced 13 % decrease of D1R ligand binding and a significant decrease in apical dendritic length. We therefore propose that stress-induced downregulation of D1Rs with concurrent impairment of D1R-modulated IL synaptic plasticity is a consequence of stress-induced apical dendritic remodeling. This hypothesis is supported by data from Liu and Aghajanian (2008). Along with dendritic retraction within layer I, Liu and Aghajanian (2008) found that chronic stress impaired serotonin (5HT)-mediated generation of excitatory responses, yet inhibiting stress-induced remodeling with the glucocorticoid receptor antagonist RU486 successfully prevented the reduced 5HT response, thereby demonstrating that preventing stress-induced dendritic remodeling successfully prevents stress-mediated decreases in monoamine receptor modulation of neuronal activity (Liu and Aghajanian, 2008). Although the low-resolution autoradiographic images prevent the isolation of specific layers in our analyses, we suspect that a targeted investigation of postsynaptic D1Rs within the deeper mPFC layers would reveal a greater CRS-mediated difference between groups. As such, electron microscopic analyses of D1R regulation are required and planned.

A remarkable feature of the *in vitro* D1R modulation of LTP seen here is the fact that it results in enhanced post-HFS potentiation. This enhancement perhaps stems from the effect of D1Rs on receptor dynamics, as D1R activation, via downstream effects of its G_s/G_q protein-coupled receptor mechanism, yields AMPAR phosphorylation (Mangiavacchi and Wolf, 2004), insertion into the perisynaptic membrane (Sun et al., 2005), as well as subsequent movement

into the synaptic space following synaptic depolarization. D1R activation also affects postsynaptic excitability, which is increased in a D1R-dependent manner by adjusting ion flux through membrane channels (Seamans and Yang, 2004). It therefore appears that D1Rs modulate neuronal responses by priming synapses for activity and increasing AMPAR availability; as AMPARs move into the synaptic space following HFS, the larger synaptic ion flux is recorded as enhanced post-HFS potentiation.

We found a surprising lack of difference between Control and Stress LTP in the absence of a D1R agonist, in contrast to *in vivo* findings that show a stress-induced impairment of hippocampal to mPFC LTP in the absence of pharmacological manipulation (Cerqueira et al., 2007). Perhaps HFS at 300 Hz, a frequency chosen to replicate the Huang et al. (2004) study, was a stimulus strong enough to ablate subtle differences between the three. As such, future studies using a lower frequency tetanization may discern *in vitro* LTP maintenance differences. On the other hand, no differences were seen in I/O curves, suggesting that neuronal excitability was equivalent between groups. Moreover, *in vivo* stimulation of hippocampus to mPFC pathways is known to stimulate VTA release of DA in the mPFC, aiding in the maintenance of *in vivo* mPFC LTP (Jay et al., 2004). This raises the intriguing possibility that the stress effect noted in the Cerqueira et al. (2007) study was in fact a reflection of impaired DA modulation of mPFC function. It is therefore possible that the *in vitro* data presented here further our understanding of the *in vivo* findings of Cerqueira et al. (2007) by suggesting that a post-stress reduction of PFC LTP capacity results, at least in part, from decreased D1R availability and subsequent impairment of synaptic plasticity. Taken together, this implies that the impact of spine loss, neuronal morphologic alterations, and dysregulated DA input disrupts PFC neuronal communication and ultimately interferes with overall PFC function. Given that almost 30% of mPFC layer V neurons project to the hypothalamus, and 10% percent to the amygdala (Gabbott et al., 2005), it follows then that layer V mPFC remodeling may contribute to known stress-induced alterations of hypothalamic (Diorio et al., 1993, Sullivan and Gratton, 2002, Radley et al., 2006, Raone et al., 2007) and amygdala regulation (Conrad et al., 1999, Miracle et al., 2006, Wood et al., 2008).

By recording from the mPFC during fear conditioning, extinction, and extinction recall, Milad and Quirk (2002) showed that IL neurons were active only in extinction recall (Milad and Quirk, 2002), suggesting active IL suppression of amygdala output during this behavior. Elements of synaptic plasticity have further been shown to underlie extinction recall, as the generation of *in vivo* mPFC long-term depression (via low frequency stimulation of the mediodorsal (MD) thalamus) abolishes extinction recall capabilities whereas establishment of mPFC LTP (via MD thalamic HFS) enhances extinction recall performance (Herry and Garcia, 2002). As mentioned earlier, long-term synaptic plasticity, as modulated by D1Rs, appears to be an integral element of mPFC-dependent behavior (Mizoguchi et al., 2000, Baldwin et al., 2002). It follows then that animal models of chronic stress frequently display significant impairments in extinction recall (Miracle et al., 2006, Garcia et al., 2008), implying a specific stress-induced deficit in IL-mediated inhibitory control over amygdala output. The data presented herein, demonstrating CRS-induced impairments in D1R-mediated IL layer V pyramidal cell LTP, may indeed be an *in vitro* reflection of this stress-induced behavioral impairment. Clearly presynaptic availability and regulation of DA release are integral elements of neurocommunication in these behavioral experiments. While some chronic stress paradigms have demonstrated decreased mPFC presynaptic DA availability (Mizoguchi et al., 2000), others have shown that DA release is paradoxically increased when chronically stressed animals are exposed to a novel stressor (Di Chiara et al., 1999, Murphy et al., 2003). Although this presynaptic DA dysregulation is not replicated in our current *in vitro* experiments, future experiments with varying concentrations of D1 agonists are planned to clarify the interaction between dysregulated release and altered D1R availability.

Perhaps rest-promoted dendritic extension and spine growth are important factors in regional functional recovery (Castrén, 2005). Indeed, we present the novel finding that mPFC function returns to control levels following a post-stress rest period, which is accompanied by the finding that rest allows for IL layer V dendritic extension, spine growth, and increased D1R availability. Whether this post-stress structural and functional plasticity will translate into restoration of extinction recall capacities has yet to be investigated. Although little is known about the mechanisms of recovery, we believe that these results support the theory that pharmacological or psychotherapeutic manipulations that encourage network recovery and long-term circuitry stabilization may prove particularly advantageous with respect to mood and anxiety disorder treatment (Castrén, 2005).

Furthermore, these results raise the possibility that ‘recovered’ neurons may have different susceptibility to a second round of stressors as the over-extension seen here suggests inherent differences between ‘recovered’ and stress-naïve neurons. Whether these structural differences result in increased susceptibility to, or enhanced protection from, future stressors is unknown. Nevertheless, there is an almost 50% chance of mood disorder relapse following disease remission (Bockting et al., 2008, Kornstein, 2008). We believe that a comprehensive analysis of both rest-promoted dendritic extension as well as the stress susceptibility of extended neurons are critical next steps in understanding the cellular underpinnings of mood disorder relapse and recovery.

In conclusion, with respect to the pathophysiology underlying the precipitation of disease by chronic stress, we have characterized the effects of chronic stress and recovery, both structurally and functionally, on layer V pyramidal neurons of IL mPFC. Investigating important follow-up questions, such as whether glucocorticoid receptor antagonists will inhibit CRS remodeling in the mPFC as they do in the hippocampus, and, in turn, whether inhibiting stress-induced retraction prevents mPFC dysfunction will help elucidate the role of chronic stress in disease. Finally, although little is known regarding the mechanisms of disease recovery, our finding of rest-promoted growth could serve as the foundation for extensive investigations into the relationship between dendritic structural plasticity and functional recovery from disease.

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Abbreviations

ACg, Anterior cingulate cortex
 ACSF, Artificial cerebrospinal fluid
 AMPA, α —amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
 ANOVA, Analysis of variance
 CORT, Glucocorticoid
 CRS, Chronic restraint stress
 D1R, Dopamine (D1) receptor
 DA, Dopamine
 DAPI, 4',6-diamidino-2-phenylindole
 fEPSPs, Field excitatory postsynaptic potentials
 HFS, High frequency stimulation
 IL, Infralimbic cortex
 LTP, Long-term potentiation

LY, Lucifer Yellow
 MD, Mediodorsal nucleus
 mPFC, Medial prefrontal cortex
 N.A., Numerical aperture
 NMDA, *N*-methyl-D-aspartate
 OD, Optical density
 PBS, Phosphate buffered saline
 PFC, Prefrontal cortex
 PL, Prelimbic cortex
 RT, Room temperature
 VTA, Ventral tegmental area

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Figure 1.

Methodology for comprehensive IL layer V neuronal morphology analyses. IL layer V pyramidal cells were iontophoretically injected with LY (A) and dendrites were reconstructed in 3-D using Neurolucida software such that neuronal traces extend both in the x-y (B) and z-y (C) axes. Three high-resolution confocal laser-scanning image stacks of apical dendritic segments were obtained per layer per cell (D, image of dendritic segment within small box in A). The Rayburst algorithm allowed for automated acquisition of spine density (E). Arrows (D,E) highlight spine visualization in confocal imaging and during the process of dendritic reconstruction.

Figure 2.

Layer V IL pyramidal neurons demonstrated CRS-induced apical dendritic retraction as well as rest-promoted proximal dendritic over-extension that is evident in representative confocal images of LY-loaded neurons from Control (A), Stress (B), and Rest (C) groups. The haze of LY surrounding the representative Stress neuron resulted from LY dye leakage from the soma during loading (arrow). One-way ANOVA analysis revealed a trend toward a CRS effect on total apical dendritic length ($p = 0.08$) (D). However, Sholl analyses demonstrated significant CRS-mediated dendritic retraction at 240 μm from the soma as well as dramatic rest-promoted proximal dendritic length increases as compared to both Control and Stress at 120 μm from the soma (E). Control vs. Stress, $p < 0.05$ (*), Stress vs. Rest $p < 0.01$ (++). Error bars indicate SEM. Scale bar, 100 μm .

Figure 3.

Examples of dendritic segments used in layer V spine density analyses as imaged from Control (A, D, G), Stress (B, E, H), and Rest (C, F, I) neurons, organized by layer I (A-C), layers II/III (D-F), and layer V (G-I). While no significant spine density differences were found per group, there were significant spine density differences across layers (layer I: 1.9 spines/ μm , layers II/III: 3.8 spines/ μm , layer V: 2.6 spines/ μm). Scale bar, 5 μm .

Figure 4.

Repeated restraint stress and recovery did not result in significant differences in spine density between groups. However, significant differences were seen within groups between layers with spine density highest within layers II/III and lowest within layer I .

Figure 5.

Effects of CRS and subsequent rest period on *in vitro* mPFC LTP in the absence (A,B) and presence (C,D) of the D1R agonist SKF38393 (0.3 μ M). Slices were stimulated in IL layer II and fEPSPs were recorded from layer V. In the absence of drug infusion, LTP is seen in all groups with no significant differences between groups. Addition of a D1R agonist does not alter LTP induction. However, post-HFS excitation is enhanced in Control and Rest group as compared to Stress. Columns (B,D) represent mean $\pm$ SEM of normalized fEPSP amplitude in consecutive 10-min periods before and after HFS. Arrows, HFS. Horizontal bar, drug infusion. Control vs. Stress $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) ; Stress vs. Rest $p < 0.01$ (++) .

Figure 6.

The IL region was sampled from Control (A), Stress (B), and Rest (C) groups with respect to [³H]SCH23390 ligand binding density, revealing a chronic stress-induced decrease in IL D1R density with full restoration by a post-stress rest period. A magnified image of each sampled IL region (small square) is seen in the lower right hand corner of each box. CRS resulted in a 13 % reduction in IL D1R ligand binding density (D), which was entirely reversed in Rest. Control vs. Stress $p < 0.01$ (), Stress vs. Rest $p < 0.01$ (++). Error bars represent SEM.