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Repeated Stress Induces Dendritic Spine Loss in the Rat Medial Prefrontal Cortex

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The prefrontal cortex (PFC) plays an important role in higher cognitive processes, and in the regulation of stress-induced hypothalamic–pituitary–adrenal (HPA) activity. Here we examined the effect of repeated restraint stress on dendritic spine number in the medial PFC. Rats were perfused after receiving 21 days of daily restraint stress, and intracellular iontophoretic injections of Lucifer Yellow were carried out in layer II/III pyramidal neurons in the anterior cingulate and prelimbic cortices. We found that stress results in a significant (16%) decrease in apical dendritic spine density in medial PFC pyramidal neurons, and confirmed a previous observation that total apical dendritic length is reduced by 20% in the same neurons. We estimate that nearly one-third of all axospinous synapses on apical dendrites of pyramidal neurons in medial PFC are lost following repeated stress. A decrease in medial PFC dendritic spines may not only be indicative of a decrease in the total population of axospinous synapses, but may impair these neurons' capacity for biochemical compartmentalization and plasticity in which dendritic spines play a major role. Dendritic atrophy and spine loss may be important cellular features of stress-related psychiatric disorders where the PFC is functionally impaired.

Keywords: axospinous synapse, cell loading, dendritic spine, post-traumatic stress disorder, prefrontal, stress

Introduction

There is a clinically well established relationship between stressful life events and mental illnesses (Sapolsky, 1996; Heim *et al.*, 1997). Following exposure to a stressor, hypothalamic–pituitary–adrenal (HPA) activity affects brain function to produce adaptive responses for the organism (McEwen, 1998). However, in instances where behavioral stress is extreme or persists over time, brain function may be altered in a maladaptive manner. Whereas such research has focused on the hippocampus both as a regulator of the response to stress and as a target of its effects (Jacobson and Sapolsky, 1991; McEwen, 2001), recent evidence suggests that the PFC also plays a role in these contexts.

The medial PFC plays an important role in the integration of cognitive and emotionally relevant information, and has been implicated in the modulation of attention through functional imaging studies in humans (Bush *et al.*, 1998, 2000; MacDonald *et al.*, 2000; Kerns *et al.*, 2004). The medial PFC also contains high levels of glucocorticoid receptors (Ahima and Harlan, 1991; Sanchez *et al.*, 2000), and regulates HPA activity under behaviorally stressful conditions (Diorio *et al.*, 1993). Collectively, these functions underscore the role for the medial PFC in the evaluation of contextually relevant stimuli in shaping responses to salient environmental events during stressful situations. Clinical evidence suggests that medial PFC dysfunction is associated with post-traumatic stress disorder (PTSD; Rauch *et al.*, 2003) and depression (Drevets *et al.*, 1997). Moreover, medial PFC lesions result in the disinhibition of some affective behaviors and attentional impairments in rodent and primate models (Morgan and LeDoux, 1995; Dias *et al.*, 1996; Birrell and Brown, 2000). Despite this recent convergence of evidence concerning the medial PFC and stress-related mental illnesses, little is known of its cellular morphologic changes.

In a previous report, rats subjected to repeated restraint stress revealed a 20% decrease in total apical dendritic length of layer II/III pyramidal neurons of the medial PFC (Radley *et al.*, 2004). In the present study we investigated the effect of stress on dendritic spine density in the medial PFC, and correlated changes in spine density with dendritic length to estimate the overall change in axospinous synaptic input into this region. To this end, we performed intracellular iontophoretic injections of Lucifer Yellow in layer II/III pyramidal neurons in the medial PFC following 21 days of repeated restraint stress, and estimated spine densities on deconvolved image stacks, acquired on a confocal laser scanning microscope, of systematic–randomly chosen dendritic segments at progressive radial distances from the neuronal soma.

Materials and Methods

Male Sprague–Dawley rats (Charles River, Wilmington, MA), weighing 250–280 g at the onset of the experiment, were housed in groups of 2–3 per cage. Animals had unlimited access to food and water except during restraint sessions. Control rats (n = 8) were housed in a separate room from stressed rats (n = 8), and were maintained on a 12 h light/dark schedule (lights on from 07:00 to 19:00 h). All rats were handled for 7 days prior to the beginning of restraint. Rats were restrained for 6 h daily (10:00–16:00 h) for 21 days with wire mesh restrainers and then returned to their home cages throughout the restraining period. To ensure that the analysis was done blind, each animal was coded by an independent observer prior to the perfusion, and the code was not broken until the analysis was completed. On day 22, rats were given a euthanizing dose of 30% chloral hydrate and transcardially perfused with cold 1% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4), followed by fixation with cold 4% paraformaldehyde with 0.125% glutaraldehyde in PBS. Brains were dissected and postfixed for 2 h in the same fixative. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Rockefeller University and Mount Sinai School of Medicine Institutional Animal Care and Use Committees.

Coronal sections (250 μm thick) were mounted on nitrocellulose filter paper and immersed in PBS. The lamination patterns of the medial PFC subregions anterior cingulate cortex and prelimbic area (ACC and PL, respectively) were identified by briefly exposing sections to a fluorescent nucleic acid stain (4,6-diamidino-2-phenylindole; Sigma, St Louis, MO). Neurons in layer II/III were loaded with intracellular injections of 5% Lucifer Yellow (Molecular Probes, Eugene, OR) under a DC current of 1–6 nA for 10 min. Sections were coverslipped with

PermaFluor and reconstructed in 3-D at ×400 using a Zeiss Axiophot 2 microscope and Neurolucida software (MicroBrightField, Williston, VT). Entry into the analysis required that neurons: (i) lie within layer II/III and within the boundary of ACC and PL; (ii) exhibit complete filling of the dendritic tree, evidenced by well-defined endings; and (iii) display intact primary, secondary and tertiary branches. The boundaries for ACC and PL were carefully delineated in order to avoid inadvertently including filled neurons that were dorsal and ventral to the regions of interest. ACC was differentiated from the more dorsolateral frontal area 2 (Fr2) by its more tightly laminated and dense layer II/III and a less laminated and thin layer V (see Krettek and Price, 1977; Paxinos and Watson, 1986). Since this distinction was not always evident to the experimenter during cell filling, neurons near the dorsal boundary of ACC and Fr2 were often excluded from the analysis. At the ventral aspect, the boundary of the PL and infralimbic (IL) cortices was more readily distinguished by the transition from a well-defined layer II/III in PL to a lack of any such clear lamination pattern exhibited in IL. Neurons that ended up in the analysis typically had perikarya that were located 10–60 μm below the surface of the section, and had an apical dendritic field that projected parallel, or ventral to, the top of the section.

The method for sampling dendritic branches for spine density (i.e., spines per μm dendritic length) was designed to minimize any possible bias. The selection of a particular branch for optical imaging had to satisfy the following criteria: (i) the entire segment had to fall within a depth of 50 μm, owing to the vertical limit of the laser scanning microscope's imaging capacity; (ii) they had to be either parallel or at acute angles to the coronal surface of the section; and (iii) they did not show overlap with other branches that would obscure visualization of spines. For apical dendrites, segments were selected with a systematic random design at 50, 100, 150 and 200 μm from the soma for digital reconstruction. A fifth category of segments of large diameter (corresponding to diameters of >3 μm) were sampled at radial distances of 100 and 150 μm, since initial observations revealed that these main dendrites had a substantially higher number of spines per unit length than each of the other branch types analyzed. In some instances, sites were sampled redundantly. For basal dendrites, segments were randomly selected at 50 and 100 μm for digital reconstruction. Samples were collected at 150 μm wherever possible; however, there was not enough material to justify any statistical comparison. In total, ~500 reconstructed dendritic segments (320 apical, 180 basal) were analyzed (9 segments/neuron, 5 neurons/animal, $n = 8$/group).

Dendritic segment and spine reconstructions were performed using a Zeiss 410 confocal laser scanning microscope using a 488 nm excitation wavelength, at a magnification of ×1000 and a zoom of ×5. After gain and offset settings were optimized, segments were digitally reconstructed at 0.1 μm increments, throughout the entire z-axis of the branch. The digitized optical stacks were then deconvolved with AutoDeblur (AutoQuant, Troy, NY) and analyzed using Neurolucida software. The analysis for spine number and length was carried out by controlling manually the plane of focus for z-step increments and marking spines as they appeared. After the total spine numbers for each branch were recorded, the length of the branch was traced, and dendritic length and spine numbers were obtained using NeuroExplorer software (MicroBrightField). Values for each branch segment were expressed as spine number/μm. Under the present analysis, the average dendritic segment was ~30 μm in length.

Dendritic arbor measurements have been previously described in greater detail (Radley *et al.*, 2004) and were expressed here only in terms of total dendritic length. Analyses for basal dendrites were done for each individual dendrite, instead of summing them together for analysis on a cell-by-cell basis, due to truncation of at least 1–2 primary branches that occurred with nearly every neuron examined. For spine densities, individual site and total averages were obtained for each neuron, and then by averaging the number of neurons within each animal. Group averages were obtained from cell averages (~9 segments / neuron, $n = 5$ neurons/animal) within each animal. Statistical testing was performed using a two-tailed t test (significance level $\alpha = 0.05$) wherever appropriate. The grouped data for radial distances were compared using repeated ANOVA with post-hoc, pairwise comparisons (Bonferroni). Values were represented as the mean ± SEM.

Results

After 21 days of repeated restraint stress, rats from both groups were indistinguishable from each other; they appeared well-groomed and healthy. Given the numerous reports that have demonstrated that this repeated restraint stress model produces significant increases in plasma corticosterone and modest increases in adrenal weights compared with unstressed rats (Watanabe *et al.*, 1992; Magariños and McEwen, 1995), these assays were not performed in the present study. However, as also previously demonstrated (Watanabe *et al.*, 1992) stressed rats (360 ± 9 g) weighed ~15% less than the controls (412 ± 7 g) at the end of 21 days of restraint ($P = 0.004$). This difference was characterized by a slower weight gain during the first 7 days of restraint compared to control animals, followed by an equivalent rate of gain over the remaining duration of the 3 week restraining period.

Since one of the distinguishing cytoarchitectural features of the ACC and PL portions of the medial PFC is a well-defined layer II/III, pyramidal neurons that were loaded in this region were readily identified from neurons inadvertently loaded in Fr2 or IL (see plate 9 in Paxinos and Watson, 1986). A few loaded neurons in Fr2 tended to have a more 'classical' pyramidal neuron shape with a long-shaft apical dendrite extending parallel to the coronal plane (these cells were not included in the analysis), whereas apical dendrites of ACC and PL neurons exhibited greater morphological diversity (Figs 1 and 2). The longest shaft apical dendrite observed was 100 μm; the shortest was ~5 μm, and the rest varied continuously within this range. Medial PFC apical dendrites tended to project at angles that were not necessarily parallel to the coronal plane or orthogonal to the pial surface (Figs 1 and 2). This was observed in several neurons examined within single animals, ruling out the possibility that subtle variations in sectioning through the coronal plane could account for such differences. Nonetheless, apical dendrites in all neurons examined extended through layer I, and the total dendritic lengths and branch numbers were observed to be comparable within each animal. The variability of total branch number and length for all of the neurons examined was between 5 and 10% (see below), regardless of whether they were long- or short-shaft primary apical dendrites. Therefore, it seemed appropriate to perform one analysis for all pyramidal neurons according to apical dendritic morphology.

Similar to recent findings from our group (Radley *et al.*, 2004), we found a 20% decrease in overall apical dendritic length (Stress, 1839 ± 91 μm; Control, 2325 ± 131 μm; $P = 0.025$) following 21 days of restraint stress. Since these effects were uniformly present in both the ACC and PL, no further attempts were made to consider them as separate entities in the quantitative analysis. No effect was observed in basal dendrites (Stress, 468 ± 39 μm; Control, 499 ± 52 μm; $P = 0.6$).

Dendritic spines were sparse at the proximal aspect of the trunk of primary apical and basal dendritic arbors, becoming more evident within 20–70 μm of the soma. Within 20–70 μm, spines became more evident, and this varied according to the dendritic morphology, over the continuum of short- to long-shaft apical dendrites. For this reason, dendritic segments that were sampled at radial distances of 50 μm were small diameter second or third order branches, which bifurcated from the primary trunk and coursed back toward the vicinity of the soma. By 100 μm from the soma, spine densities were maximally elevated on large diameter dendritic segments, and seemed to

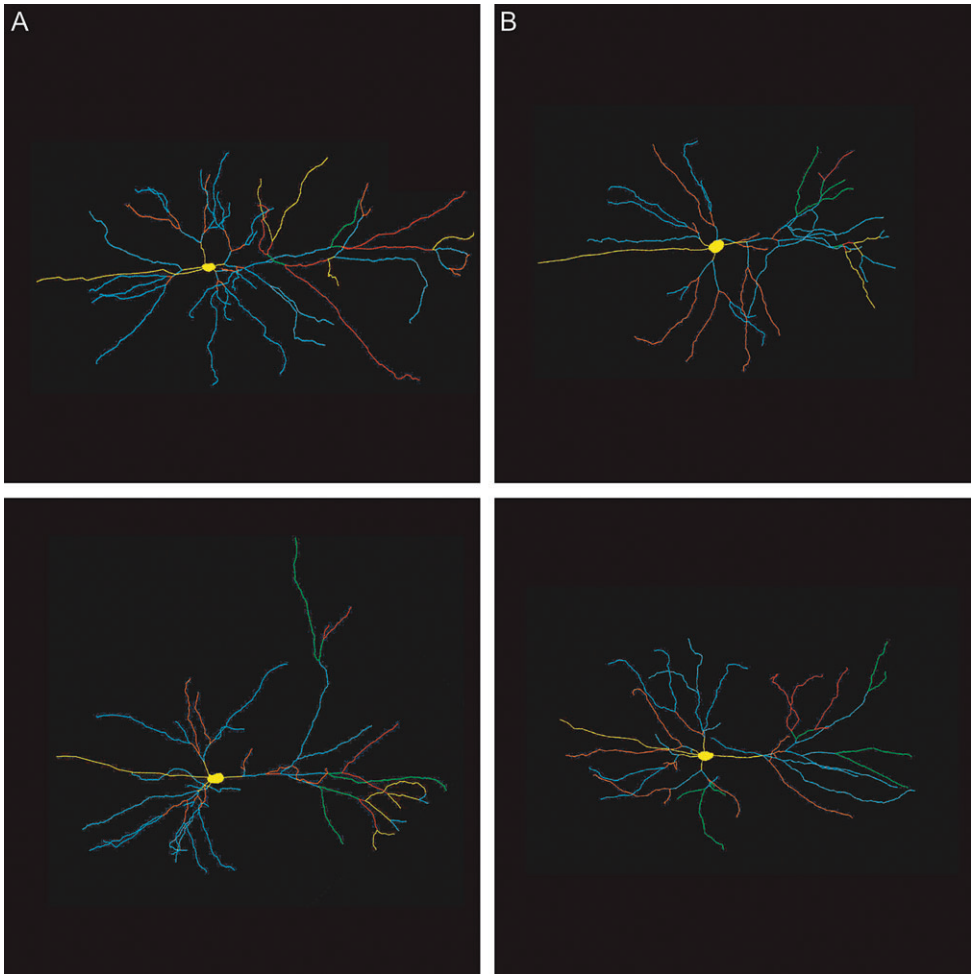

Figure 1. 3-D reconstructed neurons in rat medial PFC. Representative layer II/III pyramidal neurons from the medial PFC of control (*A*) and restraint stress animals (*B*). Apical dendrites extend to the right side of the diagram, toward the pial surface (not shown).

decrease at larger radial distances from the soma. No correlation was observed between spine density and ascending branch order (data not shown).

Most striking was the overall high density of spines (Figs 2 and 3), averaging 3.0–3.8 spines/μm on both apical and basal dendrites. On large diameter primary dendrites that were sampled, spine densities were upwards of 5 spines/μm. In control animals, the overall spine density was significantly less in basal (3.0 ± 0.1 spines/μm) compared to apical (3.7 ± 0.2 spines/μm) dendrites ($P = 0.021$). Spine density on apical dendritic arbors seemed to vary relative to the distance of the dendritic arbor from the soma, and not in terms of ascending branch order. This observation is consistent with previous reports suggesting that spine density in apical dendrites of neocortical pyramidal neurons peaks around 70–100 μm from the soma, and decreases thereafter (Peters and Jones, 1984).

Twenty-one days of repeated daily restraint stress produced an overall (16%) decrease in the apical dendritic spine density [Stress, 3.1 ± 0.1 spines/μm; Control, 3.7 ± 0.2 spines/μm; $F(1,12) = 6.0$, $P = 0.03$; Fig. 4*A*], an overall effect for radial micrometer distance from soma [$F(4,48) = 23.1$, $P < 0.0001$], and no interaction between density and distance [$F(4,48) = 1.6$, $P = 0.18$]. When separate analyses were carried out for each site (Fig. 4*B*), chronic stress produced a significant decrease in spine density at 200 μm from somata (Stress, 2.6 ± 0.1 spines/μm; Control, 3.0 ± 0.1 spines/μm; $P < 0.001$) and on large diameter dendrites (Stress, 3.9 ± 0.3 spines/μm; Control, 4.8 ± 0.2 spines/μm; $P = 0.046$). Moreover, large diameter dendrites were significantly more spiny ($P < 0.01$) than segments at other radial micrometer distances from soma. No regional differences were observed in spine densities between the ACC and PL pyramidal neurons. Basal dendritic spine density was unaffected following repeated restraint stress (Fig. 4*C*,*D*).

Discussion

The main finding of this study is a significant overall reduction in apical dendritic spine density in pyramidal neurons of the medial PFC after exposure to repeated restraint stress. This effect was most pronounced at distances of 200 μm from somata and on large diameter dendrites. We also confirmed that chronic stress produces a 20% decrease in total apical dendritic length and branch number. When these two observations are taken together, we estimate that repeated stress produces a 33% reduction in the total number of axospinous synapses on apical dendrites of pyramidal neurons in the medial PFC. Finally, we did not observe any change in basal dendritic length or spine density, suggesting that the effect of repeated stress in the medial PFC is highly selective.

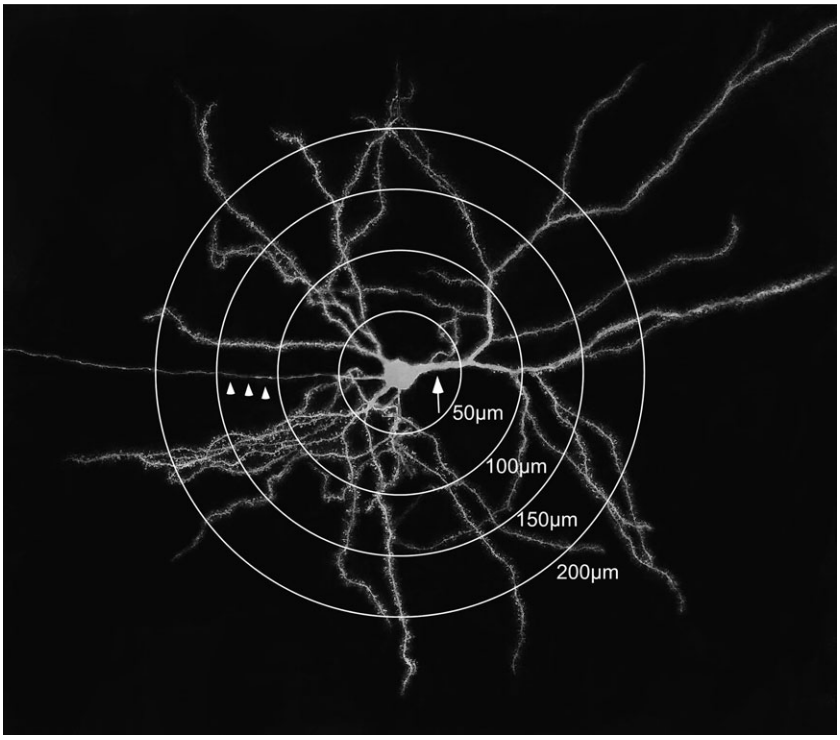

Figure 2. Digital reconstruction of Lucifer Yellow-filled neurons in medial PFC. This layer II/III pyramidal neuron was loaded on the ACC/PL border from a control rat, and imaged on a Zeiss 410 confocal laser-scanning microscope at × 160. In order to depict the entire neuron in one field, the image was merged from five separate confocal digital stacks (*z*-step = 0.1μm) that were deconvolved using AutoQuant and aligned in the *x–y–z* planes using the VIAS software (Rodriguez *et al.*, 2003). Concentric circles were drawn to select dendritic segments at radial increments of 50 μm relative to the soma for the spine density analysis. The apical dendrite is indicated by an arrow. The arrowheads point to the axon.

One issue surrounding the cell loading technique in lightly fixed tissue is whether dendrites and spines may fail to fill in stressed animals due to technical reasons. There is a convergence of evidence utilizing multiple techniques that chronic stress induces morphological and functional changes in corticolimbic structures (Watanabe *et al.*, 1992; Vyas *et al.*, 2002, 2003; McEwen and Chattarji, 2004; Radley *et al.*, 2004), and one other group has shown using a different technique that repeated restraint stress results in apical dendritic retraction of medial PFC neurons (Cook and Wellman, 2004). In our data collection, we did not encounter any problems in the cell filling procedure that suggested more difficulty filling neurons from stressed animals, and observed clear endings of dendrites that were characterized by their tapering to a well-defined tip. Finally, Lucifer Yellow is a dye of low molecular weight, making it a suitable choice for the filling of spines and dendrites for confocal microscopic analysis.

The overall high spine density observed in the medial PFC in both control and stressed animals provides further evidence that cell loading is appropriate for the examination of dendritic morphology and spine density. Our estimates of 3.0–3.8 spines/μm on medial PFC pyramidal neurons is in the range of axospinous synapse density derived from 3-D reconstructions in electron microscopic studies in other cortical brain regions (Harris *et al.*, 1992; Megías *et al.*, 2001), and substantially higher than spine density estimates in the medial PFC using the Golgi technique (Seib and Wellman, 2003; Silva-Gomez *et al.*, 2003). The disparity in our results from the latter two studies is attributable to differences in methodological and analytic approaches for estimating spine densities. Our analyses of spine density were performed on high resolution 3-D deconvolved datasets and thus included all spines rather than only those orthogonal and lateral to the dendritic shaft, and involved the systematic sampling of nine sites per neuron, computing densities from segments that were ~30 μm in length. Finally, high spine densities in medial PFC have been reported elsewhere, and may represent a specialized anatomical feature of these neurons. In the rhesus monkey, the PFC has a spine density comparable to our observations in the rodent, at ~3 spines/μm, and PFC spine densities are also much higher than in other neocortical regions (Elston *et al.*, 2001).

One of the interesting aspects of the pyramidal cell population examined was that their quantitative features seemed to be conserved regardless of their morphologic diversity. For example, the random inclusion of 40 different neurons per experimental group, comprising long- and short-shaft apical dendrites, resulted in small overall variations in total apical dendritic lengths and branch endings (see Radley *et al.*, 2004). One possible explanation for these similarities is that the location of all of the neurons examined was between 250–300 μm from the pial surface, in the more superficial aspect of layer II/III. Since medial PFC pyramidal neurons in layers II/III and V extend to layer I, layer V and deep layer II/III neurons might be expected to have larger dendritic arbors than neurons located more superficially in layer II. The distance of a pyramidal neuron from the pial surface may be a more important predictor of its quantitative features, at least for apical dendrites, such as total branch number and length. Another interesting aspect of this dendritic analysis is that the total apical dendritic lengths were nearly double what has

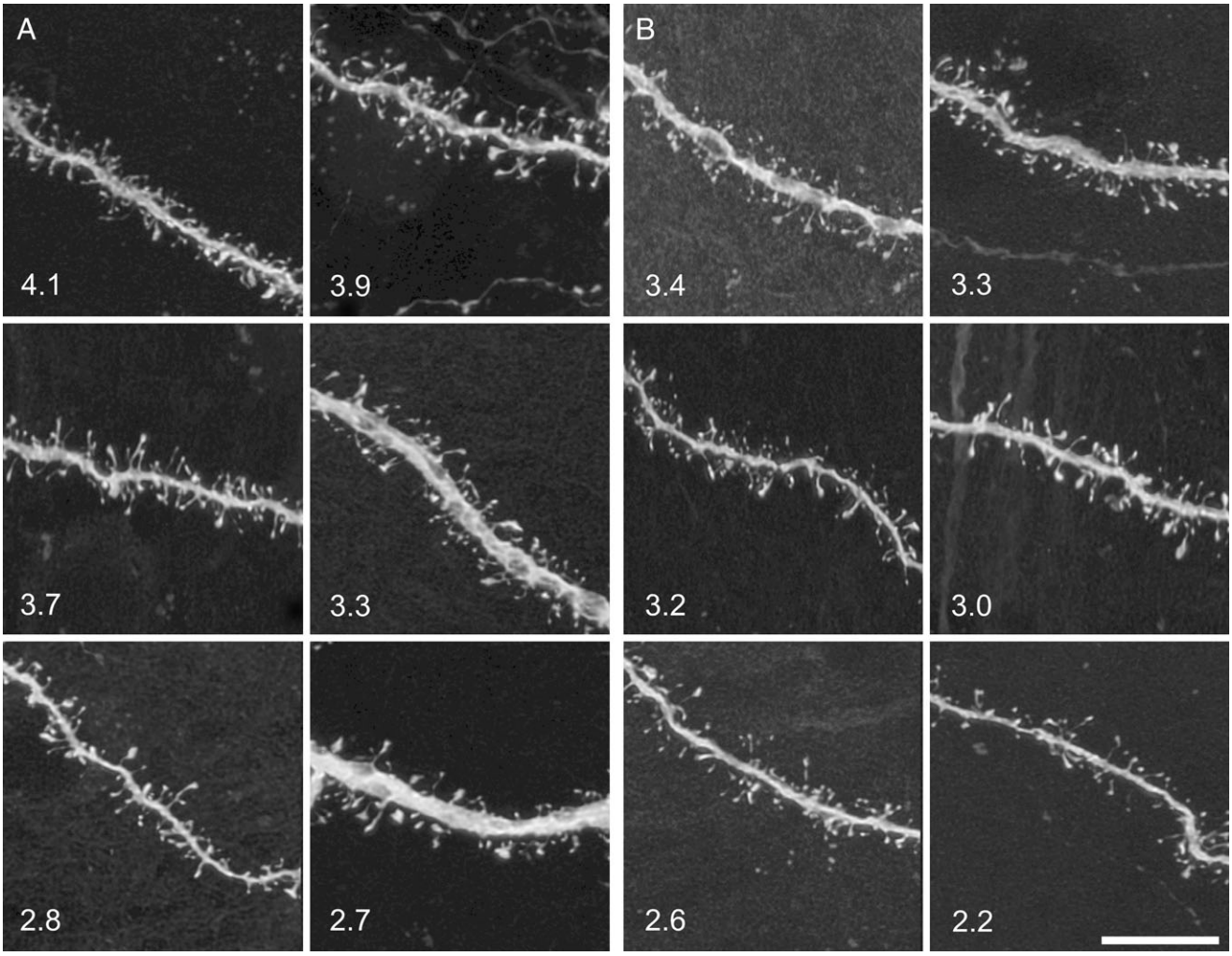

Figure 3. Digital reconstructions of dendritic segments. Examples of randomly selected dendritic segments are shown in control (*A*) and stressed (*B*) rats. Numbers shown for each segment represent spines/μm for each branch analyzed. Scale bar = 10 μm.

been previously reported (Radley *et al.*, 2004). This difference results from adopting a more stringent set of criteria for the inclusion of apical dendritic arbors in the present analysis. Whereas, in the previous study, apical dendritic arbors were included that occasionally had truncated tertiary branches, arbors were only included in this analysis if they had complete secondary and tertiary branches.

Another aspect of these results is whether stress-induced changes in dendritic spine density correlate with a net change in overall excitatory synapses. Because 95% of all cortical excitatory synapses are made onto pyramidal neurons, with each spine head receiving one excitatory terminal (Spacek and Hartmann, 1983; Harris and Stevens, 1989; Peters et al., 1991), changes in spine number may therefore represent an index of total excitatory input. One alternative is that although repeated stress might decrease the number of axospinous synapses, it may be compensated for by an increase in excitatory dendritic shaft synapses. While we cannot rule out this possibility, it is noteworthy that electron microscopy studies have shown that repeated restraint and repeated variable stress both result in a decrease in the density of excitatory synapses in hippocampal pyramidal neurons (Sousa et al., 2000; Sandi et al., 2003). The similarity in effects of stress on hippocampus and medial PFC supports the interpretation that excitatory synapse loss, through the retraction of apical dendrites and decrease in spine number, may be a compensatory reaction to elevated excitatory amino acid levels in the medial PFC. In this regard, it has been demonstrated that repeated stress is associated with a sustained increase in glutamate neurotransmission in the medial PFC (Moghaddam, 2002). Moreover, dendritic spine loss has been demonstrated to result from excitatoxic injury (Jiang et al., 1998).

Dendritic spines play a role in the sequestration of Ca^{2+}, because the spine neck prevents its exchange between the spine head and dendrite (Nimchinsky *et al.*, 2002). This barrier to Ca^{2+} exchange, along with the compartmentalization of molecules associated with postsynaptic density and spine apparatus, is important for the regulation of synaptic transmission. Ca^{2+} sequestration by spines also may be neuroprotective, preventing excitotoxicity to the dendrite and neuron by restricting excessive influxes of Ca^{2+} within the synaptic region (Segal, 1995). In the present context, repeated stress may result in morphologic changes in dendritic spines in the medial PFC that would render them more resistant to excitotoxity from excessive Ca^{2+} influx and a reduction in spine number

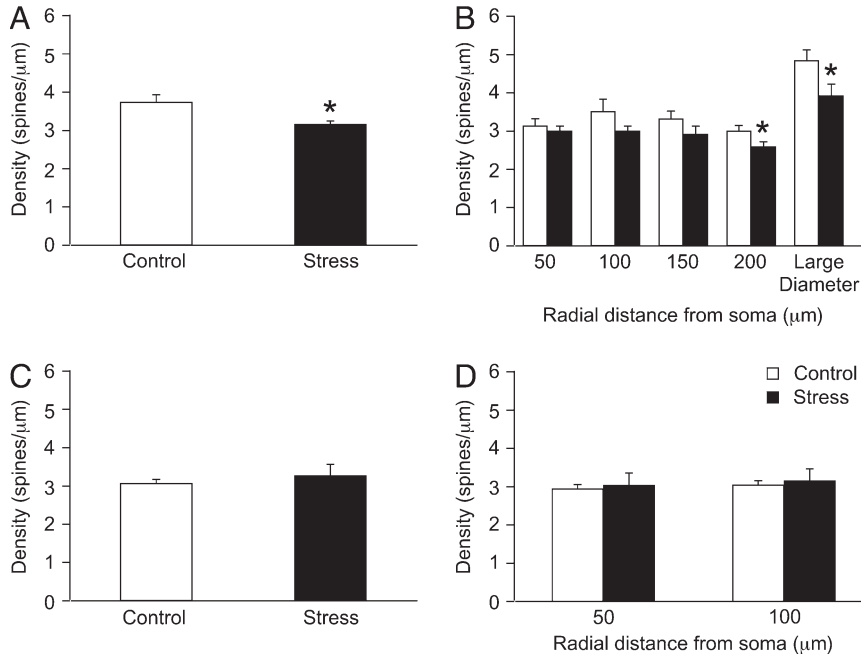

Figure 4. Spine density changes in medial PFC following repeated stress. Spine density histograms for apical (*A*, *B*) and basal (*C*, *D*) dendrites. Twenty-one days of repeated restraint stress resulted in a significant decrease in overall spine density on apical (*A*), but not on basal (*C*) dendrites (*, $P < 0.05$). In (*B*), dendritic spine density averages are depicted for each individual site sampled on apical dendritic arbors, reveal decreases at radial distance 200 μm and on large diameter dendrites. (*D*) Averages for individual sites (50 and 100 μm) sampled on basal dendritic arbors.

would also help to ameliorate excitotoxic effects from prolonged glutamate release. In this regard, it is worth noting that changes in dendritic spine morphology and number are associated with a number of neurological disorders (Fiala *et al.*, 2002; Nimchinsky *et al.*, 2002)

A decrease in axospinous synaptic input to the medial PFC by 33% may have a significant impact on the functional properties of this region. A magnitude of this effect is comparable to age-related alterations in spine number of the PFC (Duan *et al.*, 2003), and estrogen depletion (Tang *et al.*, 2004). However, it should be noted that the stress-induced decrease in dendritic spine number may not necessarily result in a permanent or irreversible loss of synapses. For example, dendritic atrophy that occurs in hippocampal neurons following repeated stress is reversible (Conrad *et al.*, 1999), and dendritic spine density in CA1 fluctuates across the estrous cycle (Woolley *et al.*, 1990).

One potential neuroantomical substrate relevant to PTSD is the medial PFC-amygdala circuit (Newport and Nemeroff, 2000). Ultrastructural evidence reveals that the basolateral amygdala makes synapses onto dendritic spines of layer II/III pyramidal neurons in the medial PFC (Bacon et al., 1996). Accordingly, the medial PFC may inhibit amygdala output through its connections onto GABAergic intercalated cells at the border of the lateral and central nucleus (McDonald et al., 1996; Quirk et al., 2003). Experimental lesions of the PFC result in enhancement of amygdala-dependent behaviors, such as emotionality and fear conditioning (Morgan and LeDoux, 1995; Dias et al., 1996). That repeated stress also results in enhanced fear conditioning (Conrad et al., 1999) suggests that stress' effects on the medial PFC may a key contributor to the disinhibition of information flow through the amygdala. Functional neuroimaging studies reveal augmented amygdala responses and diminished medial prefrontal cortex responses during the symptomatic state in PTSD (Shin et al., 2004; 2005), and also that PTSD patients show a reduction in medial PFC volume (Rauch et al., 2003; Yamasue et al., 2003). The possibility that these stress-induced alterations in brain plasticity may be ameliorated may have significant consequences for the treatment of stress-related mental illness. Future studies are needed in order to investigate the extent to which these morphologic changes are reversible, the relationship between these synaptic changes and their interconnections with the extended amygdala, and their relationship to amygdala-dependent forms of emotional learning.

Notes

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