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RAPID REPORT

CHRONIC BEHAVIORAL STRESS INDUCES APICAL DENDRITIC REORGANIZATION IN PYRAMIDAL NEURONS OF THE MEDIAL PREFRONTAL CORTEX

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Abstract—Both the hippocampus and the medial prefrontal cortex (mPFC) play an important role in the negative feedback regulation of hypothalamic–pituitary–adrenal (HPA) activity during physiologic and behavioral stress. Moreover, chronic behavioral stress is known to affect the morphology of CA3c pyramidal neurons in the rat, by reducing total branch number and length of apical dendrites. In the present study, we investigated the effects of behavioral stress on the mPFC, using the repeated restraint stress paradigm. Animals were perfused after 21 days of daily restraint, and intracellular iontophoretic injections of Lucifer Yellow were carried out in pyramidal neurons of layer II/III of the anterior cingulate cortex and prelimbic area. Cellular reconstructions were performed on apical and basal dendrites of pyramidal neurons in layer II/III of the anterior cingulate and prelimbic cortices. We observed a significant reduction on the total length (20%) and branch numbers (17%) of apical dendrites, and no significant reduction in basal dendrites. These cellular changes may impair the capacity of the mPFC to suppress the response of the HPA axis to stress, and offer an experimental model of stress-induced neocortical reorganization that may provide a structural basis for the cognitive impairments observed in post-traumatic stress disorder.

Key words: stress, prefrontal, glucocorticoid, dendrite, cell loading, post-traumatic stress disorder.

Behavioral experiences can impact the structure and circuitry of the brain, altering subsequent responses to a variety of situations. One classic example is the effect of stress-induced hypothalamic–pituitary–adrenal (HPA) activity on the brain, and the subsequent alterations in the brain that feedback on behavioral output to produce adaptive responses for the organism (McEwen, 1998). The medial prefrontal cortex (mPFC) is a regulator of HPA activity under stressful conditions (Meaney et al., 1985; Diorio et al., 1993; Brake et al., 2000), and dysfunction of this structure is associated with post-traumatic stress disorder (PTSD) in humans (Shin et al., 2001; Pissiota et al., 2003; Rauch et al., 2003; Yamasue et al., 2003). We chose to investigate the potential cellular basis of these effects in an animal model of behavioral stress. We found that 21-day repeated restraint stress resulted in a decrease in total length and branch number of apical dendrites of pyramidal neurons of layer II/III of the mPFC. We suggest that these cellular changes may impair the capacity of the mPFC to suppress the response of the HPA axis to stress, and offer an experimental model of stress-induced neocortical reorganization that may provide a structural basis for the cognitive impairments observed in PTSD. Some of these results have been reported in abstract form (Radley et al., 2003).

EXPERIMENTAL PROCEDURES

All procedures were conducted in accordance with the Rockefeller University and Mount Sinai School of Medicine Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (Charles River, Wilmington, MA, USA; 250–280 g) received restraint stress (n=6) for 6 h daily (10:00–16:00 h) with wire mesh restrainers and then returned to their home cages. Another group of unstressed controls (n=5) received no treatment. Restraint stress was performed daily for 21 days. 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. After 21 days, animals were deeply anesthetized 24 h after the restraint period, and perfused transcardially 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. Serial 250 μm-thick coronal sections were collected using a Vibratome.

The method for intracellular loading has been previously described (Duan et al., 2002). Sections were mounted on a nitrocellulose filter paper and immersed in PBS. Layer II/III pyramidal cell nuclei of the mPFC subregions of the anterior cingulate cortex (ACC) and prelimbic area (PL) were identified by briefly treating sections in a fluorescent nucleic acid stain (4,6-diamidino-2-phenylindole; Sigma, St. Louis, MO, USA). Cell loading was carried out in coronal sections that were

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Abbreviations: ACC, anterior cingulate cortex; HPA, hypothalamic–pituitary–adrenal; LY, Lucifer Yellow; mPFC, medial prefrontal cortex; PBS, phosphate-buffered saline; PL, prelimbic area; PTSD, post-traumatic stress disorder.

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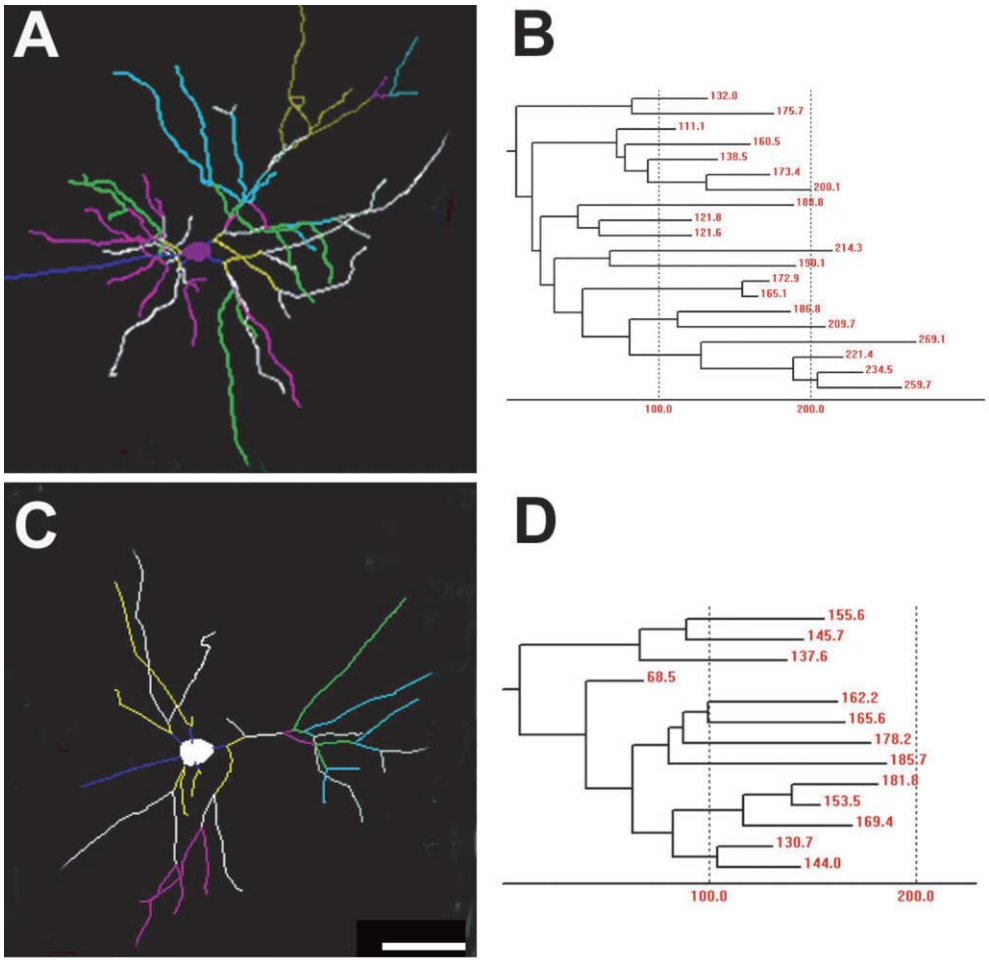

Fig. 1. Representative examples of 3-D reconstructed neurons from layer II/III of ACC and PL (A, C). The apical dendrites in each of these diagrams spread out toward the right, and axons extend (indigo-colored) at the opposite pole of the cell soma. Dendrograms (B, D) delineate the general branching patterns of the apical dendritic arbors. Note that the cell body would appear to the left of the dendrites. Scale bar=50 μm in A and C.

approximately 1.5–3.5 mm rostral to bregma. Neurons were loaded with intracellular iontophoretic injections of 5% Lucifer Yellow (LY; Molecular Probes, Eugene, OR, USA) under a DC current of 1–6 nA for 5–10 min, or until the dye had completely filled distal processes and no further loading was observed. Sections were coverslipped under PermaFluor and reconstructed in 3-D at 400× using a Zeiss Axiophot 2 microscope equipped with a motorized stage, video camera system, and Neurolucida morphometry software (MicroBrightField, Williston, VT, USA). In order for a loaded neuron to be included in the analysis, it had to satisfy the following criteria: (1) within layer II/III and within the boundary of the ACC or PL; (2) complete filling of dendritic tree, as evidenced by well-defined endings; (3) intact primary and secondary branches; (4) intact tertiary branches (except for tertiaries that extend beyond a 50 μm radial distance from its branch point). Dendrograms and 3-D Sholl analyses were prepared for each neuron with NeuroExplorer software (MicroBrightField). Analyses for basal dendrites were done for each individual dendrite, instead of summing them together for analysis on a cell-by-cell basis. An average of one to two basal dendrites per loaded cell was included in the basal dendritic analysis. Results were expressed in terms of total dendritic length, total branch number, and number of intersections and dendritic material per radial distance from the soma, in 30 μm increments (Sholl, 1953). Statistical testing was performed using a Student's *t*-test, and values were represented as the mean±S.E.M. Differences were considered statistically significant at $P<0.05$.

RESULTS

Intracellular injections of LY into layer II/III pyramidal neurons in the ACC and PL cortices revealed the complete filling of apical and basal dendrites with dendritic spines throughout the entire extent of the dendritic tree (Fig. 1). LY injections were performed at a depth of 10–50 μm below the dorsal surface of the sections. Since brains were postfixed for only 2 h, there was minimal tissue shrinkage relative to standard histological and immunocytochemical procedures. All apical dendritic arbors were analyzed regardless of their orientation relative to the coronal sections. Cells were loaded by scanning through the extent of the ACC and PL, by starting at the dorsalmost region of the ACC and moving toward the ventral boundary of PL. The apical dendritic arbors of the pyramidal neurons in ACC and PL were predominantly short shaft primary apical dendrites (2/3 of all neurons loaded), ranging from 5 to 25 μm before branching into two secondary branches, making a "Y" shape. The spatial orientation of the "Y" was not typi-

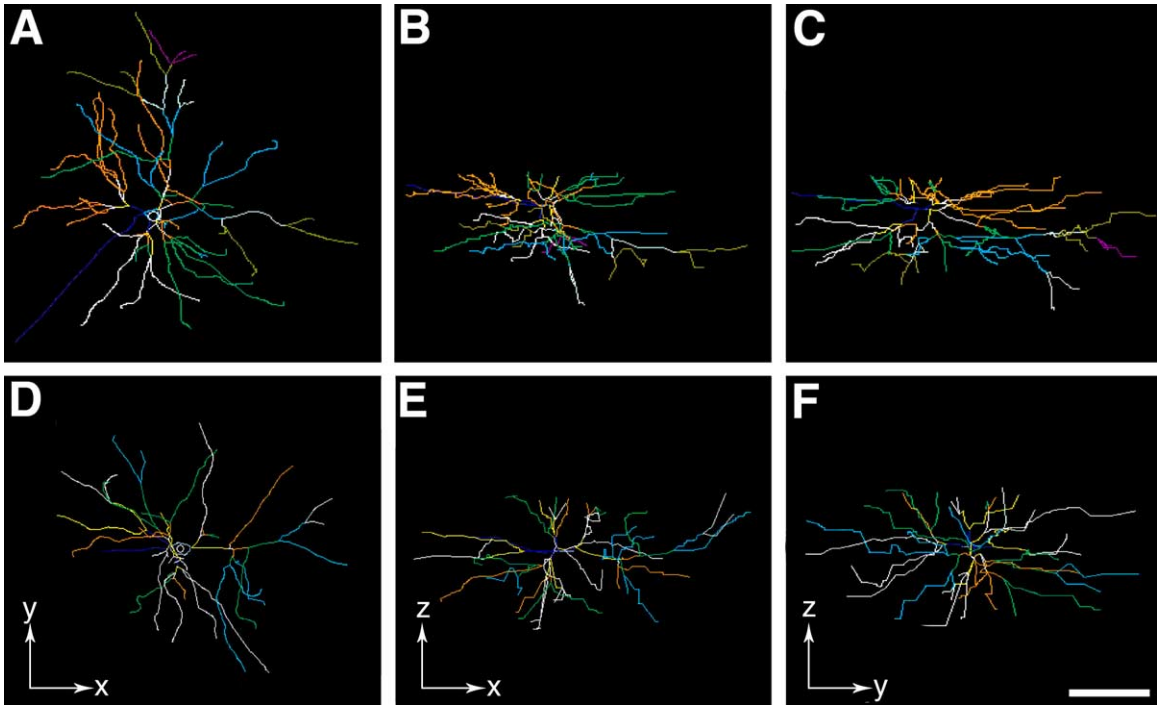

Fig. 2. Representative layer II/III pyramidal neurons from the mPFC of a control (A, C, E) and restraint stress rat (B, D, F). A and D represent neuron tracings in the coronal (*x–y*) plane. The *z*-axis is controlled by focusing up and down as branched are traced. Note, the tracing artifact in cells are rotated to the *x–z* axis (B, E) and *y–z* axis (C, F) to reveal their 3-D nature. In A, a short-shaft primary apical dendrite actually has two secondary branches that descend ventrally, whereas the basal dendrites advance toward the dorsal surface (in B and C). The soma depth is approximately 30 μm beneath the top of the section. In C, a long-shaft apical dendrite, the primary shaft gradually courses toward the dorsal surface of the section (E and F). Scale bar=100 μm in F.

cally parallel to the coronal section, although it often appeared to be the case when viewed only from the *x–y* tracing plane. In fact, when the neurons were rotated through the *y–z* or *x–z* axis, their three-dimensional morphology became evident (Fig. 2). Regardless whether short- or long-shaft, apical dendritic branches generally extended out toward the pial surface, well into layer I.

At the end of 21 days of repeated restraint stress, both groups of rats appeared 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., 1992a,b; Magariños and McEwen, 1995a,b; Magariños et al., 1997), these assays were not performed in the present study. However, as also previously demonstrated (Watanabe et al., 1992a,b), stressed rats did show a reduction in total weight compared with unstressed controls (data not shown). Light microscopic analysis of LY-filled neurons in ACC and PL revealed a significant decrease ($P=0.018$) in total apical dendritic length after 21 days of repeated restraint stress (914±51 μm; $n=24$ cells) compared with control rats (1118±86 μm; $n=23$ cells; Fig. 3A). This was also accompanied by a decrease ($P=0.017$) in total apical branch number (Stress, 12.9±0.9; Control, 15.5±0.5; Fig. 3B). Such alterations in dendritic patterning were not present in basal dendrites (Stress, $n=30$ branches; Control, $n=26$ branches; Fig. 3C, D), in terms of total length (Stress, 461±38; Control, 491±41; $P=0.574$) or branch number (Stress, 5.5±0.5; Control, 6.0±0.5; $P=0.402$). Given that there was a decrease in the total length and branch number of apical dendrites in ACC and PL pyramidal neurons, a Sholl analysis was carried out to localize better where on the dendritic arbor these changes occurred. The analysis was performed in radial unit distances of 30 μm relative to the soma. Significant decreases were found to be evident at 60–120 μm radial distances from the soma (Fig. 4).

DISCUSSION

The main findings of the present study were that chronic behavioral stress induced significant decreases in the total length and number of branches on apical dendrites of pyramidal neurons in the ACC and PL of rats. Moreover, these overall reductions were manifested by decreases in branching pattern complexity in the proximal-to-medial portions of apical dendritic arbors. This study represents a departure from the Golgi impregnation method that has been used in many previous studies that examined the effects of glucocorticoid administration and restraint stress (e.g. Woolley et al., 1990; Watanabe et al., 1992a; Magariños and McEwen, 1995a; Wellman, 2001). An advantage of the cell loading technique as employed here is that the selection criteria for cell analysis are random and unbiased (Duan et al., 2002). The other advantages are that cells can be completely filled, as evidenced by the presence of well-defined dendritic endings after an intracellular injec-

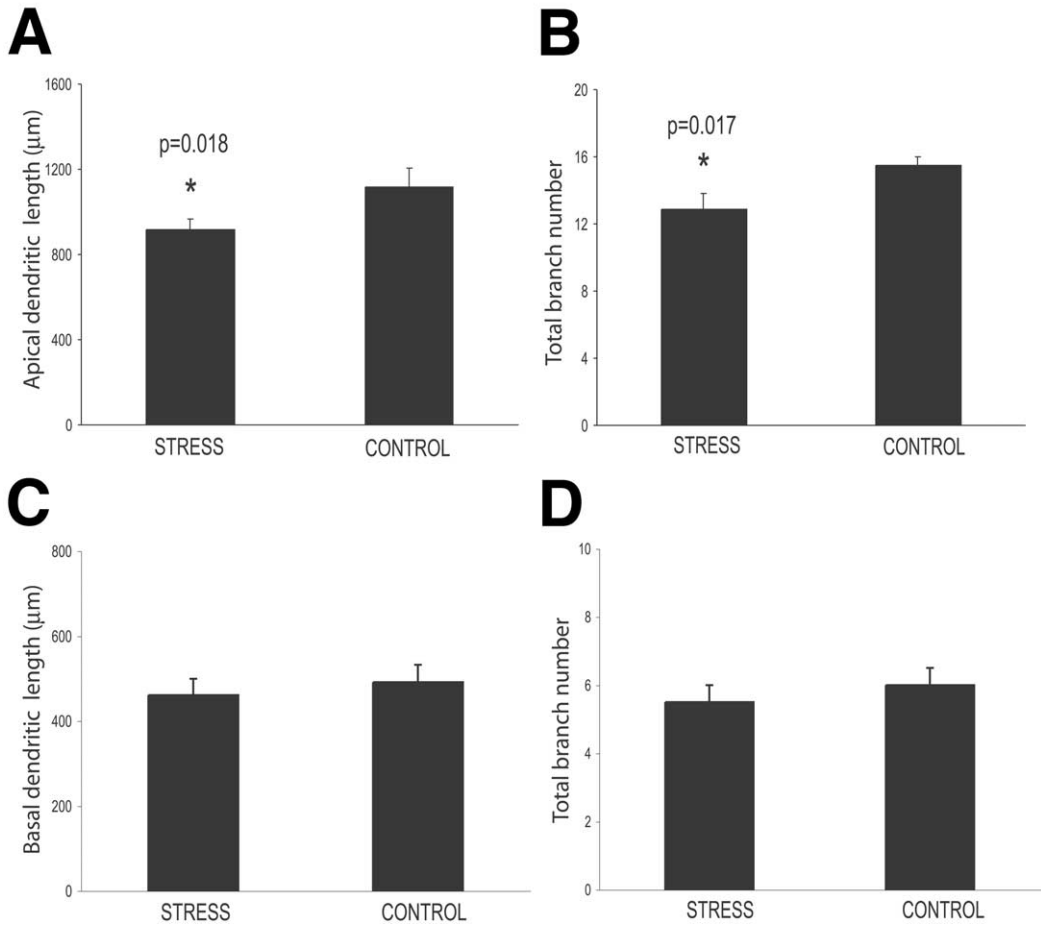

Fig. 3. After 21 days of restraint stress (n=24 cells; n=6 animals), the total dendritic length (A) of apical dendrites of layer II/III pyramidal neurons was significantly reduced by 20% compared with controls (n=23 cells; n=5 animals), and the total number of apical dendritic branches (B) was significantly reduced by 17% in stressed compared with control rats. (C, D) There were no stress-induced changes observed (Stress, n=30 cells; n=5 animals; Control, n=26 cells; n=5 animals) in total basal dendritic length (Stress, 461±38; Control, 491±41; P=0.574) or branch number (Stress, 5.5±0.5; Control, 6.0±0.5; P=0.402).

tion is performed, and the ability to prevent overlapping dendritic fields by appropriately spacing out the injection sites.

The comparable pattern of dendritic reorganization observed between the mPFC and the hippocampus following repeated restraint stress (Watanabe et al., 1992a) suggests the existence of a stereotypical effect on cortical neuronal morphologies following prolonged stressors. One implication of this is that other environmental stressors that produce dendritic reorganization in the hippocampus, such as psychosocial stress (Magariños et al., 1996; McKittrick et al., 2000), are likely to produce a similar pattern of reorganization in the mPFC. However, structural changes, such as apical dendritic retraction, in one brain region may have different functional consequences in another. For example, it has been proposed that dendritic reorganization in the hippocampus following chronic stress may be adaptive, since dominant rats show the same reduction of CA3c hippocampal dendritic reorganization as subordinates in a social dominance hierarchy paradigm (McKittrick et al., 2000). However, it remains to be shown whether the dendritic reorganization in the mPFC is adaptive or maladaptive. Given that human PTSD is correlated with the impaired functioning, and a decrease in volume, of the ACC (Shin et al., 2001; Pissiota et al., 2003; Rauch et al., 2003; Yamasue et al., 2003), this may suggest that the effects of stress on the mPFC are maladaptive.

To date, only one study has demonstrated that glucocorticoid administration results in a reorganization of apical dendrites of pyramidal neurons in layer II/III of the mPFC (Wellman, 2001). Interestingly, chronic corticosterone administration resulted in an increase in apical dendritic branching complexity at radial distances $<$130 μm relative to soma, and a decrease at radial distances $>$260 μm (Wellman, 2001). The fact that we found a decrease in apical dendritic branching complexity at radial distances between 60 and 120 μm following repeated restraint stress suggests that a different mechanism may account for these seemingly opposite effects. Indeed, repeated restraint stress has been shown previously to be more contingent on excitatory amino acids than glucocorticoids per se (Watanabe et al., 1992c; Magariños and McEwen, 1995b), however, these two experimental manipulations were not observed to

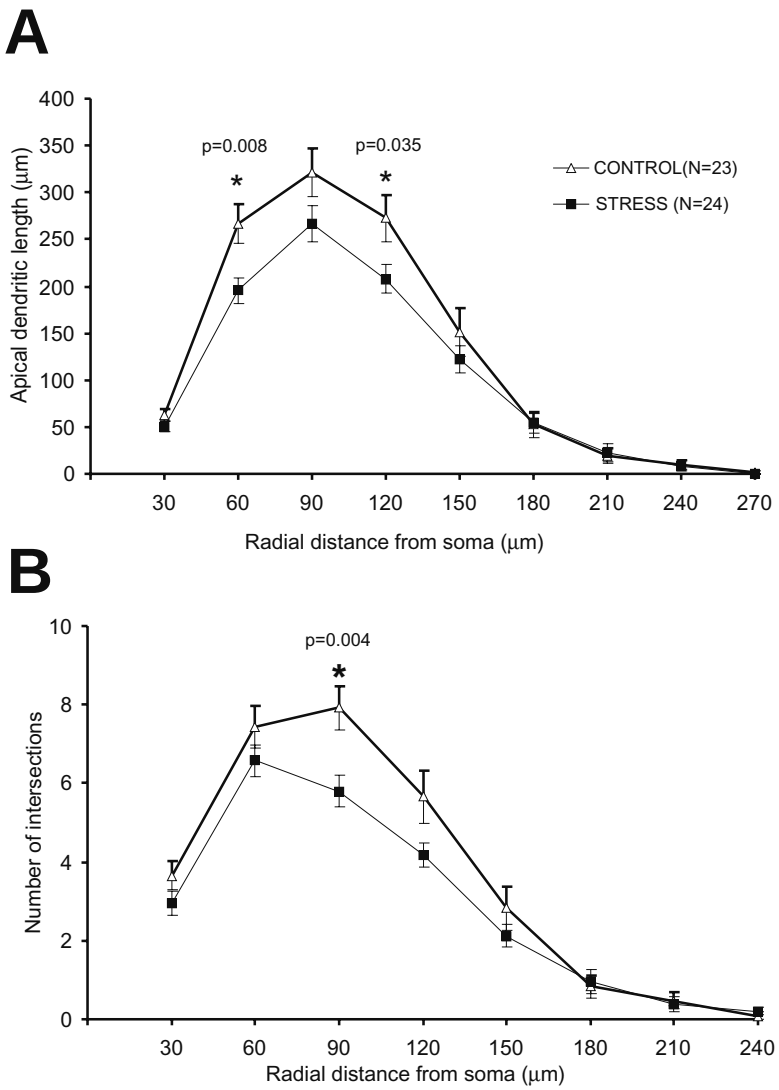

Fig. 4. Sholl analysis for mPFC pyramidal neuron apical dendritic length (A) and intersection number (B) per 30 μm radial unit distance from the soma. Restraint stress produces a reduction in branching complexity at radial distances of 60–120 μm relative to the soma.

produce opposing effects on dendritic morphology in the hippocampus in previous studies (Woolley et al., 1990; Watanabe et al., 1992a). We did not detect any decrease in apical dendritic branching complexity in the mPFC at radial distances >260 μm, as seen following chronic corticosterone administration (Wellman, 2001), though such an effect would be difficult to detect in our material, since minimal dendritic arbors extended beyond radial distances of 240 μm (Fig. 4A). Regardless of these differences with respect to proximal and distal dendritic alterations, it appears that environmental stressors are capable of producing dendritic reorganization in mPFC, that is mediated, at least in part, through glucocorticoids. It is possible that atrophy of apical dendrites of layer II/III pyramidal neurons contributes significantly to the recently observed reduction in ACC volume in patients with PTSD (Rauch et al., 2003; Yamasue et al., 2003). Future studies will examine the effects of behavioral stress on mPFC volume and synapse number.

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