Complete reversal of dendritic atrophy in CA3 neurons of the hippocampus by rehabilitation in restraint stressed rats

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We have shown previously that chronic restraint stress causes atrophy of apical dendrites and alterations in the spine density of CA3 hippocampal neurons in rats. In the present study, the effect of 45 days of rehabilitation following 21 days of restraint stress on the dendritic morphology of CA3 neurons of the hippocampus was carried out. The results revealed that 45 days of rehabilitation reverses restraint stress-induced (6 h/day for 21 days) apical dendritic atrophy in CA3 neurons of the hippocampus. Whereas prolonged repeated restraint stress during this period also resulted in an atrophy of basal dendrites, suggesting that long-term stress can affect the hippocampal neurons more severely. These results showed that chronic stress-induced structural pathology of hippocampal neurons can be completely reversed by rehabilitation.

THE role of hippocampus in mediating cognitive functions such as learning and memory is well established. The hippocampus is highly susceptible to various endogenous and exogenous insults, including stress[1–6]. Chronic stress is known to cause neuroanatomical changes in the hippocampus, such as atrophy of apical dendrites of CA3 pyramidal neurons[4,6,7], increase in dendritic spines and excrescences[4] and reduction in corticosteroid receptors[8]. A more recent study has revealed that stress and glucocorticoids alter morphology of presynaptic mossy fiber terminals in the stratum lucidum region of the CA3 neurons[9]. Long-term treatment of rats with high levels of corticosteroids also resulted in dendritic atrophy and pyramidal cell loss[10–12]. Besides glucocorticoids, excitatory amino acids and *N*-methyl-*D*-aspartate (NMDA) receptors are involved in structural plasticity as well as in neuronal death that is caused in pyramidal neurons by seizures or ischemia[13,14]. Recently, we have shown that blocking of excitatory glutamatergic inputs to the hippocampus by bilateral entorhinal cortex lesions attenuates the stress-induced atrophy of dendrites in CA3 neurons[15]. These studies suggest that the hippocampal changes associated with stress may be mediated at least in part by adrenal steroids and excitatory amino acids.

The hippocampus has been implicated as a critical structure for various aspects of learning and memory, particularly for solving tasks which require spatial memory[16]. The hippocampus has been shown to be a critical integrative centre involved in the regulation of exploratory activities and for incorporating spatial information[17]. Lesions of the CA3 region of the hippocampus have been reported to impair learning through spatial memory disturbance[18]. Involvement of CA3 region in memory is further demonstrated by using nootrophic drugs that affect the long-term potentiation (LTP) in mossy fiber–CA3 system[19]. In addition, single as well as repeated corticosterone injections inhibit LTP in the hippocampus[20]. It has been shown that rats which had higher basal levels of corticosterone throughout their life span or at old age display increased hippocampal cell loss[21]. However, our recent study showed that hippocampal neurons are protected by corticosterone at lower concentrations *in vitro*[22].

The hippocampus is a target of stress hormones, and it is an especially plastic and vulnerable region of the brain. Repeated stress causes atrophy of dendrites in the CA3 region[4,6] and both acute and chronic stress suppresses neurogenesis of dentate granule cells[23,24]. The structural plasticity is relevant to the human hippocampus, which undergoes a selective atrophy in a number of disorders, accompanied by deficits in declarative, episodic, spatial and contextual memory performance. It is important, from a therapeutic standpoint, to distinguish between a permanent loss of cells and a reversible atrophy. However, it is not yet known whether the dendritic atrophy induced by chronic restraint stress is permanent or reversible. Accordingly, the present study was undertaken to evaluate the effect of rehabilitation on the morphology of the CA3 pyramidal neurons of the hippocampus in rats subjected to chronic restraint stress.

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Materials and methods

Experimental animals

Male Wistar rats of 45 days old weighing 100–120 g were obtained from the Central Animal Research Facility of the National Institute of Mental Health and Neurosciences, Bangalore, India. They were housed individually in polypropylene cages (22.5 × 35.5 × 15 cm) and maintained in a temperature (25 ± 2°C) and light (light period, 08.00–20.00 h) controlled room with relative humidity of 50–55%. Food and water were provided *ad libitum*, except during the stress period.

Stress and rehabilitation procedures

Rats were randomly divided into five groups: (a) normal control (NC, $n = 10$); (b) stressed (ST, $n = 10$), these rats were restrained in a wire mesh restrainer, 6 h/day (10.00–16.00 h) for 21 days (for details see our earlier reports[4,15,25,26]), at the end of experiment the induction of stress was confirmed by ulcerated gastric mucosa and adrenal hypertrophy[25,27]; (c) rehabilitation (SR, $n = 6$) group, stressed rats were housed individually in standard laboratory conditions with food and water *ad libitum* for 45 days; (d) continuously stressed (CS, $n = 6$), this group of rats were stressed as that of ST group of rats for 66 days, to study whether the structural recovery is due to rehabilitation or merely an age-related effect; and (e) rehabilitation control (RC, $n = 6$), these rats served as age-matched controls for SR and CS groups of rats. Experiments were conducted in strict accordance with the NIH guidelines (*Guide for Care and Use of Laboratory Animals*, NIH Publication No. 86–23, Revised 1985) and were also approved by the local ethical committee.

Histology and quantification of dendritic morphology

After the completion of experiments, all groups of rats were sacrificed under deep anaesthesia, the adrenal weights and the presence or absence of gastric ulcers were determined as reported earlier[25]. The hippocampus was dissected and processed for rapid Golgi staining technique as described earlier[4,15,28–32]. The dorsal hippocampi were cut into 120 µm thick sections in horizontal plane using a sledge microtome. Sections were collected serially, dehydrated in absolute alcohol, cleared in xylene and coverslipped. Slides were coded prior to the quantitative analysis and the coding was broken only after the analysis was completed. The dendritic branching points and intersections were quantified from CA3 pyramidal neurons of the hippocampus. Pyramidal neurons of the CA3 region of the dorsal hippocampus were examined from the border of CA4-CA2 (refs 28, 30–32) and dendritic morphology was studied in short shaft pyramidal neurons from the CA3b subfield of the dorsal hippocampus[4,15,28–30]. Neurons were randomly viewed and those fulfilling the following criteria were selected for analysis; (i) presence of dark and consistent sliver impregnation throughout the extent of all dendrites, (ii) presence of untruncated dendrites, (iii) relative isolation from the neighbouring neurons, and (iv) cell type must be identified.

Camera lucida tracings of ten neurons from each animal were made at 625 × magnification, using a binocular Leitz microscope (Germany). At the same magnification, concentric circles were drawn on a tracing paper at 40 µm equivalent intervals, with the aid of a stage micrometer. The number of dendritic branching points and intersections was counted in successive radial segments of 40 µm distance, taking into consideration the centre of the soma as a reference point (Sholl's analysis)[28,29,31]. The points at which dendrites cross the concentric circles were considered as the points of intersection. Apical and basal dendrites were studied up to a distance of 200 and 120 µm, respectively (Figure 1).

Statistical analysis

The dendritic branching points and intersections data were subjected to two-way ANOVA followed by least significant difference (LSD), post-hoc test for the inter group comparisons[33].

Results

Qualitative observations

The photomicrographs and camera lucida tracings of CA3 pyramidal neurons from different groups of rats are depicted in Figures 2 and 3, respectively. The qualitative

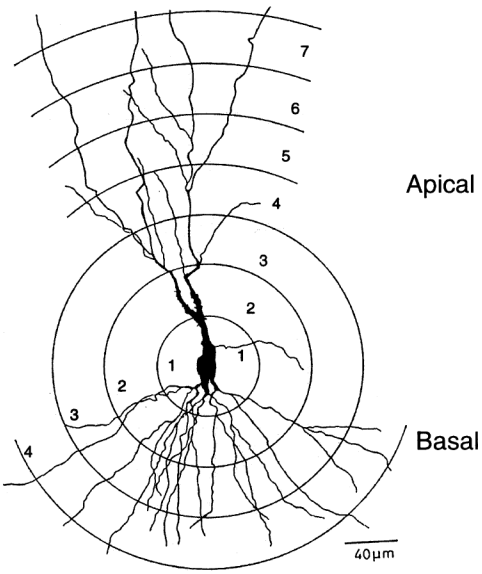

Figure 1. Schematic representation of Sholl's method for quantitative analysis of the dendritic arborization in CA3 pyramidal neurons of the hippocampus. The dendritic branching points and intersections were counted in successive radial segments of 40 µm (indicated by numbers), taking the centre of the soma as a reference point in apical and basal dendrites. The points at which dendrites cross the concentric circles were considered as intersection points (for more details, refer our earlier reports[28,29,31]).

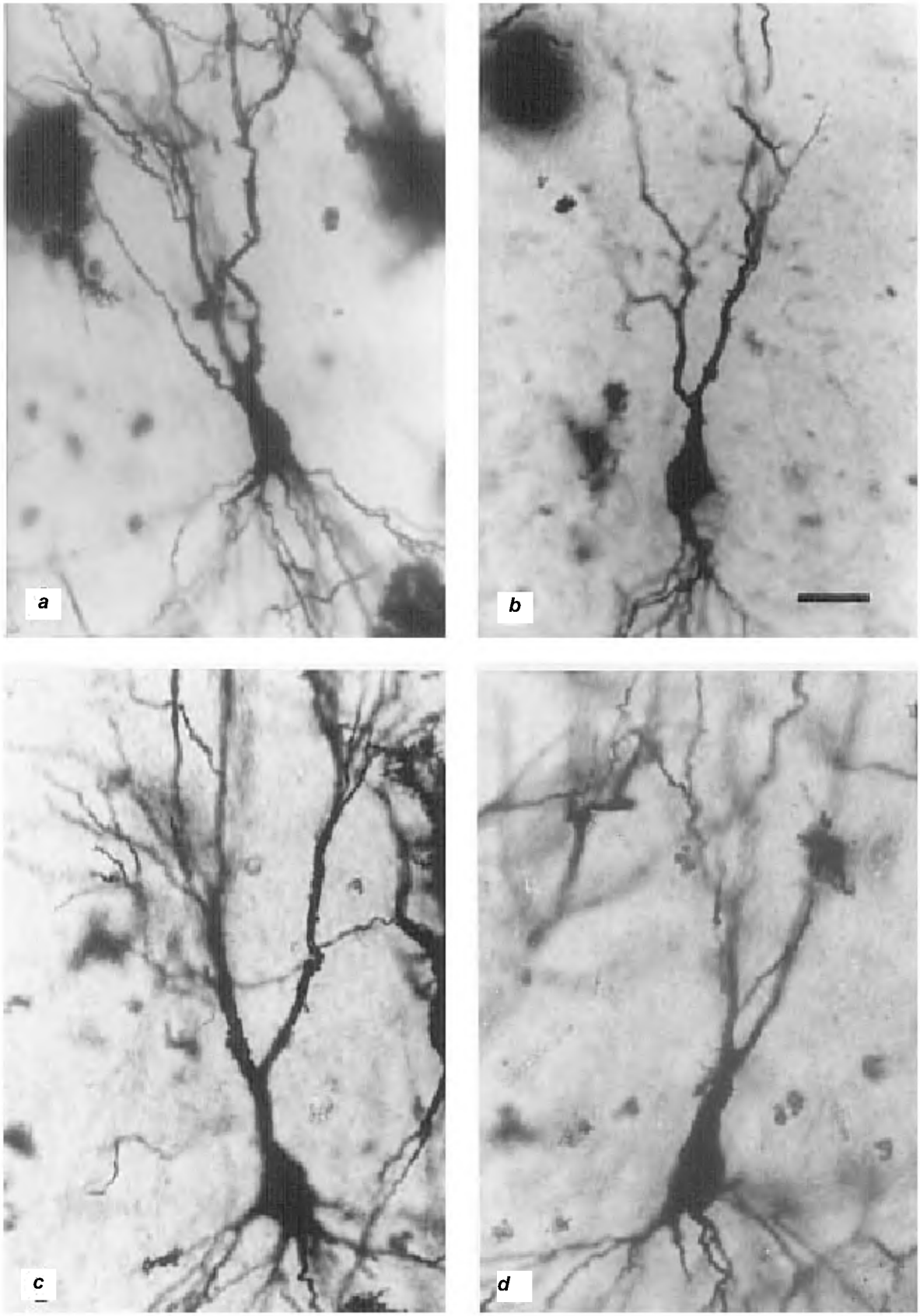

Figure 2. Golgi-impregnated short shaft CA3b pyramidal neurons of the hippocampus from (*a*) normal control (NC); (*b*) stressed (ST); (*c*) stressed, but rehabilitated (SR); and (*d*) continuously stressed (CS) groups of rats. Note decreased number of dendrites in ST (*b*) and CS (*d*) groups compared to NC (*a*) and SR (*c*) groups of rats, respectively. Scale bar in (*b*) = 40 μm and applies to all frames.

observations clearly indicate a decrease in the number of apical dendrites in stressed group (Figures 2 *b* and 3 *b*) compared to normal control group (Figures 2 *a* and 3 *a*). Whereas rehabilitation of stressed rats for 45 days resulted in a complete reversal of dendritic atrophy (Figures 2 *c* and 3 *c*). Interestingly, prolonged stress caused a further decrease in the number of apical dendrites and also affected the basal dendrites (Figures 2 *d* and 3 *d*).

Alterations in the number of dendritic branching points

Analysis of apical dendritic branching points data by two-way ANOVA revealed a significant ($P < 0.001$) difference between groups and segments. Segmental analysis of the number of branching points in apical dendrites showed a significant decrease in the 80 µm ($F_{4,33} = 5.98$, $P < 0.05$) segment in ST group compared to NC group. However, the number of branch points was decreased in the 120 ($F_{4,33} = 13.58$, $P < 0.001$), 160 ($F_{4,33} = 28.23$, $P < 0.001$) and 200 µm ($F_{4,33} = 35.06$, $P < 0.001$) segments in ST as well as CS groups compared to NC, RC and SR groups of rats, respectively. There was no significant difference in the number of dendritic branching points in RC and SR groups of rats in any of the segments, except in the 120 µm segment (Figures 2, 3 and 4 *a*).

The number of branching points in basal dendrites was not significantly different among NC, ST, RC and SR groups of rats. However, the prolonged stress resulted in a significant ($P < 0.05$) decrease in the number of branching points of basal dendrites in the 80 and 120 µm segments in CS group compared to all other group of rats (Figures 2, 3 and 5 *a*).

The total number of apical dendritic branch points was decreased significantly ($F_{4,33} = 49.93$, $P < 0.001$) in ST

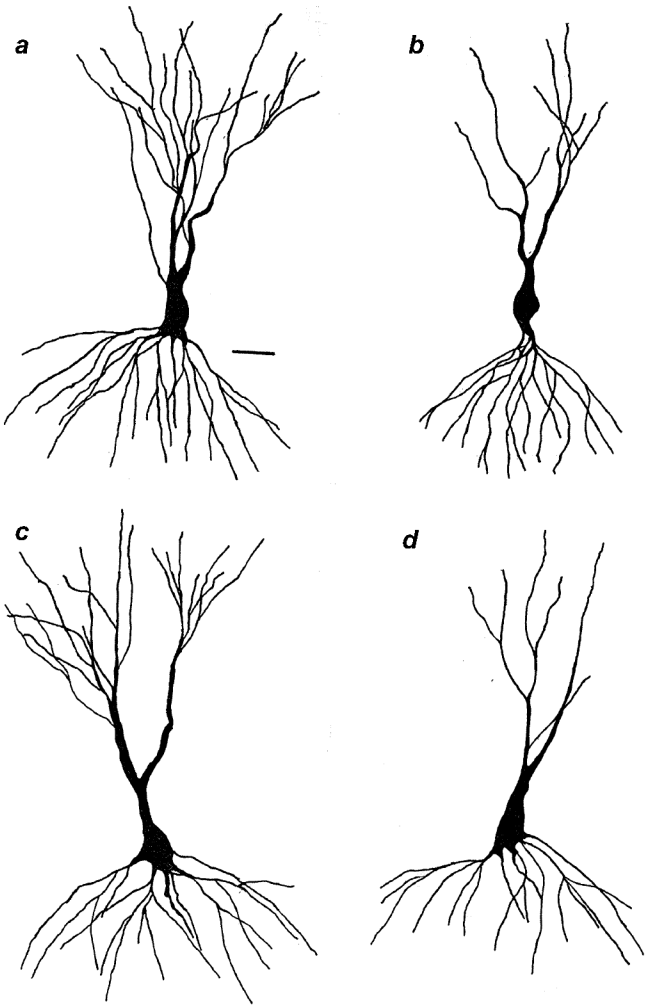

Figure 3. Camera lucida tracings of the short-shaft CA3b pyramidal neurons of the hippocampus from (*a*) normal control (NC); (*b*) stressed (ST); (*c*) stressed, but rehabilitated (SR); and (*d*) continuously stressed (CS) groups of rats depicted in Figure 2. Note decreased number of dendrites in ST (*b*) and CS (*d*) groups compared to NC (*a*) and SR (*c*) groups of rats, respectively. Scale bar in (*a*) = 40 µm and applies to all frames.

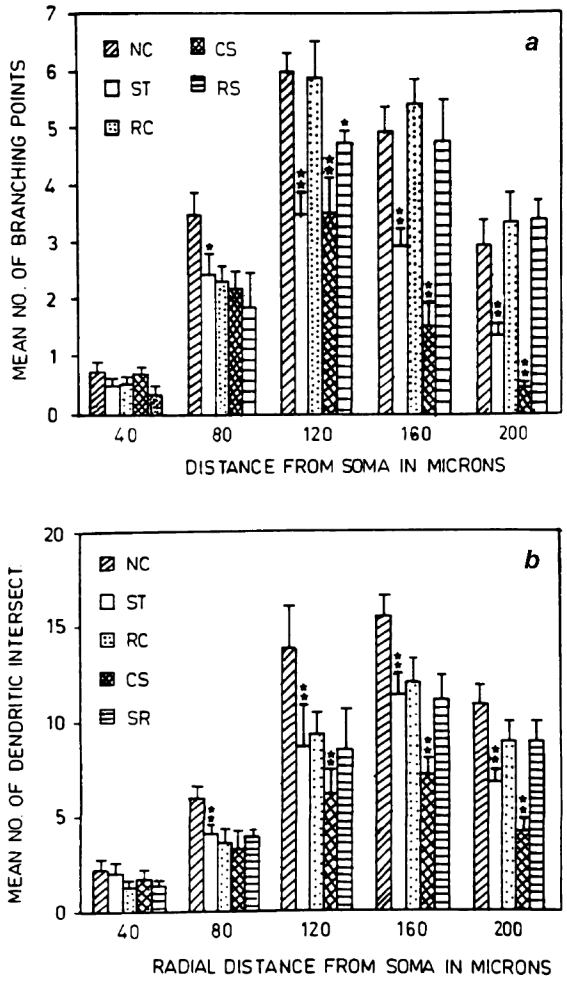

Figure 4. Number (mean ± SD) of dendritic branching points (*a*) and intersections (*b*) in apical dendrites of CA3 neurons of the hippocampus in NC, ST, rehabilitation control (RC), CS and SR/RS groups of rats (for details, refer to Figure 2). Note a significant decrease in both branching points and intersections in ST and CS groups of rats compared to other groups. $*P < 0.05$; $**P < 0.001$ (two-way ANOVA followed by LSD test).

and CS groups compared to other groups. The number of branching points in basal dendrites was decreased significantly ($F_{4,33} = 3.27$, $P < 0.05$) only in CS group (Figures 2, 3 and 6).

Alterations in the number of dendritic intersections

Statistical analysis of the number of intersections in apical dendrites showed a significant decrease in all segments, except in the 40 μm segment. The number of intersections was decreased following stress in the 80 μm ($F_{4,33} = 18.03$, $P < 0.001$) segment in ST group compared to NC. Whereas a significant ($P < 0.001$) decrease in the intersections was observed in the 120 ($F_{4,33} = 12.48$), 160 ($F_{4,33} = 62.89$) and 200 μm ($F_{4,33} = 28.85$) segments in ST and CS groups compared to NC, RC and SR groups, respectively (Figure 4 *b*).

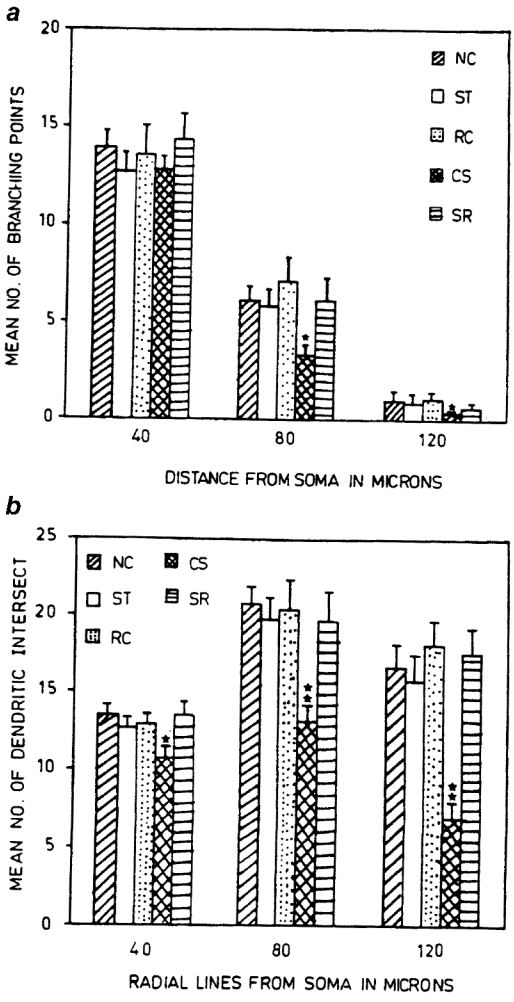

Figure 5. Number (mean ± SD) of dendritic branching points (*a*) and intersections (*b*) in basal dendrites of CA3 neurons of the hippocampus in different groups of rats (for details, refer to Figure 2). Note a significant decrease in branching points and intersections seen only in CS group of rats compared to other groups. $*P < 0.05$; $**P < 0.001$.

Data on dendritic intersections in basal dendrites revealed no significant difference among NC, ST, RC and SR groups of rats. However, a significant decrease in dendritic intersections was observed in CS group in 40 ($F_{4,33} = 9.67$, $P < 0.05$), 80 ($F_{4,33} = 26.98$, $P < 0.001$) and 120 μm ($F_{4,33} = 23.67$, $P < 0.001$) segments compared to other groups (Figure 5 *b*).

Discussion

The results of the present study demonstrated that the stress-induced selective apical dendritic atrophy of the CA3 neurons is completely reversed by 45 days of rehabilitation. In addition, the present study also showed that prolonged stress also affects basal dendrites in addition to apical dendrites. Our results are in agreement with other studies showing reversibility of dendritic atrophy in various other experimental conditions. For example, reversible atrophy of dendrites of CA3 neurons during hibernation in ground squirrels and hamsters was observed[34,35]. In addition, reversible synaptogenesis is known to be regulated by ovarian steroids and excitatory amino acids via NMDA receptors in CA1 region of female rats[36–38]. The estrogen-regulated CA1 synaptic plasticity is a rapid event. In the female rat it occurs

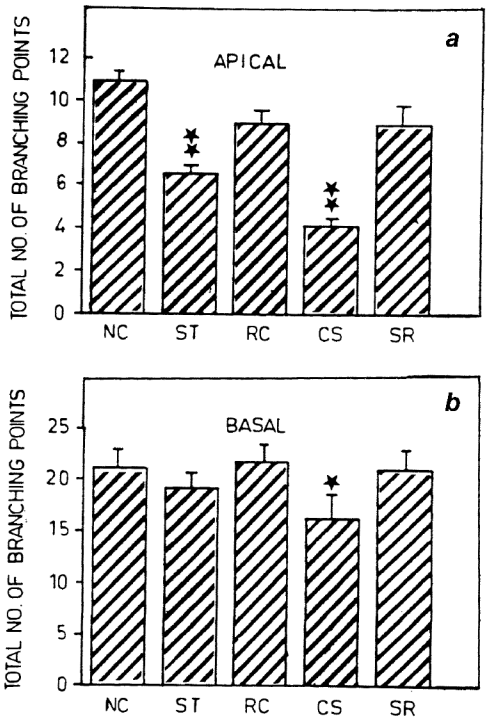

Figure 6. Total number (mean ± SD) of apical (*a*) and basal (*b*) dendritic branching points in CA3 pyramidal neurons of the hippocampus from different groups of rats (for details, refer to Figure 2). Note a significant decrease in the number of apical ($**P < 0.001$) dendrites in ST and CS groups compared to NC, RC and SR groups of rats. Whereas the number of basal dendrites was significantly ($*P < 0.05$) decreased only in CS group compared to other groups.

during the 5-day estrus cycle, with the synapses taking several days to be induced under the influence of estrogens and endogenous glutamic acid and then, disappearing within 12–24 h under the influence of the proestrus surge of progesterone[39]. Blockade of NMDA receptor blocks atrophy induced by estrogens in CA1 neurons[36,40]. However, dendritic atrophy in hibernating ground squirrels and hamsters develops as fast as the hibernating state and can be reversed rapidly within several hours[34,35]. In contrast, the CA3 atrophy found in rats is a relatively slow process, taking at least 3 weeks to develop under daily stress and 45 days to completely disappear.

The hippocampus is a principal neural target for glucocorticoids (GCs) and adrenal steroids, which are secreted during stress. Hippocampus contains high concentrations of corticosteroid receptors and has a marked sensitivity to these hormones[41,42]. Many studies have reported that prolonged supraphysiological elevations of GC can damage the hippocampal neurons. Prolonged exposure to GCs in primates caused hippocampal cell loss and damage to CA3/CA2 neurons[11,43] and also resulted in dramatic degeneration of axo-dendritic synaptic terminals in the CA3 region[44]. Daily injections of corticosterone for 3 weeks in adult rats induced atrophy of apical dendrites in CA3 pyramidal neurons and does not have any effect on CA1 or granule cells[10,12,44]. Uno *et al.*[44] have subjected foetal rhesus monkeys to higher concentrations of dexamethasone, a synthetic GC, which resulted in the loss of hippocampal pyramidal neurons, swelling of dendritic branches and axon terminals, perikaryal atrophy and dispersion of synaptic vesicles, mitochondria and microtubules. It was found that 21 days of corticosterone treatment or 21 days of restraint stress caused atrophy of apical dendrites of CA3 pyramidal neurons[4,45,46]. Psychosocial stress also causes apical dendritic atrophy of CA3 pyramidal neurons in rats[47] and tree shrews[48]. Furthermore, GCs are known to disrupt hippocampal glucose utilization[49] and it has been suggested that this leaves neurons vulnerable to metabolic insults[43]. In addition, providing alternatives to glucose can prevent the exacerbation of hippocampal damage[50].

Stress- and corticosterone-induced atrophy is prevented by the anti-epileptic drug, phenytoin[46], which blocks the glutamate release and antagonizes sodium channels and possibly also T-type calcium channels that are activated during glutamate-induced excitation. This result is consistent with the evidence that stress induces release of glutamate in the hippocampus and other brain regions[10,45,51–53]. NMDA receptor blockade is also effective in preventing stress-induced dendritic atrophy[45]. Moghaddam *et al.*[52] have shown that GCs are responsible for stress-induced glutamate accumulation in the hippocampus and prefrontal cortex. We have also shown a significant increase in the glutamate level in the hippocampus[26]. Furthermore, restraint stress also increases the glutamate high affinity uptake and release in the hippocampus, frontal cortex and septum[54]. Thus, enhanced glutamatergic transmission might be responsible for the atrophy of dendrites in CA3 neurons.

It is clear from studies carried out by us and others, that stress selectively affects the CA3 neurons of the hippocampus. The selective vulnerability of CA3 neurons might be due to lack of the calcium-binding proteins, namely calbindin D28K and parvalbumin[55]. It is known that increased concentration of intracellular-free calcium following the post synaptic action of glutamate, disassembles cytoskeletal proteins, especially microtubules[56]. In addition, widespread activation of NMDA receptors increases the levels of intracellular calcium ($[Ca^{2+}]i$), which causes the dendritic cytoskeleton to depolymerize or undergo proteolysis[45]. This may cause cytoskeletal abnormalities leading to an atrophy of dendrites, following stress. Moreover, recent studies have shown that $[Ca^{2+}]i$ concentrations regulate the dendritic spine morphology and density[57]. A large local increase in $[Ca^{2+}]i$ causes shrinkage of spines and leads to their eventual disappearance and further changes in $[Ca^{2+}]i$ in the cell body may lead to the elimination of spines throughout the dendritic tree via nuclear signalling cascades[57,58]. It has also been reported that a larger pulse application of glutamate initiates a larger rise of $[Ca^{2+}]i$, which results in the shrinkage of spines[59]. Thus, increased concentrations of glutamate following restraint stress[26] might increase the levels of $[Ca^{2+}]i$, leading to dendritic atrophy, observed in the present study. However, further studies are warranted to establish the relation between increased $[Ca^{2+}]i$ and dendritic atrophy following stress.

We have recently shown that 21 days of restraint stress results in an impaired acquisition and retention of spatial learning and memory as assessed by performance in *T*-maze tasks[25]. The impaired performance on two different types of spatial memory tasks, namely, *Y*- and radial arm-mazes in stressed rats was also reported[60,61]. In addition, Dominique *et al.*[62] have recently shown that the GCs and foot shock stress-induced impairment in the retrieval of long-term spatial memory in a water maze. Thus, atrophy of CA3 neurons caused by chronic stress may be responsible for spatial learning/memory impairment. However, the restraint stress-induced spatial memory impairments in radial arm maze are found to be reversible[61,63]. Such a reversal effect might be due to the reversal of dendritic atrophy in the hippocampal neurons following stress, as shown in the present study. However, further studies are required to correlate the complete reversal of dendritic atrophy and the extent of reversibility of stress-induced behavioural impairments.

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