

Chronic, Long-Term Social Stress Can Cause Decreased Microtubule Protein Network Activity and Dynamics in Cerebral Cortex of Male Wistar Rats

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Abstract Social stress is viewed as a factor in the etiology of a variety of psychopathologies such as depression and anxiety. Animal models of social stress are well developed and widely used in studying clinical and physiological effects of stress. Stress is known to significantly affect learning and memory, and this effect strongly depends on the type of stress, its intensity, and duration. It has been demonstrated that chronic and acute stress conditions can change neuronal plasticity, characterized by retraction of apical dendrites, reduction in axonogenesis, and decreased neurogenesis. Various behavioral studies have also confirmed a decrease in learning and memory upon exposure of animals to long-term chronic stress. On the other hand, the close relationship between microtubule (MT) protein network and neuroplasticity controlling system suggests the possibility of MT protein alterations in high stressful conditions. In this work, we have studied the kinetics, activity, and dynamicity changes of MT proteins in the cerebral cortex of male Wistar rats that were subjected to social instability for 35 and 100 days. Our results indicate that MT protein network dynamicity and polymerization ability is decreased under long-term (100 days) social stress conditions.

Keywords Social stress · Microtubule network · Microtubule dynamicity · Social instability

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Introduction

Stress is generally defined as any real or perceived physical, social, or psychological event or stimulus that causes our bodies to react or respond (Glanz and Schwartz 2008), and it is an elementary part of any living organism life cycle. Moreover, social interactions are considered as one of the important sources of chronic stress for social animals such as human (Blanchard, McKittrick, Hardy, and Blanchard 2002; Sapolsky 2005). These interactions are considered as unique impacts of the evolution for higher animal species (e.g., mammals). Competition over resources, sexual partner, and hierarchal dominance relationships are some of the good examples of stressful social interactions (Sapolsky 2005). Social stress is viewed as a factor in the etiology of a variety of psychopathologies such as depression and anxiety (Blanchard et al. 2002). Social stress in people is often evaluated in terms of the number and magnitude of life events that an individual experiences. An important index, strongly associated with the number of stressful events that are likely to be experienced, is social status. Low social status is regarded as having an impact on almost every area of the individual's life, with implications for access to resources, safe living conditions, and health care (Blanchard et al. 2002). People in low social status would have the concern about their position in the society and how it would be affected by changes in higher status positions, and this can be a continuous source of stress for them. Also, it is well known that chronic stress diminishes health and increases susceptibility to mental disorders (Blanchard et al. 2002; Moradi et al. 2012; Sapolsky 2005).

Animal models of social stress involve single, periodic, or chronic exposure of a subject animal to other member of the same species (Blanchard, McKittrick, and Blanchard 2001). Most laboratory studies of social stress effects are done on

rodents, typically laboratory rats or mice. Social instability models involve setting up social groups and later mixing them. Since intruders into an established home area are typically attacked more strongly than are subordinates within a stable social grouping, this procedure would be expected to involve a very high level of agonistic behavior (Blanchard et al. 2001, 2002).

Stress is known to significantly affect learning and memory (Bianchi, Hagan, and Heidbreder 2005; Blanchard et al. 2002; Glanz and Schwartz 2008; Radley and Morrison 2005). And this effect strongly depends on the type of the stress, its intensity, and duration. Some stressors can enhance learning and amygdala-dependent memory, which is the basis for long-term memories of traumatic events and post-traumatic stress disorder (PTSD) (Radley and Morrison 2005; Sousa, Cerqueira, and Almeida 2008). But prolonged or extreme stress could lead to decreased memory and even amnesia (Blanchard et al. 2001, 2002). This happens by molecular changes and modifications that occur in brain and can result in disrupted neuroplasticity—the fundamental mechanism of neural adaptation to external and internal stimuli and a key player of learning and memory. Previous works and experiments have shown that chronic and acute stress conditions can change neuronal plasticity, being characterized by retraction of apical dendrites, reduction in axonogenesis and decreased neurogenesis (Bianchi et al. 2005; Blanchard et al. 2002; Radley and Morrison 2005). Various behavioral studies have also confirmed a decrease in learning and memory upon exposure of animals to long-term chronic stress (Blanchard et al. 2001; Ohl and Fuchs 1998, 1999).

On the other hand, the close relationship between microtubule (MT) protein network and neuroplasticity controlling system suggests the possibility of MT protein alterations in high stressful conditions (Bianchi et al. 2005, 2006; Sousa et al. 2008; Yang, Wang, Wang, Liu, and Wang 2009). According to some recent studies, MT protein networks are proposed to be one of the best explanations for memory and intelligent behavior in living organisms (Hameroff and Watt 1982; Tuszyński, Hameroff, Satarian, Trpisova, and Nip, 1995). Microtubules have been suggested to be involved in consciousness, biological information processing, and information storage (Hameroff and Watt 1982) and also perform a wide variety of key cellular functions such as chromosome segregation, cellular organization, axonal transportation, cell motility, and signal transduction (Nogales 2000; Stephens and Edds 1976). The final step in Alzheimer's disease, tauopathies, and other similar neurodegenerative diseases involves aggregation of tau protein, resulting in lowered stability of MT network and axonal degeneration (Falnikar and Baas 2009). All these suggest that MT network and its stability, as well as dynamicity—i.e., the ability of MT filaments to polymerize/depolymerize quickly and repeatedly—plays a crucial role in brain function, as well as proper memory formation and

learning (Bianchi et al. 2006; Jibu, Hagan, Hameroff, Pribram, and Yasue 1994). There have been some reports on the cytoskeletal network alterations following repeated stress conditions: alpha-tubulin which is the major isoform of microtubules in brain can go through post-translational modifications during chronic stress periods (Bianchi et al. 2006; Bianchi et al. 2005). Chronic stress results in an increase in hippocampal acetylated-tubulin and decrease in tyrosinated tubulin, suggesting that the cytoskeletal network undergoes stabilization as an adaptive response that may be protective (Bianchi et al. 2003; Radley et al. 2004). The MT-associated protein, MAP-1, which may also be important for stabilizing microtubules (Tucker 1990; Tucker, Garner, and Matus 1989), has been seen to increase in the hippocampus following repeated stress (Xu, He, Richardson, and Li 2004). In addition, the non-phosphorylated form of neurofilament proteins are increased in dentate granule cells following social deprivation in prepubescent rhesus monkeys (Siegel et al. 1993).

In this work, we have studied the kinetics, activity, and dynamicity changes of MT proteins in the cerebral cortex of male Wistar rats that were subjected to social instability for 35 and 100 days. Since medial prefrontal cortex plays a key role in integration of higher cognitive information and it regulates stress-induced responses (Bianchi et al. 2005; Blanchard et al. 2002), we have decided to focus on the alterations that occurs in this area to check whether we can correlate observed behavioral changes in learning and memory with changes in MT protein network.

Materials and Methods

Chemicals Blood corticosterone level was analyzed using “enzo life sciences” rat corticosterone ELISA kit. Salts and other chemicals were mainly purchased from Merck.

Animals In this study, male Wistar rats at the age of around 3–4 months and weighting 300–350 g were used. The animals were housed 6 per each Plexiglas cage in a room with a 12/12-h light/dark cycle (lights on 07:00 a.m.), and controlled temperature (22 ± 2 °C) with free access to food and water. All experiments were carried out during the light phase between 9:00 and 12:00. The procedures were performed in accordance with international guidelines for animal care and use (NIH publication #85-23, revised in 1985). Animals were divided into three groups ($n=6$): two stress-treated groups and one control. At first, animals were given a week to get used to the new environment and each other. Afterwards, they were weighed and the stress period was started. In stress-treated groups, two animals were randomly switched between the groups every other day (three times a week). In control groups, animals were just handled at the same time to omit the effect of human handling. At the end of each period, animals

were anesthetized, blood was collected from their ventricle and animals' brains were extracted, cerebral cortex—including all cortical regions as well as hippocampus—was dissected and kept on ice cold saline, and quickly used in next experiments. Blood serum was extracted and stored at -20 for further analysis.

Microtubule Polymerization Assay in Brain Extract

Brain extracts containing microtubules, as well as interacting proteins and GTP was prepared as described previously (Qian, Burton, and Himes 1993). Briefly, rat brains were removed quickly, cerebral cortex was isolated and homogenized in 0.1-M pipes containing 1 mM EGTA and 1 mM MgSO₄ (PEM buffer), pH 6.90 at 4 °C. The homogenate was kept on ice for 30 min and then centrifuged at $100,000\times g$ for 30 min. The supernatant after this centrifugation step is referred to as the extract. The total protein concentration of extracts used in all microtubule assembly studies was adjusted to 8 mg/ml. Microtubule assembly was detected by monitoring the increase in turbidity at 350 nm and 37 °C in a Cary 100 spectrophotometer. Depolymerization was done by decreasing the temperature in situ to 4 °C, and after, at least 15-min repolymerization was analyzed by increasing the temperature to 37 °C.

Microtubule Purification and Polymerization Assay

Tubulin was freshly purified extracted brain samples after two cycles of assembly and disassembly (Miller and Wilson 2010; Williams Jr and Lee 1981). Brains were homogenized in a buffer comprising 0.1 M piperazine—1,4-bis(2-ethanesulfonic acid), 1 mM ethylene glycol bis(2-aminoethyl ether) tetra acetic acid, 2 mM MgCl₂, and 1 mM adenosine triphosphate (PEM buffer), and the resulting homogenate was centrifuged at 30,000 g for 30 min. The tubulin in the supernatant was polymerized at 37 °C for 45 min with 0.5 mM GTP and 33 % glycerol. Microtubules were pelleted by centrifugation (120,000g, 45 min), re-suspended in PEM buffer, depolymerized at 4 °C, and the mixture was centrifuged at 85,000 g (4 °C) for 45 min. The supernatant was collected and the polymerization and depolymerization steps were repeated. The final sample contained tubulin dimers, MAPs, etc. (Fig 1)

Tubulin Endogenous GTPase Activity Assay Using HPLC

GTPase activity of tubulins was analyzed as described previously (Qian et al. 1993). Briefly, tubulin samples were incubated at a protein concentration of 0.5 mg/ml (lower than critical concentration so that they will not polymerize) with 0.5 μ M GTP for 90 min at 37 °C, stopping the reaction with

2.5 % perchloric acid. Then, the samples were centrifuged at $30,000g$ and the supernatant was extracted. This supernatant included GTP molecules that were not polymerized by microtubules. The supernatant obtained after centrifugation was then neutralized with 4 M potassium acetate-10 M KOH, and immediately injected into HPLC column. To make sure that GTP hydrolysis was not a result of incubation at 37 °C, we designed a negative control including the same amount of GTP (5 μ M) in PEM buffer and in the absence of tubulin protein. This control was also incubated at 37 °C for 90 min. For analysis of the amount of GTP, a C18 reverse-phase column (150 mm \times 3.9 mm inside diameter) was used with a 4 μ m Nova-Pack C18 cartridge (Water, Millford, MA) (Meshkini, Yazdanparast, and Nouri 2011). The mobile phase consisted of 8 % acetonitrile, 50 mM K₂HPO₄, 50 mM KH₂PO₄, and 10 mM ion-pair reagent tetra-n-butyl ammonium bromide and run isocratically at a flow rate of 0.8 ml/min. The injection volume was 30 μ l. The elution was monitored at 254 nm. HPLC grade nucleotide standards were used to calibrate the signals. The Millennium workstation (version 3.05) chromatography manager was used to process the data (area integration, calculation, and plotting of chromatograms) throughout the method validation and sample analysis.

Results

Weight Loss and an Increase in Blood Corticosterone Levels Confirms Stress Conditions in Animals

Weight changes and blood corticosterone levels were chosen as indicators of stress according to literature. Both elements were significantly changed in stress-treated animals (Fig. 1a, b). Animals under social instability stress in both 35- and 100-day period, seem to gain less weight than control littermates. Blood corticosterone levels are also significantly higher in both stress-treated groups. These confirm that the model has caused enough stress in animals, resulting in activation of HPA axis and increased levels of corticosterone.

Microtubule Activity in Brain Extracts (in the Presence of Cellular GTP Pool)

Microtubule polymerization kinetics in brain extracts as well as purified samples was analyzed in the absence and presence of colchicine (Fig. 2a, b) to make sure that it is the polymerization, not aggregation that is being recorded. Polymerization kinetics in brain extracts of 35-day stress-treated animals in the absence of any external GTP, did not show much difference in comparison to control groups (2c). The slope of log phase (i.e., how quickly the curve reaches the plateau state),

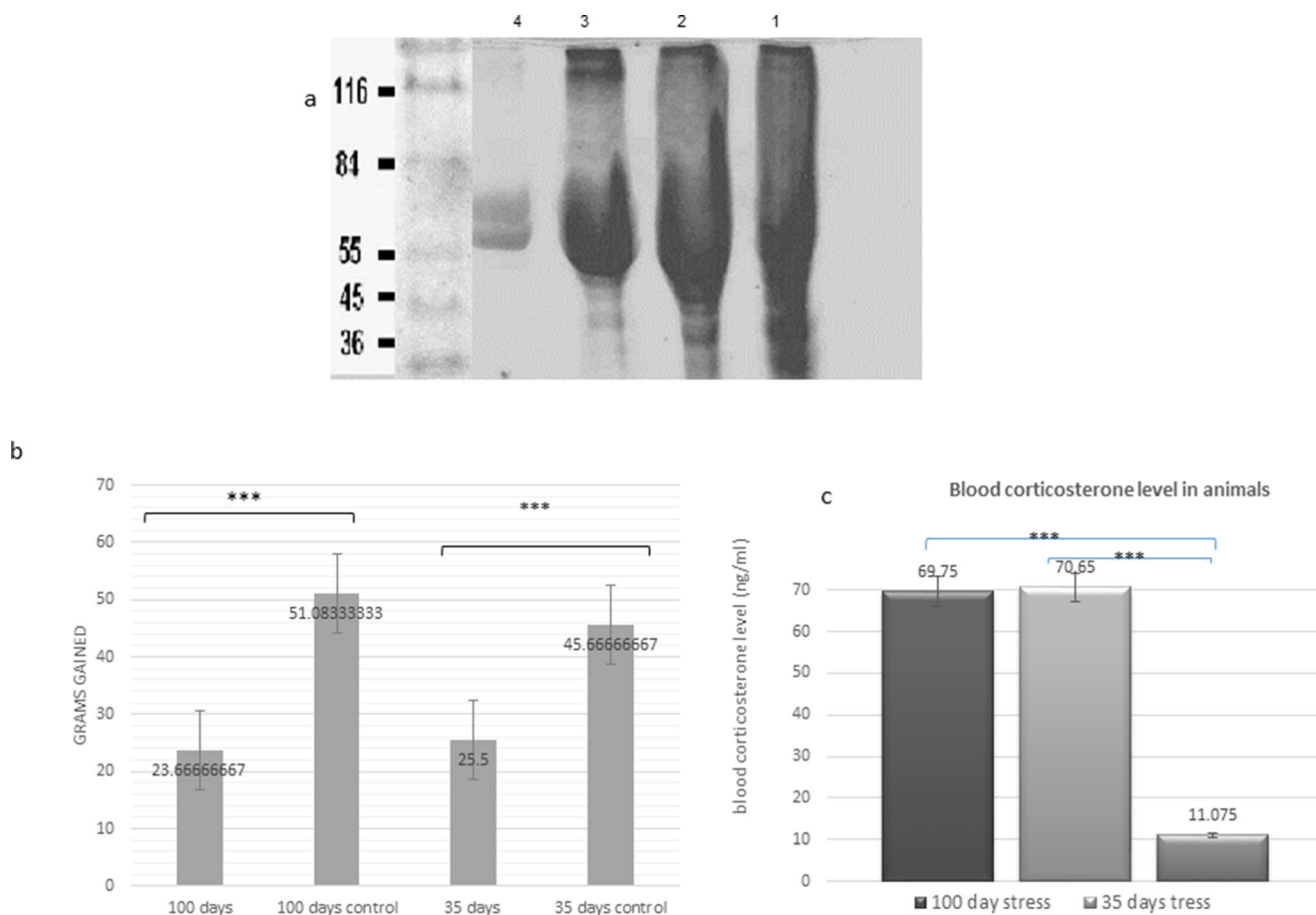


Fig. 1 **a** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of MT purification steps. **1** brain homogenate, **2** brain extract, **3** after first round of centrifugation, and **4** pure MT without

MAPs. **b** Weight gain diagram of 35- and 100-day stress-treated animals. (P value < 0.001). **c** Blood corticosterone levels in stress-treated vs. control animals (P value < 0.001)

the nucleation time as well as the depolymerization, repolymerization capacity of the extract (suggesting the dynamicity of microtubules) seems to be pretty much the same in both groups. However, in 100-day stress-treated animals, polymerization kinetics looks quite different compared to control littermates (2d). In these groups, the slope of log phase is lower and the curve does not fully reach plateau state even after 25 min (which is more than enough for microtubules to polymerize). Microtubules also show low dynamicity, i.e., they cannot repolymerize very well after first depolymerization step.

Microtubule Activity in Purified Brain Extracts in the Presence of External GTP

Purified microtubule polymerization kinetics in the presence of 1 mM GTP in 35-day stress-treated groups is very similar to control littermates. There is not much difference between nucleation step and the log phase slope in stress-treated groups compared to the controls (Fig. 3a). However, 100-day stress-treated animals microtubule show a different scenario

(Fig. 3b). In these groups, the microtubule proteins seem to need almost twice longer time for nucleation. And they also need more time to reach the plateau (very noticeable in group 2). All these factors suggest that 100-day stress-treated animals tend to have a less dynamic microtubule, that is weakly capable of forming active, stable polymers. So to further investigate this, we decided to look into the endogenous GTPase activity of tubulin in these samples.

Endogenous GTPase Activity of 100-Day Stress-Treated animals' Tubulin

To check whether the lower polymerization ability of 100-day stress-treated microtubules was a consequence of their endogenous GTPase activity changes, we did a GTPase activity assay as described earlier and detected the unconsumed GTPs by HPLC. The results indicate that there is a significant difference between tubulin GTPase activities of stress-treated groups compared to control groups. The negative control confirms that incubation in 37 °C doesn't have a noticeable effect on the hydrolysis of GTP (less than 50 nM hydrolysis)

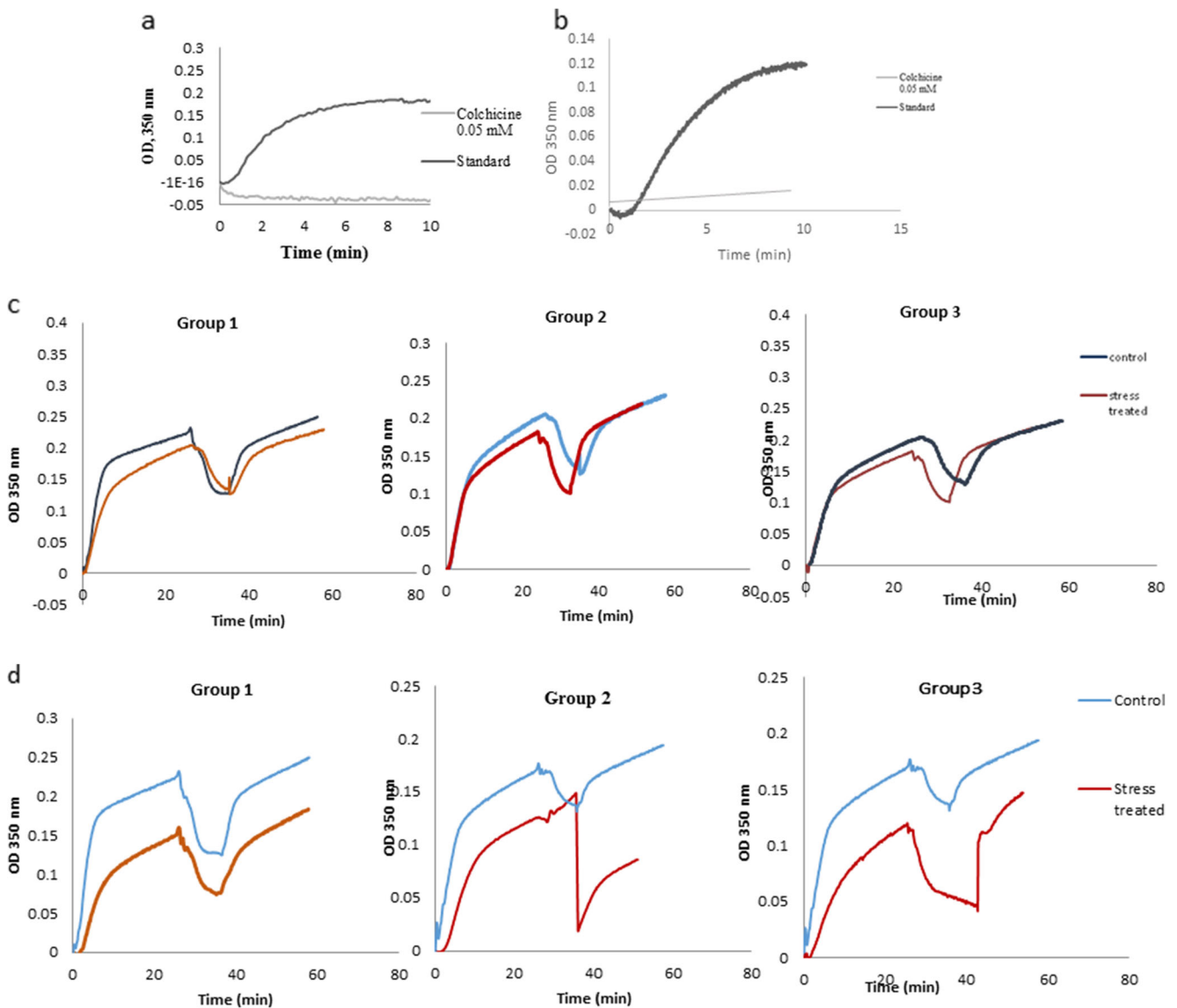


Fig. 2 MT polymerization in the presence and absence of colchicine in **a** brain extract and **b** purified MT. Microtubule polymerization kinetics in brain extracts of **c** 35-day stress-treated animals and **d** 100-day stress-treated animals, in the absence of any external GTP. In all three groups, the solid blue curve represents the control group. After reaching plateau

state, the temperature was decreased to 4 to trigger depolymerization and after at least 15 min, it was again increased to 37 to record repolymerization. Protein concentration was adjusted to 8 mg/ml in all samples. Groups 1–3 represent the repeat of experiments done with 3 animal groups

(Fig. 4a–d). Seven GTP solutions in PEM with known concentration (between 10–500 nM) were measured, and the standard curve was graphed (Fig. 4e). As the results indicate, the 100-day stress-treated tubulins have hydrolyzed 12 times less GTP compared to the control groups (Table 1).

Discussion

Chronic stress can have various effects on nervous system and it has been shown previously that memory and learning can be significantly affected in long-term stressful conditions

(Bianchi et al. 2006; Ohl and Fuchs 1998, 1999). Other works from our group have also shown the effect of social instability on brain tissue and increased formation of lipofuscin in this organ which can accelerate the aging process (Mahdi Dust et al. 2013; Moradi et al. 2012).

The modulation of neuronal function by corticosteroids is well documented. One example is the induction of long-term potentiation (LTP) in the hippocampus showing impaired LTP in the presence of elevated levels of corticosteroids (e.g., during stress). Importantly, hypercorticalism is also known to facilitate long-term depression (LTD) in the hippocampus (Diamond, Bennett, Fleshner, and Rose 1992; Krugers et al. 2005). And recent studies suggest that

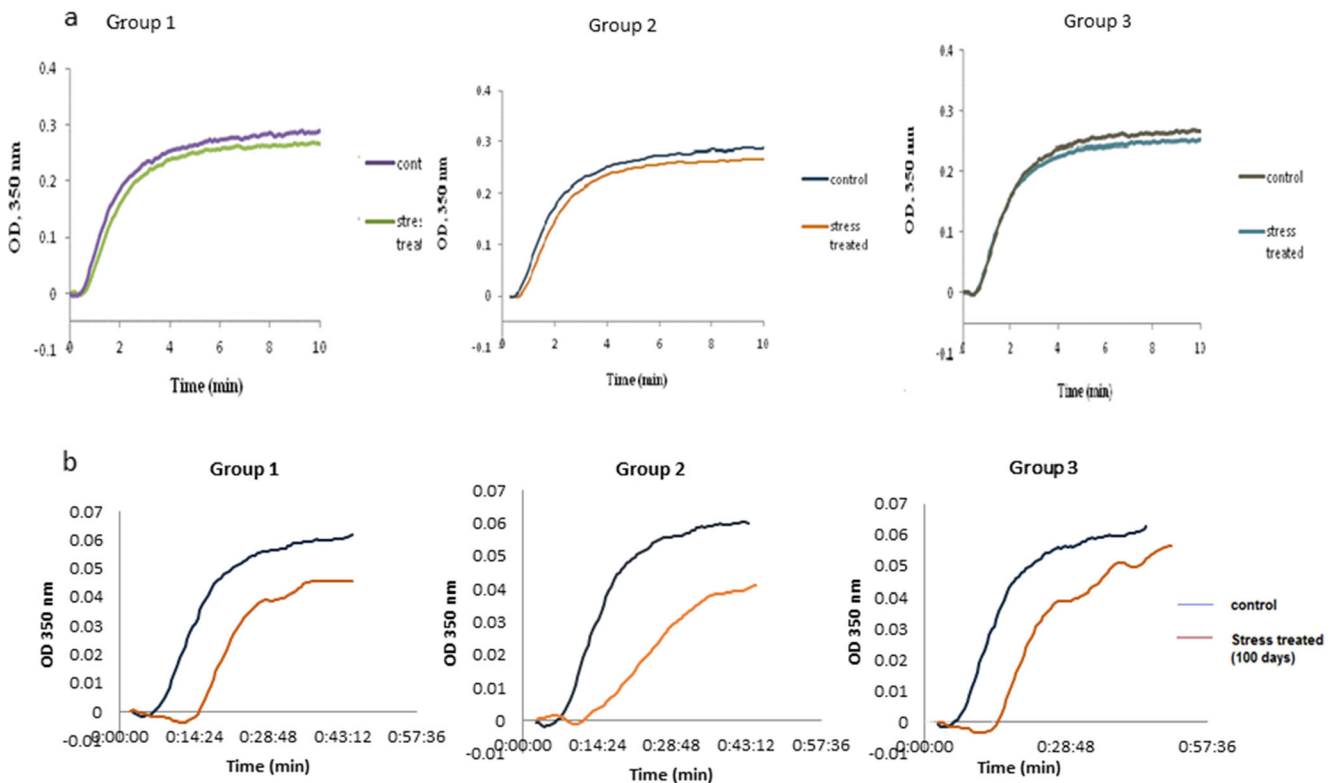


Fig. 3 Purified microtubule polymerization kinetics in PEM buffer, in the presence of 1-mM GTP. **a** 35-day stress-treated animals, **b** 100-day stress-treated animals. Protein concentration was adjusted to 2 mg/ml for all assays. Groups 1–3 represent the repeat of experiments done with 3 animal groups

other brain regions such as prefrontal cortex are also affected by chronic stress. Although the underlying mechanism through which high corticosteroids/ stress exert their electrophysiological effects is still largely unknown, there is evidence for an involvement of glutamatergic transmission in this phenomenon (Bianchi et al. 2005). Indeed, increased glutamate and calcium influx, both of which impair synaptic plasticity, have been described in conditions of hypercorticalism (Blanchard et al. 2002). On the other hand, several researchers have demonstrated that repeated stress can cause an increase in hippocampal acetylated-tubulin and decrease in tyrosinated tubulin, suggesting that the cytoskeletal network undergoes stabilization and lowered dynamicity as an adaptive response that may be protective (Bianchi et al. 2006; Bianchi, Heidbreder, and Crespi 2003; Radley et al. 2004). Axonal degeneration and dendritic debranching of neurons have been observed under chronic stress conditions and this could be a protective behavior of neuron in response to high levels of glutamate (M. Bianchi et al. 2005; Radley and Morrison 2005).

Our results indicate that MT protein network dynamicity and polymerization ability is decreased under long-term chronic stress conditions. After 35 days of chronic stress, the activity and dynamicity of brain-

derived MT proteins did not show much difference with control groups. It has been mentioned in previous reports that the toxic effects of stress is time dependent. In a study on tree shrews, 1 month of social stress was not sufficient to induce neuronal loss (Vollmann-Honsdorf, Flügge, and Fuchs 1997). However, MT polymerization assays in brain extracts of 100-day stress-treated animals show distinguishable difference from control groups. There are two general factors that can affect MT polymerization ability: GTP concentrations and MT protein modifications (other factors such as MT-associated protein (MAPs) modifications can also affect dynamicity and polymerization ability of MT proteins). To further investigate whether the difference observed in 100-day stress-treated animals' brain extracts is because of a change in MT proteins or a result of different cellular GTP concentrations, we analyzed the purified MT polymerization in the presence of 1 μ M GTP. Again, 100-day stress-treated animals MT proteins showed the same decreased dynamicity and polymerization ability in comparison to control animals. Since interaction of tubulin with GTP, and hydrolysis of GTP to GDP by tubulin protein plays a crucial role in its assembly and disassembly (Dadras et al. 2013; Nogales 2000; Stephens and Edds 1976), we decided to check whether 100-day stress treatment can cause any changes in tubulin

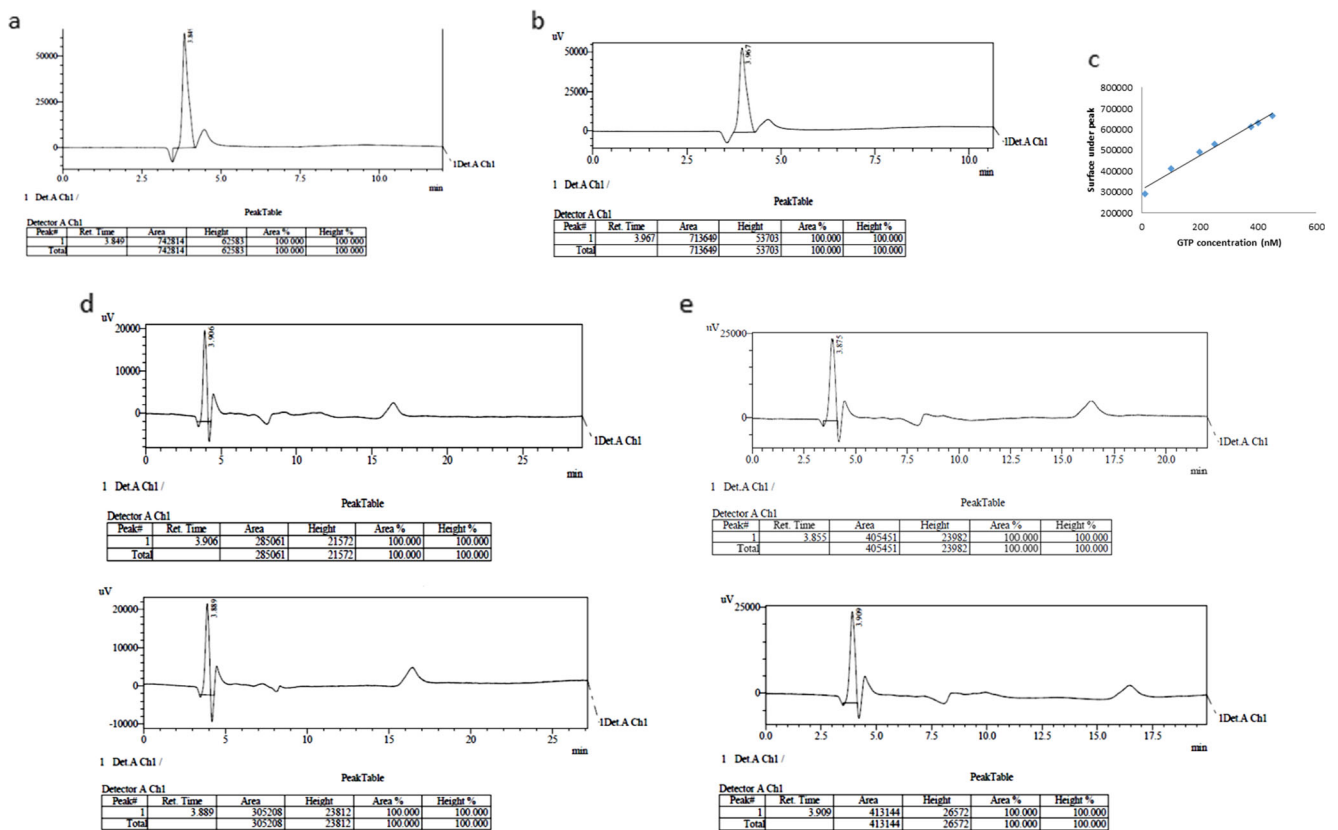


Fig. 4 Analyzing the amount of unconsumed GTP in GTPase assays by HPLC, **a** HPLC graph obtained from injection of 0.5 μM GTP, **b** 5 μM GTP after incubation at 37°C for 90 min, **c** standard graph obtained from seven known concentrations of GTP, **d** amount of unconsumed GTP from 100-day stress-treated groups' assays, **e** amount of GTP detected from

control groups assays. These results show that there is much more unconsumed GTP in assays from 100-days stress-treated animals, compared to the control groups, suggesting that the tubulin from these animals' brains has a reduced GTPase activity. For control and stress-treated groups, two repeats are being shown

endogenous GTPase activity. And our results indicate that there is a 12fold decrease in endogenous GTPase activity of 100 days stress treated groups MT proteins. The importance of MT protein network in memory and cognition, as well as the variety of important functions that MTs do in cell suggests that lowered dynamicity and polymerization ability of MT proteins could have some negative effects on memory. In another research from our group (data unpublished), mice treated colchicine (which inhibits microtubule polymerization) or paclitaxel (which keeps microtubules in GTP-bound form, inhibiting their depolymerization) performed poorly in Morris water maze in comparison to saline-treated groups (control). Moreover, β-boswellic acid which enhance MT dynamics increased

memory and learning in mice. The MT protein network disruption is also a consequence in neurodegenerative diseases such as Alzheimer's disease and tauopathies, where hyperphosphorylation of tau, a MT-associated protein that stabilizes MT filament results in lower affinity of tau for filaments and disruption of MT network (Falnikar and Baas 2009). All these strongly support the importance of MT dynamicity in memory and learning and suggest that decreased polymerization ability and dynamics could result in memory deficiencies.

We therefore suggest that the decreased dynamicity of MT protein network could be one of the mechanisms through which social stresses as a chronic form of stress can affect memory and cause memory deficiency and amnesia.

Table 1 GTP concentration in endogenous GTPase activity assay

GTP concentration from control groups assay	GTP concentration from stress-treated groups assay	GTP concentration from negative control (tube incubated at 37°C)
9.195±2.396 nM	120.774±6.704 nM	≈490 nM

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