Cytoskeletal Changes in the Hippocampus Following Restraint Stress: Role of Serotonin and Microtubules

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ABSTRACT The aetiology of depression is associated with depletion in central levels of serotonin (5-HT). Hence, a major effect of antidepressant drugs is to increase synaptic 5-HT levels. Stressful conditions have also been shown to affect neuronal plasticity and 5-HT neurotransmission in the hippocampus. Neuronal plasticity, which is typically referred to as a structural adaptation of neurons to functional requirements, requires more dynamic forms of microtubules (cytoskeletal component). The α-tubulin, which is the major component of microtubules, can be postranslationally modified and both the tyrosinated (tyr-tub) and acetylated (acet-tub) forms are considered markers of more dynamic or more stable microtubules, respectively. The aim of the present work was to investigate the expression of tyr-tub and acet-tub in the hippocampus of rats submitted to either acute (6 h for 1 day) or sub-chronic (6 h for 4 days every day) restraint stress. In addition, ex vivo hippocampal 5-HT levels were monitored by differential pulse voltammetry to analyse the influence of both stress conditions upon 5-HT levels. Our results showed that the expression of tyr-tub in the hippocampus was significantly decreased to 70 ± 7% following sub-chronic restraint stress ($P < 0.01$). In contrast, acute and sub-chronic restraint stress increased the hippocampal expression of acet-tub to 139 ± 11% and 145 ± 11% of control, respectively. Finally, 5-HT levels were significantly increased ($P < 0.05$) to 142 ± 15% and 135 ± 11% following acute and sub-chronic restraint stress, respectively. The stress-induced cytoskeletal changes observed in the present study suggest that the microtubular network is a potential new pathway that may increase our understanding of stress-related events. **Synapse 49:188–194, 2003.**

INTRODUCTION

Unipolar depression is among the most common and life-threatening illnesses in Western society by affecting 20% of women and 12% of men (Murray and Lopez, 1997). Depressive disorders cause a marked impairment in physical function and work capacity and 15% of suicides in the United States can be related to unipolar depression (Maris, 2002). Several pieces of evidence link the pathogenesis of depression to a reduced activity of monoamine neurotransmission in the CNS. For example, decreases in serotonin (5-HT) neurotransmission have been reported in sub-groups of patients with major depression (Blier and de Montigny, 1994). To date the 5-HT system is the major target of antidepressant drugs and Selective Serotonin Reuptake Inhibitors (SSRIs) lead to increased 5-HT function by preventing the reuptake of 5-HT presynaptically (Blier and de Montigny, 1994).

Neuronal plasticity, which refers to the elongation or shortening of cell branches, is the structural adaptation of neurons to functional requirements and plays a significant role in brain development as well as learning and memory processes in the mature brain (Friston, 1998). Repeated stress in animals results in neuronal plasticity failure in limbic/cortical area, which consists in neuronal atrophy (retraction of apical den-

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drites), cell death, and decreased neurogenesis particularly in the hippocampus (Duman et al., 2000). Indeed, the hippocampus seems to be the most stress-vulnerable among the limbic regions. For example, chronic restraint stress (McEwen, 1999) and psychosocial stress (McKittrick et al., 1996) in rats as well as tree shrew (Magarinos et al., 1996) can produce failure in neuronal plasticity in the hippocampus. These stress-induced anatomical changes in the hippocampus can be reversed by chronic treatment with the atypical antidepressant tianeptine (Magarinos et al., 1999; Czeh et al., 2001), which is a modified tricyclic antidepressant that increases 5-HT uptake (Wagstaff et al., 2001).

Since stress has been associated with the onset of depression (Post, 1992) and loss in hippocampal volume (Sapolsky, 2000), the deficit in cognition and memory observed in human depressed patients (Duman et al., 2000) could be partially explained by stress-induced failure in neuronal plasticity (Czeh et al., 2001).

In addition, it is well known that stressful conditions can increase 5-HT levels in the whole hippocampus (for a review, see Chaouloff, 2000). In particular, restraint stress promotes a significant increase in hippocampal 5-HT levels (Thorre et al., 1997). Altogether, these findings suggest that not only neurotransmitter systems such as 5-HT, but also neuronal morphological events are involved in the pathogenesis of depression.

Neuronal plasticity requires cytoskeletal changes for synaptic remodelling such as more dynamic forms of microtubules (Popoli et al., 2000). Microtubules are highly dynamic polymers that exchange rapidly the polymerized tubulin dimers with a soluble subunit pool. A more dynamic microtubule, which can be defined as the major presence of the soluble form vs. the insoluble form, is essential for rapid intracellular mechanisms that result in elongation or shortening of cell processes, which are two major morphogenetic events occurring during neurogenesis (Meninger and Binet, 1989; Rochlin et al., 1996; Kalil et al., 2000; Popoli et al., 2000). Microtubules derive from the polymerization of tubulin proteins (heterodimers consisting of α- and β-subunits); they are abundant in neurons and exhibit high heterogeneity of components (Cleveland, 1987). The α-tubulin subunit of the microtubule is expressed in mammalian species in various isoforms that are related to different primary sequences and several post-translational modifications (MacRae, 1997). Among these modifications, the cyclic tyrosination/detyrosination of the C-terminus and the acetylation at Lys 40 have been shown to occur in many cell types and to be related to microtubule dynamics (MacRae, 1997). Highly dynamic microtubules are tyrosinated (Gundersen et al., 1987) whereas stable microtubules are acetylated (Contin and Arce, 2000). The expression of tyrosinated α-tubulin (tyr-tub) and acetylated α-tubulin (acet-tub) is currently used as markers of more dynamic or stable forms of microtubules, respectively (Gundersen et al., 1987; Rochlin et al., 1996; Farina et al., 1999; Contin and Arce, 2000; Farina et al., 2001).

Accordingly, the aim of the present work was to investigate by using Western Blot analyses of tyr- and acet-tub the possible microtubular changes occurring in the hippocampus of rats submitted to restraint stress. In addition, differential pulse voltammetry was used to assess ex vivo 5-HT levels in the hippocampus of the same animals as a marker of stress in order to confirm the validity of the restraint stress protocols used in the work.

MATERIALS AND METHODS

Animals

Adult male (250–300 g, Charles River, Wilmington, MA) Wistar rats were employed in this experiment. One week before the start of the experiment, rats were individually housed in controlled conditions (20 ± 2°C, 12 h/12 h light/dark cycles, with food and water available ad libitum). The animals were weighed and randomly assigned to experimental groups. The experimental procedures were in complete accordance with the guidelines of the "Principles of Laboratory Animal Care" (NIH publication No.86-23, revised 1985) as well as with the regulation of the Italian laws. All experiments were pre-reviewed and consented to by a local animal care committee and all efforts were made to minimize the number of animals used and their suffering.

Restraint stress

The animals were exposed to stress between 10:30 and 16:30 h. Restraint stress was performed by immobilising the animals by using a cylindrical plastic rodent restrainer. The animals were subdivided into three groups: (1) *Unstressed control group* (n = 4): rats were not subjected to stress, but were accustomed to handling 5 min every day for 4 days. (2) *Acute restraint stressed group* (n = 4): rats were submitted to restraint stress 6 h for 1 day. (3) *Sub-chronic restraint stressed group* (n = 4): rats were submitted to restraint stress 6 h for 4 days every day.

The animals were killed immediately after the last immobilisation period. Each brain was then rapidly removed, the hippocampus dissected on an ice-chilled plate and immediately homogenised in lysis buffer [5 mM Tris-HCl, 2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM pepstatin A, 1 mM leupeptin, 1 mM aprotinin, pH 8.0[(Cambray-Deakin and Burgoyne, 1987). The hippocampus homogenate was processed for Western Blot analyses of tyr- and acet-tub expression and *ex-vivo* Differential Pulse Voltammetry (DPV) analyses of 5-HT levels.

Western blot analysis of tyr- and acet-tub expression

The Western Blot analyses of tyr- and acet-tub expression were performed as previously described (Farina et al., 1999). Briefly, protein concentrations were determined by using a colorimetric assay (Bradford protein assay; Bio-Rad, Hercules, CA), and by setting the spectrophotometer to 595 nm. Samples (2 μg of total proteins) were diluted in a Laemmli buffer [62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 0.5% bromophenol blue (BPB)] (Laemmli, 1970). Protein samples were heated for 3 min at 99°C and proteins were separated on SDS—12% polyacrylamide gel electrophoresis, at 40 mA for 1 h and then transferred onto nitrocellulose membranes using a dry transfer unit at 50 mA overnight. Molecular size markers (205–6.5 kDa range, Sigma, St. Louis, MO; 182–8.4 kDa, Invitrogen, Paisley, UK) were used to locate tubulin (55 kDa) on the basis of its position on the gel. The membranes were blocked for 2 h with 5% skimmed powder milk in Tris-buffered saline (TBS) and incubated 1 h at 4°C with monoclonal antibodies clone TUB-1A2 (Sigma) specific to tyr-tub at 1:1,000 dilution or clone 6-11B-1 specific to acet-tub at 1:2,000 dilution in 0.1% skimmed powder milk in TBS. The membranes were then incubated with a 1:5,000 IgG anti-mouse alkaline phosphatase antibody (Promega, Madison, WI) for 30 min and the reaction developed using a stabilized substrate for alkaline phosphatase (Western Blue; Promega). The immunoreactivity of the tyr-tub bands obtained was quantified by densitometry (Molecular Analyst Software; Bio-Rad, Richmond, CA).

Ex vivo DPVoltammetry analysis of the 5-HT levels

5-HT levels were selectively measured within 200 μl of hippocampal homogenate using DPV associated with carbon fibre microelectrodes (mCFE) (Crespi, 1990) coated with Nafion in order to monitor selectively serotonin levels (Crespi et al., 1988). The three-electrode potentiostat system needed to perform voltammetry was prepared as described previously (Crespi, 1990; Stamford et al., 1992). Briefly, the reference electrode was silver/silver chloride (Ag/AgCl) and the auxiliary (counter) electrode was a silver wire, both approximately 100 μm in diameter. The DPV was applied by means of a μAUTOLAB polarograph (EcoChemie, The Netherlands) linked to an IBM PC computer equipped with a General Purpose Electrochemical System Software (GPES) package.

Data analyses

Results are presented as means ± S.E.M. Data were analysed by one-way analyses of variance (ANOVA) with a main factor of stress (acute, sub-chronic, control) and dependent variables of tyr-tub expression, acet-tub expression, and ex vivo 5-HT levels. The post-hoc Dunnett's test was used to evaluate significant differences between stress conditions. Statistical significance was set at a probability level of $P < 0.05$.

RESULTS

Western blot analysis of tyr and acet-tub expression

The bands obtained by Western Blot immunodetection were located between the bovine serum albumin (66 kDa) and the chicken egg ovalbumin (43 kDa) of the pre-stained color protein weight marker or between the bands at 63.8 and 49.5 kDa of the pre-stained blue protein weight marker. This finding is consistent with the molecular weight of tubulin, which is 55 kDa (Figs. 1A, 2A). ANOVA performed on the expression of tyr-tub in the hippocampus yielded a significant effect of stressful conditions ($F_{2,17} = 5.13$, $P < 0.05$). The densitometric analyses of the bands showed that tyr-tub expression was decreased to 90 ± 11% following acute restraint stress and significantly decreased to 70 ± 7% following sub-chronic restraint stress ($P < 0.01$, Dunnett's test) in comparison with unstressed control rats (Fig. 1B).

ANOVA also showed a significant effect of stress conditions ($F_{2,11} = 6.34$, $P < 0.05$) upon the expression of hippocampal acet-tub. The densitometric analyses of the acet-tub expression showed a significant increase ($P < 0.05$) to 139 ± 11% in rats submitted to acute restraint stress and a significant increase ($P < 0.01$) to 145 ± 11% following sub-chronic restraint stress (Fig. 2B).

Ex vivo DPVoltammetry analysis of 5-HT levels

In vitro tests using Nafion-covered mCFEs showed that in lysis buffer the hippocampal 5-HT potential was between 250–300 mV. Only peaks that fell between this potential range were considered for analyses (Fig. 3A). Restraint stress produced a significant increase in 5-HT levels in the hippocampus ($F_{2,11} = 5.42$, $P < 0.05$). Specifically, both acute and sub-chronic restraint stress significantly increased 5-HT levels compared with controls ($P < 0.05$, Dunnett's test) to 142 ± 15% and 135 ± 11%, respectively (Fig. 3B). In unstressed control rats, the real current value was 0.172 ± 0.006 nA, which corresponds to a 5-HT concentration of approximately 0.7 pg/μl as assessed by the in vitro calibration of the Nafion-covered mCFEs.

DISCUSSION

The main result of the present study is that restraint stress changes the status of the microtubular dynamics in the hippocampus. Specifically, the observed decrease of tyr-tub and increase of acet-tub expression indicates a less dynamic microtubular network. In addition, ex

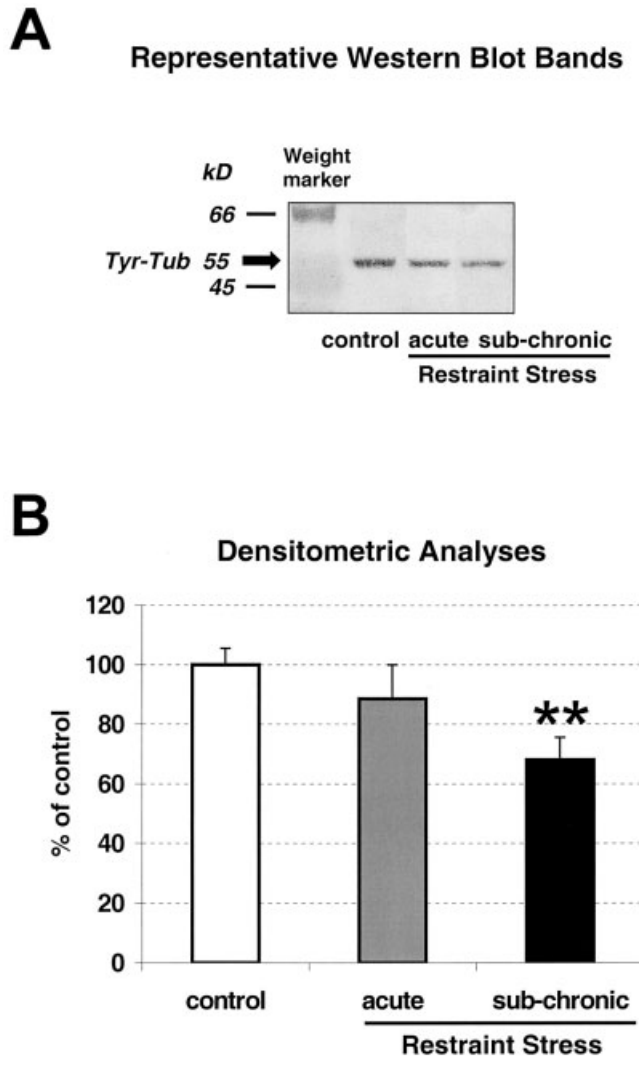

Fig. 1. **A:** Representative Western Blot showing the colour weights marker and the tyr-tub immunoreactivity in the hippocampus of rats submitted to acute or sub-chronic restraint stress. **B:** Densitometric analysis of the bands obtained via Western Blot. Results are expressed as percent of control (unstressed control rats). Mean ± S.E.M. $n = 4$. ANOVA: $F_{2,17} = 5.13$, $P < 0.05$. $^{**}P < 0.01$, Dunnett's test.

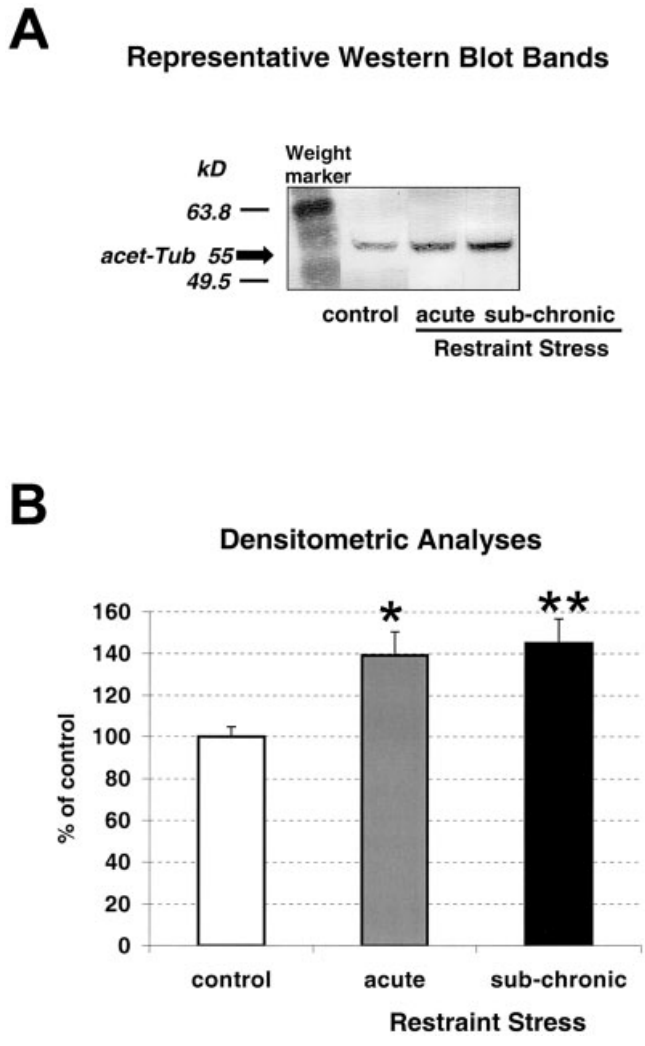

Fig. 2. **A:** Representative Western Blot showing the pre-stained blue protein weights marker and the acet-tub immunoreactivity in the hippocampus of rats submitted to acute or sub-chronic restraint stress. **B:** Densitometric analysis of the bands obtained via Western Blot. Results are expressed as percent of control (unstressed control rats). Mean ± S.E.M. n = 4. ANOVA: $F_{2,11} = 6.34$, $P < 0.05$. $^{*}P < 0.05$ $^{**}P < 0.01$, Dunnett's test.

vivo levels of 5-HT in homogenates from the hippocampus were increased both under the acute and sub-chronic conditions of restraint stress. Changes in neuronal morphology are strictly related to the dynamic status of cytoskeletal microtubules (Rochlin et al., 1996; Kalil et al., 2000; Azmitia, 2001). The microtubular network in neurons is dynamic, capable of assembly, disassembly, and rearrangement. These properties are due to the intrinsic dynamic properties of the polymers, which are determined by the biochemical properties of the tubulin αβ-heterodimer that is the microtubule building block. Axonal and synaptic remodelling requires more dynamic forms of microtubules (Meninger and Binet, 1989; Rochlin et al., 1996; Kalil et al., 2000; Popoli et al., 2000). When the microtubular network is stable (i.e., less dynamic), neuronal plasticity events such as axon extension are disrupted (Rochlin et al., 1996). The expression of tyr- and acet-tub in neurons is considered as an index of the dynamic status of microtubules (Rochlin et al., 1996; Contin and Arce, 2000; Farina et al., 2001). It has been shown that during CNS development, when plasticity requires more dynamic forms of microtubules, the expression of tyr-tub is increased (Cumming et al., 1984; Farina et al., 1999). In contrast, neurons treated with a microtubular stabilising drug such as nocodazole, show a decrease in the expression of tyr-tub and an increase in the expression of acet-tub (Rochlin et al., 1996).

Chronic restraint stress induces failure in neuronal plasticity in the rat and tree shrew hippocampus consisting in the retraction of apical dendrites in the CA3 region and in the suppression of neurogenesis of den-

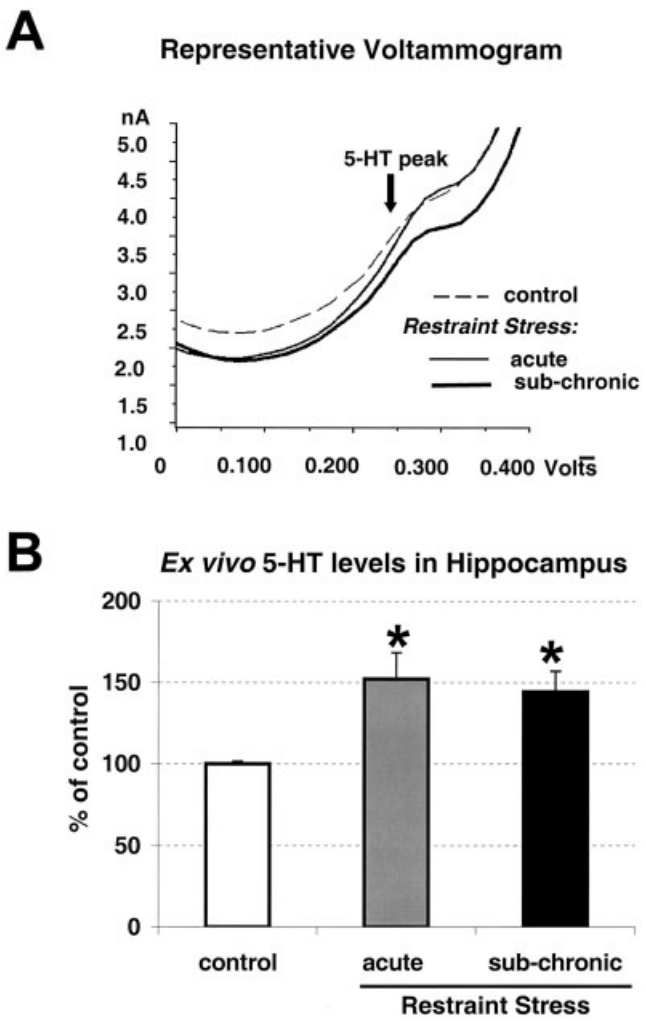

Fig. 3. **A:** Representative voltammogram showing the 5-HT peak in homogenates from the hippocampus of unstressed control rats (dashed line) vs. rats submitted to either acute (thick dashed line) or sub-chronic (solid line) restraint stress. **B:** 5-HT levels monitored by means of DPVoltammetry in homogenates from the hippocampus of unstressed control rats vs. rats submitted to either acute or sub-chronic restraint stress. Results are expressed as percent of control (unstressed control rats). Mean ± S.E.M. n = 4. ANOVA: $F_{2,11} = 5.42$, $P < 0.05$. *$P < 0.05$, Dunnett's test.

tate gyrus granule neurones (McEwen, 1999, 2000). These failures in neuronal plasticity are mimicked by both the systemic and oral administration of glucocorticoids (Wooley et al., 1990; Magarinos et al., 1998) and can be prevented by inhibiting the synthesis of adrenal steroids (Magarinos and McEwen, 1995). Furthermore, a 14-day treatment with glucocorticoids has been recently shown to increase microtubular stability in the cortex of mice (Liu and Brady, 2002). Taken together, these data suggest that changes in microtubular dynamics may be responsible for the failure in neuronal plasticity observed in the hippocampus after chronic restraint stress.

In the present study, the observed alterations in the microtubular network are faster events compared with the morphological changes reported by McEwen et al. (McEwen, 1999, 2000). In fact, morphological alterations take more than 14 days to be produced and become evident only after 21–35 days of 6 h of daily restraint (McEwen, 2000). These results lead to the hypothesis that a model of sub-chronic restraint stress induces molecular changes, which in turn will trigger a cascade of neuronal cytoskeletal changes that will become morphologically evident only later on.

The results of the present study also showed that 5-HT levels in homogenates from the hippocampus are elevated following both acute and sub-chronic restraint stress. These data confirmed the validity of the restraint stress model performed in this work and consequently the stress condition of the animals used to analyse the α-tubulin isoforms.

It is well established that the 5-HT system can be changed by acute or chronic stressful conditions in different brain regions (for a review, see Chaouloff, 2000). Although acute vs. chronic (long-term) stress may have opposite effects on 5-HT reuptake, 5-HT_{1A} receptor densities, or 5-HT_{1A} mRNA, both stressful conditions seem to have the same effect on 5-HT levels (for a review, see Chaouloff, 2000). For example, 45 min of acute restraint stress can increase ex-vivo 5-HT in the whole brain (Muller et al., 1976). Furthermore, mild stress such as tail pinch or handling, but also short exposures to new environment (Wright et al., 1992; Cadogan et al., 1994) or saline injection (Adell et al., 1997) can increase 5-HT levels throughout the hippocampus. Finally, stressful conditions characterised by a huge release of corticosterone such as restraint stress have been shown to promote a rapid and significant increase in hippocampal extracellular 5-HT levels (Vahabzadeh and Fillenz, 1994; Thorre et al., 1997). The latter findings could be explained by previous results showing that restraint stress can increase brain tryptophan levels (Kennet and Joseph, 1981) without altering the activity of tryptophan hydroxylase (Palkovits et al., 1976), thus resulting in increased intraneuronal synthesis of 5-HT. In contrast, it is also reported by a few authors that restraint stress decreased (Lee et al., 1987) or had no effect on hippocampal 5-HT levels (Amat et al., 1998).

Our data show that 5-HT levels increased in a similar manner under both the acute and sub-chronic restraint stress conditions, suggesting a long-term effect of stress upon this monoaminergic system. This effect could be related to the reported desensitisation of 5-HT_{1B} auto-receptors following restraint stress as it has been shown that these autoreceptors exert a negative control on release and metabolism of 5-HT (Bolanos-Jimenez et al., 1995; Seguin et al., 1997). It has also been shown that 5-HT-moduline, an endogenous tetrapeptide that specifically interacts with 5-HT_{1B} auto-receptors (Massot et al., 1996), increases in the hippocampus following acute restraint stress and con-

tributes to desensitisation of 5-HT_{1B} auto-receptors (Bonnin et al., 1999).

To the best of our knowledge, the findings reported in the present study represent the first molecular evidence of cytoskeletal alterations following restraint stress. A previous study (Kuroda and McEwen, 1998) failed to demonstrate any suppressive effect of restraint stress on Microtubule Associated Protein 2 (MAP2) gene expression. These findings suggest a lack of effect of stress on gene expression of cytoskeletal component. Since the expression of both tyr- and acet-tub is regulated by a post-translational cycle, such isoforms may be useful markers to evaluate stress-induced cytoskeletal changes.

We have recently shown that in rats treated with colchicine (microtubules disrupter), the in vivo raphe dorsalis cell firing and striatal 5-HT levels were increased whereas treatment with para-chlorophenylalanine (specific depletor of 5-HT) but not alpha-methyl para tyrosine (blocker of the synthesis of catechols) decreased tyr-tub expression in the whole brain (Crespi and Bianchi, 2002). Furthermore, the Forced Swimming Test decreased both tyr-tub expression and ex vivo 5-HT levels in the rat whole brain (Crespi and Bianchi, 2002). Thus, the results obtained in the whole brain suggest a possible relationship between the 5-HT and microtubules and a link between 5-HT and the expression of different isoforms of α-tubulin may be hypothesised also in the hippocampus. This possible link and the identification of the receptors involved will be subject of future studies.

In conclusion, the observed changes in the serotonergic and microtubular systems in the hippocampus following restraint stress confirm the structural (McEwen, 1999; Duman et al., 2000) and biochemical (Chaouloff, 2000) vulnerability of this limbic area to stressful conditions. Furthermore, the present findings support the idea that cytoskeletal changes occurring in the hippocampus represent a potential new pathway that may increase our understanding of stress-related events. The question of whether or not changes in 5-HT levels are *casually* related to changes in the expression of α-tubulin needs to be assessed in future studies.

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