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# Chronic stress, as well as acute stress, reduces BDNF mRNA expression in the rat hippocampus but less robustly

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**Abstract**

Daily restraint for 3 weeks was shown to atrophy dendrites of hippocampal pyramidal neurons in rats. Brain-derived neurotrophic factor (BDNF), which maintains neuronal survival and morphology, has been shown to decrease in response to acute stress. Plasma glucocorticoid (GC) and serotonergic projections from the raphe nuclei play major roles in reducing BDNF synthesis in the hippocampus. We investigated BDNF mRNA levels there, together with plasma GC levels, GC receptors in the hippocampus/hypothalamus and 5-HT synthesizing enzyme, tryptophan hydroxylase in the raphe nuclei, in animals chronically stressed for 1–3 weeks, using in situ hybridization and immunohistochemistry. In these animals, BDNF mRNA levels were significantly decreased in the hippocampus after 6 h of restraint, but the ability of restraint to reduce BDNF synthesis seemed less robust than that seen in acute stress models. HPA axis response to stress in these animals assessed by plasma GC levels was delayed and sustained, and the GC receptor in the paraventricular hypothalamic nucleus was increased at 1 week. Tryptophan hydroxylase immunoreactivity was increased in the median raphe nucleus at 2–3 weeks. Repetitive stress-induced reduction of BDNF may partly contribute to the neuronal atrophy/death and reduction of hippocampal volume observed both in animals and humans suffering chronic stress and/or depression.
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Brain-derived neurotrophic factor (BDNF), a member of neurotrophin family, has the widest distribution among the neurotrophins in the central nervous system (CNS), and is expressed at highest levels in the hippocampus and cerebral cortex (Schmidt-Kastner et al., 1996; Conner et al., 1997). BDNF is one of the key contributors in the development, survival, maintenance and plasticity of CNS neurons (Thoenen, 1995; Lewin, 1996). Since it plays an important role in the maintenance of dendritic arborization of various CNS neurons (McAllister et al., 1997; Schwartz et al., 1997; Horch et al., 1999), its deficiency may result in retraction or atrophy of dendrites.

It has been well established that repeated daily restraint stress for 21 days or long-term corticosterone administration cause dendrites of CA3 pyramidal neurons to retract (Woolley et al., 1990; Watanabe et al., 1992). It is reasonable to assume that stress-induced decrease of BDNF synthesis in the hippocampus may contribute to the pathogenesis of dendritic retraction. Therefore, we analyzed the change of BDNF mRNA expression in the hippocampus of rats subjected to repeated restraints for 3 weeks.

In recent clinical studies, smaller hippocampal volume was observed in patients with recurrent depressive disorder and post-traumatic stress disorder (Bremner et al., 1995; Sheline et al., 1996). Patients with depressive or eating disorders show low serum BDNF levels, and treatment with antidepressants restores those levels (Nakazato et al., 2003; Shimizu et al., 2003). These studies suggest that BDNF deficiency plays a pivotal role in the pathophysiology of psychiatric disorders and associated hippocampal atrophy.

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In animal models, acute stress elicits distinct down-regulation of hippocampal BDNF mRNA expression (Smith et al., 1995; Ueyama et al., 1997). However, BDNF synthesis has not been precisely assessed in chronically stressed animals and contribution of BDNF deficiency on stress-induced dendritic atrophy is still obscure. Especially, a previous study excluded the possibility of BDNF hypothesis showing that at 21 h after the termination of the last session of restraint repeated daily for 3 weeks hippocampal BDNF mRNA expression did not differ from that of control rats (Kuroda and McEwen, 1998). In the present study, we applied the same stress paradigm to rats of the same strain as Kuroda et al. did, and assessed hippocampal BDNF mRNA levels both at earlier and later time points, in addition to the time course change in expression during 3 weeks, in order to re-evaluate the role of BDNF on hippocampal atrophy in this chronic stress model.

The synthesis of BDNF in the hippocampus is regulated by various neurotransmitters and hormones (Thoenen et al., 1991; Lindholm et al., 1994). Corticosterone and 5-HT are the candidates that are well known and best studied. Serotonin fibers innervating the hippocampus are originated from the midbrain raphe nuclei (Azmitia and Segal, 1978). Biosynthetic levels of serotonin can be monitored by measuring its rate-limiting enzyme, tryptophan hydroxylase (TPH). Our previous study has revealed that repeated restraint stress increased TPH protein levels in the rostral ventromedial medulla (RVM), which includes the raphe magnus (Imbe et al., 2004). However, the effect of repeated stress on TPH levels in the midbrain raphe nuclei is not fully examined. Therefore, the time course changes in plasma glucocorticoid (GC) levels, GC receptors (GRs) in the hippocampus/hypothalamus and a key enzyme of 5-HT synthesis, TPH, in the midbrain raphe nuclei were also investigated in these animals.

## 1. Materials and methods

### 1.1. Experimental animals

Male Sprague–Dawley rats were purchased from SLC Japan, and housed two rats per cage at least for 1 week before they were used for experiment. All animals were maintained with free access to food and tap water and under standard conditions, with lights on between 08:00 h and 20:00 h and room temperature controlled at 24 °C. All the experiments were approved by the Animal Research Committee of Wakayama Medical University and carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

At 8 weeks of age, 6 h (10:00–16:00 h) of restraint stress was applied to rats following the previously reported procedure (Kuroda and McEwen, 1998), using wire-mesh restrainers. Some of these rats were sacrificed immediately after the termination of the stress session (acute-stress group), and others were returned to their home cages and allowed to recover until 16:00 h on the next day and sacrificed (acute-stress + 1 day group). Other groups of rats received the same 6 h of restraint daily for 1–3 weeks. In repeated stress groups, stress period began at the age of 7–5 weeks, and all rats were sacrificed at the age of 8 weeks immediately after the termination of the last stress session at 1 week, 2 weeks or 3 weeks, respectively (1–3W-stress groups). To assess the basal conditions of chronically stressed rats, other groups of rats were sacrificed at 16:00 h on the last day without being subjected to the last stress session (1–3W-baseline groups). Untreated rats of 8 weeks of age served as the control group. The procedure of repeated restraint stress is summarized in Fig. 1.

For plasma corticosterone assays, rats were killed at two different time points, 12:00 h and 16:00 h. Being transferred to the testing room, rats were allowed to remain there for 2 h (Control 2 h) or 6 h (Control 6 h), or received a single stress session for 2 h (Acute 2 h) or 6 h (Acute 6 h). Rats of chronic stress groups, which had been subjected to daily restraint for 1–3 weeks, were transferred to the testing room. They were allowed to remain there for 6 h (1–3W-baseline groups), or received a single stress session for 2 h (1–3W 2 h) or 6 h (1–3W 6 h).

### 1.2. In situ hybridization (ISH) histochemistry

Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and sacrificed by decapitation. Brains were rapidly removed, frozen on dry ice and stored at −80 °C. Coronal sections of 12 μm thickness were cut on a cryostat and thaw mounted onto silane-coated glass slides. Tissue sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 15 min, acetylated and dried. Levels of BDNF mRNA were examined by probing with $^{35}$S-labeled riboprobes as previously described (Ueyama et al., 1997). Briefly, antisense- and sense-strand mouse BDNF cRNA probes were prepared from *Eco*RI- and *Bam*HI-linealized pGEM-mBDNF, provided by Dr. Shoei Furukawa (Gifu Pharmaceutical University, Gifu, Japan), using SP6 RNA polymerase and T7 RNA polymerase (Promega), respectively, and [α-$^{35}$S]UTP (Dupon/NEN Research Product). The sections were hybridized for 16 h at 55 °C with $1 \times 10^6$ cpm of labeled probe per slide in 100 μl of hybridization buffer containing 50% formamide, 600 mM NaCl, 10 mM Tris, 1× Denhardt's solution, 1 mM EDTA, 100 mM DTT, 10% dextran sulfate, 500 μg/ml tRNA and 0.25% SDS. After hybridization, sections were washed with 2× SSC/50% formamide at 68 °C for 30 min, then treated with 25 μg/200 ml ribonuclease A (Sigma) for 30 min in 10 mM Tris–HCl buffer containing 0.5 M NaCl and 1 mM EDTA. The slides were then washed in 2× SSC for 15 min at 37 °C and 0.1× SSC for 15 min at 55 °C, dehydrated in graded ethanol and air-dried. The hybridized sections were dipped in NTB-2 liquid emulsion (Kodak, Rochester, NY,

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