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The Effects of Repeated Restraint Stress on the Synaptic Plasticity in the Inner Molecular Layer of Mouse Dentate Gyrus

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Stress induces degeneration of brain structures and functions. Particularly, hippocampus is sensitive to stressful stimulations. In the present study, the change of synaptic related molecules in the mouse dentate gyrus was examined with immunohistochemistry after restraint stress.

We subjected mice to restraint stress for 6 h per day for 4 days. As a result, the number of Ki-67, a marker for proliferation, and doublecortin (DCX), a marker for neurogenesis, immunoreactive cells was decreased in the stress group. On the other hand, the intensity of calbindinD-28k, a marker of pre-existing granule cells, immunoreactivity was increased in the granule cell layer after 4 days restraint stress.

As well as, the immunoreactivity of synaptic related molecules, postsynaptic density-95 (PSD-95), growth association protein-43 (GAP-43) and β-NADPH-d reactivity were increased in the inner molecular layer of dentate gyrus after 4 days restraint stress.

In conclusion, this study shows that repeated restraint stress suppresses neurogenesis in dentate gyrus and strengthens synaptic plasticity of existing granule cells.

Key words : Restraint stress, Dentate gyrus, Inner molecular layer, Post synaptic density-95, Doublecortin, Growth associated protein-43, β-NADPH-d

Introduction

Several studies have shown that acute and chronic stress produces structural and functional changes in the brain, especially in the hippocampus (McEwen 2000, Kim and Diamond 2002). The chronic stress-induced hippocampal structural changes include atrophy of apical dendrites of CA3 pyramidal neurons which can be prevented by antidepressant or benzodiazepine treatment (Watanabe et al. 1992, Magarinos et al. 1999). The functional changes may include decreased mRNA levels of specific proteins linked to neural and synaptic plasticity, such as growth-associated protein 43 (GAP-43), brain-derived neurotrophic factor (BDNF), and synaptophysin (Kuroda and McEwen 1998, Nibuya et al. 1999, Thome et al. 2001). Interestingly, these structural and functional changes have also been shown in subjects suffering from psychiatric illnesses, like major depression and bipolar disorders (Sheline et al. 1996, Hrdina et al. 1998, Bremner et al. 2000, Fuchs and Gould 2000, Reid and Stewart 2001). Therefore, such findings suggest that stress may have linkage with synaptic

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plasticity and mental disorders.

Stress also has been known to regulate adult neurogenesis in animals (Fuchs and Gould 2000, Jacobs et al. 2000, Duman et al. 2001). The process of neurogenesis occurs mainly in the subventricular zone adjacent to the lateral ventricles and in the subgranular zone of the dentate gyrus of the hippocampus. In the dentate gyrus, the newly generated cells integrate into the granule cell layer and develop the morphological characteristics of mature granule neurons able to form functional synapses (van Praag et al. 2002). In the rat, adrenal steroid hormones, chronic restraint stress, inescapable foot-shock stress, and chronic restraint stress has been shown to suppress this adult hippocampal neurogenesis, measured by the BrdU labeling method (Cameron and Gould 1994, Czeh et al. 2002, Malberg and Duman 2003, Pham et al. 2003). Moreover, antidepressant treatment increases neurogenesis in animals (Malberg et al. 2000), and depression reduces hippocampal volume (Sheline et al. 1996, Bremner et al. 2000). Thus, it was hypothesized that the change of adult hippocampal neurogenesis are important factor in the precipitation and the recovery from episodes of depression (Jacobs et al. 2000).

Nitric oxide (NO) is a free radical with signaling functions in the central nervous system (Garthwaite and Boulton 1995). In the brain, NO is produced enzymatically in postsynaptic structures, and is made from L-arginine by NO in response to the activation of N-methyl-D-aspartate (NMDA) receptors by the excitatory amino acids (Moncada et al. 1991). NMDA receptor activity appears to play a role in some neurophysiological phenomena (Croucher et al. 1982). Neuronal NOS, a Ca^{2+}-activated form of NOS, can bind to postsynaptic density-95 (PSD-95) (Sattler et al. 1999). Therefore, PSD-95 may concentrate nNOS near the NMDA receptor at postsynaptic sites in the neurons. Granule cells in the dentate gyrus establish functional connections in the dentate molecular region, and receive excitatory synaptic input from entorhinal cortex perforant path afferents (van Praag et al. 2002). The localization of postsynapse signaling molecules, PSD-95 and nNOS, in the inner molecular layer of the dentate gyrus may represent the synapse between dendrites of granule cells and NMDA fibers from the medial entorhinal cortex.

The nuclear protein Ki-67, which is expressed in all phases of the cell cycle except the resting phase, can be used for determination of progenitor cells of adult neurogenesis. Recent studies showing increased Ki-67-positive cells in the hippocampus after chronic antidepressant treatment similar to what was found by using the BrdU labeling method (Malberg et al. 2000, Rosenbrock et al. 2004).

The present study examined the effect of stress on the cell proliferation and differentiation using Ki-67 and doublecortin (DCX) immunohistochemistry (Couillard-Despres et al. 2005) in the dentate gyrus, and then the effect of stress on the synaptic related molecules, nitric oxide synthase (NOS), using nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d; used to detect the presence of NOS neurons) reactivity (Dawson et al. 1991, Hope et al. 1991), PSD-95 and growth associated protein-43 (GAP-43) immunohistochemistry in the dentate gyrus.

Materials and Methods

1. Animals

Three-month-old male C57BL/6 mice were used in all the experiments. The mice were treated and maintained in accordance with the animal care guidelines of the NIH and the Korean Academy of Medical Sciences. The mice were maintained at a controlled temperature (20±2°C), and they were group-housed (12 hr light/dark cycle) with access to food and water *ad libitum*.

The mice were divided into two groups of 10 mice each consisting of one stress and one sedentary con-

trol group. The sedentary control group animals were placed in standard mouse cages at 5 animals/cage for 4 days, whereas the stress group animals were kept in restraint tube for 6 h a day for 4 days (10:00AM and 4:00PM). The mice of stress group were individually placed in a room adjacent to their colony in an independent plastic compartment and immobilized in a 12 × 3 cm plastic bottle, with a plastic taper on the outside and a 0.5 cm hole at one end for breathing. After the termination of each daily restraint stress session, the mice were returned to their home cages. The sedentary control group mice were handled as their tested littermates except that they were not immobilized.

2. Histological procedures

The mice were fully anesthetized with pentobarbital (50 mg/kg, i.p.), and were transcardially perfused with 50 mM phosphate-buffered saline (PBS), followed by a freshly prepared solution of 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brains were removed and post-fixed in the same fixative overnight, and then the brains were placed into 30% sucrose in 50 mM PBS. Serial 30 μm-thick coronal sections were sliced on a freezing microtome (Leica, Nussloch, Germany), and the sections were stored in cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05 M PB, pH 7.4) at −20˚C until further use.

3. Immunohistochemistry

Every 12th brain section was taken from the region between Bregma −1.06 mm and Bregma −3.28 mm, and this was done for each brain (Paxinos and Franklin 2004). The free-floating sections were pre-incubated for 15 min in 1% H_2O_2. Following extensive washes in phosphate buffered saline (PBS), sections were incubated with the primary antibodies. Sections were incubated with the primary antibodies [rabbit anti-Ki-67 (Novocastra laboratories, New castle, UK, 1 : 1000), goat anti-doublecortin (Santa cruz, USA, 1 : 1000), mouse anti-calbindinD-28k (Sigma, USA, 1 : 1000), mouse anti-PSD-95 (Upstate, NY, USA, 1 : 1000), mouse anti-GAP-43 (Sigma, USA, 1 : 1000)] overnight at 25˚C in 0.3% Triton X-100 and 0.5 mg/mL bovine serum albumin. The sections were then incubated with the anti-rabbit or anti-rat secondary antibodies (1 : 200 dilution; Vector, Burlingame, CA, USA) for 90 min. For chromogenic immunodetection, sections were treated with an avidin-biotin-peroxidase complex (1 : 200 dilution; Vestastatin Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Peroxidase activity was visualized by incubating the sections with 0.02% 3, 3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2, in 0.5 M Tris buffered saline, (pH 7.6). After several rinses, sections were mounted on gelatin coated slices, dehydrated and then cover-slipped using Histomount medium. For epifluorescence immunodectection, sections were washed extensively and incubated with fluorochrome-conjugated species-specific secondary antibodies (anti-rabbit Cy3 and anti-rat Cy2 (1 : 200; Jackson ImmunoResearch, USA). Sections were placed on gelatin coated slices and mounted in Prolong Antifade kit (Molecular Probes, USA). Epifluorescence observation and photo-documentation were accomplished using a confocal microscope (Zeiss Axiovert LSM 510 META, Germany).

4. NADPH-d histochemistry

The sections were stained to detect NADPH-d according to a NADPH-d-specific histochemical method. Briefly, the free-floating sections were incubated at 37˚C for 30 ~ 60 min in 0.1 M PB (pH 7.4) containing 0.3% Triton X-100, 0.1 mg/mL nitroblue tetrazolium and 1.0 mg/mL β-NADPH.

5. Quantitative evaluation of staining

The optical density of the NADPH-d reactivity PSD-95, and GAP-43 in the inner molecular layer was

quantitatively assessed according to a microdensitometrical method using an image analysis system (Multiscan, Fullerton, CA, USA). The boundary of the inner molecular layer were manually traced before being measured. Digitally fixed images at 200× magnification were analyzed under an optical microscope equipped with an image analyzer. Pixel values of 0 and 255 correspond to white and black colors, respectively. The number of Ki67-, and DCX-immunoreactive cells in the inner rim of the granule cell layer of the dentate gyrus were counted stereologically with Stero Investigator software (MicroBrightfield williston, VT) by using an optical fractionator. The total dentate gyrus volume was determined using Cavalieri's principle, and the cross-sectional areas were calculated using an image analysis system (Multiscan, Fullerton, CA, USA). We observed no changes in the volume of the granule cell layer or the volume of the dentate gyrus during any of the experiments that were conducted. Hence, the results are expressed as good estimates of the total numbers of immunoreactive cells. At this point, each sample was given a coded identification number so that the data could be analyzed in a "blind" manner. The raw numbers and optical density data were subjected to one-way ANOVA testing followed by Dunnett's test of post hoc comparisons; the results were expressed as mean±S.E.M. The differences were considered significant when p values were <0.05.

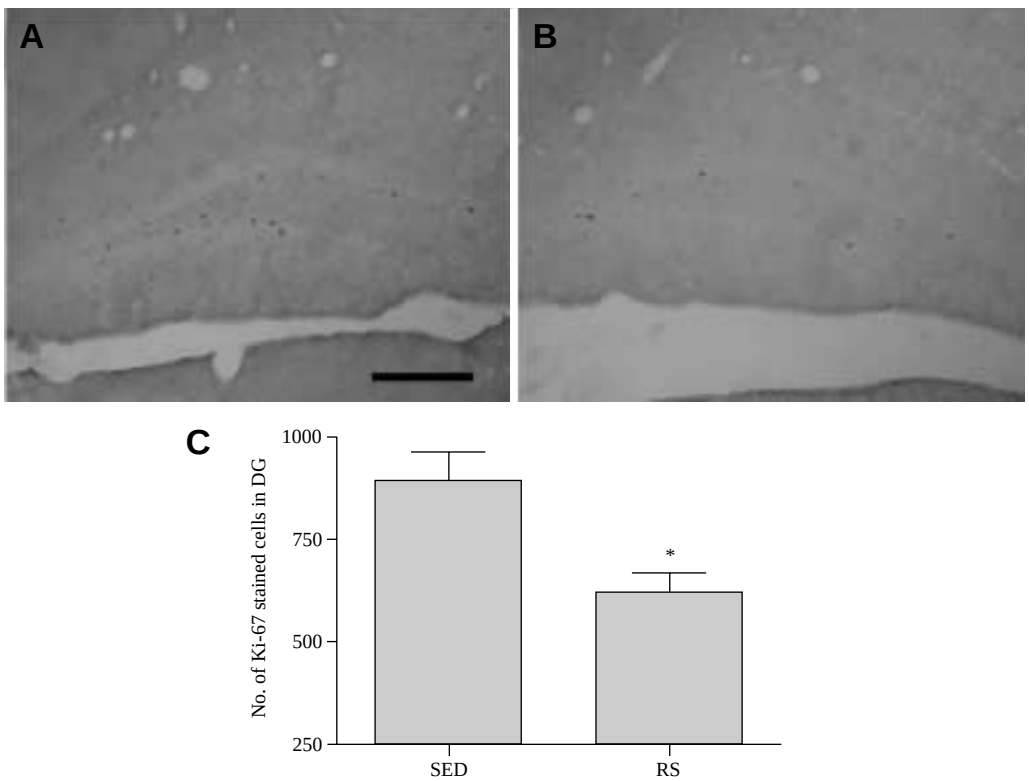

Fig. 1. Stress decreases the number of Ki-67 immunoreactive cells in the dentate gyrus. A, sedentary control group; B, 4 days restraint stress group. SED, sedentary; RS, restraint stress. Values indicate the mean number of Ki-67 immunoreactive cells ±S.E.M. * vs. SED ($p<0.05$) by Dunnett's post hoc comparisons. Scale bar: A-B, 250 μm.

Results

The number of Ki-67 immunoreactive cells in the dentate gyrus was significantly decreased in the stress group (618±52) than in the control group (893±75) ($p<0.05$) (Fig. 1). The number of DCX-stained cells in the dentate gyrus was significantly decreased in the stress group (2,833±49) than in the sedentary control group (3,610±61) ($p<0.05$) (Fig. 2).

In an attempt to investigate the change of the acti-

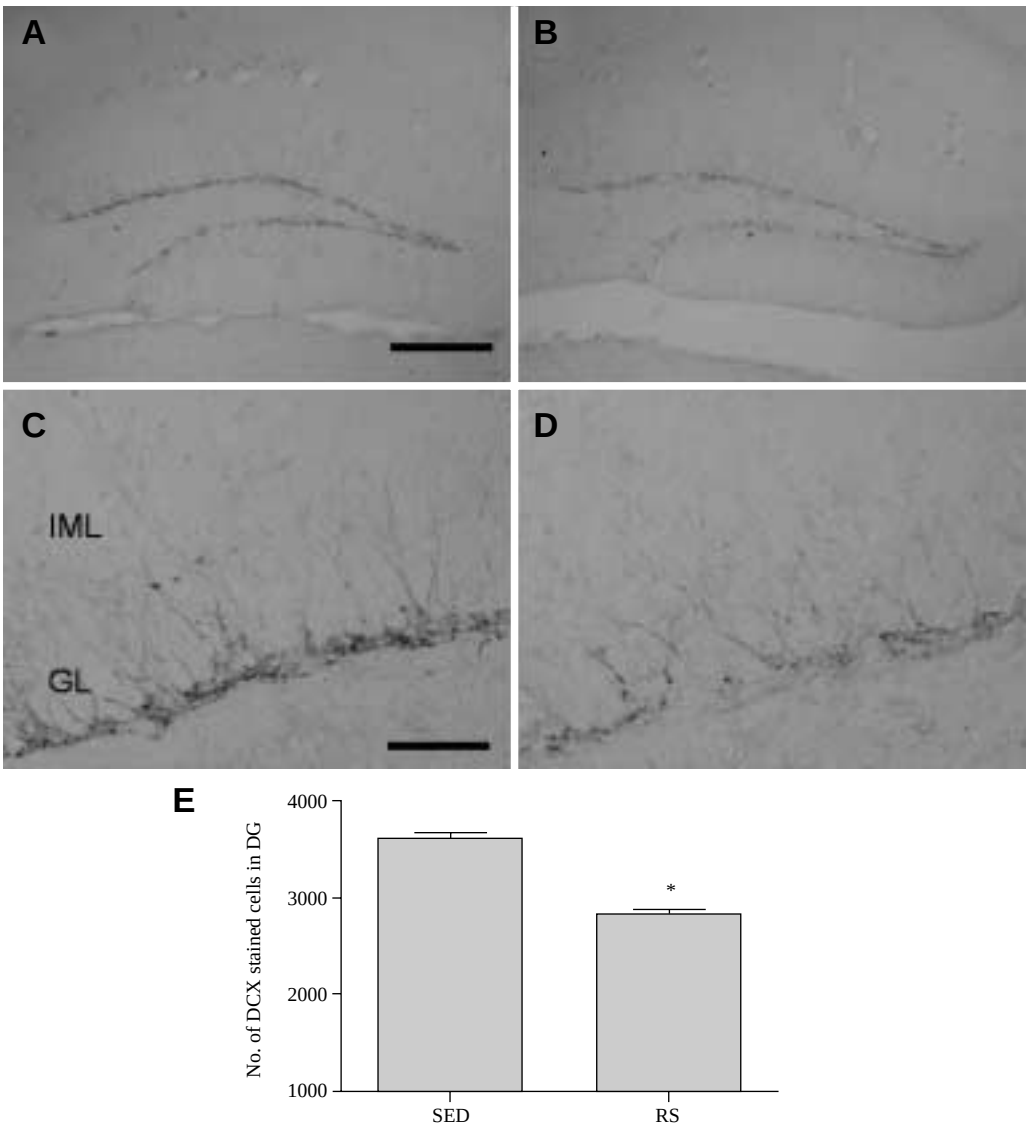

Fig. 2. Stress decreases the number of DCX immunoreactive cells in the dentate gyrus. A and C, sedentary control group; B and D, 4 days restraint stress group. SED, sedentary; RS, restraint stress, IML, inner molecular layer; GL, granule cell layer. Values indicate the mean number of DCX immunoreactive cells ±S.E.M. * vs. SED ($p<0.05$) by Dunnett's post hoc comparisons. Scale bar: A-B, 250 μm; C-D, 50 μm.

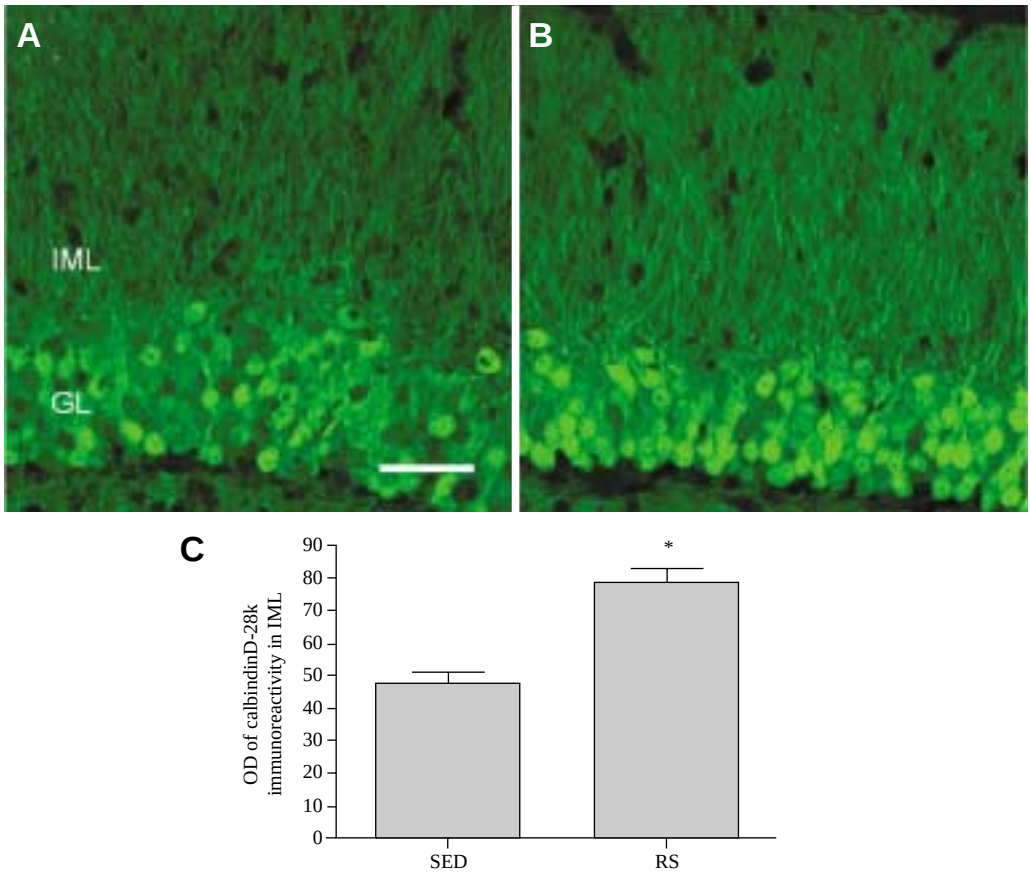

Fig. 3. Stress increases the optical density (OD) of calbindinD-28k immunoreactive cells in the dentate gyrus. A, sedentary control group; B, 4 days restraint stress group. SED, sedentary; RS, restraint stress, IML, inner molecular layer; GL, granule cell layer. Values indicate the mean OD of calbindinD-28k immunoreactive cells ±S.E.M. * vs. SED ($p < 0.05$) by Dunnett's post hoc comparisons. Scale bar: A-B, 50 μm.

vity of pre-existing granule cells in the hippocampus, the immunoreactivity of calbindinD-28k was analyzed (Dumas et al. 2004). The optical density of calbindinD-28k in the granule cell layer was increased in the stress group (78.8±4.25) compared with the sedentary control group (47.7±3.5) ($p < 0.05$) (Fig. 3).

PSD-95 and nNOS were analyzed in the inner molecular layer of dentate gyrus with using immunohistochemistry and NADPH-d histochemistry. The optical density of PSD-95 in the inner molecular layer was increased in the stress group (91.7±2.3) compared with control group (67.8±1.5) ($p < 0.05$) (Fig. 4). The intensity of the NADPH-d staining of the dentate gyrus was increased in the inner molecular layer of the dentate gyri of the stress group (107.9±1.8) when compared with the control group (83.6±2.38) ($p < 0.05$) (Fig. 5).

GAP-43 was analyzed in the inner molecular layer of dentate gyrus with immunohistochemistry. The optical density of GAP-43 in the inner molecular layer was increased in the stress group (115±1.6) compared with control group (98.7±1.4) ($p < 0.05$) (Fig. 6).

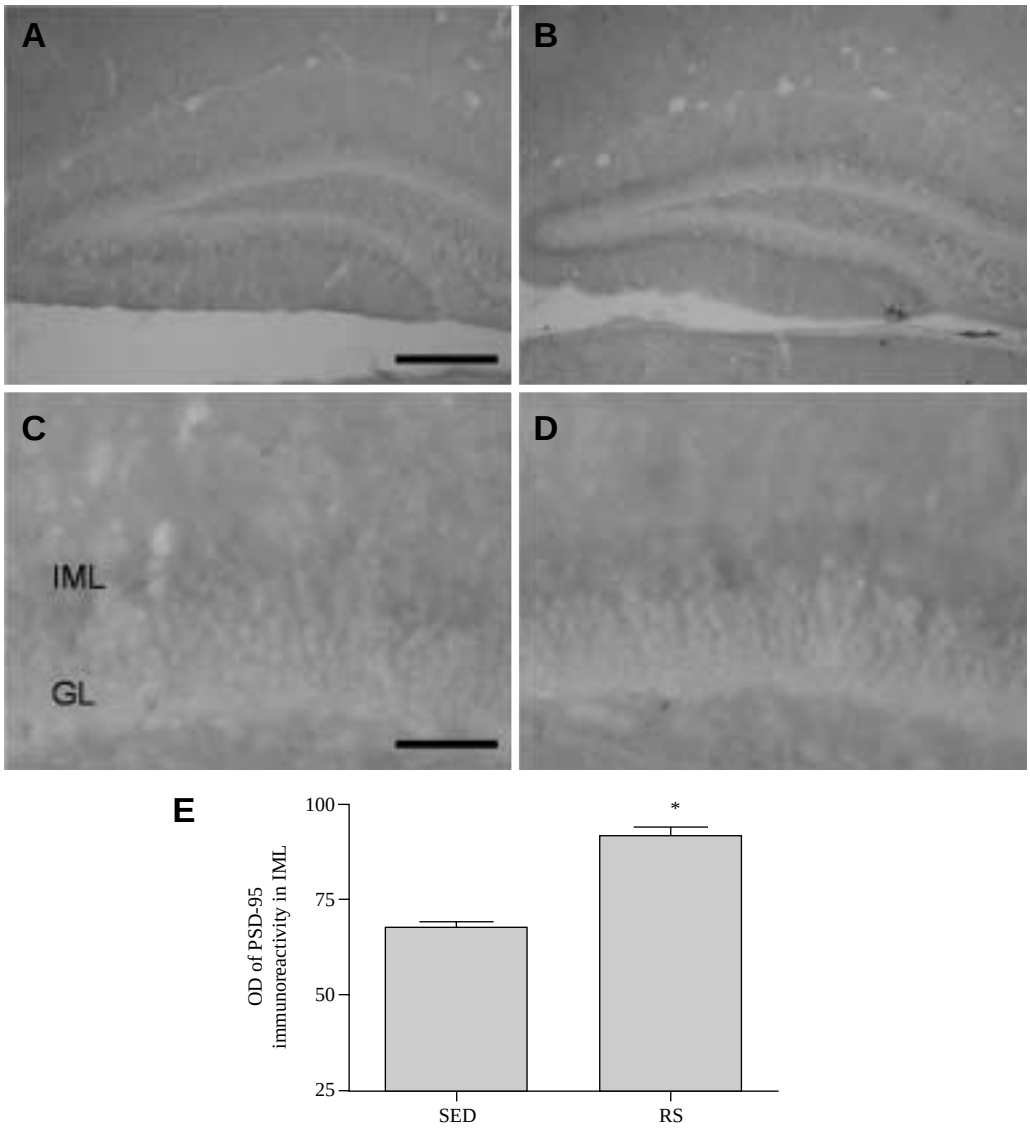

Fig. 4. Stress increases the optical density (OD) of PSD-95 immunoreactivity in IML of dentate gyrus. A and C, sedentary control group; B and D, 4 days restraint stress group. SED, sedentary; RS, restraint stress, IML, inner molecular layer; GL, granule cell layer. Values indicate the mean OD of PSD-95 immunoreactivity ±S.E.M. * vs. SED ($p < 0.05$) by Dunnett's post hoc comparisons. Scale bar: A-B, 250 μm; C-D, 50 μm.

Discussion

The present study was designed to follow the change of neurogenesis and synaptic activity of granule cells after stress. We have found that the decrease of neurogenesis after stress is concomitant with the increase of synapse activity of the granule cells under stress con-

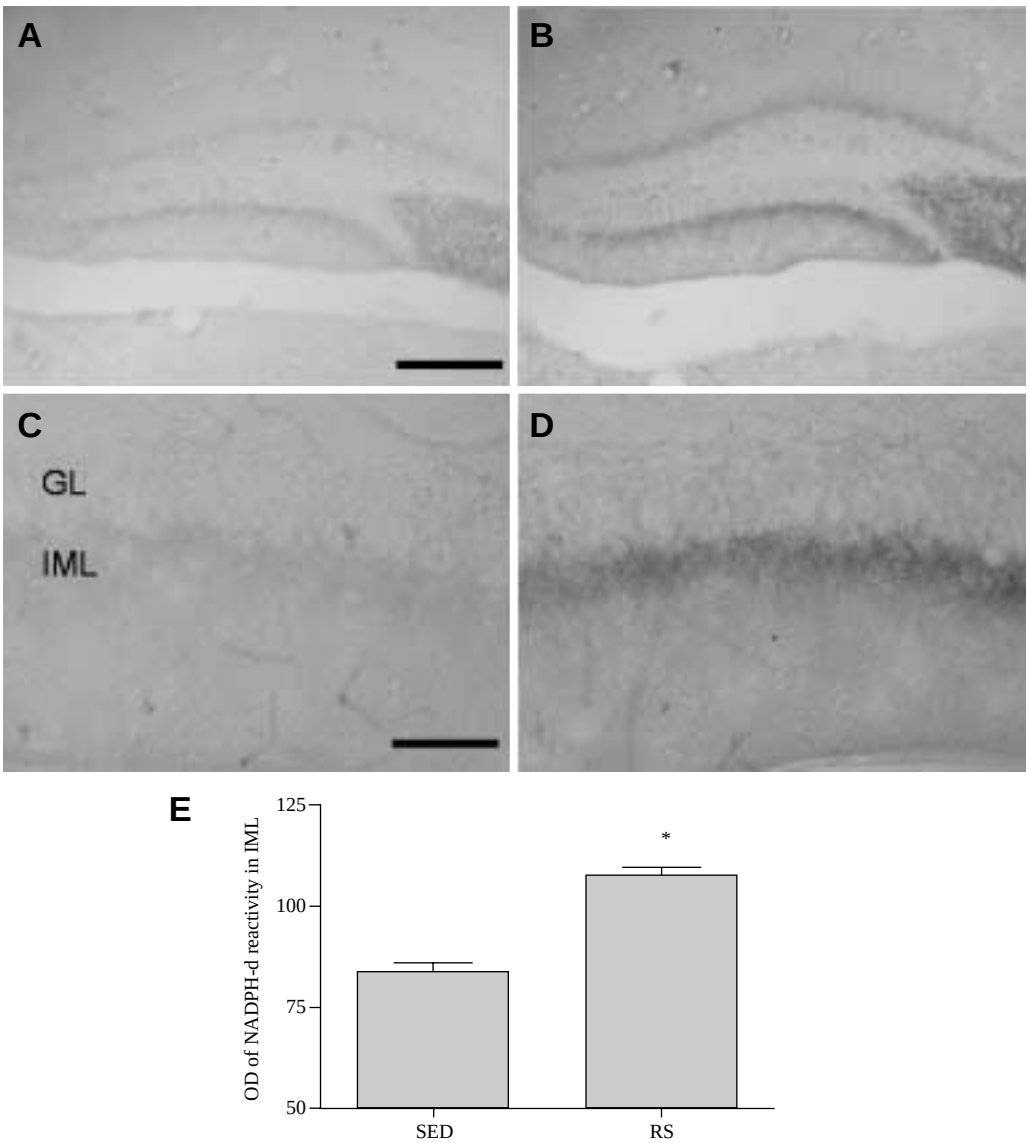

Fig. 5. Stress increases the optical density (OD) of NADPH-d reactivity in IML of dentate gyrus. A and C, sedentary control group; B and D, 4 days restraint stress group. SED, sedentary; RS, restraint stress, IML, inner molecular layer; GL, granule cell layer. Values indicate the mean OD of NADPH-d reactivity ±S.E.M. * vs. SED ($p < 0.05$) by Dunnett's post hoc comparisons. Scale bar: A-B, 50 μm; C-D, 50 μm.

dition. The present results may be the first morphological evidence that intermittent stress may induce synaptic activity of granule cells of dentate gyrus.

Preclinical studies have suggested that restraint stress may change brain structure and activity (McEwen 2000, Kim and Diamond 2002). These changes may depend on stress intensity and/ or on de novo gene transcription and synthesis of proteins involved in

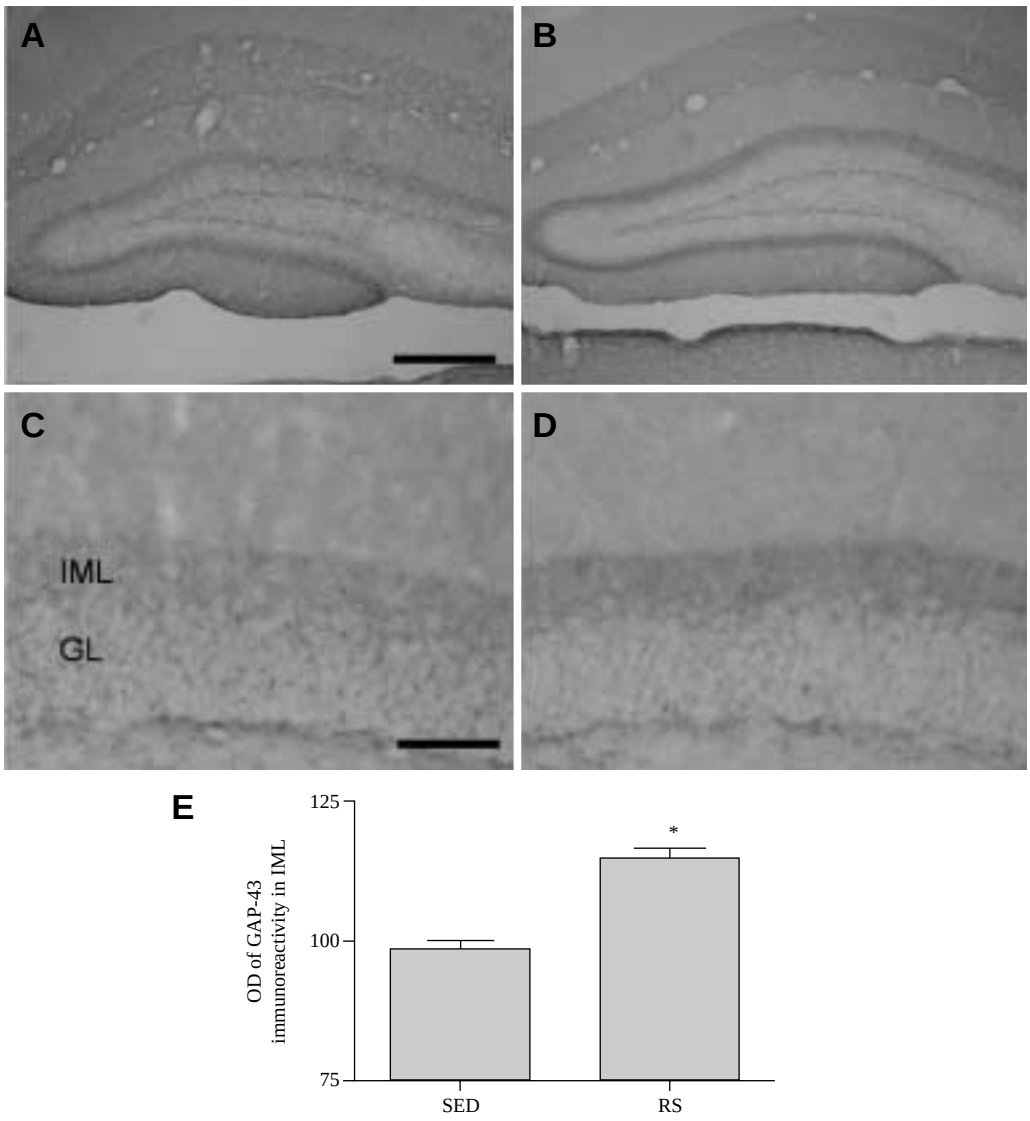

Fig. 6. Stress increases the mean optical density (OD) of GAP-43 immunoreactivity in IML of dentate gyrus. A and C, sedentary control group; B and D, 4 days restraint stress group. SED, sedentary; RS, restraint stress, IML, inner molecular layer; GL, granule cell layer. Values indicate the OD of GAP-43 immunoreactivity ±S.E.M. * vs. SED ($p < 0.05$) by Dunnett's post hoc comparisons. Scale bar: A-B, 250 μm; C-D, 50 μm.

neuronal and synaptic plasticity. Using in situ hybridization histochemistry, mRNA expression levels of synaptophysin and GAP-43, markers for synaptic plasticity, were shown to be slightly decreased in the CA3 areas of the hippocampal formation after chronic restraint stress for 1 h per day for 5 days and 6 h per day for 21 days, respectively (Kuroda and McEwen 1998). On the other hand, the expressions of these

molecules in the dentate gyrus were not changed after the stresses. Differentially from these findings, Rosenbrock et al. (2005) observed the increase in hippocampal expression of those markers on their protein levels after acute restraint stress, although the change was not significant. In the present study, the 6 h per day for 4 days restraint stress induced the increase of synaptic related molecules, PSD-95 and GAP-43, in the inner molecular layer of dentate gyrus, which suggests that the intermittent restraint stress could increase synaptic activity (Altrock et al. 2003, Ehrlich and Malinow 2004) in the specific region of hippocampus.

Another important effect of stress treatment is its suppressing influence on adult neurogenesis in the hippocampus. This was demonstrated after chronic psychosocial and chronic restraint stress by using the BrdU labeling method (Czech et al. 2002, Pham et al. 2003). Some reports demonstrated that the proliferation marker Ki-67 can be used as a valuable alternative to BrdU labeling for determination of brain progenitor cells linked to adult neurogenesis (Heine et al. 2004). In addition, the present study showed that the decrease of Ki-67 protein correlated with the change of doublecortin immunoreactivity in the dentate gyrus of stressed animals. The reduction of hippocampal progenitor cells after restraint stress as measured in this study indicates a suppression of hippocampal neurogenesis in stress animals.

On the post-synaptic side, chemical synapses contain a large number of diverse proteins involved in synaptic structure, neurotransmitter and signal transductions (Kennedy 2000). These proteins are organized in to a dense, macromolecular assembly visible in electron micrographs as a post-synaptic density (PSD). One prominent component of the PSD at synapses in the hippocampus is PSD-95 (Hunt et al. 1996). PSD-95 interacts with a number of other post-synaptic proteins, including NMDA receptors (Kennedy 2000). In the brain, NO is produced enzymatically in postsynaptic structures, and is made from L-arginine by NO in response to the activation of N-methyl-D-aspartate (NMDA) receptors by the excitatory amino acids (Moncada et al. 1991). NMDA receptor activity appears to play a role in some neurophysiological phenomena (Croucher et al. 1982). Neuronal NOS, a Ca^{2+}-activated form of NOS, can bind to PSD-95 (Sattler et al. 1999). Therefore, PSD-95 may concentrate nNOS near the NMDA receptor at postsynaptic sites in the neurons. In the present study, the change of PSD-95 and nNOS expression demonstrates that the change of synaptic activity of granule cells occur in the inner molecular layer of dentate gyrus after stress. To the best of our knowledge, the present study is the first to demonstrate the morphological evidence that mild restraint stress increases the synaptic activity specifically in the inner molecular layer of the hippocampal dentate gyrus of mice.

In the normal brain, the major afferent projections to the molecular layer of the dentate gyrus originate in the entorhinal cortex (Steward 1976). These entorhinal afferents display a topographical arrangement, with axons from medial entorhinal areas terminating in the inner molecular layer onto the proximal apical dendrites of the dentate granule cells. Granule cells establish functional connections in the dentate molecular region, and receive excitatory synaptic input from perforant path afferents (van Praag et al. 2002). We also evaluated the immunoreactivity of growth associated protein-43 (GAP-43), which is a neuronal phosphoprotein localized in growth cones (Skene et al. 1986). The concomitant change of GAP-43 and PSD-95 in the inner molecular layer of the dentate gyrus could indicate that the synapse between dendrites of granule cells and NMDA fibers from the medial entorhinal cortex are changed after restraint stress.

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반복적인 부동스트레스가 치아이랑 속분자층의 연접 가소성에 미치는 영향

윤미숙[1], 문민호, 정주원, 김정혜, 안희경, 허영범, 박 찬
경희대학교 의과대학 해부학교실
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간추림 : 스트레스는 뇌의 구조적 기능적 변성을 유발한다. 특히 해마는 이러한 반응에 가장 민감한 부위이다. 본 연구는 반복적인 억제 스트레스에 의한 치아이랑의 속분자층에서의 연접 관련 분자를 면역조직화학을 이용하여 관찰하였다.

이 실험을 위하여 C57/BL6계 생쥐를 4일 동안 매일 6시간씩 부동스트레스를 가하였다. 스트레스 후에 분열하는 세포는 Ki-67에 대한 면역조직화학을, 새로 형성되는 과립세포는 doublecortin에 대한 면역조직화학을 이용하였고, 기존에 존재하는 과립세포는 calbindinD-28k에 대한 면역조직화학을 이용하였으며, 연접전 표지물질로는 growth associated protein-43 (GAP-43), 그리고 연접후 표지물질은 postsynaptic dentsity-95 (PSD-95)에 대한 면역조직화학을, 그리고 nitric oxide synthase (NOS)의 활성은 nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-diaphorase)조직화학을 이용하여 관찰하였다.

그 결과 Ki-67에 염색된 세포의 수는 스트레스 군에서 감소하였고, 새로 형성된 과립세포의 가지돌기를 보여주는 doublecortin의 염색성은 스트레스 군에서 감소하였다. 반면 기존 과립세포의 가지돌기를 보여주는 calbindinD-28k의 염색성은 스트레스 군에서 증가하였다. 또한 연접에 관련된 GAP-43, PSD-45그리고 NADPH-diaphorase의 염색성도 스트레스 군에서 증가하였다.

이상의 결과는 반복적 부동스트레스에 대하여 해마 치아이랑의 과립세포의 새롭게 형성되는 신경세포의 증가는 억제하고, 기존에 형성된 과립세포의 연접을 보다 강화시킴을 보여준다.

찾아보기 낱말 : 부동스트레스, 치아이랑, 속분자층, post synaptic density-95, doublecortin, growth associated protein-43, β-NADPH-d

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