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IV. 研究成果の刊行物・別刷

Neonatal isolation changes the expression of IGF-IR and IGFBP-2 in the hippocampus in response to adulthood restraint stress

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Abstract

Early adverse experiences are thought to contribute to the development of stress vulnerability, and to increase the onset of stress-related psychiatric disorders in stressful environments in adulthood. One plausible molecular mechanism of stress vulnerability is the modulation of neurotrophic factor signal transduction in the hippocampus by early adversity. In the present study we investigated the influence of neonatal isolation (NI) with or without adulthood single restraint stress (SRS) on the expression of several growth factor-related genes in the rat hippocampus using a cDNA microarray, real-time quantitative PCR, and Western blot. We found that hippocampal insulin-like growth factor-I receptor (IGF-IR) mRNA levels and immunoreactivity, and IGF binding protein-2 (IGFBP-2) mRNA levels were significantly lower in response to SRS in NI rats compared with rats without NI. Immunohistochemical analyses revealed that hippocampal IGF-IR immunoreactivity in the CA1 and CA3 pyramidal cell layers, and in the dentate gyrus granule cell layer of NI rats subjected to SRS was significantly lower compared with rats subjected to SRS. In addition, the hippocampal levels of IGF-IR mRNA were significantly lower in adult rats subjected to NI. These findings indicate that NI down-regulates IGF signal transduction under basal and stressful conditions in later life. Since the activation of IGF signalling plays a role in the development and neuroprotection of the central nervous system, the down-regulation of IGF signal transduction induced by NI may be, at least in part, involved in the development of adulthood stress vulnerability, which in turn precipitates the onset of depression.

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Key words: Hippocampus, insulin-like growth factor-I receptor, insulin-like growth factor binding protein-2, neonatal isolation, stress vulnerability.

Introduction

Numerous epidemiological studies reveal that early adverse experiences are closely associated with an increased risk of stress-related psychiatric disorders in adulthood, such as major depression and post-traumatic stress disorder (PTSD) (Bifulco et al., 1991; Harris et al., 1986; Widom, 1999). Although the precise mechanism for the precipitation of the onset

of stress-related psychiatric disorders remains to be determined, it has been postulated that early life adversity causes the development of stress vulnerability and subsequently induces the onset of psychiatric disorders under stressful environmental conditions in adulthood. For example, various studies using rats and non-human primates have demonstrated that early adverse experience including neonatal isolation (NI), maternal separation, and poor maternal care is associated with enhanced activity of the hypothalamic-pituitary-adrenal (HPA) axis in response to adulthood restraint stress (Liu et al., 1997; McCormick et al., 2002; Meaney et al., 1996; Plotsky and Meaney, 1993), anxiety-like behaviour (Huot et al., 2001; Wigger and Neumann, 1999), or impairment of spatial memory acquisition (Huot et al., 2002) in adulthood.

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In addition to hippocampal dysfunction, morphological changes in the hippocampus may also play an important role in the pathophysiology of depression and PTSD. Clinical studies using MRI-based volumetric analysis showed significant reduction of the hippocampal volume of patients with PTSD or major depression both with (Bremner et al., 2003; Vythilingam et al., 2002) and without an early adverse experience (Bremner et al., 1995, 2000; Sheline et al., 1999). Similarly, hippocampal atrophy in animals in response to stress has also been demonstrated. Prior studies have demonstrated that exposure to stress induces marked neuronal degeneration of hippocampal neurons in vervet monkeys (Uno et al., 1989), atrophy of the apical dendrites of CA3 pyramidal neurons in the rat hippocampus (Watanabe et al., 1992), and a reduction in the proliferation of granule cell precursors in the dentate gyrus (Gould et al., 1997, 1998). Further studies on the mechanism of stress-induced neurobiological changes in the hippocampus proposed that the modulation of neurotrophic factors and their receptors was closely associated with altered hippocampal structure (Molteni et al., 2001; Scaccianoce et al., 2000; Smith et al., 1995; Ueyama et al., 1997). Based on these findings, it is conceivable that early adversity affects the expression of neurotrophic factors and their receptors in the rat hippocampus during maturation, and consequently leads to stress vulnerability in adulthood. Some studies have examined the influence of early adverse experience on the expression of neurotrophic factors, members of the neurotrophin gene family, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3) in rodent hippocampus (Cirulli et al., 2000; Greisen et al., 2005; MacQueen et al., 2003; Roceri et al., 2004; Roceri et al., 2002).

Growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF), or insulin-like growth factor (IGF) are also reported to be involved in the development and survival of the central nervous system (CNS) (Cameron et al., 1998). One study recently demonstrated a significant reduction of IGF-II mRNA in the hippocampus with the choroid plexus of adult rats subjected to maternal separation (Kohda et al., 2006), however, the influence of NI on the expression of growth factors, their receptors, or their binding proteins in adult rat hippocampus has not yet been fully elucidated. Furthermore, it is also unknown as to whether adulthood stress can produce a significant change in the expression of growth factors, and their receptors and binding proteins in the hippocampus of

rats subjected to early adversity. In this study, we investigated the influence of NI with or without adulthood restraint stress on the expression of several growth factors, and their receptors and binding proteins in the rat hippocampus using a cDNA microarray, real-time quantitative PCR, Western blot, and immunohistochemistry.

Materials and methods

Animals

Pregnant female Sprague–Dawley rats were purchased from Charles River Japan (Yokohama, Japan). The rats were housed individually in a breeding colony at constant room temperature (23 ± 2 °C) and humidity (60%) with a 12 h light/dark cycle (lights on 08:00 to 20:00 hours). Food and water were provided ad libitum. The litters were culled to 12 pups on postnatal day 1 (PN day 1).

NI and single restraint stress (SRS) paradigm

The mothers and pups of the non-isolated group were left undisturbed until weaning (Figure 1). NI was conducted according to the method of Kehoe and Bronzino (1999). Pups were isolated from the dam, nest, and siblings and placed in individual round containers for 1 h per day on PN days 2–9 (Figure 1). All litters were weaned on PN day 21, separated on the basis of sex, and maintained with ad libitum access to food and water. Only the male rats were subjected to the following experimental procedure. On PN day 90, half of both the NI and non-isolated rats were subjected to a SRS for 2 h (Figure 1). The restraint stress was conducted as described previously (Suenaga et al., 2004). Briefly, rats were restrained using a clear polyethylene disposable bag (Asahikasei, Tokyo, Japan). Animals were sacrificed by decapitation after completion of the restraint stress. On PN day 90, NI and non-isolated rats (sham) were sacrificed by decapitation (Figure 1). Two groups of adult rats [one group subjected to repeated NI followed by SRS (NI+SRS); the second group subjected to SRS (SRS)] were used for DNA microarray analysis, Western blot analysis, and immunohistochemistry. Four groups of adult rats [subjected to NI followed by SRS (NI+SRS); NI alone (NI); SRS alone (SRS); or sham treatment (sham)] were used for real-time quantitative PCR. For the measurement of plasma corticosterone levels, both sham and NI rats were subjected to SRS for different durations. A total of 116 rats were used in the study and a different set of rats was used for each of the methods (i.e. DNA microarray, real-time quantitative PCR, Western

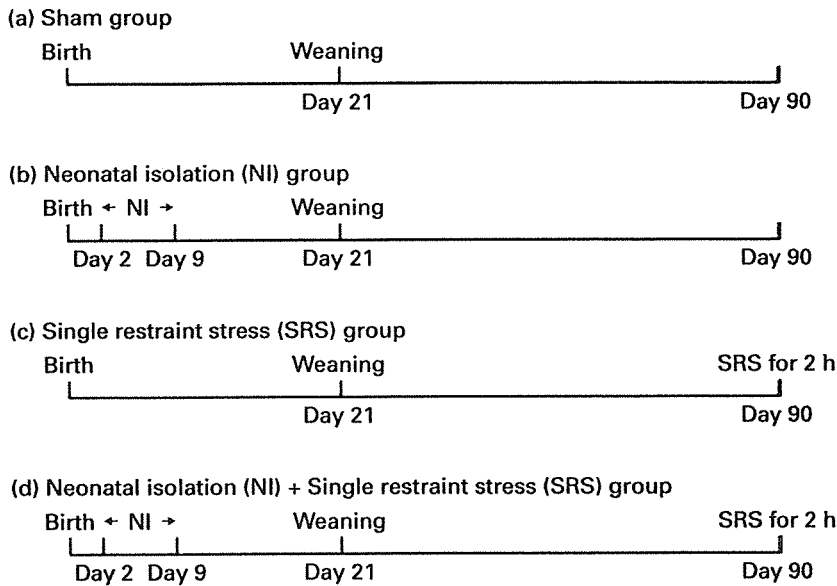


Figure 1. Experimental paradigms. (a) Sham treatment: Sham-treated rats were undisturbed until weaning (PN day 21) and were not subjected to a single restraint stress on PN day 90. (b) Neonatal isolation (NI): Pups were isolated from the dam, nest, and siblings for a period of 1 h per day on PN days 2–9, and were not subjected to restraint stress. (c) Single restraint stress (SRS): Pups were undisturbed until weaning and were subjected to SRS for 2 h on PN day 90. (d) NI + SRS: Pups were isolated from the dam, nest, and siblings for a period of 1 h per day on PN days 2–9, and on PN day 90, they were subjected to SRS for 2 h.

blotting, immunohistochemistry, and plasma corticosterone levels). The hippocampus was isolated and used for DNA microarray, real-time quantitative PCR, and Western blot analysis. In this procedure, the lateral choroid plexus was carefully removed from the hippocampus. For immunohistochemistry, brains were removed rapidly, immediately frozen, and stored at -70°C . All animal procedures were conducted in accordance with the Guiding Principles on Animal Experimentations in Research Facilities for Laboratory Animal Science Hiroshima University and approved by Hiroshima University Animal Care Committee.

DNA microarray

For the DNA microarray analysis, the ExpressChip™ DNA Microarray System RO1 (Mergen, San Leandro, CA, USA) containing 1152 genes was used. The ExpressChip arrays were pre-spotted with oligonucleotide sequences designed to determine the expression of target genes with the highest specificity. The experiments were carried out according to the manufacturer's protocol. In brief, total RNA was extracted from the hippocampi of five adult rats in each group (NI+SRS, SRS) with an RNAqueous Phenol-free Total RNA Isolation kit (Ambion, Austin, TX, USA), and pooled. After treatment with RNase-free

DNase I (Takara, Kusatsu, Japan) for the removal of DNA, first-strand cDNA was synthesized using oligo[(dT)₂₄T7promoter]₆₅ primer from DNase-treated total RNA, and then double-stranded cDNA was synthesized with T4 DNA polymerase. Biotin-labelled cRNA probes produced from the double-stranded cDNA template were hybridized to microarrays overnight at 30°C . The arrays were washed, and incubated with streptavidin, anti-streptavidin antibody, and finally, secondary antibody conjugated Cyanine-3 fluorescent dye (Cy3). Signals were detected with a GMS417 array scanner (Affymetrix, Santa Clara, CA, USA), and spot intensity was analysed using ImaGene (BioDiscovery, El Segundo, CA, USA). The relative expression level of each gene was normalized against housekeeping gene controls.

Real-time quantitative PCR

Total RNA was extracted with an RNAqueous Phenol-free Total RNA Isolation kit (Ambion). After treatment with RNase-free DNase I (Takara), real-time quantitative PCR was performed with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA) to quantitate relative mRNA levels in samples. Real-time quantitative PCR was performed to amplify insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II),

Table 1. Primer and probe sequences used for real time PCR

	Forward primer (5' to 3')	Reverse primer (5' to 3')	Taqman probe (5' [FAM] to 3' [TAMRA])
IGF-I	TCCAGCATTCGGAGGGC	TCACAGCTCCGGAAGCAAC	CCACAGACGGGCATTGTGGATGAG
IGF-II	GCCGTACTTCCGGACGACT	TCCAGGTGTGCAATTTGAAGAA	CCCCAGATACCCCGTGGGCAA
IGF-IR	CGCTCTGGCCGACGAGT	CTGCTGATAGTCGTTGCGGA	AGAAATTTGTGGGCCCGGCATTGAC
IGF-IIR	CGGAATGGAAGCTCGATTATTG	GTGTCGTCTCACTCTCATCGTAT	TTCATCCACCGCACTGGTGGTTATGA
IGFBP-2	CAGGTCCTGGAGCCGCATC	ATGTTCCAGAGGACCCCGAT	CCACCATGCGCCTTCCGGA

insulin-like growth factor-I receptor (IGF-IR), insulin-like growth factor-II receptor (IGF-IIR), and insulin-like growth factor binding protein-2 (IGFBP-2). The primers and TaqMan hybridization probe were designed using Primer Express software (PE Applied Biosystems). Table 1 shows the sequences and fluorescent dye of PCR primers and TaqMan probes for each molecule. The TaqMan probes, which were designed to hybridize to the PCR products, were labelled with a fluorescent reporter dye at the 5'-end and a quenching dye at the 3'-end. PCR was carried out with TaqMan Universal PCR Master Mix (PE Applied Biosystems). All standards and samples were assayed in triplicate. Each plate contained the same standard. Thermal cycling was initiated with an initial denaturation at 50 °C for 2 min and 95 °C for 10 min. After this initial step, 40 cycles of PCR (heating at 95 °C for 15 s and 60 °C for 1 min) were performed. The PCR assay for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using TaqMan Rodent GAPDH Control Reagents (PE Applied Biosystems). PCR assays for unknown samples were performed simultaneously with standard samples (rat brain tissue) to construct a standard curve. The relative concentrations of GAPDH and IGF-I, IGF-II, IGF-IR, IGF-IIR, or IGFBP-2 in unknown samples were calculated from this standard curve and the ratio of the relative concentration of IGF-I, IGF-II, IGF-IR, IGF-IIR, or IGFBP-2 was calculated relative to the concentration of GAPDH.

Western blot

Immunoblot analyses for IGF-IR and IGFBP-2 were performed by the method of Cardona and colleagues (2000) with a minor modification. In brief, the rat hippocampus was homogenized on ice with a Polytron homogenizer at top speed (30 000 rpm) in homogenization buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 2 mM DTT, 150 mM NaCl, 0.5% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 100 µM phenylmethylsulphonyl fluoride. The insoluble material was removed by centrifugation at

10 000 g at 4 °C for 10 min. Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (60 µg) for each group were fractionated using sodium dodecyl sulphate (SDS) gel (7.5% for IGF-IR, and 12.5% for IGFBP-2; Atto, Tokyo, Japan) and transferred to a PVDF membrane (for IGF-IR) or a nitrocellulose membrane (for IGFBP-2) using a semi-dry blotting apparatus (Bio-Rad). Nitrocellulose membrane was incubated with Miser™ Antibody Extender Solution NC (Pierce, Rockford, IL, USA) before blocking. All membranes were blocked at room temperature for 1 h in TBS containing 5% non-fat dry milk and 0.05% Tween-20 (TBST-MLK), and then incubated overnight with anti-IGF-IR β-chain antibody (1:500 dilution; sc-713, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST-MLK, or anti-IGFBP-2 antibody (1:1000 dilution; no. 06-107, Upstate Biotechnology, Lake Placid, NY, USA) in Solution 1 of Can Get Signal™ (Toyobo, Osaka, Japan) as a primary antibody, overnight at 4 °C. The membranes were washed at room temperature in TBST four times for 5 min per wash, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2000 dilution for IGF-IR, 1:20000 dilution for IGFBP-2; Zymed, San Francisco, CA, USA) in TBST-MLK (for IGF-IR) or Solution 2 of Can Get Signal™ (for IGFBP-2). IGF-IR blots were detected by a colorimetric method (PerkinElmer, Wellesley, MA, USA), and IGFBP-2 blots were developed using an Enhanced Chemiluminescence (ECL) Western Blotting Detection System (Amersham Pharmacia Biotech, Buckinghamshire, UK). The blots were reprobbed with anti-β-actin antibody (Sigma Chemical Co., St. Louis, MO, USA) to ensure equal protein loading. The density of the immunoreactive bands was quantified with Atto Image analysis software (version 4.0 for Macintosh; Atto).

Immunohistochemistry

Freshly frozen coronal brain sections (15 µm) through the hippocampus were cut in a cryostat, thaw-mounted onto slides and fixed with 4%

paraformaldehyde for 5 min. Sections were washed three times with PBST (PBS-0.1% Triton X-100) and pretreated with 10% H₂O₂ in methanol to neutralize the endogenous peroxidase activity. Then, the sections were washed twice in PBST for 10 min. After being blocked in 10% sheep serum in PBST for 60 min, the sections were incubated overnight at 4 °C with anti-IGF-IR monoclonal antibody (1:100 dilution; MAB1123, Chemicon, Temecula, CA, USA) or anti-IGFBP-2 antibody (1:1000 dilution; no. 06-107, Upstate Biotechnology) in 10% sheep serum in PBST. After four 10 min washes in PBST, the sections were incubated at room temperature for 180 min with goat anti-mouse IgG (H+L) HRP conjugate (1:200 dilution for IGF-IR, 1:1000 dilution for IGFBP-2; Zymed) in 10% sheep serum in PBST. The sections were then washed four times in PBST for 10 min per wash and exposed with liquid DAB+substrate chromogen solution (Dako, Carpinteria, CA, USA). The immunohistochemical signal for IGF-IR and IGFBP-2 was detected using a digital video image analyser (Keyence BZ-8000, Osaka, Japan). The mean density of sections from the SRS and NI+SRS groups was measured in the CA1 and CA3 regions, granule cell layer and hilus of the dentate gyrus using the NIH Scion Image analysis program.

Measurement of plasma corticosterone levels

Blood samples were collected before SRS, and 30 min and 2 h after the beginning of SRS in NI and non-NI rats. After centrifugation (500 g at 4 °C for 30 min), plasma samples were frozen and stored at -70 °C until the day of analysis. The plasma corticosterone level was determined using a rat corticosterone [¹²⁵I] assay system (Amersham Pharmacia Biotech).

Statistical analyses

Results were expressed as mean ± S.E.M. The results of real-time quantitative PCR were analysed by two-way analysis of variance (ANOVA) (NI × SRS) followed by Scheffé's test. The levels of plasma corticosterone were analysed by 2 × 3 ANOVA (NI × time after the beginning of SRS) followed by Scheffé's test. For results analysed by ANOVA, the degrees of freedom were presented. For Western blot and immunohistochemical analyses the results of experiments containing two groups of rats were analysed by Mann-Whitney *U* test, and the results of the experiment on IGFBP-2 immunoreactivity at different intervals after the termination of SRS for 2 h was analysed by one-way ANOVA followed by Scheffé's test. Significance was set at $p < 0.05$.

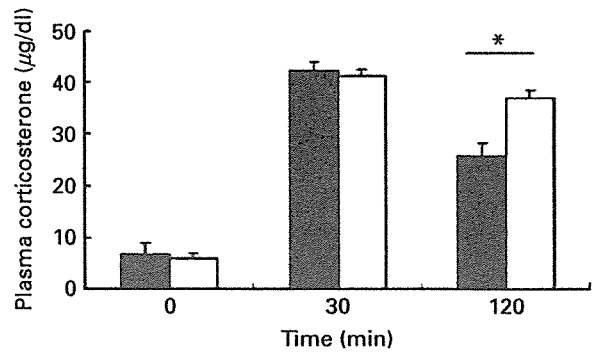


Figure 2. Plasma corticosterone concentrations before (0 min) and after the beginning of single restraint stress (SRS) (30 min, 2 h) in neonatal isolation (NI; □) and non-NI (■) adult rats. The mean ± S.E.M. ($n=6$) is shown. * $p < 0.01$ compared to non-NI group (2 × 3 ANOVA followed by Scheffé's test).

Results

The influence of NI on the levels of plasma corticosterone

Statistical analysis of the plasma corticosterone levels revealed that there was a significant interaction between NI and restraint time [$F(2, 30) = 7.697$, $p = 0.002$]. While there were no differences in the plasma corticosterone levels between NI and non-NI rats before SRS or 30 min after the beginning of SRS, there was the significant difference in the levels of plasma corticosterone between NI and non-NI rats 2 h after the beginning of SRS ($p < 0.01$) (Figure 2).

The influence of NI on the levels of IGF-I, IGF-IR, IGF-II, IGF-IIR, and IGFBP-2 mRNA in response to a SRS in adulthood

Differences in the hippocampal expression of growth factors and related genes between the SRS and NI+SRS groups at PN day 90 were analysed using an ExpressChip™ (Table 2). With a cut-off of 2.0-fold change, the expression of several genes involved in the IGF signal transduction (IGF-I, IGF-IR, IGF-II, IGF-IIR, and IGFBP-2) in the NI+SRS group was different from that in the SRS group. To confirm the difference in the mRNA levels of these genes between the SRS and NI+SRS group, we performed real-time quantitative PCR analysis using a different set of animals from the microarray analysis. For IGF-I mRNA expression, two-way ANOVA showed no significant effect of NI [$F(1, 28) = 0.109$, $p = 0.744$], or SRS [$F(1, 28) = 1.125$, $p = 0.298$], and no significant interaction between NI and SRS [$F(1, 28) = 0.035$, $p = 0.853$] (Figure 3a). For

Table 2. Hippocampal expression of growth factors and related genes

Genbank ID	Gene	Fold change SRS/NI+SRS
U04842	Epidermal growth factor (EGF)	1.1
M37394	EGF receptor	1.1
X14232	Fibroblast growth factor (FGF) 1 (heparin binding)	8.7
M22427	FGF2	1.0
D64085	FGF5	1.0
D14839	FGF9	0.7
D79215	FGF10	1.3
AB004638	FGF18	0.9
S54008	FGF receptor 1, complete cds	1.5
L19109	Heparin binding FGF receptor 2 (intracellular domain) mRNA	1.8
L19104	Heparin binding FGF receptor 2 (extracellular domain) mRNA	2.3
U57715	FGF receptor activating protein (FRAG1) mRNA, complete cds	1.4
D90102	Hepatocyte growth factor	1.0
M15651	Insulin-like growth factor (IGF)-I	2.2
X14834	IGF-II	2.4
M37807	IGF-I receptor	4.1
U59809	IGF-II receptor	3.0
M58634	IGF-binding protein (IGFBP) 1	0.5
J04486	IGFBP2	4.8
M31837	IGFBP3	1.3
M62781	IGFBP5	1.3
M69055	IGFBP6	1.0
M29014	Insulin receptor	0.8
X56551	Keratinocyte growth factor	1.6
L14447	Neurotrophic tyrosine kinase, receptor, type 3 (Ntrk3)	1.3
M36589	Beta-nerve growth factor gene, last exon	1.3
M55291	Neuronal receptor protein-tyrosine kinase (trkB)	1.6
L06238	Platelet-derived growth factor (PDGF) A-chain mRNA	0.6
M63837	PDGF receptor alpha	0.9
M31076	Transforming growth factor (TGF) alpha	1.1
X52498	TGF beta 1	2.0
U03491	TGF beta 3	0.7
M77809	TGF beta receptor III	3.2
M55431	TGF-beta masking protein large subunit, complete cds	1.0
L00981	Tumour necrosis factor (TNF) alpha	1.5
M63122	TNF receptor	1.0
X05137	NGF receptor, fast	1.1
M32167	Vascular endothelial growth factor (VEGF) mRNA, partial cds	0.9
AF014827	VEGF-D mRNA, complete cds	1.9

Total RNA was isolated from the hippocampi of five rats in the NI+SRS and SRS groups, and subjected to the ExpressChip™ (Mergen, San Leandro, CA, USA) followed by analyses with the ImaGene (BioDiscovery).

IGF-IR mRNA expression, there was a significant effect of NI [$F(1, 28) = 19.972, p < 0.001$] as well as SRS [$F(1, 28) = 30.890, p < 0.001$] but there was no significant interaction between NI and SRS [$F(1, 28) = 0.062, p = 0.806$] (Figure 4a). Post-hoc analysis revealed that the level of IGF-IR mRNA ($p < 0.05$) in the NI+SRS group was significantly lower than that in the SRS group (Figure 4a). In addition, the level of IGF-IR

mRNA in the NI group was significantly lower ($p < 0.05$) than that in the sham group (Figure 4a). In the SRS group, the level of IGF-IR mRNA was significantly increased compared to that in the sham group ($p < 0.01$) (Figure 4a). In the NI+SRS group, the level of IGF-IR mRNA was significantly increased compared to that in the NI group ($p < 0.01$) (Figure 4a). For IGF-II mRNA expression, two-way ANOVA showed

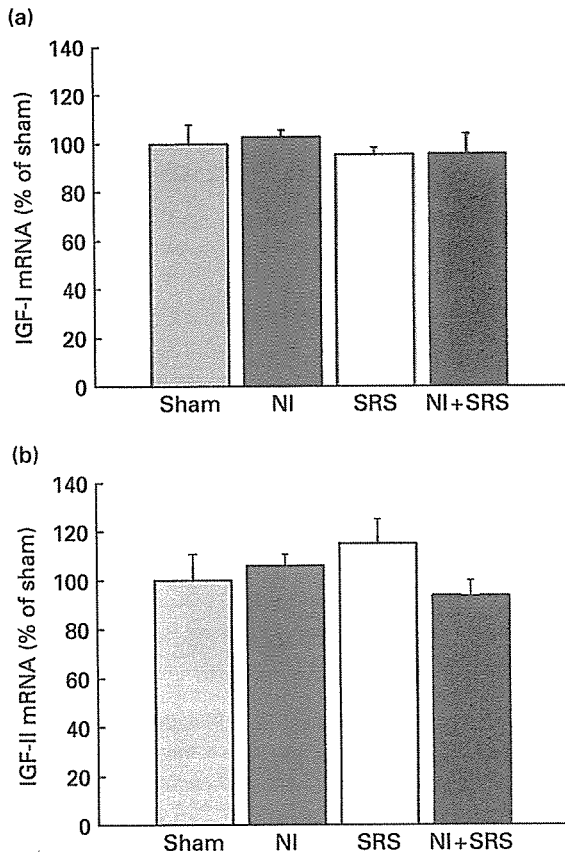


Figure 3. Expression of IGF-I and IGF-II mRNA in the hippocampus of adult rats subjected to sham treatment (sham), neonatal isolation (NI) alone, single restraint stress (for 2 h) on PN day 90 (SRS) alone, and neonatal isolation followed by a single restraint stress (for 2 h) on PN day 90 (NI+SRS). (a) IGF-I mRNA levels were determined by real-time quantitative PCR as described in the Materials and Methods section. Results are expressed as the percentage of sham. The mean \pm S.E.M. ($n=8$) is shown. (b) IGF-II mRNA levels were determined by real-time quantitative PCR as described in the Materials and Methods section. Results are expressed as the percentage of sham. The mean \pm S.E.M. ($n=6-8$) is shown.

no significant effect of NI [$F(1,23)=0.444$, $p=0.512$], or SRS [$F(1,23)=0.034$, $p=0.854$], and no significant interaction between NI and SRS [$F(1,23)=2.440$, $p=0.132$] (Figure 3b). For IGF-IIR mRNA expression, two-way ANOVA showed no significant effect of NI [$F(1,28)=0.439$, $p=0.513$], or SRS [$F(1,28)=0.582$, $p=0.452$], and no significant interaction between NI and SRS [$F(1,28)=0.010$, $p=0.921$] (Figure 4b). For IGFBP-2 mRNA expression, two-way ANOVA showed that there was a significant interaction between NI and SRS [$F(1,24)=19.098$, $p<0.001$] (Figure 5). Post-hoc analysis revealed a significant

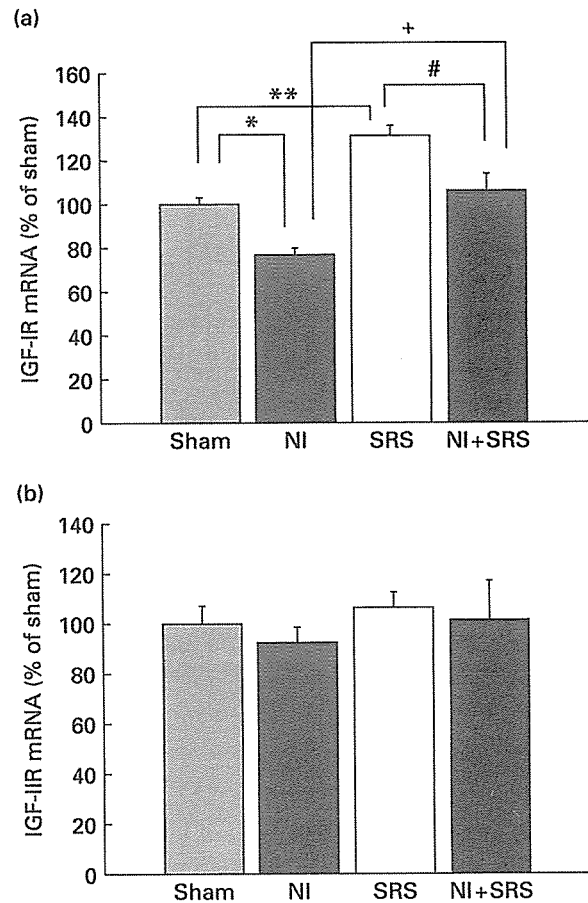


Figure 4. Expression of IGF-IR and IGF-IIR mRNA in the hippocampus of adult rats subjected to sham treatment (sham), neonatal isolation (NI) alone, single restraint stress (for 2 h) on PN day 90 (SRS) alone, and neonatal isolation followed by a single restraint stress (for 2 h) on PN day 90 (NI+SRS). (a) IGF-IR mRNA levels were determined by real-time quantitative PCR as described in the Materials and Methods section. Results are expressed as the percentage of sham. The mean \pm S.E.M. ($n=8$) is shown. * $p<0.05$ compared to sham, ** $p<0.01$ compared to sham, # $p<0.05$ compared to SRS, + $p<0.01$ compared to NI (two-way ANOVA followed by Scheffé's test). (b) IGF-IIR mRNA levels were determined by real-time quantitative PCR as described in the Materials and Methods section. Results are expressed as the percentage of sham. The mean \pm S.E.M. ($n=8$) is shown.

($p<0.01$) increase in the levels of IGFBP-2 in the SRS group as compared with the other groups (Figure 5).

Hippocampal IGF-IR and IGFBP-2 immunoreactivity in SRS and NI+SRS rats in adulthood

To elucidate whether the significant differences in IGF-IR and IGFBP-2 mRNA expression between the SRS and NI+SRS groups (shown by real-time PCR

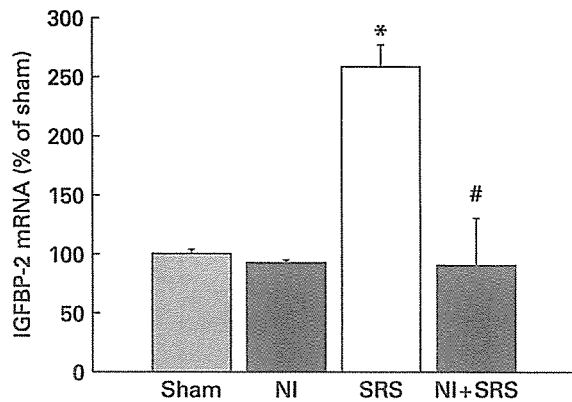


Figure 5. Expression of IGFBP-2 mRNA in the hippocampus of adult rats subjected to sham treatment (sham), neonatal isolation (NI), a single restraint stress (for 2 h) on PN day 90 (SRS), and neonatal isolation followed by a single restraint stress (for 2 h) on PN day 90 (NI+SRS). IGFBP-2 mRNA levels were determined by real-time quantitative PCR as described in the Materials and Methods section. Results are expressed as the percentage of sham. The mean \pm S.E.M. ($n=6-8$) is shown. * $p < 0.01$ compared to sham, # $p < 0.01$ compared to SRS (two-way ANOVA followed by Scheffé's test).

analysis) affected the protein level of these genes, the levels of IGF-IR and IGFBP-2 protein were measured by Western blotting. Western blot analysis revealed significantly lower IGF-IR immunoreactivity in the NI+SRS group than in the SRS group ($p < 0.05$) (Figure 6). However, there was no significant difference in the immunoreactivity of IGFBP-2 between the SRS and NI+SRS groups (Figure 7).

Since no significant difference in IGFBP-2 immunoreactivity was found between the NI and non-NI rats immediately after SRS for 2 h, we determined the levels of hippocampal IGFBP-2 immunoreactivity at different intervals after the termination of SRS for 2 h in adult non-isolated rats. IGFBP-2 immunoreactivity in adult rats sacrificed at 8 h, but not 2 h or 4 h, after the termination of SRS was significantly higher than in rats without SRS ($p < 0.05$) (Figure 8).

To identify the hippocampal cell layers in which IGF-IR immunoreactivity in the NI+SRS group was down-regulated, we examined IGF-IR immunoreactivity by immunohistochemistry. Hippocampal IGF-IR immunoreactivity in the CA1 and CA3 pyramidal cell layers and in the granule cell layer and hilus of the dentate gyrus was significantly decreased in NI+SRS rats compared with SRS rats ($p < 0.05$) (Figures 9, 10). We also conducted immunohistochemical analysis to determine regional differences in IGFBP-2 immunoreactivity between the SRS

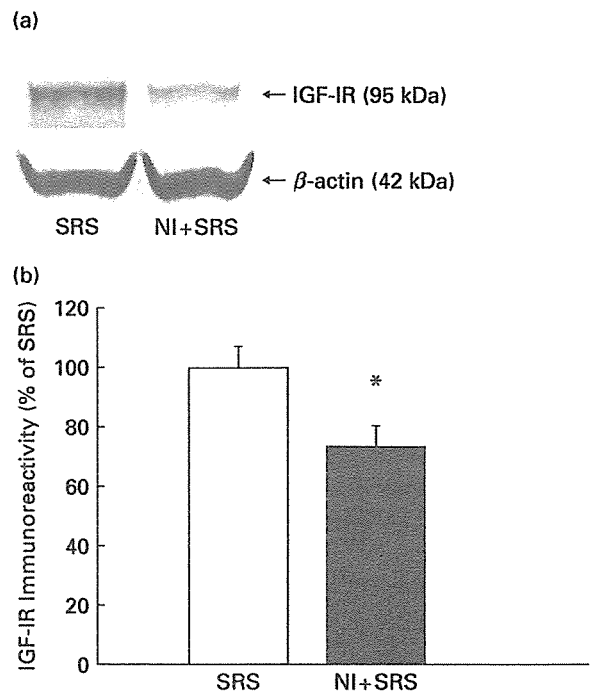


Figure 6. Western blot analysis of IGF-IR expression in the hippocampus of adult rats subjected to a single restraint stress (for 2 h) on PN day 90 (SRS), and neonatal isolation followed by a single restraint stress (for 2 h) on PN day 90 (NI+SRS). Results are expressed as the percentage of SRS. Immunoreactivity was determined by Western blot analysis as described in the Materials and Methods section. (a) Representative immunoblots for IGF-IR, and β -actin as a positive control for loading error, in the rat hippocampus. (b) Comparison of IGF-IR protein levels in the hippocampus of SRS and NI+SRS groups. Results are expressed as the percentage of SRS. The mean \pm S.E.M. ($n=6$) is shown. * $p < 0.05$ compared to SRS (Mann-Whitney U test).

and NI+SRS rats (Figure 11). However, hippocampal IGFBP-2 immunoreactivity in the CA1 and CA3 pyramidal cell layers and granule cell layer and hilus of the dentate gyrus did not differ between the two groups (data not shown).

Discussion

Since a stressful environment in later life readily disrupts the homeostasis of neurotransmitters and hormones in individuals with early adversity (Heim et al., 2000; Meaney et al., 2002; Plotsky and Meaney, 1993), and subsequently may induce changes in gene expression in the CNS, early adverse experiences are thought to precipitate stress vulnerability in adulthood. However, the precise molecular mechanism for the development of the stress vulnerability remains to be determined. In this context, we

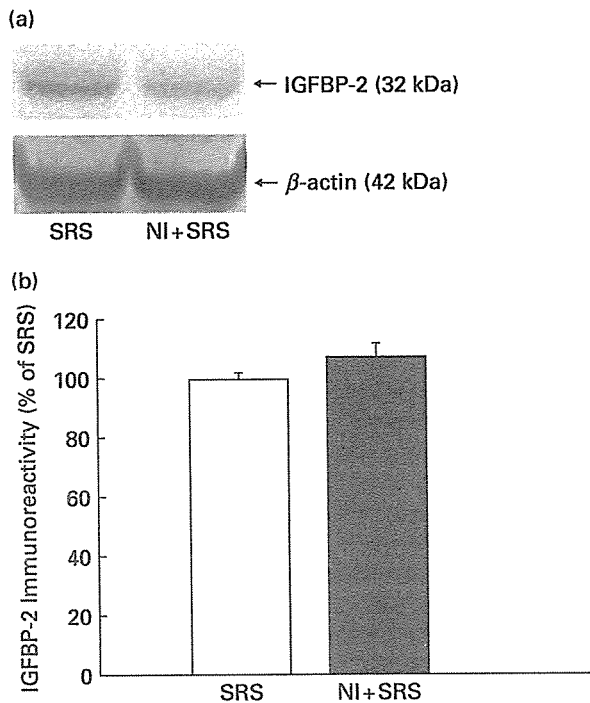


Figure 7. Western blot analysis of IGFBP-2 in the hippocampus of rats subjected to a single restraint stress (for 2 h) on PN day 90 (SRS) alone, and neonatal isolation followed by a single restraint stress (for 2 h) on PN day 90 (NI+SRS). Western blot analysis was performed as described in the Materials and Methods section. (a) Representative immunoblots for IGFBP-2, and β -actin as a positive control for loading error, in the hippocampus are shown. (b) Comparison of IGFBP-2 protein levels in the SRS and NI+SRS groups. Results are expressed as the percentage of SRS. The mean \pm S.E.M. ($n=6$) is shown.

examined differences in hippocampal gene expression profiles between rats, with and without NI, in response to adulthood SRS for 2 h. As shown in the present study, SRS for 2 h led to a significant difference in plasma corticosterone levels between SRS and NI+SRS rats, indicating that stress vulnerability induced by NI occurred in this stressful condition. We showed that repeated NI affected IGF-IR mRNA levels and immunoreactivity, and IGFBP-2 mRNA levels in the hippocampus in response to SRS for 2 h in adult rats. In addition, the levels of IGF-IR mRNA in the hippocampus were significantly decreased in adult rats subjected to NI.

Since it is well known that neurotrophic factors play an important role in synaptic formation and cell differentiation during development (Berninger and Poo, 1996; Henderson, 1996), several studies were previously undertaken to examine the influence of maternal separation on the expression of BDNF

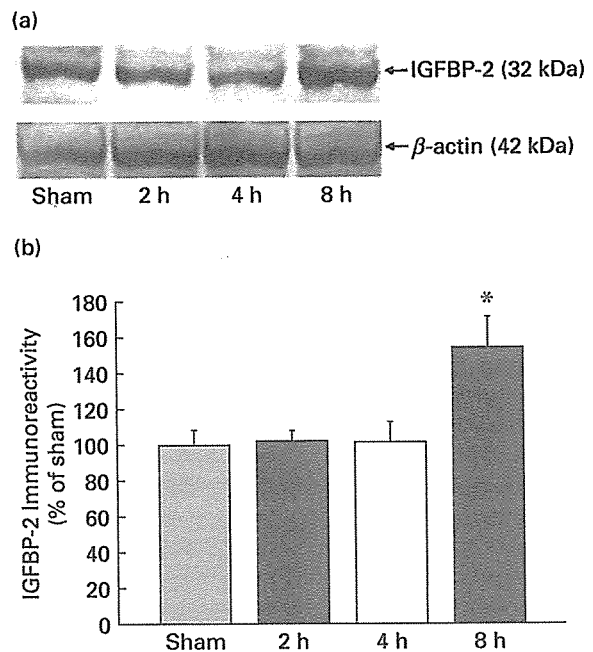


Figure 8. Western blot analysis of IGFBP-2 in the hippocampus of adult rats at three intervals (2 h, 4 h, and 8 h) after the termination of SRS for 2 h. Western blot analysis was performed as described in the Materials and Methods section. (a) Representative immunoblots for IGFBP-2, and β -actin as a positive control for loading error, in the hippocampus are shown. (b) Comparison of IGFBP-2 protein levels at three intervals (2 h, 4 h, and 8 h) after the termination of SRS for 2 h. Results are expressed as the percentage of the sham group. The mean \pm S.E.M. ($n=5$) is shown. * $p < 0.05$ compared to sham (one-way ANOVA followed by Scheffé's test).

mRNA in the brain, and to elucidate the mechanism for the development of stress vulnerability in response to early adversity. Almost all studies showed that maternal separation significantly decreased the levels of BDNF in adult rodents (Greisen et al., 2005; MacQueen et al., 2003; Roceri et al., 2002, 2004). In contrast to BDNF, only one study, to our knowledge, has examined the influence of maternal separation on IGF-II mRNA expression in adult rats (Kohda et al., 2006). Kohda and his associates have demonstrated that IGF-II mRNA expression in the hippocampus with the choroid plexus is significantly reduced in adult rats subjected to maternal separation. On the other hand, the result of the present study demonstrates that NI causes no change in the levels of IGF-II mRNA in the hippocampus of adult rats. While the reason for this difference is unclear, the difference in experimental procedures and brain regions may be associated with this discrepancy.

The IGF signalling system consists of IGFs (IGF-I and IGF-II), membrane receptors (IGF-IR and IGF-IIR),

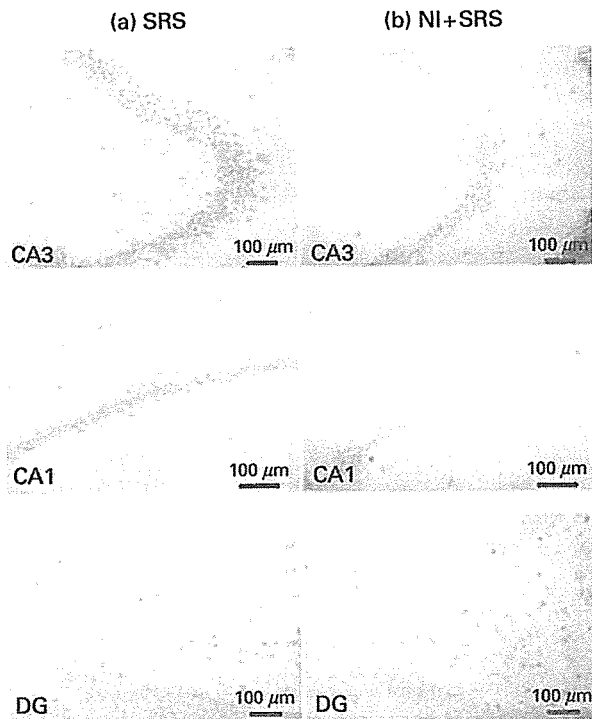


Figure 9. Immunohistochemical analysis of IGF-IR expression in the adult rat hippocampus. (a) IGF-IR immunoreactivity in rats subjected to a single restraint stress (SRS). (b) IGF-IR immunoreactivity in rats subjected to neonatal isolation followed by a single restraint stress (NI+SRS). CA, cornu ammonis; DG, dentate gyrus.

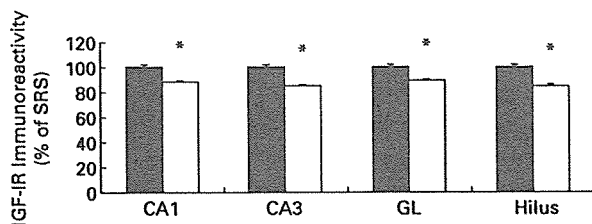


Figure 10. Mean density of IGF-IR immunoreactivity at the CA1 and CA3 pyramidal cell layers, and the granule cell layer and hilus of the dentate gyrus in the hippocampus of single restraint stress (SRS, ■) and neonatal isolation followed by a single restraint stress (NI+SRS, □) rats. Results are expressed as the percentage of SRS. The mean \pm S.E.M. ($n=4$) is shown. * $p < 0.05$ compared to SRS (Mann-Whitney U test). CA, cornu ammonis; GL, granule cell layer.

and IGF binding proteins, and plays a pivotal role in the growth and development of various tissues (LeRoith and Roberts, 2003), including the CNS (D'Ercole et al., 1996; Feldman et al., 1997; Torres-Aleman, 1999). With respect to the function of the IGF system, IGF-IR is the primary mediator of IGF-I action

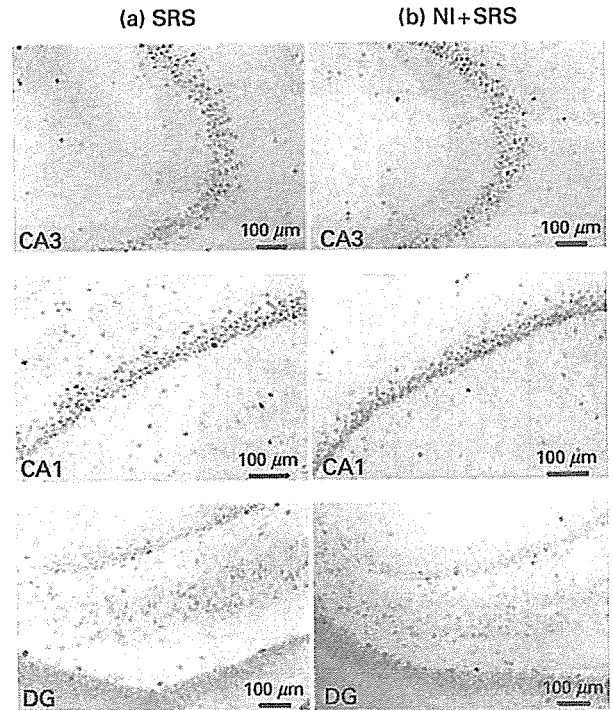


Figure 11. Immunohistochemical analysis of IGFBP-2 expression in the adult rat hippocampus. (a) IGFBP-2 immunoreactivity in rats subjected to a single restraint stress (SRS). (b) IGFBP-2 immunoreactivity in rats subjected to neonatal isolation followed by a single restraint stress (NI+SRS). CA, cornu ammonis; DG, dentate gyrus.

(LeRoith et al., 1995). Transgenic mice lacking IGF-I or IGF-IR show severe brain growth retardation (Baker et al., 1993; Liu et al., 1993). Furthermore, transgenic mice overexpressing IGF-I in the brain show an increase in the total number of neurons and synapses in the dentate gyrus (O'Kusky et al., 2000), while IGF-I $-/-$ mice show a decrease in the number of granule cells in the dentate gyrus (Cheng et al., 2001). Previous studies have shown that peripheral infusion of IGF-I induces neurogenesis in adult rat hippocampus (Aberg et al., 2000), and that IGF-I stimulates proliferation in adult rat hippocampal progenitor cells (Aberg et al., 2003). In this context, it is plausible that the significant decrease in hippocampal IGF-IR expression in response to NI with or without restraint stress may, at least in part, contribute to the disturbance of hippocampal function through the decrease of IGF-I signal transduction.

IGFBPs act as carriers of IGFs, affect the half-lives of IGFs, and modulate the action of IGFs (D'Ercole et al., 1996; Jones and Clemmons, 1995). In the present study, the levels of IGFBP-2 mRNA, but not IGFBP-2 immunoreactivity, immediately after the termination

of adulthood restraint stress for 2 h, were significantly lower in the hippocampus of rats subjected to NI. IGFBP-2 immunoreactivity was significantly increased at 8 h after the termination of SRS in adult rats without NI, and the slower response of IGFBP-2 protein to SRS in the hippocampus might be involved in this difference. Similarly, it was demonstrated that the induction of IGFBP-2 protein was slower than that of IGFBP-2 mRNA in primary astroglial cells in response to IGF-I (Bradshaw and Han, 1993).

It has been reported that the expression of IGFBP-2 and IGF-I mRNA is increased around the site of CNS injury. After hypoxia-ischaemia or cerebral cortical contusion in rats, the expression of IGFBP-2 mRNA as well as IGF-I is induced in the hippocampus (Beilharz et al., 1998; Sandberg Nordqvist et al., 1996). Injection of colchicine into the hippocampus of rats increases the expression of IGFBP-2 mRNA in the hippocampus and damaged cortex (Breese et al., 1996). These findings suggest that not only IGF-I but also IGFBP-2 responds to brain damage and produces neuroprotective effects. It appears that the lack of an increase of IGFBP-2 mRNA expression in response to adulthood restraint stress in rats subjected to NI may be, at least in part, involved in the lack of protection against adulthood stress.

Administration of venlafaxine or fluoxetine for 2 wk has been shown to up-regulate IGF-I protein levels in the hippocampus and to significantly increase the proliferation and survival of progenitor stem cells in the dentate gyrus (Khawaja et al., 2004). Recently, Hoshaw et al. (2005) have shown that central administration of IGF-I or BDNF exerts antidepressant-like effects in the forced swim test, and that the duration of effects is longer than that of traditional antidepressants. We hypothesize that the decrease in hippocampal IGF signal transduction by NI is one plausible mechanism by which early adverse experiences precipitate the onset of depression in adulthood under environmental stress conditions. Further studies examining whether NI with additional adulthood stress affects the activity of the intracellular signal transduction mediated by IGFs and IGF-receptors, are required to verify our hypothesis.

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Statement of Interest

None.

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Importance of early lighting conditions in maternal care by dam as well as anxiety and memory later in life of offspring

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Keywords: early lighting conditions, emotionality, licking and grooming, object recognition memory, rat

Abstract

Rodent studies have revealed that the early rearing environment plays an important role in the development of stress vulnerability, memory and cognition. Although early lighting conditions (ELC) are involved in these neuronal developments through both maternal and offspring behavior, their influence has not been fully elucidated. Thus, by using Sprague-Dawley rats, we examined whether ELC affected maternal care by the dam and the subsequent neurodevelopment of the offspring. Prolonged dark phase conditions (PDC) (light/dark, 6/18 h) and prolonged light phase conditions (light/dark, 18/6 h) were administered from postnatal day 2 to postnatal day 14. Throughout this period, maternal care and the circadian rhythmicity of dams were investigated. In adolescence and adulthood of the offspring, we measured anxiety-like behavior, social interaction, object recognition memory, activity rhythm and corticosterone response to stress with hippocampal expression of *N*-methyl-D-aspartate and glucocorticoid receptor mRNAs. PDC altered maternal care and circadian rhythmicity in the dam compared with normal lighting conditions and prolonged light phase conditions. PDC markedly increased anxiety-like behavior, decreased social interaction and object recognition memory, and inhibited corticosterone feedback in offspring later in life. Furthermore, hippocampal levels of glucocorticoid receptor mRNA and *N*-methyl-D-aspartate receptor 2B mRNA in rats subjected to PDC were significantly lower than in animals subjected to normal lighting conditions. In the adult offspring, the circadian rhythm of locomotor activity was not affected. These findings suggested that ELC affect mother–infant interactions and subsequently at least partially alter the neurobehavioral development of offspring.

Introduction

Rodent studies have shown that early life experiences based on mother–infant interactions have a long-lasting influence on neuronal development and subsequently regulate behavioral, cognitive, and neuroendocrinological function in adulthood. For example, early adversity such as maternal separation or low maternal care was demonstrated to be closely involved in enhanced activity of the hypothalamic-pituitary-adrenocortical (HPA) system in response to stress in later life (Ader & Grotta, 1970; Rosenfeld *et al.*, 1992; Pihoker *et al.*, 1993; Liu *et al.*, 1997, 2000a; Biagini *et al.*, 1998; Caldji *et al.*, 1998; Kalinichev *et al.*, 2002; Levine, 2005). In addition, a series of studies by Meaney and colleagues showed that maternal care, especially licking/grooming (LG), which forms the basis for tactile stimulation (Jutapakdeegul *et al.*, 2003), is critical for the development of emotionality (Liu *et al.*, 1997; Caldji *et al.*, 1998, 2003; Menard *et al.*, 2004; Zhang *et al.*, 2005) and memory function in pups (Liu *et al.*, 2000b; Bredy *et al.*, 2003a,b, 2004). In line with this maternal mediation hypothesis earlier postulated by Levine (1967) and Smotherman & Bell (1980), Meaney predicted that variations in maternal care can account for the effects of various postnatal manipulations on the phenotype of offspring (Champagne & Meaney, 2001; Cameron *et al.*, 2005).

It has been reported that the peripartum environment, involving such factors as poverty, food availability, stress exposure, etc., directly affects characteristics of maternal care and mother–infant interaction in many species (Gray, 1994, 1995; Champagne & Meaney, 2001; Cameron *et al.*, 2005; Pryce *et al.*, 2005). Although it is conceivable that lighting conditions are among the major environmental factors affecting rearing behavior, few studies have examined the influence of lighting conditions on maternal care and neuronal development in mammals. There is a clear circadian rhythm to nursing behavior in many species, including mouse (Hoshino *et al.*, 2006), rat (Ader & Grotta, 1970; Grotta & Ader, 1974; Lee & Williams, 1977; Coble *et al.*, 1994) and rabbit (Jilge, 1993, 1995). In rats, maternal care changes over the diurnal cycle, with the amount of time spent nursing being relatively high during the light phase (Ader & Grotta, 1970; Grotta & Ader, 1974). Recently, it was demonstrated that the Clock mutation in the suprachiasmatic nucleus in the hypothalamus impaired nurturing behavior by destroying the circadian secretion of prolactin in the mouse (Hoshino *et al.*, 2006). As the biological clock is strongly controlled by light, whether the lighting environment affects nurturing behavior may be an important question with regard to the early neonatal environment. It was suggested that entrainment to a new lighting condition was accompanied by stress (Stephens, 1980; Munck *et al.*, 1984) and therefore it is also likely that circadian change-induced stress may affect maternal care by dams. Thus, it could be predicted that altered photoperiodic conditions would change the acrophase in the dam and subsequently influence mother–infant

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interaction. Also, it could be predicted that offspring exposed to various levels of maternal care might correspondingly exhibit altered development of emotionality and memory functioning.

In this context, to elucidate whether early lighting conditions (ELC) affect maternal care and the circadian rhythm of the dam, we examined whether prolonged dark phase conditions (PDC) as well as prolonged light phase conditions (PLC) during the postpartum period in rats changed active nursing behavior and locomotor activity. Furthermore, we also examined whether alterations in ELC during the neonatal period of rats induced anxiety-like behavior and dysfunction of hippocampus-dependent memory or the HPA system in response to stress in later life to elucidate the importance of ELC in the development of stress vulnerability.

Methods and materials

Animals

One hundred and four pregnant Sprague-Dawley rat dams were housed individually in standard polycarbonate cages. Environmental conditions were consistent among the different manipulated groups with the exception of lighting (temperature 23 ± 2 °C, humidity 60%). The light intensity at cage level was approximately 100 lux. Rat chow and tap water were available at all times. Pregnant dams were inspected daily in the morning for delivery and the day of birth was designated as postnatal day (PND) 0. Only litters with 11–15 pups were used in this study and furthermore, there were no differences in

mean litter size among groups of mothers exposed to various light conditions. Litters were left intact and, on PND 10, pups were gently counted, sexed and weighed. As another parameter of somatic change, we observed the day of eye opening. On PND 22, all litters were weaned and counted again. Male rats under similar lighting conditions and from the same litters were housed in groups of three in standard cages with free access to food and water under a 12-h light/dark cycle (lights on 08:00–20:00 h).

All procedures involving animals were conducted in accordance with the Guiding Principles on Animal Experimentations in Research Facilities for Laboratory Animal Science, Hiroshima University and approved by the Hiroshima University Animal Care Committee.

Experimental schedule (Fig. 1)

For the behavioral evaluation of dams, the amount of maternal care from PND 2 to PND 14 and the circadian rhythm of locomotor activity (PND 4–6 and 10–12) were examined. Additionally, a 1-h focal observation of maternal care was undertaken on PND 5. For the behavioral assessment of offspring, the elevated plus-maze test, social interaction test and object recognition test were administered during the dark active phase (21:00–01:00 h) on PND 42 (adolescence) and PND 84 (adulthood). Additionally, the circadian rhythm of locomotor activity in offspring was measured on PND 42–44 and PND 84–86. Raters unaware of the lighting conditions conducted data analysis. No more than two animals per experimental group were from any single

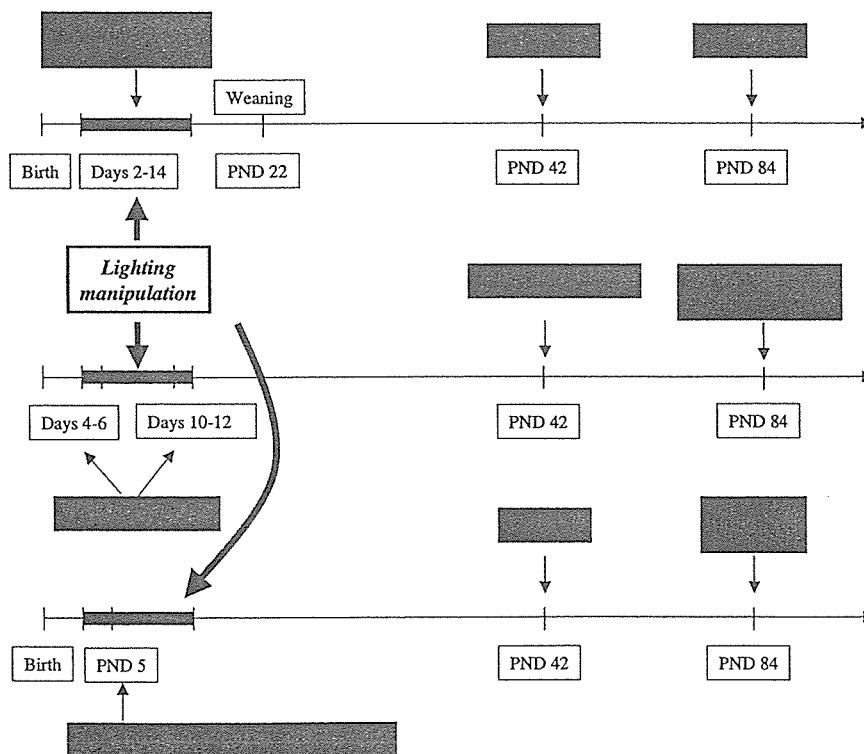


FIG. 1. Schematic representation of the experimental design. Lighting manipulations were administered to dams and pups from postnatal day (PND) 2 to PND 14. 1- and 24-h observations of maternal behavior and measurement of circadian rhythm of locomotor activity were performed in three sets of animals. After weaning, the elevated plus-maze test (EPM), social interaction test and object recognition test (ORT) were administered and locomotor activity was measured on PND 42 and PND 84. Corticosterone response to restraint stress and hippocampal glucocorticoid receptor and *N*-methyl-D-aspartate receptor mRNA expressions were investigated on PND 84. Each rat was used only once in one experiment, including all behavioral and molecular experiments. HPA, hypothalamic-pituitary-adrenocortical; RT-PCR, real-time-quantitative polymerase chain reaction.

litter. Particularly for real-time-quantitative polymerase chain reaction (RT-PCR), there was only one pup per litter representing seven to nine litters per group. No animal was used in more than one experiment. A total of 413 rats were used in these behavioral analyses and a different set of rats was used for each behavioral test. The test apparatus and arena were wiped down with 10% ethanol between each test session. Each group consisted of non-fasted male rats that had not been acclimatized to the experimental apparatus (15–19 for the elevated plus-maze test, 8–9 pairs for the social interaction test, 11–12 for the object recognition test and 10–12 for the locomotor test; each group was comprised of animals from 10 different litters). Only male rats were used to eliminate the effects of the estrous cycle in female rats on anxiety and memory functioning. In conjunction with the object recognition test, the hippocampal levels of *N*-methyl-D-aspartate receptor (NR) subunits were measured by RT-PCR in adult offspring. Furthermore, in adult offspring we measured changes in plasma corticosterone levels by radioimmunoassay and mRNA levels of hippocampal glucocorticoid receptor (GR) by RT-PCR in response to a single immobilization stress.

Manipulations of lighting conditions

From PND 2 to PND 14, dams and pups were exposed to the following postnatal lighting manipulations: normal lighting conditions (NLC) ($n = 35$), continuing maintenance of NLC; PDC ($n = 34$), prolonging the dark phase by a 6-h delay of light onset (light/dark, 6/18 h; lights on at 14:00 h and off at 20:00 h) and PLC ($n = 35$), prolonging the light phase cycle by a 6-h delay of light offset (light/dark, 18/6 h; lights on at 08:00 h and off at 02:00 h). Dams and pups were then transferred back to NLC.

24-h intermittent observation of maternal behavior

We examined maternal behavior using a version of the procedure described by Myers *et al.* (1989). Throughout PND 2–14, all cages were continuously video-recorded for behavioral scoring. Small infrared cameras (one per cage) with adjustable lenses were mounted on a standard laboratory rack to face the short side of the cages. Maternal behavior was scored blindly at 3-min intervals for 1 h (20 samples/h) every third hour, starting at 09:00 h. Thus, for each dam a total of 160 samples/day (8×20) were obtained. The following behaviors were scored: (i) mother off pups; (ii) mother licking and grooming pups; mother nursing pups in either (iii) an arched-back posture, (iv) a 'blanket' posture in which the mother lies over the pups, (v) a passive posture in which the mother is lying either on her back or side while the pups nurse or (vi) an undetectable state when poor visibility prevented unambiguous identification of the behavior. Overall, the latter accounted for 3.2% of all observations. Maternal observations were converted to a percentage of the times that the dam engaged in each of these behaviors over the total number of time points observed. Licking and arched-back nursing were combined into a single category of active nursing according to the work of Meaney's group (Champagne *et al.*, 2003a). Contact was defined as any behavior that involved physical contact or close proximity to pups and almost invariably implied nursing and/or LG (Champagne *et al.*, 2003a).

1-h focal observation of maternal behavior

As scores by the above method do not necessarily reflect either the number or length of LG bouts but emerge as a function of both

frequency and duration, other groups of individual NLC, PDC and PLC mothers (NLC, $n = 11$; PDC, $n = 10$; PLC, $n = 11$) were observed continuously for 1 h between 15:00 and 19:00 h on day 5 of lactation. The observer noted the onset and offset of each individual bout of pup LG, which provided a direct measure of duration.

Dams' circadian rhythm of locomotor activity

The circadian rhythm of locomotor activity was measured by automatic actography in which the level of activity was estimated as the number of interruptions of near-infrared rays; the counterinterface was connected to a personal computer. Individual standard cages, in which dams and pups were maintained, were set within the apparatus and monitored. In order to measure the locomotor activity only of dams, the infrared sensors were set at a height of 10 cm from the floor. Movements detected were recorded every 10 min throughout the experiment. Activity counts were summed over 60 min and mean values over the 72 h were calculated. The resulting 12 mean patterns (72 data points each, expressed as percentages of the total mean value of the corresponding subject) were averaged at each time point during the day. Additionally, the mean values for three continuous days were depicted in each group.

Elevated plus-maze

The plus-maze consisted of two open arms (50×10 cm) and two enclosed arms ($50 \times 10 \times 38$ cm) opposite each other at a height of 73 cm above the floor. Lighting in the open arms was 55 lux. At the beginning of each test, the rat was placed in the center facing a closed arm. During the 5-min exposure, the number of entries into each of the arms and the time spent therein were monitored by a video camera. An entry was scored when two forepaws passed over the open or closed dividing line. Data were processed to yield the ratio of time spent in the open arms vs. total time and the number of entries into each arm of the maze.

Social interaction test

The social interaction test was performed in an open-field arena (48 cm square) dimly lit with white light (65 lux). All sessions were recorded by a video camera located above the apparatus. A male rat was placed simultaneously with an unfamiliar partner rat into the social interaction apparatus. During the 10-min test period, the number and duration of social interactions (sniffing, crawling over, following, etc.) were scored by a rater unaware of the lighting conditions to which the animal had been subjected.

Object recognition test

The object recognition test using two different colored bottles as novel objects was performed according to the method of Ennaceur & Delacour (1988) with a minor modification. The male rats were handled twice per day for 1 min for 5 days and, on the subsequent day, habituated to the empty open-field arena (48 cm square) for 1 h. The training session began 24 h after habituation. During the training session, the two identical objects described above were placed into the arena where habituation had taken place and each rat was allowed to explore freely for 3 min. During the retention test, the rat was placed back into the same apparatus 1 or 24 h after the training and one of the familiar objects was replaced by a new object. The rat was allowed to explore freely for 3 min. The time spent exploring