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Early postnatal repeated maternal deprivation causes a transient increase in OMpg and BDNF in rat cerebellum suggesting precocious myelination

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ABSTRACT

Repetitive maternal deprivation (MD) of neonatal rats during early life is known as one of the strongest stressors to pre-weaned animals. There is increasing evidence that the cerebellum is involved in cognition and emotion. In the present study, we examined how neurotrophic factors and myelin-associated molecules and their receptors (NGF, BDNF, OMgp, TrkA, TrkB, p75 NTR, and NgR) in the cerebellum are affected by early postnatal maternal separation. Rat pups were separated from their mothers for 3 h/day during postnatal days (PND) 10–15. At PND 16 and 30, the levels of mRNA and protein in the cerebellum were determined using real-time PCR and Western blot analysis. Cerebellar mRNA and protein levels of BDNF, TrkB, and OMgp were significantly increased in MD rats at PND 16. However, by PND 30 these variables normalized to control levels. In contrast, the levels of mRNA and protein for NGF, TrkA, p75 NTR, and NgR were unchanged at both ages examined. Transient enhancement of neurotrophic system and myelin-associated molecule expression may cause interference of normal development of the cerebellum such as precocious myelination, which may lead to functional and cognitive deficits later in life.

1. Introduction

Adverse life events during early postnatal life are thought to disrupt normal brain development and neural processing, as well as predisposition to learning, behavioral, and mood disorders in later life. Maternal deprivation during the pre-weaning period is one of the strongest stimuli to the neonate. Laboratory animals manipulated with maternal deprivation have been widely used as an experimental paradigm to examine the neuromolecular-based etiological mechanism of psychiatric diseases induced by adverse experiences in early life [1,2]. Recent clinical and laboratory investigations indicate that maternal separation during critical periods of brain development can cause cytoarchitectural disruption of various brain regions, including the cerebral cortex, hippocampus, and cerebellum, which are closely linked to learning, memory, cognition, and behavior [3–5]. Conventionally, the cerebellum is thought to control motor coordination. However, accumulating evidence has revealed that the cerebellum contributes to not only motor function but also cognitive processing and emotional control [6–9]. Therefore, studies examining these additional functions will improve the understanding of the effects of maternal separation stress on brain development.

The neurotrophin family of proteins is widely distributed in the central nervous system (CNS) throughout the fetal, neonatal, and adult periods and plays critical roles in brain development, maintaining neuronal functions, and structural integrity [1,10–12]. Various neurotrophic factors are thought to be present at downstream targets of stress, implying that neurotrophic factors are important endogenous mediators of stress responses in the brain [13]. Neurotrophic factors, particularly nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are involved in survival differentiation, plasticity, and maintenance of neuronal functions in the developing brain mainly through high-affinity receptor tyrosine kinase tropomyosine-related kinase (Trk) A and TrkB, and low-affinity receptor p75 neurotrophin receptor (p75 NTR) [14–17]. In contrast to the trophic effects elicited by neurotrophin activation through Trk receptors, p75 NTR is involved in an apoptotic signaling pathway to induce cell death. Recent reports indicate that the interaction between p75 NTR and the Nogo receptor (NgR) inhibits neurite outgrowth [18–20]. Oligodendrocyte myelin glycoprotein (OMgp) is a glycosylphosphatidylinositol-anchored protein

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localized on the myelin membrane and expressed on the surface of both neurons and oligodendrocytes in the CNS [21]. OMgp that is derived from glial cells has distinct roles in myelination and neurite growth during central nervous system (CNS) development [22]. In normal brain development, OMgp expression increases concurrently with CNS myelination and reaches a maximum during the late stages of myelination [23,24]. OMgp is well known to inhibit axon growth through the NgR. Furthermore, recent studies by Kikusui et al. reported that early weaning of mice results in abnormal development of myelin formation, and that the animals show increased anxiety-related behaviors [25,26].

The purpose of the present study was to evaluate how maternal separation stress in early life affects growth promotion and inhibition system in the cerebellum, with respect to the alteration of mRNA and protein expression levels of NGF, BDNF, OMgp, TrkA, TrkB, p75 NTR, and NgR. This study provides a framework within which to understand how maternal separation stress affects cerebellar development.

2. Materials and methods

2.1. Animal and maternal deprivation manipulation

Six pregnant Wistar rats obtained from CLEA Japan (Tokyo, Japan) were used in the present study. The day of birth was designated postnatal day 0 (PND0). On PND2, all pups were placed together and randomly assigned back to 6 lactating mothers so that each mother received 12 pups (male:female = 6: 6). These 6 sets of "litters with mother" were then randomly assigned to either maternal deprivation (MD) or mother-reared control (MRC) groups. MD rats were separated from their mothers for 3 hours (9:00 AM–12:00 AM) per day during PND10 and PND15. During this period of separation, the pups within the MRC groups were allowed to remain with their mothers. After the separation period, the pups were returned to their mothers each day. During this period of separation the pups were placed in a holding cage under the same conditions as the MRC groups. Animals were weaned on PND21. This study was carried out in compliance with the guidelines for experimental use and care of laboratory animals set forth by the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Kagawa University Animal Ethics Committee.

On PND 16 and 30, male pups of MD and MRC groups (2 litters each from 3 sets of "litters with mother"; $n = 6$ for each group) were anesthetized with sodium pentobarbital (60 mg/kg, i.p) and perfused intracardially with medical grade physiological saline. The brains were sectioned in the horizontal plane on a vibratome to yield 1-mm-thick slices. The sampling level coincided with plate 200 of the rat brain atlas [27]. The cerebellar region was removed from the brain and then cut at the midsagittal plane in chilled physiological saline with an aid of a dissection microscope. Right cerebellar specimens for reverse transcription (RT)-PCR were processed with RNAlater (Ambion, Austin, TX, USA). For Western blot analysis, left side tissues were quickly frozen in liquid nitrogen and then stored at −80 °C until use.

2.2. Real-time RT-PCR

Total RNA was extracted from RNAlater-processed cerebellar slices using the guanidinium isothiocyanate acid-phenol method with commercially available TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) was reverse-transcribed to cDNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Little Chalfont, UK) following the manufacturer's instructions. Real-time RT-PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics, Basel, Switzerland). Reactions were performed in a 20 μL volume with 2 μL cDNA, 0.5 μM primers, and reagents included in the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics). The forward (F) and reverse (R) primers used for this study are shown in Table 1. The amplification protocol consisted of one cycle at 95 °C for 10 min, followed by 45 cycles of PCR as follows; 10 s of denaturing at 95 °C, 10 seconds of annealing at 59 °C (NGF), 56 °C (BDNF), 56 °C (OMgp), 62 °C (Trk A), 62 °C (Trk B), 56 °C (p75 NTR), and 58 °C (NgR), with 20 seconds of extending at 72 °C. Detection of fluorescent products was carried out at the end of the extension period. As an internal control, β-actin mRNA was co-amplified in each sample.

To confirm amplification specificity, PCR products from each primer pair were subjected to melting curve and subsequent sequence analysis. For melting curve analysis, PCR products were melted by gradually increasing the temperature beginning at 65 °C in 0.2 °C increments. To exclude genomic contamination, electrophoresis of the PCR products amplified from cDNA using each primer was carried out on 2% agarose gel and stained with ethidium bromide. Similar electrophoresis of the amplification product without RT was also performed for each sample as a negative control. Quantification data were analyzed using the LightCycler analysis software. mRNA levels for each gene were expressed as compared to the mRNA level for the housekeeping gene β-actin. Each sample was analyzed in duplicate without prior knowledge of the experimental group to which the samples belonged. This blinding was achieved by allocating a code number to each cerebellum sample. The sample identity was not revealed until all the samples in a given experiment had been analyzed.

2.3. Western blotting

Tissues were processed for homogenization and sonication on ice in lysis buffer containing 50 mM Tris-base (pH 7.4), 150 mM NaCl, 0.5 mM sodium vanadate, 1% NP-40, 0.1 mM phenylmethylsulfony fluoride,

Table 1
Forward and reverse primer sequences for real-time RT-PCR.

Gene	Primer set	PCR product (base pairs)	Gene accession number	Position
NGF	5′-CAGACCCGCAACATCACTGTG-3′	131	NM_001277055.1	5′: 378–398
	5′-CCATGGGCCTGGAAGTCTAA-3′			5′: 508–489
BDNF	5′-GGTATCAAAAGGCCAACTGA-3′	183	M61175.1	5′: 591–610
	5′-CTTATGAACCGCCAGCCAAT-3′			5′: 773–754
OMgp	5′-TGGAAGACGACATTATGGCT-3′	187	NM_001005898.3	5′: 130–149
	5′-TCTTGCAGTCCAGGTGGTAA-3′			5′: 316–297
TrkA	5′-GAGGTCTCTGTCCAAGTCAGCG-3′	387	NM_021589.1	5′: 890–911
	5′-GCTGTTAGTGTCCACTGGCGA-3′			5′: 1276–1256
TrkB	5′-CGCCCCGTGAGCTGAACTCCT-3′	172	NM_001163168.2	5′: 2156–2176
	5′-CTGCTTCTCAGCTGCCTGACC-3′			5′: 2327–2307
p75 NTR	5′-CCTGCCTGGACAGTGTTACA-3′	583	NM_012610.2	5′: 313–332
	5′-GCCAAGATGGAGCAATAGAC-3′			5′: 895–876
NgR	5′-ATCTTCCTGCACGGCAACCGAAT-3′	532	AF462390.1	5′: 184–206
	5′-AGAGGTTGTTGGCAAACAGGTAG-3′			5′: 715–693
β-actin	5′-AGCCATGTACGTAGCCATCC-3′	250	NM_031144.3	5′: 468–487
	5′-TTTAATGTCACGCACGATTT-3′			5′: 717–698