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Developmental changes in concentrations and distributions of neurotrophins in the monkey cerebellar cortex

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Abstract

Neurotrophins are involved in the survival, differentiation, migration and neurite outgrowth of various neuronal populations. Neurotrophins and their receptors are widely expressed in the developing cerebellum of various experimental animals. To gain some insight into the possible roles played by these molecules in monkey cerebellum, we examined the protein levels of BDNF, NT-4/5 and NT-3 and distributions of those neurotrophins and TrkC, a high affinity receptor for NT-3, in the cerebellum of developing macaque monkeys using ELISAs and immunohistochemical methods. We found that the level of BDNF increased during development, while the level of NT-3 was higher during embryonic stages and decreased toward adulthood. The level of NT-4/5 increased from embryonic stages to infant stages and gradually declined with age. Among the three neurotrophins, BDNF immunoreactivity was found in all kinds of cerebellar neurons, including all inhibitory interneurons, throughout the postnatal periods examined, indicating that BDNF may be an essential factor for the maintenance of cerebellar neural functions. The Bergmann glial fibers and the internal part of the external granule cell layer were strongly NT-3 immunopositive at the early postnatal stages, and more weakly immunoreactive toward adulthood. In addition, we found that the premigratory precursors of the granule cells were TrkC immunopositive at early postnatal stages. These findings suggest that NT-3 in Bergmann glial fibers may be involved in the migration of the premigratory granule cells.

Keywords: Cerebellar cortex; Primate; BDNF; NT-3; TrkC; ELISA; Immunohistochemistry

1. Introduction

The mammalian cerebellar cortex is comprised of six distinct neuronal cell types, Purkinje cells, granule cells, stellate cells, basket cells, Golgi cells and unipolar brush cells. The distributions of those cells and the synaptic connections between them have been thoroughly elucidated (Palay and Chan-Palay, 1974; Ito, 1984). In addition, several developmentally critical events, such as neurogenesis, neuroblast migration and neuronal differentiation occur extensively in postnatal stages in the cerebellum. The cerebellum has therefore been used as a model system to study the role of epigenetic factors in the development of the central nervous system (CNS).

Despite the fact that the cerebellum has been considered to control mainly motor systems, recent studies have demonstrated that it may also be involved in higher cognitive functions. For example, cerebellar output through thalamic neurons has been shown to extend to the prefrontal cortex (Middleton and Strick, 1994) which is of importance for planning and working memory. Several functional neuroimaging studies have also demonstrated activation of the cerebellum during various cognitive tasks, such as verbal tasks, passive sensory tasks and visual attention shifting (Raichle et al., 1994; Gao et al., 1996; Allen et al., 1997).

Neurotrophins are a family of structurally related proteins, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT-4/5) and neurotrophin-3 (NT-3). Neurotrophins and their receptors have been reported to be expressed widely in the developing and adult cerebellum of various experimental animals (Maisonpierre et al., 1990; Rocamora et al., 1993; Kawamoto et al., 1996; Lindholm et al., 1997; Friedman et al., 1998; Das et al., 2001; Dieni and Rees, 2002; Ohira and Hayashi, 2003; Ohira et al., 2004). Studies using primary cultures of rat cerebellar neurons have demonstrated promotion of survival, movement and neurite extension by neurotrophins (Segal et al., 1992;

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Lindholm et al., 1993b; Gao et al., 1995; Kubo et al., 1995; Nonomura et al., 1996; Tanaka et al., 2000). Furthermore, gene-targeting studies have shown that BDNF (Schwartz et al., 1997) and NT-3 (Bates et al., 1999) are required for normal foliation. Although developmental changes in levels and localizations of neurotrophins have been investigated intensively in the rodent cerebellum (Lindholm et al., 1997), less is known about the primate cerebellum.

In view of the above, we considered it worthwhile to investigate developmental patterns in the levels and cellular distributions of neurotrophins in the primate cerebellum. In this study, we examined developmental changes in the protein levels of BDNF, NT-4/5 and NT-3 by enzyme-linked immunosorbent assays (ELISAs) and the distributions of those three neurotrophins and TrkC, a high affinity receptor for NT-3, in the cerebellar cortex of macaque monkeys. Most previous reports have focused on the localizations of neurotrophins in the Purkinje cells and granule cells, and precise studies on the cerebellar interneurons have not been performed. Therefore, we also analyzed the localizations of neurotrophins in inhibitory interneurons, such as stellate cells, basket cells, Golgi cells and unipolar brush cells, which are of importance in the cerebellar circuitry.

2. Materials and methods

2.1. Experimental animals and tissue preparation

Twenty-three crab-eating monkeys (*Macaca fascicularis*) and six rhesus monkeys (*Macaca mulatta*) from embryonic day 120 (E120) to 15 years old were the subjects for ELISAs and twelve rhesus monkeys and three crab-eating monkeys from postnatal day 1 (P1d) to 15 years old were used for immunohistochemical analysis (Table 1). The method used to determine the embryonic day of age of fetal monkeys was previously described by Shimizu (Shimizu, 1988). Embryonic monkeys were obtained by Caesarian section under anesthesia with ketamine hydrochloride (10 mg/kg, i.m.) and maintained with halothane (1%)–N_2O–oxygen inhalation.

For non-survival procedures, all monkeys were pretreated with ketamine hydrochloride (10 mg/kg, i.m.) and deeply anaesthetized with pentobarbital sodium (25 mg/kg, i.v.). For ELISAs, monkeys were killed by bloodletting from the carotid artery. The skulls were quickly opened and dissection of cerebellar tissue was performed on ice. The dissection of cerebellum included cerebellar cortex of lobule VI to VIII, but not cerebellar nuclei. The dissected tissues were stored at −80 °C until use. For immunohistochemistry, monkeys were perfused through the heart with 0.15 M NaCl followed by one of the fixation buffers (1) 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 (PB), (2) 2% PFA and 0.5% glutaraldehyde (GA) in PB, (3) 4% PFA in PB or (4) 4% PFA and 0.1% GA in PB. After perfusion, the cerebellum were immediately removed and immersed in 2% PFA and 5% sucrose in PB at 4 °C for 24 h, followed by successive immersion in 10% sucrose in 0.1 M phosphate-buffered saline, pH 7.4 (PBS), 20% sucrose in PBS and 30% sucrose in PBS containing 0.02% sodium azide. The cerebellum was sagittally cut into blocks 5 mm thick and mounted in Tissue-Tek (Miles, Elkhart, IN, USA) and frozen rapidly in a dry-ice/acetone bath, and stored at −80 °C until dissection. All procedures were carried out in accordance with The Guide for the Care and Use of Laboratory Animals established by the NIH (1985) and The Guide for the Care and Use of Laboratory Primates established by the Primate Research Institute of Kyoto University (2002).

Table 1
Monkeys used in this study

Age (abbreviation)	*Macaca facicularis*		*Macaca mulatta*	
	Male	Female	Male	Female
ELISAs				
Embryonic day 120 (E120)	3	1		
Embryonic day 140 (E140)	3	1		
Postnatal day 1 (P1d)	2	2		
Postnatal 2 months (P2m)	3	1		
Postnatal 2 years (P2y)	1			2
Postnatal 4 years (P4m)		1	1	1
Postnatal 7–15 years (AD)	3	2	1	1
Immunohistochemistry				
Postnatal day 1 (P1d)			2	1
Postnatal 1 month (P1m)			2	
Postnatal 6 months (P6m)			3	
Postnatal 5–15 years (AD)	1	2	2	2

2.2. Antibodies

For ELISAs, we used each of three kinds of antibodies as the coating antibody: anti-BDNF mouse monoclonal IgG, R43-01 (Radka et al., 1996), anti-NT-4 mouse monoclonal IgG (Promega) and anti-NT-3 mouse monoclonal IgG (Promega). For the primary antibody, we used the following: anti-BDNF chick polyclonal IgY (Promega), anti-NT-4 chick polyclonal IgY (Promega), anti-NT-3 chick polyclonal IgY (Promega) and normal chick IgY (Promega). Horseradish peroxidase (HRP) conjugated anti-chick IgY antibody (Promega) was used as secondary antibody.

For immunohistochemistry, we used anti-BDNF rabbit polyclonal IgG (1:1600, sc-546, Santa Cruz Biotech., Calif., USA), NT-4/5 (1:3200, sc-545, Santa Cruz Biotech.), NT-3 (1:3200, sc-547, Santa Cruz Biotech.) and TrkC (1:400, sc-117, Santa Cruz Biotech.) as the primary antibody. As the secondary antibody, we used biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA).

2.3. ELISAs

ELISA methods we used in this study were based on the procedure described by Mori et al. (2002, 2004). The monkey cerebellar tissue was homogenized in 10 volumes of extraction buffer (1 mM EDTA, 1 M guanidine hydrochloride, 10 μg/ml aprotinin, 0.2 mM benzethonium chloride, 2 mM benzamidine and 1 mM PMSF in 0.1 M phosphate buffer, pH 7.2). The homogenate was centrifuged for 30 min at 8000 × *g* at 4 °C, the supernatants (first extraction) were collected and the sediments were rehomogenized and centrifuged as described above. The supernatant (second extraction) were collected, added to the first extraction and stored at −80 °C until use. Hundred microliters of the supernatant was used to determine the concentrations of neurotrophins. To estimate the recovery of exogenous neurotrophins in our ELISA methods, we added recombinant human (rh) neurotrophins (200 pg/ml of BDNF, 50 pg/ml of NT-3 and 50 pg/ml of NT-4; Peprotech, London, UK) into the cerebellar extract. Ninety six-well plates (Nunc, Roskilde, Denmark) were coated with 100 μl of the coating antibody in coating buffer (50 mM sodium carbonate–bicarbonate buffer, pH 9.6) and incubated overnight at 4 °C. After aspiration of excess antibody and three washes with washing buffer (50 mM Tris–HCl, 0.6 M NaCl and 0.1% Triton X-100), 300 μl of coating buffer containing 1% BSA was added to each well and the plates were incubated at room temperature for 1 h. The standard amounts of rh-neurotrophins were diluted in sample buffer (1 M guanidine hydrochloride and 1% BSA in washing buffer). After aspiration and three washes with washing buffer, 100 μl of standards or duplicated test samples were added per well and wells were incubated overnight at 4 °C. The plates were then washed three times with washing buffer, followed by incubation with 100 μl of the primary antibody in washing buffer overnight at 4 °C. After three washes, 100 μl of a 1:1000 dilution of the secondary antibody in washing buffer were added per well, and the plates were incubated for 6 h at 4 °C. After further washing, the reaction was developed for 15 min with 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB; Kirkegaard and Perry Laboratories, Gaithersburg, MD) and