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Immunohistochemical Localization of Nerve Growth Factor, Glial Fibrillary Acidic Protein and Ciliary Neurotrophic Factor in Mesencephalon, Rhombencephalon, and Spinal Cord of Developing Mongolian Gerbil

Il-Kwon Park, Kyoug-Youl Lee, Chi-Won Song, Hyo-Jung Kwon, Mi-Sun Park, Mi-Young Lee, Keun-Jwa Lee[1], Young-Gil Jeong[2], Chul-Ho Lee[3], Kwon-Soo Ha[4], Man-Hee Rhee[5], Kang-Yi Lee[6] and Moo-Kang Kim*

College of Veterinary Medicine, Chungnam National University
[1]*Chungnam Veterinary Service Laboratory,* [2]*College of Medicine, Konyang University*
[3]*Korean Research Institute of bioscience and biotechnology (KRIBB),* [4]*College of Medicine, Kangwon National University*
[5]Department of Cell Biology & Physiology College of Medicine Washington University
[6]*College of Oriental Medicine, Daejeon University*

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Abstract

The distribution of the nerve growth factor (NGF), the glial fibrillary acidic protein (GFAP) and the ciliary neurotrohic factor (CNTF) was performed in coronal sections of the mesencephalon, rhombencephalon and spinal cord in the developing Mongolian gerbils. Generally, NGF specifically recognizes neurons with the NGF receptor, whereas GFAP does the glia, and CNTF does the motor neurons. The receptor expression was examined separately in gerbils between embryonic days 15 (E15) and postnatal weeks 3 (PNW 3). The NGF-IR was first observed in the spinal cord at E21, which might be related to the maturation. The GFAP reactivity was peaked at the postnatal days 2 (PND2), while the highest CNTF-reaction was expressed at PNW 2. The GFAP stains were observed in the aqueduct and the spinal cord, which appeared to project laterally at E19. The CNTF was observed only after the birth and found in both the neurons and neuroglia of the substantia nigra, mesencephalon, cerebellum and the spinal cord from PND1 to PNW3. These results suggest that NGF, GFAP and CNTF are important for the development of the neurons and the neuroglia in the central nervous system at the late prenatal and postnatal stages.

Key words : NGF, GFAP, CNTF, Mongolian gerbil, immunohistochemistry

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* Corresponding author: Moo-Kang Kim
Tel : +82-42-821-6752, Fax : +82-42-825-6752,
E-mail : mgkim@cnu.ac.kr

Introduction

This study was based on optical microscopy examinations and an analysis of the induced fluorescence in order to localize the nerve growth factor (NGF), the glial fibrillary acidic protein (GFAP) and the ciliary neurotrophic factor (CNTF) in the mesencephalon, rhombencephalon and the spinal cord. This localization for the neurotrophins suggests a role for antibodies in the formation of the neuronal and glia developmental pathways. Among these neurotrophins, the neurons require NGF in order to continue maturation until the early prenatal days. Therefore, NGF may be used as a possible therapeutic agent for treating neurodegenerative disorders such as Alzheimers disease [2, 24]. In contrast to NGF, GFAP acts on glial growth [6, 10], CNTF has an influence on the motor neurons [4]. In this paper, an attempt was made to derive some general conclusions from the rather divergent distributional patterns observed throughout the CNS except the forebrain, which are described elsewhere. The distribution of NGF, GFAP and CNTF-immunoreactive (IR) cells in the rhombencephalon and spinal cord were investigated using immunohistochemical methods.

Materials & Methods

The Mongolian gerbil (*Meriones unguiculatus*) was used for experimental animals. The experimental groups composed of embryonic days 15, E17(E15), E19, E21, postnatal day 1 (PND 1), PND 2, PND 3, postnatal week 1 (PNW 1), PNW 2 and PNW 3. The embryos were dissected from pregnant gerbils from 15 to 21 days during gestation after sacrificing with a thiopental sodium injection (IP, 40mg/kg). The embryos were then immersed in 4% paraformaldehyde in a 0.1M phosphate buffer saline (PBS, 0.9% NaCl, pH 7.4). The gerbil offspring were transcardinally perfused with the same

fixatives. The brain tissue blocks were transferred to a 0.1 M phosphate buffer (PB, pH 7.4) containing 30% sucrose overnight and then stored at -70℃ deep freezer. The cryosections were used to obtain the coronal sections (45㎛) for the free floating methods. Alternate sections were pre-incubated in PB containing 0.3% Triton X-100, 1% normal goat serum and 1% bovine serum albumin (BSA) for 2 hours. The sections were then incubated in the primary antibody solution (working solution of 1:200) with the NGF (rabbit, Biogenesis), CNTF (rabbit, Biogenesis) and GFAP (rabbit, DAKO) antiserum PB containing to 1% BSA and 0.3% Triton X-100 at 4℃ for a overnight. As the kind of antiserum was not varied, double labeling could not be applied. The sections were 3 times washed in 0.1M PBS for 10 minutes, and then the sections were incubated with the secondary antibodies (biotinylated swine anti-rabbit Ig G, Vector). All incubation steps were carried out at 4℃. These sections were subsequently incubated in peroxidase-conjugated avidin (Vector, 1:100) for 1h. The sections were then incubated at room temperature in 0.05% 3.3- DAB-4HCl (40㎎/100㎖) and the floating immunostained sections were then mounted onto a slide glass. In the controls, the antiserum was pre-absorbed with GFAP and applied in this form to the control sections. The sections were also incubated omitting the primary antibodies, with peroxidase conjugate only. The immunofluorescent procedures were similar to the same immunohistochemical methods until incubation of the primary antibody solution. After incubation in the primary antibody solution, the tissues were washed 3 times in 0.1M PBS. Thereafter, the tissues were incubated for 12h with the secondary antibodies, consisting of fluroescein isothiocyanate (FITC, 1:200). They were then washed, coverslipped and examined using confocal microscopy (Leica).

Results

The NGF, GFAP and CNTF were found in the fewer part of the mesencephalon, rhombencephaolon and spinal cord compared to the forebrain. The immunopositive areas of NGF, GFAP and CNTF are shown in each Fig 1-3.

By E19, NGF was not expressed in any region. As expected, NGF-IR was associated with the neurons. NGF-IR neurons first appeared in the spinal cord weakly at E21 (Fig. 1G and Table 1). In the mesencephalon, a few NGF-IR cells were observed in the superior colliculus from PND1 (Fig. 1A) to PNW3, with a slight increase in the staining density. The positive cells were observed only in the cell body of the superior olivary nucleus of the ventral periaqueductal gray after PND3 (data not shown). Some diffuse NGF-IR staining was found in the inferior olive forward PND1 (Fig. 1B). By PNW3, the extent of the reactivity decreased in the midbrain, adding to the potency of the reactivity. In addition to the dorsal portion of the midbrain, NGF-IR was found in the internal geniculum of facial nerve after PND 1 (Fig. 4D), observed well fine at PNW2 (Fig. 1C) and the nucleus of the spinal tract of the trigerminal nerve (Fig. 1D). In cerebellum, the positive reaction began to be expressed in the Purkinje cell layer at PND1 (Fig. 1E, 4A), which was clearly seen from PNW1 to PNW3 (Fig. 1F). In the spinal cord, the neurons were examined in the posterior root (Fig. 5A) under high magnification at PND1 (Fig. 5D) and PND2 (Fig. 1H), the number of NGF-IR increased, which increased the intensity of positive neurons. This is in contrast to that observed in the white and gray matter of the spinal cord at the PNW2. Many processes were observed in the white matter of the spinal cord at PNW 2 (Fig. 1I).

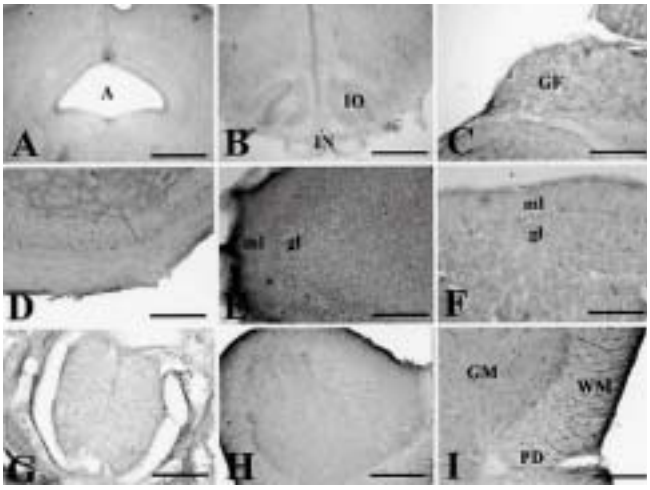

Fig. 1. NGF-IR neurons were found in the mesencephalon, rhombencephanlon and spinal cord of the developing brain. NGF-IR initiated to be found in the aqueduct (A), Inferior olive nucleus (B) and the internal geniculum of facial nerve (C) at PNW2. The NGF-IR was found in th pons at PNW3 (D). Not yet developed the cerebellum at PND1 (E), and changed to distinguish the cerebellar layer at PNW3 (F). A: PND1, B: PND1, C: PNW2, D: PNW3, E: PND1, F: PNW3, G: E21, H: PND2, I: PNW2. A: aqueduct, gl: glomerular layer, GF: the internal geniculum of facial nerve, GM: gray matter, ml: molecular layer, IN: the interpeducular nucleus, IO: inferior olive nucleus, PD: pyramidal decussation, WM: white matter. Scale bar =100㎛(A-C,G-I), 50㎛(E,F), 25㎛(D).

GFAP was first observed in the spinal cord at E19 (Table 2), In mesencephalon, GFAP-IR was observed around the ventricle at E21 (data not shown) and developed the marginal portion by the projecting fibers and the continuously also found in the superior colliculus after PND1 (Fig. 2A and Table 2). In the ventral part of mesencephalon, a slightly higher number of GFAP-stained elements were observed (Fig. 2B). A weak reaction was found around the aqueduct until PND3 (Fig. 2C), and preserved the staining by PNW3. The most notable GFAP-IR glia was observed on the margin of the aqueduct and the 4th ventricle. The cortex of the midbrain proper was

Table 1. Distribution of NGF-IR in the developing Mongolian gerbil brain[a].

Tissue	E17	E19	E21	PND1	PND2	PND3	PNW1	PNW2	PNW3
Superior colliculus	−	−	−	±	+	+	+	+	+
Periaquaduct	−	−	−	−	−	+	+	+	+
Midbain cortex	−	−	−	±	±	+	+	+	+
Pons	−	−	−	±	+	++	++	+	+
Cerebellum	−	−	−	±	+	+	++	++	++
White matter of S.C.	−	−	−	−	−	−	−	+	+−
Gray matter of S.C.	−	−	±	+	+	++	++	++	+

[a]Relative intensities of NGF-IR are graded:-, absent; ±, barely detectable;+, moderate to weak; ++, strong; +++, very strong. S.C. : spinal cord.

poorly stained at E21. GFAP-staining was observed somewhat more GFAP-IR cells in the periaquaduct compared to the facial nerve of the pons at PND3W (Fig. 2D). There were more GFAP-stained cells in the bundles of cranial nerve fibers than in the pons. Nevertheless, the motor nerve fiber tracts could also be followed readily in the medulla due to an arrangement of IR parallel to the course of the nerve fibers. In contrast, caudal to the decussation, the former place of pyramidal tract was filled with an abundance of GFAP-IR fibers running to the surface. Some distinguished areas nevertheless contained high amounts of immunoreactivity such as the substantia nigra and interpeduncular nucleus and to a lesser extent, the central gray matter (Fig. 4E). The increase in the number and packing density of the GFAP-immunostained elements was encountered in the medulla, and particularly in the area postrea. Another prominently GFAP-labeled region was the spinal trigerminal nucleus. The intense staining of this region continued caudally into the Rolando substance. Fiber tracts were devoid of immunoreactive GFAP. In cerebellum, GFAP expression was not observed until PND2 and typically found in the granular cell layer (Fig. 2E). GFAP-stained fibers were found in astrocytes of the molecular layer after PND2 (Fig. 2F, 4B). In the spinal cord, the fiber-like structure was found in the marginal portion after E19 (Fig. 5B). It began to be detected in the boundary between the white and gray matter at E21 (Fig. 2G), identified by the confocal images peakly at PNW1 (Fig. 5E). The nucleus appeared as dark stain stripes, which upon higher magnification proved to be composed of thick, irregular fibers. The overall distribution of the GFAP-IR was characterized by the population of immunostained stellate astrocytes in the gray matter at PND1 (Fig. 2H), and by a coarse radial GFAP-fiber system in the white matter. In addition, the midline structures and dorsal bundle septa contained an accumulation of labeled fibers and cells at PNW3 (Fig. 2I).

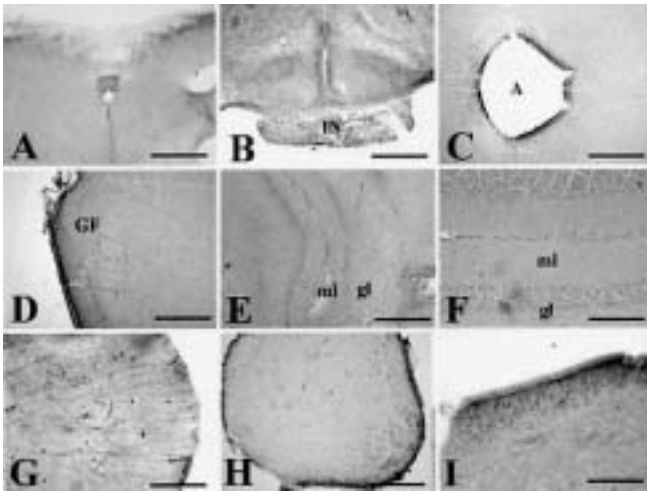

Fig. 2. The pattern of the developing GFAP-IR was like projecting the surface. Immunoreative developing astrocytes can be identified during early postnatal days. In mesencephalon, GFAP-IR fibers was seen in the collculus (A) and the inferior olive nucleus from PND1, increasing in the number and density at PND3 (B). GFAP-IR was first observed in the periaqueductal gray matter at E21 (data not shown), the fibers were progressed the cortex (C). In pons (D) and cerebellum (E, F), the reaction was found lately. By PNW3, the glial reaction was its greatest part filled with the stained stellate astrocytes in spinal cord (H, I). A: PND3, B: PND3, C: PND3, D: PNW3, E: PNW1, F: PNW3, G: E21, H: PND1, I: PNW3. A: aqueduct, gl: glomerular layer, IN: the interpeducular nucleus, GF: the internal geniculum of facial nerve, ml: molecular layer, Scale bar=100㎛(A-D, I), 50㎛(E,F), 25㎛(G,H).

Table 2. Distribution of GFAP-IR in the developing Mongolian gerbil brain[b]

Tissue	E17	E19	E21	PND1	PND2	PND3	PNW1	PNW2	PNW3
Superior colliculus	−	−	−	±	+	+	±	±	±
Periaquaduct	−	−	+	+	+	+	+	+	±
Midbain cortex	−	−	−	±	+	+	+	±	±
Pons	−	−	−	+	+	+	+	+	±
Cerebellum	−	−	−	−	−	±	+	++	++
White matter of S.C.	−	+	+	+	++	++	+++	++	++
Gray matter of S.C.	−	−	−	+	+	+	+	+	+

[b]Relative intensities of GFAP-IR are graded:-, absent; ±, barely detectable;+, moderate to weak; ++, strong; +++, very strong. S.C. : spinal cord.

CNTF was observed in both neurons and neuroglia only after birth (Table 3). Fig. 3 shows the CNTF protein expression in developing brain sections taken from the PND1 to the PNW3 in the mesencephalon, rhombencephalon and the spinal cord. Positive neurons were observed in the cerebellum and the subcortical regions as well as in the spinal cord. CNTF-IR cells were first observed in the marginal region of the pons at PND1 (Fig. 3A), the spinal cord (Fig. 3G), and around the cerebral aqueduct slightly (Fig. 3C). CNTF didnt show the shape of neurons in the mesencephalon at early postnatal days (Fig. 3B). On the other hand, CNTF-IR glial cells were observed throughout the CNS although not with the same frequency as with the CNTF-IR neurons, suggesting that possibly only a subset of glia are immunopositive. Note again there were a strong nuclear positive reaction at all postnatal ages and an apparent increase in the cortical neurons with age. By PNW2, CNTF-IR appeared to be more widely distributed throughout the cytoplasm with an increased density. In the pons, the reaction was weak, however the neuron-like structure was found in the trigerminal nerve (Fig. 3D) and the facial nerve (Fig. 3E) after PNW1. This pattern persisted to PNW3 and appeared to be a common theme throughout the cortex of the mesencephalon (Fig. 4F). In the cerebellum, CNTF appeared in the granular layer at PNW1 (Fig. 4C) and developed more strongly with age within the Purkinje cell layer at PNW3 (Fig. 3F). In the spinal cord, CNTF was observed in the cell bodies and processes at PND2 (Fig. 3G). At PNW1, the white matter and gray matter was distinguished (Figs. 5C, 5F). The neurons were found in the ventral white matter portion of the spinal cord after PND1, especially well defined at PNW2 (Fig. 3H), which stained in the process in the white matter at PNW3 (Fig. 3I).

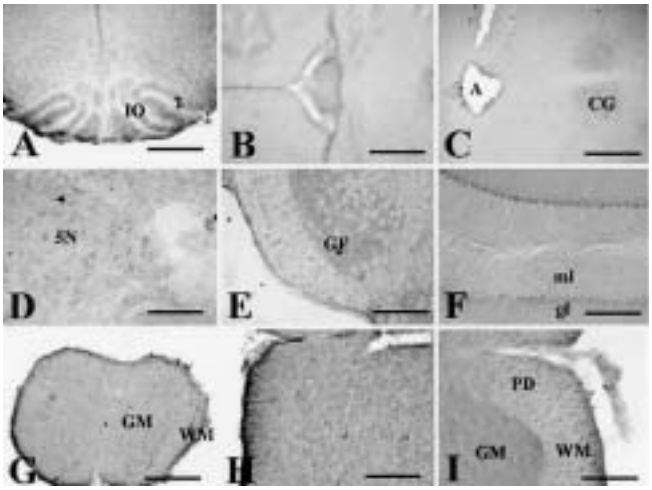

Fig. 3. CNTF-IR was first found in the neuron slowly at PND1 (A), expressed in the central gray at PNW1 (C). In the pons, CNTF-IR neurons and glia were observed in the subtantia nigra and the facial nerves from PNW1 to PNW3 (D, E). Immunoreative developing neurons and astrocytes can be identified in the spinal cord (G-I). A: PND1, B: PND3, C: PNW1, D: PNW2, E: PNW3, F: PNW3, G: PND2, H: PNW2, I: PNW3. 5N: the trigerminal nucleus in the pons, A: aqueduct, gl: glomerular layer, GF: the internal geniculum of facial nerve, GM: gray matter, ml: molecular layer, IO: inferior olive nucleus, PD: pyramidal decussation, SN: substantia nigra, WM: white matter. Scale bar=250㎛ (G), 100㎛(A-C), 50㎛(D-F), 25㎛(H,I)

Table 3. Distribution of CNTF-IR in the developing Mongolian gerbil brain[c].

Tissue	E17	E19	E21	PND1	PND2	PND3	PNW1	PNW2	PNW3
Superior colliculus	−	−	−	−	−	−	−	−	−
Periaquaduct	−	−	−	±	±	±	±	−	−
Midbain cortex	−	−	−	±	±	±	±	−	−
Pons	−	−	−	+	+	+	+	+	+
Cerebellum	−	−	−	−	−	±	+	+++	++
White matter of S.C.	−	−	−	+	+	+	+	++	++
Gray matter of S.C.	−	−	−	−	±	+	+	+	+

[c]Relative intensities of CNTF-IR are graded:-, absent; ±, barely detectable;+, moderate to weak; ++, strong; +++, very strong. S.C. : spinal cord.

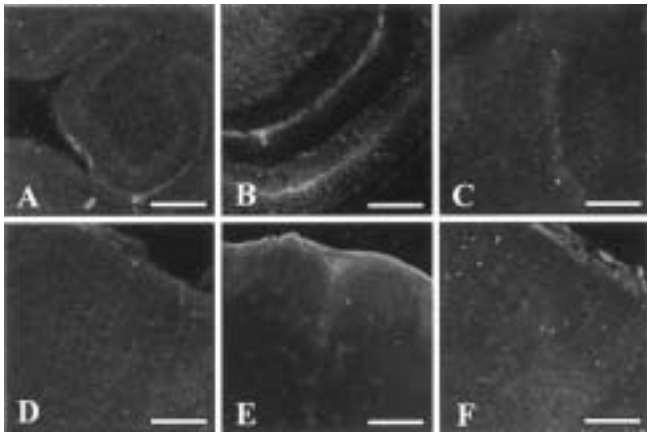

Fig. 4. Confocal images of NGF-, GFAP- and CNTF-immunofluorescent in the cerebeullum and pons. A, D: NGF (+), B, E: GFAP (+), C, F: CNTF (+). A, B, C: cerebellum, D, E, F: pons, A: PND1, B: PND3, C: PNW1, D: PND1, E: PND3, F: PNW1. Scale bar= 100㎛ (D-F), 200㎛(A-C).

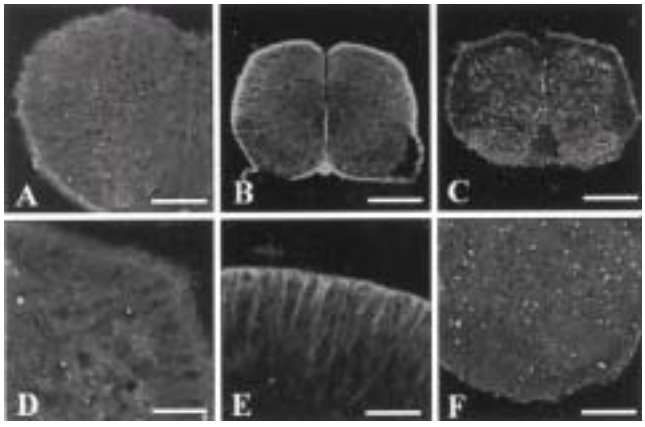

Fig. 5. Confocal images of NGF-, GFAP- and CNTF-immunofluorescent in the spinal cord at P3. A, D: NGF (+), B, E: GFAP (+), C, F: CNTF (+). A: PND1, B: PND1, C: PNW1, D: PND1, E: PNW1, F: PNW1. Scale bar=50㎛(D,E), 100㎛(A,F), 500㎛(B,C).

DISCUSSION

In this study, the presence of NGF-, GFAP-, and CNTF-IR cells in the mesencephalon, rhombencephalon and spinal cord in developing Mongolian gerbils was established. This localization suggests a role for antibodies in the formation of the neuronal and glial pathways. Different neurotrophic factors and proteins affected neurons and glia during developmental. In the former study regarding the distribution of NGF-, GFAP- and CNTF-IR cells in the forebrain, the investigations with observations of the mesencephalon, rhombencephalon and spinal cord were reported. The following discussion will encompass the findings from this paper as well as some points relevant to the whole CNS. Observations concerning of the forebrain are contained in the first of our two papers (Park et al, 2002).

NGF was expressed in the developing brainstem and spinal motor neurons. The function of NGF can be distinguished from the cellular sites of the NGF [21, 26]. In case of rats, NGF-IR expression in these neurons is transient and largely disappears by PND10 [27]. This transport of NGF from the spinal cord is currently under investigation and may differ in adults and embryos [7]. In this study, NGF-IR was expressed more slowly throughout the mesencephalon, whereas a weak stained fiber for NGF was initiated in the olfactory bulb in the forebrain at E21. It appears to be differentiated during the growth of the developing nervous system in gerbils. NGF-IR increased suddenly at PND3. The location of the NGF-IR cells in gerbils was related to the sympathetic neurons like other animals [3, 8, 12, 15, 21, 26, 27]. The NGF-IR was widely expressed among the mesencephan and rhombencephalon, and substantial amounts of NGF were also found in the striatum, thalamus, caudate putamen, ventral premammillary nucleus, mesencephalic trigerminal nucleus, prepositus hypoglossal nucleus, raphe nucleus, nucleus ambiguous, and Purkinje cells of the cerebellum with lower levels found in the cerebral cortex. The localization of NGF-IR neurons was

similar to that in rats and mice. However, the spinal cord gray matter, whilst being positive, was far less positive than the surrounding marginal zone white matter. A positive reaction was found in the developing cerebellar analge, but not in either the molecular layer or the glomerular layer, as was reported in a previous study using mice. Multiple positive fiber tracts were seen running through the pons, medulla oblongata and spinal cord. The spinal cord expressed a positive reaction both at the cervical and thoracic levels, with intense IR in the marginal zone. To a lesser extent, immunoreactive material was observed in the developing spinal cord gray matter. Similar response patterns to NGF have also been reported for rat neurons at similar developmental ages. This suggests that the neurons require other factors such as the other neurotrophins or even non-soluble factors, at this stage (E16-E18) in their development [26].

The greater part of mesencephalon lacked GFAP-IR cells [10]. The GFAP-reaction by staining the axial filament bundles clearly reveals a skeleton of astrocytes [1, 9, 10, 13, 22]. Although GFAP-IR began to be observed in the lateral ventricle and the third ventricle at E17, expressed in the periaquaduct and spinal cord slight slowly at E19. They were observed to project into the cerebral cortex that time. As expected, the shape of GFAP-IR was similar to glial cells. The staining for GFAP was constantly highly intense at PND2. However, the GFAP-intensity decreased in the forebrain as the fetus developed. This is in contrast to that observed in the cerebellum and spinal cord. Therefore GFAP within the intermediate filaments might take charge of developing the glia at the early postnatal stages in gerbils.

CNTF-IR neurons and the glia were widely distributed throughout the rat and mouse CNS and are known to prevent the 'programmed' death of the spinal cord motor neurons and oligodendrocytes after birth [4, 11, 14, 21, 25]. In gerbils, CNTF-IR neurons were first observed primarily in the glia after birth. Although neurotrophic factors were originally isolated on the basis of their ability to support neuron survival, these molecules are now thought to influence many aspects of CNS development and maintenance [25]. Therefore, CNTF-IR neurons are present within the facial nucleus, dentate gyrus, locus coeruleus, cortex and substantia nigra in the adult rat [11]. The neurons through the Purkinje cells within the cerebellum also have CNTF-IR cells. There is a paucity of reports on CNTF-IR neurons prior to 1995. However, Seniuk-Tatton et al. [23] suggested that the pattern of hybridization signals revealed in their lower micrographs through the midibrain showed a positive neuronal signal. As expected, CNTF-IR was observed only after birth, and was found in both the neurons and neuroglia in the CNS like rats. However, the there were a few differences between gerbils and rats, for example expression time. A gradual increase in the density of the CNTF-reaction was observed with increasing age after PNW2 in gerbils. The neuronal and glial distribution of the trophic factors may represent an important component of their actions on the neural cells. The CNTF-IR neurons may be separated from a glial signal. The location of the CNTF suggests the possibility that CNTF might have an effect on maturing neurons and glia as suggested by Henderson et al [11]. This study didnt deal with the double localization of-NGF, GFAP and CNTF, therefore we had not found the co-localization of them.

In summary, NGF-, GFAP- and CNTF-IR was found in many areas in the developing brain by the immuno-histochemical methods

1. The reactivity was no more specific to NGF, GFAP and CNTF than that reported in other studies using the general antibodies. NGF-IR neurons were widely distributed throughout the gerbil CNS, and were expressed in most neurons like the results of the other rodents from E21 to PNW3. The reactivity was found in the neurons that developed to their fibers and the somata in the central nervous system (CNS).
2. The GFAP-IR was observed in small numbers in the cortex, for example, the cerebral corticle, the lateral ventricle, the 3rd ventricle, pons, the cerebellum and the spinal cord. GFAP-IR seems to be produced from the ventricle, and was seen the peak at PND2. It declined to a density of staining after PND3 and expressed only the glial fibers after PNW2. GFAP-IR was found in the glial cells in the CNS from the late embryonic days to early postnatal days.
3. The CNTF-IR cells were located in the glia-like structures from PND1 to PNW1. The intense CNTF-IR was found in the neurons after PNW2, and expressed more slowly than other neurotrphins. CNTF-IR was found in glial-like structure at early postnatal days, changed to locate into the neurons as growing up. This may relate with the formation site and action sites of CNTF.

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