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Distribution of trkA in cerebral cortex and diencephalon of the mongolian gerbil after birth

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TrkA is essential components of the high-affinity NGF receptor necessary to mediate biological effects of the neurotrophins NGF. Here we report on the expression of trkA in the cerebral cortex and diencephalon of mongolian gerbils during postnatal development. The expression of trkA was identified by immunohistochemical method. In parietal cortex and piriform cortex, higher levels of trkA-IR (immunoreactivity) were detected at 3 days postnatal (P3) and at P9. Although trkA was not expressed till P3 in the parietal cortex, it was detectable at birth in the piriform cortex. Several regions, such as Layers I, IV & VI, did not show much expression. Layer I showed especially weak labeling. In the hippocampus, thalamus, and hypothalamus, higher levels of trkA-IR were detected at P6 and P12 than earlier days. But trkA was not expressed at birth in the hippocampus, at P3 in the reticular thalamic nucleus, or neonatally in the dorsomedial hypothalamic nucleus. This data shows that expression of trkA is developmentally regulated and suggests that high affinity neurotrophin-receptors mediate a transient response to neurotrophines in the cerebral cortex and diencephalon during mongolian gerbil brain ontogeny.

Key words: trkA, NGF, mongolian gerbil, cerebral cortex, diencephalon

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Introduction

In the developing mammalian nervous system, redundant neurons are eliminated during the period of naturally-occurring cell death [10]. The remaining cells form part of the adult neuronal network and the formation of this system depends on target-derived neurotrophic factors [5]. Nerve growth factor (NGF) is the prototype for a family of structurally related neurotrophic factors called neurotrophins. Neurotrophins include brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neuotrophin 4/5 (NT-4/5) [1,4]. In the peripheral nervous system, NGF supports the development and maintenance of sympathetic neurons and neural crest-derived sensory neurons [16]. In the central nervous system, NGF promotes the survival of basal forebrain cholinergic neurons. It has been shown the NGF plays a crucial role in synaptic plasticity during brain development and adulthood by activating a dual receptor system composed of trkA and p75 receptors, also known as high and low affinity receptors, according to their ligand binding affinity [3,7,12]. The trkA protein, a tyrosine kinase receptor of 140 kDa (gp140trk), acts as the specific functional receptor for NGF [13]. NGF provides trophic support for the basal forebrain cholinergic system consisting of acetylcholine-synthesizing neurons distributed across several distinct areas: the medial septal nucleus, the vertical and horizontal limbs of the diagonal band of Broca, and the magnocellular preoptic area [6,17]. NGF secreted in target regions is taken up by cholinergic nerve terminals and is then retrogradely transported to the neuronal body [22]. Loss of p75NTR or trkA leads to cholinergic neuronal loss in basal forebrain neurons, an effect resembling a lack of NGF support [9,18,20]. Recent findings indicate that increased levels of NGF in the cerebral cortex and hippocampus must be reflected by an enhanced availability

of trkA and p75NTR for more efficient transport to the basal forebrain [23]. Thus, expression of trkA, which has been identified as a specific functional receptor for NGF [13], dictates the biological activity of NGF. The biological effects of NGF are mediated via high-affinity receptor gp140trk that binds NGF and has intrinsic tyrosine protein kinase activity [14]. Taken together, trkA expression is key to neurotrophin responsiveness, and localization of trkA expression can be used further to define the biological functions of NGF and other neurotrophins. Localization of trkA expression is an important clue to neurotrophin responsiveness. To investigate the time course of NGF and trkA, we examined trkA expression in the cerebral cortex and diencephalon of postnatal Mongolian Gerbil brain. This study provides further evidence that expression of trkA detects the biological activity of NGF and is a marker for NGF-responsive CNS neurons.

Materials and Methods

Mongolian gerbil (*Meriones ungulitus*) was used for all studies. Experimental animals were divided into the following age groups: neonatal, postnatal 3 days (P3), P6, P9, P12, P15, P21, P28, P42, and adult. Gerbils were deeply anesthetized with methylether, sacrificed, and perfused transcardially with 0.9% NaCl in 0.1 M phosphate buffer saline (PBS, pH 7.4). This was followed by 150 ml 4.0% paraformaldehyde in 0.1 M PBS. The brain was then removed, postfixed in the same fixative solution overnight, transferred to 30% sucrose in PBS until sunk, and then frozen on dry ice. All samples were store at −20°C until used.

TrkA immunohistochemistry was carried out following ABC standard procedure as described before. Several frozen brain sections (45 μm) were cut with a cryostat and collected in PBS. All sections were washed with 0.1 M PBS (pH 7.4) 3 times, blocked for endogenous peroxidase activity with 1% hydrogen peroxide in PBS at room temperature for 30 min, and washed with PBS 3 more times. Sections were then incubated with blocking solution containing 1% normal goat serum (NGS, Vector, USA) in 0.3% Triton X-100 (Sigma, USA) at room temperature for 2 hours or at 4°C overnight to reduce nonspecific staining. After further washing with PBS, a rabbit anti-trkA primary antibody directed against the specific trkA were used at a dilution of 1 : 50. Three-day incubations with the primary antibodies were carried out at 4°C in PBS containing 1% fetal calf serum and 0.3% Triton X-100. The immunohistochemical reaction was developed with Vectastain ABC Kit (Vector, USA). Sections were then washed with PBS and incubated with biotinylated goat anti-rabbit IgG (Vector, USA) diluted 1 : 100 in PBS at 4°C for 12-24 hours. Sections were immunostained using a standard biotine-avidin detection system (Vectastain, USA). Visualization of immunobinding was carried out with DAB solution (0.04% diaminobenzidine and 0.05% H_2O_2 in PBS). After staining the sections were mounted on silane-coated slides (3-amino propyltriethoxy silane, Sigma, USA).

Results

We only describe those areas in which marked changes in the levels of trkA were seen in cerebral cortex and diencephalon during postnatal gerbil brain development. TrkA was localized to several neuronal populations. Generally, trkA expression increased with age.

Parietal cortex

There are six layers to the parietal cortex. TrkA expression was widespread in layers of the parietal cortex, but undetectable in neonatal and P3 brains. At P6, trkA-positive reaction could be detectable but the intensity was very low. Although trkA-immunoreaction was lower at P9, it was very clear in layer II, III, V, and VI. TrkA immunoreactivity was shown that the similar higher levels were seen in II, III and V cortex zones from 6 days to adult, reaching maximal levels at P21 and the strongest intensity was seen over parietal cortex layers II and III, as well as V among all six layers. However, the intensity was always much lower in layer I (Fig. 1, 2). The sections showed that immunoreactive cell bodies were larger in layers II, III, and V than in layers IV and VI and dendritic vertically direct to the outside layer at the time when the process were carefully observed with light microscope. However, the cell bodies in layer I were small and trkA immmunoreactivity was weak (Fig. 2).

Piriform cortex

There was a higher spread of trkA in the piriform cortex at all ages. In these areas, the density of cells displayed stronger trkA immunoreactivity with increasing ages. A low level of labeling in the piriform cortex was observed even from newborn mongolian gerbils. The positive intensity became stronger after P3, and the strongest intensity was seen at P12 (Fig. 3).

Hippocampus

TrkA expression increased with age in the hippocampus. However, no trkA-positive reaction existed until 3 days after birth. TrkA immunoreactivity began to be seen clearly in CA and dentate gyrus(DG) regions at P6. Similar levels were seen in regions CA1, CA2, and CA3, which was stronger than DG (Fig. 4). There was a much lower level of trkA-positive reaction at P6 and P9 (Figs. 4 A and B). The positive reactions increased after ages (C~H) and reached higher levels at 28 days of age (Fig. 4).

Thalamus

TrkA positive reactions were not detectable until 6 days, and increased with age in the reticular thalamic nucleus (Rt).

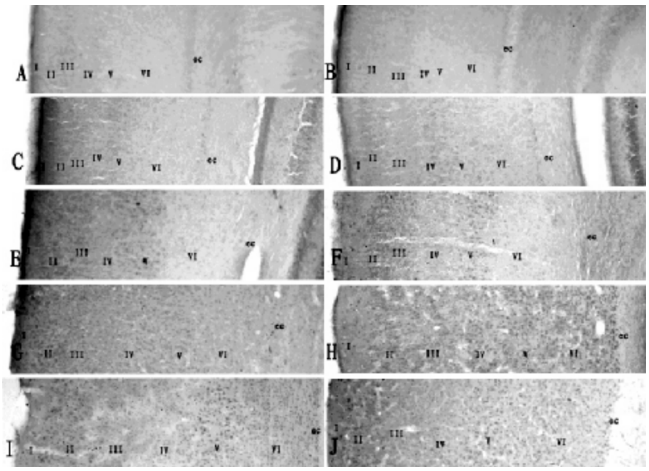

Fig. 1. TrkA immunoreactivity in parietal cortex. The strongest intensity is seen over parietal cortex layers II, III,and V among all 6 layers from P9 to adult (D~J). At P0 and P3 (A and B), it is hardly observed, while it is much lower at P6 (C). TrkA immunoreactivity is clearly detectable at the age of 9 days (D) and it increases with age. A~J: neonatal, P3, P6, P9, P12, P18, P21, P28, P42 and adult, respectively. I-VI: 6 layers in parietal cortex, EC: external capsule. ×100.

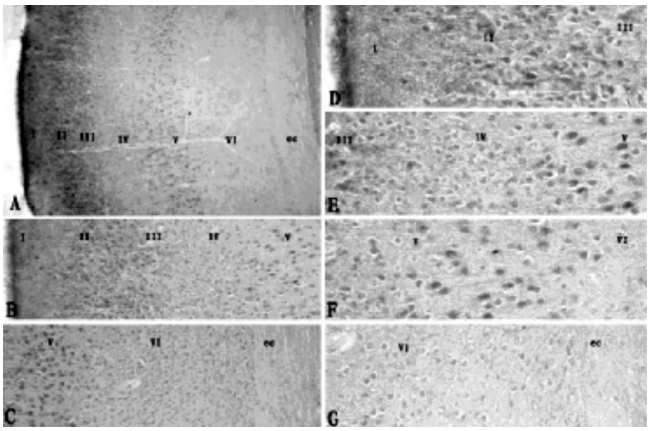

Fig. 2. TrkA immunoreaction in parietal cortex sections. Comparison of all layers at different magnification. TrkA-immnoreactivity is stronger in layers II, III, and V than that in layers I, IV, and VI. All layers are enlarged in (B~G) to illustrate the immunoreactive cell bodies in (D~G). Cell bodies are larger in layers II, III, and V than that in layers IV and VI and the cell processes vertically direct to the outer layers. A: ×100, B, C: ×200, D~G: ×400.

Very similar low levels were observed at P6 and P9 (Figs. 5 A and B). After P12 (C), trkA-IR was clearer and stronger (C~G), reaching the strongest level at the adult stage(H). The intensity in the thalamus was weaker than that in the hypothalamus regions (Fig. 5).

Hypothalamus

In the hypothalamus, there were more strongly reactive cells exhibiting trkA immunostaining. Very strong labeling in trkA immunoreactivity was observed from P6 to adult in the

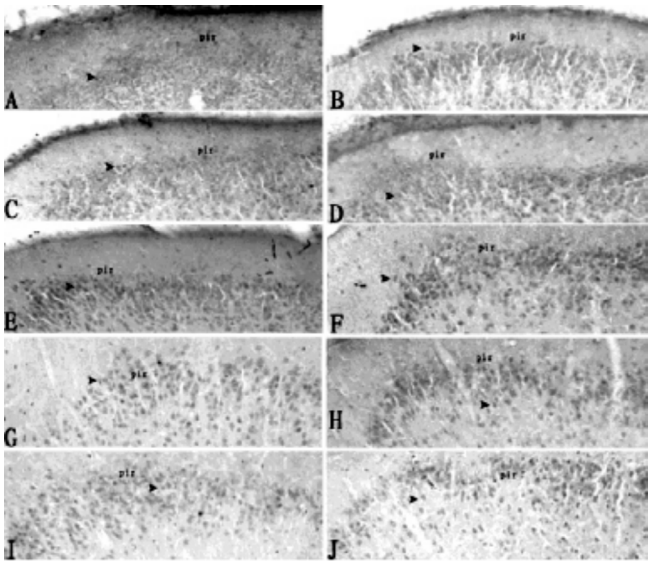

Fig. 3. TrkA immunoreactivity in piriform cortex. TrkA-positive reaction is observed clearly at birth (A). Since then, the expression gradually increases till adult (B~J). A~J: neonatal, P3, P6, P9, P12, P18, P21, P28, P42, and adult. ×200.

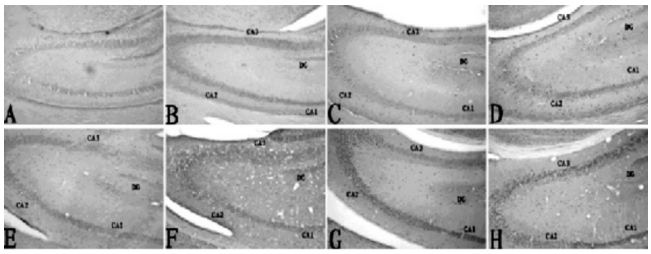

Fig. 4. TrkA immunoreactivity in hippocampus. TrkA expression was not present when mongolian gerbils were born and was very low at P3 (data not shown). It was expressed after P6 (A), but there was a much lower level of trkA-positive reaction at P6 and P9 (A and B). Positive reactions increased after ages (C~H). There was lower level of expression in the DG region than in the CA1, CA2, and CA3 regions. CA1, CA2, and CA3: hippocampus, DG: dentate gyrus, A~H: P6, P9, P12, P15, P21, P28, P42, and adult. ×100.

dorsomedial hypothalamic nucleus (DM), while cell labeling was not displayed at birth. There was weak trkA-IR at P3, but the strongest intensity was seen as early as at P12 (Fig. 6).

Discussion

In this report, we have only studied the developmental expression of trkA in the mongolian gerbil brain. The localization indicated by the trkA antibodies correlates with the developmental expression of NGF and the formation of the neuronal and glial pathways.

All the data on trkA expression in gerbil brains showed developmentally-regulated patterns after birth. These patterns are summarized in Table 1. Generally, trkA

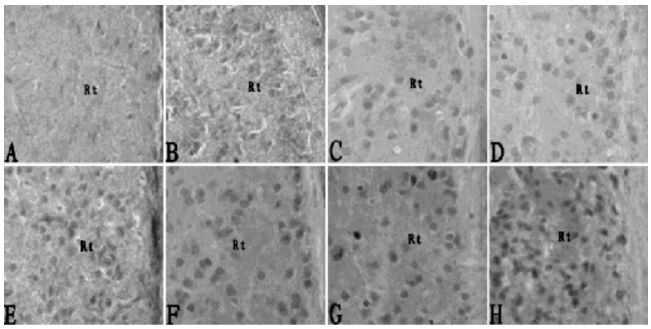

Fig. 5. TrkA immunoreactivity (IR) in reticular thalamic nucleus (Rt). Positive labeling was hardly seen till P3 (data not shown). Very similar low levels were observed at P6 and P9 (A, B). After P12 (C), trkA-IR was clearer and stronger (C~G), reaching the strongest level at adult (H). A~H: P6, P9, P12, P15, P21, P28, P42, and adult. ×400.

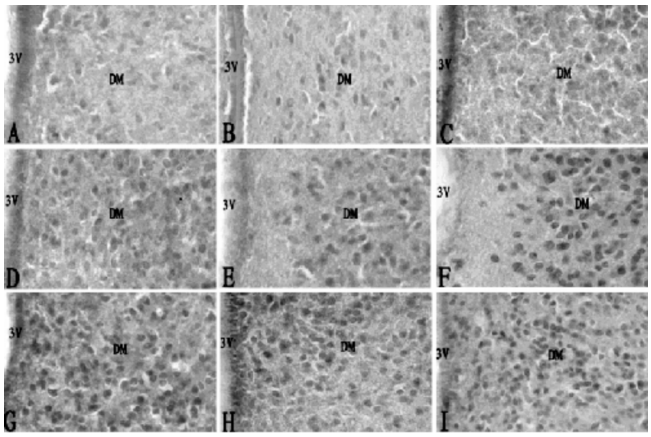

Fig. 6. TrkA immunoreactivity in dorsomedial hypothalamic nucleus (DM). No trkA was expressed neonatally (data not shown). TrkA-IR began to be clearly detectable at P3 , but only slightly, the same as at P9 (A and B). The peak presented itself at P12 and persisted to later ages (D~I). 3V: third ventricle. A~I: P3, P6, P9, P12, P15, P21, P28, P42, and adult. ×400.

expression increased with age and was found in similar locations to that in brains of other kinds of murine and rat. TrkA immunoreactivities were observed in newborn rat piriform cortex sections. These observations indicate that trkA expression is initiated in the piriform cortex during the embryonic stage. However, trkA-immunoreaction is detectable in the parietal cortex after P6. It seems like that trkA development occurs later in the parietal cortex of postnatal mongolian gerbils.

The main results of our studies can be summarized as follows. In the parietal cortex, no trkA was detected up to P3, but it was found at P6. The same trkA levels were seen in layers II, III, and V between P9 and P15 and increased to their adult level. However, there are differences in the piriform cortex in that trkA expression was detected when mongolian gerbils were born and gradually increased with age. Furthermore, trkA-positive cell bodies were larger in size and trkA intensity was stronger in layers II, III, and V than in layers IV, VI, & I. This may be the reason why layers II, III and V show clear trkA expressions. Analysis of trkA immunoreactivity (IR) was carried out using a trkA-specific antibody that recognized only trkA and was carried out by determining relative levels of trkA-IR in the parietal and piriform cortexes. The expression of trkA has been shown to be up-regulated by NGF in the parietal and piriform cortexes. Cholinergic cells containing high affinity NGF receptors are mainly interneurons in the caudate putamen (CPu) [14,17]. Only a subset of these neurons project outside of the CPu, particularly to the parietal cortex [2]. Therefore, trkA expression in the parietal cortex is developmentally regulated in a manner similar to that in hippocampus and CPu. Because the availability of NGF receptors on cholinergic neurons is essential in determining NGF biological activity [15], we have measured levels of trkA-IR to examine the availability of endogenous NGF in two areas of the mongolian gerbil brain.

Cholinergic neurons in the septum project mainly to the hippocampus, where NGF is synthesized [2] and retrogradely

Table 1. Overview of areas with a developmentally regulated expression of trkA. The intensity of the labeling was graded

	P0	P3	P6	P9	P12	P15	P21	P28	P42	adult
pc										
Layer I	-	-	+	+	+	+	+	+	+	+
Layer II , III & V	-	-	+	++	++	++	+++	+++	+++	+++
Layer IV & VI	-	-	+	+	+	+	++	++	++	++
pir	+	++	++	++	+++	+++	+++	+++	+++	+++
CA1, CA2 & CA3	-	+*	+	+	++	++	++	+++	+++	+++
DG	-	-	+	+	+	+	+	++	++	++
Rt	-	-	+	+	++	++	++	++	++	+++
DM	-	+	++	++	+++	+++	+++	+++	+++	+++

*Where-: no labeling, +: low, but clear and consistent labeling: ++: strong labeling: +++: very strong labeling. pc: parietal cortex, Layer IVI: cortical layer. Pir: piriform cortex, CA1, CA2 & CA3: hippocampus, DG: dentate gyrus, Rt: reticular thalamic nucleus, DM: dorsomedial hypothalamic nucleus. P0~P42, adult: postnatal ages of mongolian gerbil, P0: neonatal rat.

transported to septal cholinergic cells [21], which respond to NGF *in vivo* [8] and can be rescued by NGF after axotomy [11]. From embryonic Day 17 in rat, the beginning of the differentiation of hippocampal pyramidal cells, NGF gene expressions have been detected in the hippocampus [17]. During embryonic and postnatal development, the expression of NGF mRNA in the hippocampus increases until it reaches its adult level [19]. Our results are different. TrkA-positive cells were not present when the mongolian gerbil was born. Data indicated that these expressions were later in the hippocampus, reticular thalamic nucleus, and dorsomedial hypothalamic nucleus of mongolian gerbils. Immunohistochemical analysis of trkA showed the presence of trkA-IR in the granule layer as well as in the molecular layer of the dentate gyrus. This result suggests that trkA IR is localized mainly in axonal terminals around granule cells, which are known to synthesize NGF. TrkA in hippocampus has been shown to increase progressively after birth and peak at P21 [15]. Thus it appears that expression of NGF in the target-fields of the NGF-responsive cholinergic neurons and expression of trkA receptor in these sections are developmentally regulated in a similar fashion. A related phenomenon is seen in the developmental expression of NGF and trkA in the thalamus and hypothalamus of postnatal mongolian gerbil brains, but in Rt, trkA expressions are weaker than in other regions.

In summary, our data shows a developmentally regulated expression of trkA in many areas of the postnatal mongolian gerbil brain: the cerebral cortex, the hippocampus, the reticular thalamic nucleus, and the dorsomedial hypothalamic nucleus. Different intensities are shown in different brain areas of postnatal mongolian gerbils.

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