

## Expression of tyrosine kinase A in the cerebral cortex of postnatal developing rat

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Tyrosine kinase A (TrkA) is an essential component of the high affinity nerve growth factor (NGF) receptor necessary to mediate the biological effects of the neurotrophins, NGF. This study examined the distribution of TrkA-immunoreactivity (IR) cells in the postnatal rat cerebral cortex and the changes that occur in postnatal development as a result of the expression of this protein. TrkA-IR was detected at postnatal day (PD) 3, PD6, PD9 and PD15. Base upon their somatodendritic morphology, the most commonly labeled cell type was the pyramidal neurons. At PD3 and PD6, layer I, II, III and V was immunopositive for TrkA, at PD9, not only at layer I, II, III, and V but also at layer VI. At PD15, the TrkA-positive cells were distributed in all layers. These TrkA-positive cells were not detected at PD0. In contrast, there was significant increase in the percentage of cells exhibiting TrkA-IR with development and the highest level was detected at PD15. These results suggest that the cerebral cortex expresses TrkA strongly during the postnatal period. Moreover, the postnatal development-related increase in the expression of TrkA-cells shows that NGF may have a trophic effect on these cerebral cortex neurons from the postnatal period.

**Key words:** cerebral cortex, development, immunohistochemistry, TrkA, NGF

### Introduction

Neurotrophins are a group of neurotrophic factors that play an essential role in neuronal development, differentiation, survival, regeneration and function in both the central and peripheral nervous system [16]. This neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin 4/5

(NT-4/5) and neurotrophin-6 (NT-6) [3,21,22,32]. The effects of neurotrophins are mediated by an interaction with specific cell surface receptors, which are divided into two classes according to binding affinity to the ligand. The low-affinity neurotrophin receptor binds all members of the neurotrophin family with a similar low affinity. It is a transmembrane glycoprotein of approximately 75,000 Da, which is referred to as p75 [5,23] and plays an essential role in the formation of high-affinity NGF-receptor [13,30,33]. The second type of neurotrophin receptors is represented by a family of high-affinity receptors, which are known as tyrosine kinase (Trk) receptors. This Trk family includes tyrosine kinase A (TrkA), which binds NGF specifically, TrkB, which appears to mediate the biological effects of both BDNF and NT-4/5 and TrkC, which serves as the primary receptor for NT-3 [2,4,17].

NGF is a dimer of two 118 amino acid polypeptides and target-derived neurotrophic factor, which supports the survival and growth of peripheral sympathetic and primary sensory neurons. NGF also induces a variety of effects in CNS cells, including protein phosphorylation [12], the activation of gene expression [20], the promotion of axonal [8,9], the dendritic branching [18], the reduction neuronal loss after injury [15,19] and the chemotropic guidance of axons [11]. Two of the main NGF-responsive CNS neurons that have been characterized extensively are the cholinergic neurons of the basal forebrain and the striatum [10,26,27]. The basal forebrain cholinergic neurons (BFCN) are the projection neurons, which extend throughout the hippocampus and neocortex and are important for attention, learning and memory functions [29]. NGF is transported retrogradely from the terminals of the magnocellular neurons in the neocortex and hippocampus to the cell bodies within the basal forebrain nuclei [7,31]. It is believed that these biological effects of NGF are mediated via the high-affinity receptors, TrkA. It is localized most exclusively on the cholinergic neurons of the basal forebrain and mediate the retrograde transport of NGF to these neurons from distant neocortical and hippocampal structures. Several studies have shown that NGF binding promotes the autophos-

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phosphorylation of the TrkA protein, which suggests that TrkA plays an important role in the signal transduction of NGF. Therefore TrkA expression is essential to the neurotrophin responsiveness. Moreover, the localization of TrkA expression can be used further to define the biological functions of NGF and other neurotrophins.

Recent studies have shown that NGF is also expressed by the cortical neurons throughout the cortex and TrkA is widely produced by the postsynaptic target [28,25,1,24]. In immunohistochemical studies, it was reported that the TrkA-responsive neurons are expressed in the mature cerebral cortex by the post-synaptic profiles, somata and dendrites. Anti-TrkA antibody essentially labeled the neurons with cell bodies in the layer V pyramidal neurons and dendrites in the supragranular laminae of motor-somatosensory cortex.

In order to determine if NGF is also critical for regulating the cortical activity during development, this study analyzed the expression and age-related changes of TrkA in the cerebral cortex of postnatal rats using immunohistochemistry.

## Materials and Methods

### Experimental Animals

Sprague Dawley rats were used in all experiments. At each of the different developmental time point, including postnatal day (PD) 0, PD3, PD6, PD9 and PD15, rats were killed by decapitation under light ether anesthesia and perfused through the left ventricle of the heart with 0.1 M phosphate buffered saline (PBS, pH7.2), followed by fixative. 4% paraformaldehyde in 0.1 M PBS, pH 7.2, were used. Tissues were post-fixed in this same fixative overnight at 4°C and then cryoprotected in 30% sucrose in PBS for 48 hr. After freezing, each brain was cut into a complete set of 30 micrometer coronal sections.

### Immunohistochemistry

Immunohistochemistry was carried out by using the avidin-biotin-peroxidase complex (ABC) method as described below. After immersion with 1% hydrogen peroxide in PBS for 30 min at room temperature (RT) to inhibit endogenous peroxidase, section were preincubated with 1% normal goat serum (Vector, USA) and 1% bovine serum albumin for 2-3 hr at RT. They were incubated for 24-48 hr at 4°C with rabbit anti-TrkA immunoglobulin, diluted 1 : 100 (Santa Cruz, USA). This primary antibody is affinity purified polyclonal antibodies and recognizes an epitope corresponding to amino acids 763-777 mapping adjacent to the carboxy terminus of trk gp140 of human origin. This antibody reacts with TrkA of mouse, rat and human origin and do not show cross-reactivities with TrkB or TrkC. The section were washed 3 times in PBS, 10 min each at RT (same wash procedure was performed between each of the following steps), and incubated overnight at 4°C with biotinylated secondary anti rabbit antibodies (Vector, USA), dilution

1 : 200. The section were incubated for 3 hr at RT with gently agitation with a solution containing a preformed avidin-biotin-peroxidase complex (Vector, USA), at a dilution of 1 : 200. The section were then incubated in a solution containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.04% hydrogen peroxide for 10 min. The peroxidase reaction was stopped by rinsing the section. For light microscopy, the section were mounted on gelatin-coated slides, dehydrated through a graded series of ethanols, cleared in xylene and coverslipped. Section were viewed and photographed under brightfield.

### Control

Three controls for the immunohistochemical reactions were performed. In two of these controls, the sections were treated according to the methods described above except that either the primary or secondary antibodies were omitted. In the third control, the amount of endogenous staining was assessed; both the primary and secondary antibodies were eliminated from the processing.

### Analysis

The number of cells in the dorsal primary somatosensory cortex (area 3) at the level of the rostrum of the corpus callosum were counted in order to provide a quantitative estimate of the relative changes in TrkA immunolabeled cell levels during postnatal development. In addition, the labeling frequency of the TrkA-positive cells was assessed separately for each cortex layer using an image analysis program. Briefly, the images were captured using x20 objective lens via a CCD color video camera attached to a Zeiss microscope (Carl-Zeiss, Swiss) and the labeled cells were then counted. Three sections from each of the five rats were used. The mean labeling ratios were calculated as the density of the immunolabeled cells divided by the density of the cresyl-violet stained cells in the adjacent section. The laminar differences in the labeling frequency were assessed using an analysis of variance.

## Results

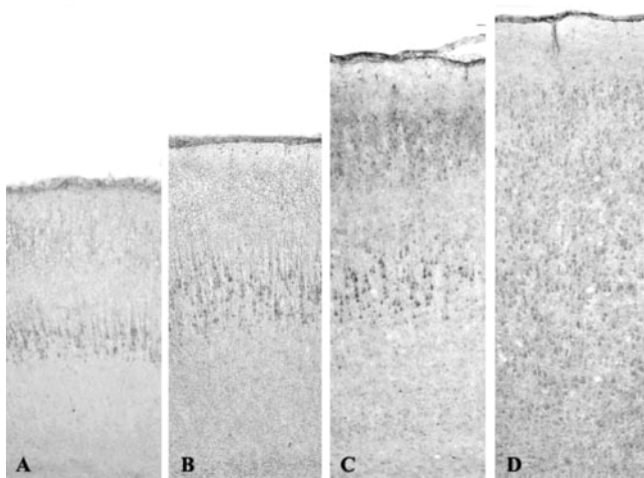
During first 15 days of postnatal life, a period of rapid cerebral cortex development involving, changes in TrkA-IR cells occurred in the developing cerebral cortex. TrkA-positive cell was not detected at PD0. In contrast, there was significant increase in the percentage of TrkA-IR cells with increasing age and the highest level was found at PD15 (Table 1).

The labeling frequency of the TrkA-positive cells was determined at each postnatal cortical layer. At PD3 and PD6, TrkA-positive cells were not evenly distributed in all cortical laminae (Fig. 1). Layer I, II, III and V were immunopositive for TrkA, but the intensity of layer I, II and III was very low and most labeled cells were in layer V.

**Table 1.** Immunohistochemical expression of TrkA in the cerebral cortex of postnatal developing rat

	PD0	PD3	PD6	PD9	PD15
Layer I	-	+	+	+	+
Layer II	-	+	+	+	+
Layer III	-	+	+	++	++
Layer IV	-	-	-	+	++
Layer V	-	++	++	+++	++
Layer VI	-	-	-	-	+

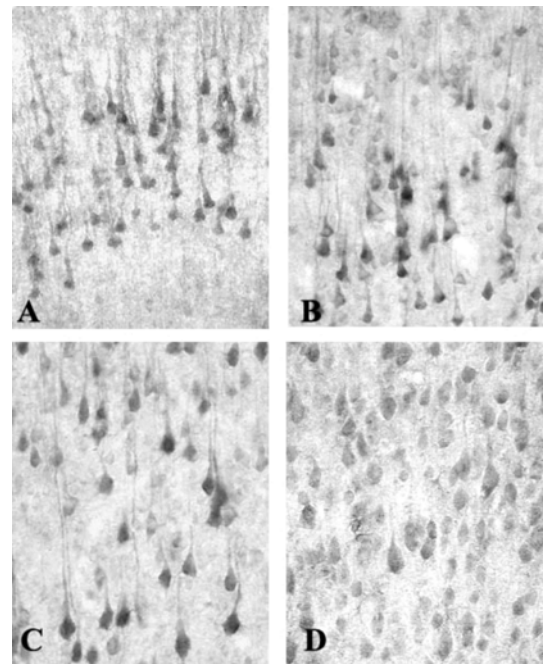
\*Immunoreactivity is shown as follows; -, absent; +, moderate to weak, ++, strong, +++; very strong. PD ; postnatal day



**Fig. 1.** TrkA immunoreactivity in cerebral cortex at each day. From postnatal day (PD) 3 (A), there was significant increase in the percentage of TrkA-positive cells with increasing age (A~D) and the highest level was found at PD15 (D). TrkA-positive cells start to be present in layer I, II, III and V at PD3 (A) and PD6 (B), but most labeled cells were in layer V. At PD9 (C), layer VI as well as layer I, II, III and V were immunopositive, primarily in layer III and V. At PD15 (D), the TrkA-positive cells were distributed in all layers, but the number of labeled cells in layer III, IV and V was much more than in the other laminae. A~D:  $\times 40$ .

Based upon their somatodendritic morphology, the most commonly labeled cell type in layer V was the pyramidal neurons (Fig. 2). Typically, the labeled neurons exhibited a dense immunoreaction product in the triangular cell bodies and in the proximal dendrites. Many of the neurons involved a large process arising from the apex of the cell body, which branched into the supragranular laminae. In addition to layer V, TrkA immunostaining was in a dense zone of the neuropil and some of the neuronal cell bodies of layer I, II and III.

At PD9, layer VI as well as layer I, II, III and V were immunopositive for TrkA. However, the intensity of layer I, II and IV was very low and most labeled cells were in layer III and V (Fig. 1). The distribution and shape of the TrkA-positive cells in layer V was similar to PD3 and PD6. An apical process of pyramidal neurons was well developed



**Fig. 2.** TrkA immunoreactivity in layer V of cerebral cortex at each day. The most commonly labeled cell type was the pyramidal neurons typically exhibiting a dense immunoreaction product in the triangular cell bodies and in the proximal dendrites (A~C). Many of the neurons involved a large process arising from the apex of the cell body, which branched into the supragranular laminae. At postnatal day (PD) 15, positive reaction increased, but processes were well not developed. A(PD3), B(PD6), C(PD9), D(PD15).  $\times 400$ .

(Fig. 2), which could be traced into layer I, II and III. In addition to layer V, TrkA-IR cells were found in a number of neuronal cell bodies in layer I, II, III and VI. The labeled neurons were stained strongly in the perikaryon and in the proximal dendrites similar to layer V but the neuronal processes were not well developed.

At PD15, the TrkA-positive cells were distributed in all layers from I through VI but were not evenly distributed in all cortical laminae (Fig. 1). The number of labeled cells in layer III, IV and V was much more than in the other laminae. The morphology of the TrkA-positive cells in layer V was similar to PD3, PD6 and PD9. Bodies of the neurons were strongly immunostained, but it was different from PD3, PD6 and PD9, in that the processes were well not developed (Fig. 2). In addition, the immunopositive of layer I, II, III, IV and VI was confined to the cell bodies, so the only the nuclei of neurons were immunolabeled.

Negative results were generated in each of the three control studies. No labeling was obtained in preparations in which the primary, secondary, or both the primary and secondary antibodies were eliminated. Therefore, the labeling with each anti-TrkA antibody was specific.

## Discussion

The TrkA expression in the developmental period has been widely analyzed. However, most of these studies were centered in the BFCN. NGF is the most potent growth factor in regulating the cholinergic phenotype during development [14]. Maturation of the BFCN is associated with the increased movement of the NGF receptor, TrkA, from the cell bodies to the processes. Therefore, various studies show that TrkA is commonly expressed within the cholinergic neurons located in the basal forebrain and the media septum during development and adulthood [10]. This study demonstrated different developmental programs of TrkA-IR cells in a postnatal rat cerebral cortex using immunohistochemistry. The results showed that the cerebral cortex cells were TrkA-immunoreactive from PD3 throughout the examination period. In addition, the development-related changes were statistically significant. All TrkA-positive cells exhibited elevated levels during the early postnatal development period. From PD3 to PD15, the cerebral cortex cells expressed TrkA, but not in PD0. With aging, there was significant increase in the percentage of cells exhibiting TrkA-immunoreactive. This result is similar to that reported in a previously study that TrkA expression in different brain areas (BFCN) was low at birth and then increased substantially with age [10,14].

Immunohistochemical studies showed that the high affinity receptors are commonly expressed in the mature cerebral cortex [1,24,25,28]. The distribution of cells expressing each of the three receptors was virtually identical. Each anti-Trk antibody primarily labeled the pyramidal neurons within the cell bodies in layer V and the dendrites in the supragranular cortex. More than 65% of the layer V neurons tested positive for the high affinity Trk receptor. Few immunoreactive somata (1-5%) were in the other layers. Recent data has shown that ligand-receptor colabeling is also common among the cortical neurons. For example, nearly 70% of the NGF, BDNF, and NT-3-positive neurons in layer V were colabeled with their respective high affinity receptors. This indicates that mature cortical neurons are responsive to more than one growth factor.

In this study, bases upon their somatodendritic morphology, the most commonly immuno-labeled cell type was the pyramidal neurons. Large triangular neurons had a pronounced process that arose from its apex and branched into the supragranular laminae. At PD3 and PD6, layer I, II, III and V were immunoreactive for TrkA and at PD9, layer VI as well as layer I, II, III and V were immunopositive. At PD15, the TrkA-positive cells were distributed in all layers. Moreover, there was significant increase in the percentage of cells exhibiting TrkA-IR with development and the highest level was detected at PD15. This shows that TrkA regulation of the cerebral cortex increases with aging and the other layers, I, II, III, IV and VI as well as V require

TrkA control differ from mature cortex [1,24,25,28]. However, the TrkA-positive cells were not evenly distributed in all cortical laminae. Most labeled cells were in layer V and the number of labeled cells in layer IV and VI was much less than in the other laminae. The layer V neurons are particularly important because they are the gatekeepers of cortical activity. Neurons in layer Va project callosally to the contralateral hemisphere and neurons in layer Vb project to the brainstem and spinal cord [1]. Thus, the maintenance and activity of the layer V pyramidal neurons are critical for the passage of cortical information. Layer IV is the primary terminal field of thalamic projections and the thalamus is notably poor in p75 or Trk expression [1]. These data suggest that the thalamocortical system of the early developing rat is not regulated by NGF.

This study provides immunochemical evidence that the cerebral cortex expresses TrkA strongly during postnatal development and there is a development-related increase in the expression of TrkA-IR cells. This suggests that NGF may have a trophic effect on these cerebral cortex neurons from the postnatal period.

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