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Expression of Trk A receptors in the mammalian inner ear

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Abstract

Tyrosine kinase receptors, including Trk A, Trk B and Trk C, participate in many different biological processes that are regulated by neurotrophic factors. Nerve growth factor (NGF)-triggered Trk A signaling is involved in growth, survival and differentiation of neurons in the central nervous system and in neural crest-derived cells. Trk A, Trk B and Trk C expression has been reported in the rat ventral cochlear nucleus. In the present study, we explored the immunocytochemical distribution of Trk A in the rodent inner ear. Rat and mouse cochleae were immunolabeled with a rabbit anti-Trk A polyclonal antibody (Chemicon) that has no reported cross-reactivity with Trk B and Trk C. In embryonic day 16 mice, no Trk A immunolabeling could be detected in the developing neuroepithelium. At postnatal day 6, weak Trk A labeling could be observed in both inner and outer hair cells. At postnatal day 12, enhanced punctate Trk A immunoexpression was present in hair cells. In adult mice and rats, intense Trk A labeling was observed in outer and inner hair cell bodies, in supporting cell bodies throughout the cochlea, and in spiral ganglion neurons. Trk A was not observed in stria vascularis, hair cell stereocilia, nor in the Trk B- and Trk C-rich cerebellum. This distribution pattern of Trk A suggests that its ligand, NGF, exerts significant trophic effects in the rodent inner ear.

1. Introduction

Nerve growth factor (NGF) is one member of a superfamily of neurotrophins that includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). These neurotrophins all share significant structural homology and have some overlapping functional effects. They exert their effects through a family of tyrosine kinase (Trk) receptors, comprising Trk A (selective for NGF), Trk B (selective for BDNF and NT-4/5), and Trk C (selective for NT-3; see Table 1). The production of neurotrophins in primary sensory neurons is essential for the development, maturation and maintenance of the nervous system (Pirvola et al., 1994, 1997; Kaplan and Miller, 1997; Cochran et al., 1999). It is also known that NGF directly affects the capsaicin response of dorsal root ganglion (DRG) cells (Hu-Tsai et al., 1996; Winter et al., 1988), which suggests that NGF should be able to directly sensitize the response to noxious agonists of TRPV1 receptors found in the organ of Corti (Zheng et al., 2003). This raises the possibility that NGF may play a role in inflammation-triggered events, such as those that may result from loud sound stress (Shi and Nuttall, 2002, 2003). NGF levels are increased in inflamed tissues, and this neurotrophin

plays an important role in generating inflammatory hyperalgesia (Woolf et al., 1994). NGF can also induce TRPV1 upregulation in the DRG via p38 activation (Ji et al., 2002).

Trk B and C and their associated ligands are expressed in the chick auditory system throughout development. Trk B and Trk C are highly expressed in the embryonic auditory brainstem, cochlear ganglion neurons and in peripheral fibers that directly innervate the base of hair cells in the basilar papilla. BDNF was the major neurotrophin identified in these locations. In contrast, Trk A immunolabeling is distinctly absent in all tissues at the ages studied (Pirvola et al., 1997; Cochran et al., 1999). Thus, it appears that both Trk B and Trk C, but not Trk A, are essential for development of the avian peripheral auditory system.

In the developing mammalian inner ear, the distribution and function of neurotrophins and their receptors suggests that they play a critical role in the maturation of the mammalian central and peripheral auditory system (Pirvola et al., 1994; Hafidi et al., 1996). In the developing rat inner ear, NT-3 and BDNF mRNA, but not NT-5 and NGF mRNA, are expressed in the sensory cells of auditory and vestibular end-organs (Pirvola et al., 1992). Attempts to clarify the function of neurotrophins and their associated receptors in development of the murine inner ear have been made. Mice lacking either BDNF or its associated receptor, Trk B, show a severe reduction in the number of vestibular neurons and a loss of all innervation to the semicircular canals. Mice lacking NT-3 or its receptor, Trk C, show a severe reduction of spiral neurons in the basal turn of the cochlea (Fritzsche et al., 1997a; Silos-Santiago et al., 1997). Mice lacking both BDNF and NT-3 or Trk B and Trk C reportedly lose all innervation to the developing inner ear (Fritzsche et al., 1997a,b; Liebl et al., 1997). Furthermore, BDNF was the most potent stimulator of neurogenesis in maturing spiral ganglion neurons (SGN) in vitro. NT-3 provided the strongest support for neuronal survival, while NGF supported only limited neurogenesis (Malgrange et al., 1996). In the cochlea, auditory type I neurons require NT-3 for their survival, whereas type II neurons depend on BDNF (Agerman et al., 1999; Ernfors et al., 1995). Thus, BDNF and NT-3 are crucial trophic factors for the survival of vestibular and auditory neurons, respectively, during development.

However, Trk A is not essential for inner ear development, as Trk A homozygous mutant mice show normal numbers of neurons and no Trk A expression was detected in neurons of *trkB*^{-/-}, *trkC*^{-/-} double mutant mice (Schimmang et al., 1997). Thus, the early development of the inner ear is largely determined by two members of the neurotrophic family, BDNF and NT-3. In postnatal rat inner ears, mRNAs of the low affinity NGF receptors, Trk B and Trk C, are expressed in the vestibular and spiral ganglia, respectively (Ylikoski et al., 1993). However, Trk A, NGF or NT-5 mRNA could not be detected in the inner ear neuroepithelium nor in the innervating neurons. Thus, BDNF is the predominant neurotrophic factor in the adult vestibular labyrinth, and NT-3 in the mature organ of Corti. In postnatal gerbils and adult rats, Trk A, Trk B and Trk C receptors are expressed in the ventral portion of the cochlear nucleus (Hafidi et al., 1996; Burette et al., 1997; Pirvola et al., 1997).

The expression and function of NGF and its associated receptor, Trk A, in mammalian inner ear remains poorly understood and requires further investigation. The present study determined the immunohistochemical distribution of Trk A in rat and mouse cochleae using confocal microscopy, to explore further the potential role of the Trk A receptor and its ligand neurotrophins in the peripheral auditory system.

2. Methods

Healthy adult male or female rats and mice with Preyer's reflex, postnatal mice (1, 6 and 12 days old) and embryonic day 16 mice were used. Animals were anesthetized with ketamine hydrochloride (100 mg/kg, i.m.; Abbott Labs) and xylazine (2 mg/kg, i.m.; Phoenix Scientific), and a cardiac perfusion with saline followed by 4% paraformaldehyde in 0.02 M phosphate-buffered saline (PBS) was performed on adult rats and mice. The cochleae of rats, and young, neonatal or embryonic mice were removed, opened and fixed in 4% paraformaldehyde after decapitation. Stria vascularis, basilar membrane, spiral ganglion, DRG and cerebellum were excised and fixed in 4% paraformaldehyde in 0.02 M PBS for 2 h.

For immunocytochemistry, fixed tissues were washed in 0.02 M PBS (pH 7.4), permeabilized with 0.5% Triton X-100 (Sigma) for 1 h and immunoblocked in 10% goat serum in 1% bovine serum albumin in 0.02 M PBS (BSA-PBS) for 1 h. Tissues were incubated with rabbit Trk A polyclonal antibody (Chemicon; diluted 1:1000 in 1% BSA-PBS) for 48 h. After washing in 1% BSA-PBS, tissues were subsequently incubated with Alexa-488-conjugated goat anti-rabbit IgG for 3 h (Molecular Probes; 1:100 in BSA-PBS). The labeled tissues were mounted and observed on a Nikon Eclipse TE 300 inverted microscope fitted with a Bio-Rad MRC 1024 confocal scanning laser microscopy system. Confocal microscopy of the organ of Corti was conducted perpendicular to the luminal surface of the sensory epithelium, resulting in horizontal optical fields or sections of the cochlear surface preparation. For positive controls, Trk A-positive DRG (Carroll et al., 1992; Smeyne et al., 1994; Verge et al., 1992) tissues were incubated with primary antibodies. For negative controls, Trk A-negative (but Trk B- and Trk C-positive) cerebellum (Courtney et al., 1997; Savaskan et al., 2000) tissues were incubated with primary antibodies. For all other tissues, the primary antibody was replaced by 1% BSA-PBS. This study was approved by the Institutional Animal Care and Use Committee at the Oregon Health-&Science University and conformed to NIH guidelines for the care and use of laboratory animals.

3. Results

3.1. Immunohistochemical controls

Positive labeling for Trk A was observed in the cell bodies of neurons in the DRG (Fig. 1A), known to contain Trk A receptors (Carroll et al., 1992; Verge et al., 1992; Smeyne et al., 1994), and in the organ of Corti (Fig. 1C). Negligible fluorescence was observed in these same tissues when the primary antibody was replaced with BSA-PBS (Fig. 1B,D). Furthermore, the cerebellum, which is widely recognized to be deficient in Trk A expression and rich in Trk B and C receptors (Courtney et al., 1997; Savaskan et al., 2000), displayed no immunolabeling in the presence of Trk A antiserum (Fig. 1E) and negligible non-specific labeling in the absence of Trk A antiserum (Fig. 1F).

3.2. Distribution of Trk A receptor in the rat inner ear

Expression of Trk A was observed in hair cells, supporting cells and innervation throughout the organ of Corti, but not in the stria vascularis. In hair cells, positive immunolabeling was observed in the soma of both inner hair cells (IHC) and outer hair cells (OHC) (Fig. 2A), but was excluded from the hair cell nucleus (Fig. 2B,C). No obvious differences in the intensity of immunolabeling between OHC and IHC, or between hair cells of the apical turn and basal turn, could be detected. No Trk A expression could be detected in the stereocilia of IHC or OHC (Fig. 3).

In supporting cells, weak immunolabeling (compared to hair cells) was observed in the Hensen's cells, and in the inner and outer pillar cell bodies in the organ of Corti (Figs. 2D,F and 4A), although distinct labeling of the inner pillar cell was often shrouded by the fluorescence emitted by the IHC (Fig. 2F). Labeling of Deiters' cells could not be distinguished above background levels. Immunolabeling was also observed in the interdental cells of the spiral limbus (Fig. 2E).

Trk A immunolabeling was also observed in various neural structures within the cochlea, at fluorescent intensities below that observed in hair cells (Fig. 4). Trk A immunolabeling was present in the tunnel crossing fibers (Figs. 2F and 4A) and in the inner spiral bundle within the organ of Corti (Fig. 4A). Labeling was also present in the neural structures proximal to the habenula, presumably the dendrites of SGN (Fig. 4B), and in the soma of SGN within the bony modiolus of the cochlea (Fig. 4C).

3.3. Distribution of Trk A receptor in the mouse inner ear

Trk A immunolabeling was located in mature hair cells, and supporting cells throughout the adult organ of Corti (Fig. 5), and no difference could be observed in the intensity of labeling between the basal and apical turns (data not shown). The expression of Trk A appears to be upregulated during postnatal development. In embryonic day 16 mice, no Trk A immunolabeling could be detected in the developing neuroepithelium (Fig. 5A). At postnatal day 6, faint Trk A labeling could be observed in both IHC and OHC (Fig. 5B). At postnatal day 12, enhanced punctate Trk A immunoexpression was present in hair cells, with weak Trk A immunoexpression in supporting cells (Fig. 5C). In adult mice, intense Trk A immunoexpression was obviously enhanced in the soma of both OHC and IHC (Fig. 5D,E), and was also present in SGN (Fig. 5F).

4. Discussion

NGF is the best-characterized member of the neurotrophin family. Its primary biological function is the maintenance and survival of the peripheral and central nervous systems (Snider, 1994). NGF exerts its effect through binding to two distinct classes of cell surface receptors: the shared p75 neurotrophin receptor (p75NTR; also known as the low affinity neurotrophin receptor) and the Trk A receptor (Yano and Chao, 2000). Activation of the p75NTR receptors can only be achieved through NGF binding. Activation of p75NTR receptors in neurons that are also Trk A-negative initiates apoptosis, but if Trk A is also co-expressed on the cell surface, even at low concentrations, then NGF promotes neuron survival (Kaplan and Miller, 2000).

DRG neurons that mediate nociception require NGF and the expression of its signaling receptor, Trk A, for survival. Trk A is expressed in 40–50% of the total DRG neuronal population in the mature rat (Carroll et al., 1992; Verge et al., 1992; Smeyne et al., 1994). In contrast, cerebellar granule neurons and Purkinje cells express Trk B, Trk C and p75NTR, but not Trk A receptor (Courtney et al., 1997; Savaskan et al., 2000). In the current work, we present data showing positive Trk A immunolabeling in cytoplasm of neurons in the DRG of rats, but not in the Trk A-negative cerebellum, confirming these previous studies (Table 2). Thus, the rabbit anti-Trk A polyclonal antibody from Chemicon used in this study is specific for the Trk A receptor, and does not cross-react with Trk B or C (Barker-Gibb et al., 2001). Moreover, this antiserum from Chemicon specifically recognizes rat Trk A on Western blots and in immunoprecipitations and blocks the binding of NGF to Trk A receptors expressed by PC12 cells (Clary et al., 1994). Finally, it has been well documented that Trk B and Trk C, but not Trk A, are expressed in the cochlear sensory epithelium during inner ear development (Ylikoski et al., 1993; Pirvola et al., 1994). If our commercial antibody was

cross-reactive with Trk B and Trk C, immunolabeling should be present in cochlear tissues of embryonic day 16 mice, however, this was not observed.

In a preliminary study we reported Trk A immunolabeled fibers on blood vessels within the cochlea (Vass et al., 2001a,b) and here extend our observations to describe Trk A immunoexpression patterns in the organ of Corti of postnatal rodents.

The antibody used in this study is directed against the extracellular domain of Trk A. In embryonic day 16 mice, no Trk A immunoreactivity was detected in the inner ear neuroepithelium. At postnatal day 6, weak Trk A immunolabeling could be observed in the murine organ of Corti. At postnatal day 12, increased Trk A immunoreactivity was observed in both IHC and OHC, and also weakly in supporting cells in the murine organ of Corti. In the mature mouse and rat inner ear, intense Trk A expression was present in both IHC and OHC, supporting cells and cochlear neurons. The neuronal types included tunnel crossing fibers, fibers of the inner spiral bundle, the SGN, and neural structures proximal to the habenula, presumably the dendrites extending from the soma of Trk A-positive SGN.

Our results demonstrate Trk A receptor labeling in the postnatal and adult rodent organ of Corti for the first time. Previous studies failed to identify Trk A receptor expression in the postnatal and adult rodent inner ears, possibly because these *in situ* hybridization or immunohistochemical studies employed histological sections of the cochlea using transmitted light microscopy (Ylikoski et al., 1993; Pirvola et al., 1994). However, we examined whole mounts of the organ of Corti using immunofluorescence combined with confocal microscopy, which is more sensitive than detection of enzymatic chromogenic deposits using transmitted light microscopy.

The Trk A receptor is located at the cell membrane, and theoretically, Trk A immunolabeling should be observed at the cell membrane. However, in our study, Trk A labeling was visualized within the cytoplasm, where it would be synthesized. In immunocytochemical studies, often only the cytoplasmic antigen is visualized because the membranous fraction is reduced compared to the cytoplasmic fraction, and therefore, the membranous label can be swamped by the cytoplasmic label and remains undetected. The phenomenon is common for other membranous proteins, such as in our other study, documenting the distribution of TRPV1 in the cochlea (e.g., Zheng et al., 2003).

Neurotrophins and their cognate receptors are critical to normal auditory system development. The absence of Trk A immunolabeling in the embryonic rodent inner ear is consistent with previous studies (Pirvola et al., 1997; Cochran et al., 1999; Schecterson and Bothwell, 1994). The increasing expression of Trk A between embryonic day 16 and adulthood in mice suggests that Trk A expression is upregulated as the inner ear matures. This is similar to gerbil brainstem expression of Trk A, Trk B and Trk C at postnatal days (Evans and Dallos, 1993) 0, 6, 15, 21 and 30 (Hafidi et al., 1996). Immunoreactivity to Trk A was observed in non-neuronal cells during the first two postnatal weeks. Then in mature animals, Trk A-positive labels were identified in a subpopulation of cochlear nucleus neurons. In contrast, at postnatal day 0, both Trk B and Trk C labeling was virtually absent from auditory brainstem nuclei. By postnatal day 7, Trk B- and Trk C-positive neurons were present in most brainstem auditory neurons. Furthermore, the number of Trk B-positive neurons increased as the auditory system matured with increasing age.

In our study, no difference was observed in Trk A expression within basal and apical cochlear turns. Other studies have shown a longitudinal gradient of NT-3 or of both BDNF and NT-3 in cochlea with the highest expression in the apex (Pirvola et al., 1992; Wheeler et al., 1994). NT-3 null mutants lose all neurons in the basal turn of the cochlea and have incomplete neuronal loss in the middle and apical turns. However, immunocytochemical

studies of Trk B and Trk C receptor expression in cochlear spiral neurons revealed no gradients along the length of the cochlea. It has been suggested that the expression pattern of neurotrophins rather than their receptors contributes to the spatial loss of spiral sensory neurons in NT-3 null mutants (Farinas et al., 2001).

Our results are consistent with the observation that Trk A is not required in the developing inner ear (Schimmang et al., 1997). However, it may exert functional roles in postnatal and adult inner ears. The mechanisms by which neurotrophin receptors exert their influences on neurons are not fully understood. Trk A is activated primarily by extracellular NGF that initiates autophosphorylation of Trk A triggering a cascade of specific signal transduction mechanisms, including those controlled by Ras, the Cdc42/Rac/RhoG protein family, mitogen-activated protein, phosphoinositide 3'-kinase and phospholipase C- α , thereby affecting both development and function of the nervous system (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). During the last decade, research in the field of neurotrophic factors has produced a vast amount of data on how they function, provide support and organize neuronal networks during development, and significantly, how they participate in maintaining nervous system function. NGF deprivation is also associated with depleted nociceptive terminals and reduced nociceptor sensitivity (Snider and McMahon, 1998). NGF levels are known to be increased in a variety of inflammatory conditions, contributing to hyperalgesia (McMahon, 1996; Woolf et al., 1996). Our previous studies have established a role for primary sensory fibers to the cochlea in a capsaicin-stimulated inflammatory-like response (Vass et al., 1995, 2001a,b).

It is now well established that NGF provides a retrograde survival signal to autonomic and sensory ganglion cells (Miller and Kaplan, 2001). The expression of Trk A receptor in the central auditory system implies that NGF has a functional role in the central auditory system (Burette et al., 1997). Our data demonstrate that the Trk A receptor is present in the adult rodent organ of Corti, suggesting that NGF may exert specific functions in the peripheral auditory system. Cochlear perfusion with NGF enhances preservation of the auditory nerve and has protective effects on auditory neural degeneration induced by neomycin ototoxicity (Schindler et al., 1995). Thus NGF may promote mature auditory neuronal survival.

NGF binding to Trk A alters the sensitivity of the TRPV1 receptor to heat and protons (Bonnington and McNaughton, 2003). We have shown that TRPV1 receptors are present and active on cochlear hair cells (Zheng et al., 2003). Although the roles of NGF, Trk A and TRPV1 receptors in cochlea physiology are unknown, we speculate that there could be a connection to inflammatory mechanisms which are known to increase NGF (Woolf et al., 1994). The cochlea does exhibit some hallmarks of inflammation following loud sound exposure (Shi and Nuttall, 2002, 2003). It is also possible that physiologically relevant levels of NGF in the blood may bind to cochlea Trk A and have some role in sensory neural hearing loss (Salvinelli et al., 2002).

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Abbreviations

NGF	nerve growth factor
DRG	dorsal root ganglion
OHC	outer hair cell

IHC	inner hair cell
SGN	spiral ganglion neurons

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Fig. 1.

Positive and negative control study of Trk A. Trk A immunolabeling (green) in the soma (arrow) of cells in the DRG (A) and in the soma of OHC and IHC in the organ of Corti (OoC) (C). Negligible fluorescence is present in the DRG (B) and organ of Corti when the primary antibody was replaced with BSA-PBS (D). Red fluorescence in C and D is Alexa-568-conjugated phalloidin specific for F-actin. Negligible fluorescence occurs when Trk B- and Trk C-rich cerebellar tissues are incubated with Trk A antiserum (E) or when the primary antibody is replaced with BSA-PBS (F).

Fig. 2.

Distribution of Trk A in the rat cochlea. In hair cells (A-E), Trk A immunolabeling occurs in the soma of both IHC and OHC, and is largely excluded from the nucleus of IHC and OHC (B,C). No labeling was observed in the hair cell stereocilia (see also Fig. 3 below). Supporting cell immunolabeling is weak compared to hair cells, and is present in the Hensen's cells (HC in D), interdental cells of the spiral limbus (IC in E), and in the outer pillar cell body (OPC in F).

Fig. 3.

Trk A and actin co-localization in the rat cochlea. (A) Trk A immunolabeling (green) occurs in the soma of IHC and OHC, but not in the stereocilia as indicated by the absence of labeling (arrow) compared to the Alexa-568-conjugated phalloidin labeling of stereocilia (S) in B and the absence of co-localized (or yellow) pixels at the level of the stereocilia (S) in C.

Fig. 4.

Expression of Trk A in neural fibers and SGN in the rat inner ear. (A) Trk A immunolabeling is present in the tunnel crossing fibers (TCF) and in the inner spiral bundle (ISB) below the IHC within the organ of Corti, (B) in neural dendrites (D, arrow) proximal to the habenula and (C) labeling was also present in the soma of SGN (arrow) within the bony modiolus of the cochlea.

Fig. 5.

Distribution of Trk A in the organ of Corti (OoC) of the mouse cochlea. (A–D) Single optical sections of the murine sensory epithelium at different developmental stages. (A) At embryonic day 16, no Trk A immunolabeling can be distinguished in the inner ear neuroepithelium. (B) At postnatal day 6, weak Trk A immunolabeling occurs in the soma of both IHC and OHC. Negligible expression of Trk A is observed in supporting cells. (C) At postnatal day 12, enhanced punctate Trk A immunolabeling occurs in IHC and OHC, and labeling also occurs in supporting cells (HC, Hensen's cells). (D) In adult mice, intense Trk A immunolabeling occurs in IHC and OHC, compared to earlier time points and compared to supporting cells (HC, Hensen's cells). (E) In adult mice, combined images from several focal planes reveal clear Trk A immunoexpression in the region of the Hensen's cells (HC arrow), and (F) also in the soma of SGN.

Table 1

Neurotrophic factors and their associated receptors

	p75 ^{NTR}	Trk A	Trk B	Trk C
NGF	+	+		
BDNF	+		+	
NT-3	+	+	+	+
NT-4	+	+	+	
NT-5	+	+	+	

* Specific receptor.

From Neet and Campenot (2001).

Table 2

The intensity of Trk A expression in rats and mice

	Mouse E16	Mouse P6	Mouse P12	Mouse adult	Rat adult
IHC	-	+	++	+++	+++
OHC	-	+	++	+++	+++
SGN				++	++
Supporting cells	-	+/-	+	+	+
DRG					+++