Analysis of the Expression and Function of Ciliary Neurotrophic Factor (CNTF) in the Developing Nervous System.

Katsusuke Hata

Department of Molecular Mechanics, School of Life Science,
The Graduate University for Advanced Studies, Okazaki, Japan.
Laboratory for Speciation Mechanisms I,
National Institute for Basic Biology, Okazaki, Japan.

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I. ABSTRACT

Ciliary neurotrophic factor (CNTF) attracts much attention, because it is a unique member of the interleukin-6 (IL-6) family whose receptor subunit for ligand binding is exclusively expressed in the nervous system and muscle. CNTF supports survival and differentiation of various types of neurons and glial cells *in vitro*. The role of CNTF as a moderate neurotrophic factor on mature motor neurons has been demonstrated. However, its role in embryonic development remains unknown. I examined expression patterns of the IL-6 family members and their receptors during the rodent embryonic development, and found a specific expression of CNTF in the pineal gland and eyes. *In vitro* assays showed that newborn rat pineal extract supports some specific neurotransmitter/neuropeptide gene expressions [including substance P (SP) and vasoactive intestinal polypeptide (VIP); the gene expression of these peptides is known to be induced by CNTF *in vitro*] and survival of newborn rat sympathetic neurons. In contrast to the *in vitro* results, sympathetic innervation of the pineal gland in CNTF-deficient mice showed no apparent difference from that of wild-type mice. There were few preprotachykinin-A (SP precursor protein) and VIP-positive neurons among sympathetic neurons that innervate a pineal gland. These few VIP-positive neurons in superior cervical ganglion were not different in number between adult CNTF knockout and wild-type mice, implying that the CNTF signal in pineal glands may be usually unavailable for innervating sympathetic neurons. Finally, I discuss the role of CNTF on the photoreceptor development in pineal organ of different species.

Abbreviations used

cDNA :	complementary DNA
CG :	ciliary ganglion
CNTF :	ciliary neurotrophic factor
DAB :	dimethylaminoazobenzene
DNA:	deoxyribonucleic acid
E :	embryonic day
FGF :	fibroblast growth factor
IL :	interleukin
LIF :	leukemia inhibitory factor
M :	molar = mol/liter
ml :	mililiter
mRNA :	messenger RNA
µg :	microgram
ng :	nanogram
NGF :	nerve growth factor
P :	postnatal day
PE :	pineal extract
PB :	phosphate buffer
PBS :	phosphate buffered saline
PBST :	PBS + 0.2% Triton X-100
PCR :	polymerase chain reaction
PFA :	paraformaldehyde
PPT :	preprotachykinin-A
RNA:	ribonucleic acid
SCG :	superior cervical ganglion
SP :	substance P
SSC :	standard saline citrate.
TBS :	Tris buffered saline
TH :	tyrosine hydroxylase
VIP :	vasoactive intestinal polypeptide

II. INTRODUCTION

During the past two decades the reports on neurotrophic molecules has strikingly increased in number. There are two types of neurotrophic molecules well-studied. The one family is neurotrophin, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, (NT-3) and NT-4/5, which show a high homology (more than 50% each other), and interact with Trks of tyrosine kinase receptor (Thoenen, 1991). Another family is the interleukin (IL)-6 family, or sometimes named as neuropoietic factors (Gadient and Otten, 1997). CNTF, which belongs to the second family, is one of the most well-studied neurotrophic factor, except for neurotrophins.

The isolation of CNTF has its beginnings in the finding that about one-half of the chick ciliary neurons die during embryogenesis, apparently due to a limited trophic supply from the target tissue (Adler *et al.*, 1979; Landmesser and Pilar, 1974). The activity that supports the survival of ciliary neurons was purified as ciliary neurotrophic factor (CNTF) (Barbin *et al.*, 1984). The protein was purified and the gene was cloned (Stockli *et al.*, 1989; Lin *et al.*, 1989; Leung *et al.*, 1992). The identification of the gene and its receptors revealed that CNTF belongs to the IL-6 family (Ip *et al.*, 1993) that includes leukemia inhibitory factor (LIF) or cholinergic differentiation factor (Gearing *et al.*, 1987; Yamamori *et al.*, 1989). Now, this family includes CNTF, LIF, cardiotrophin-1 (CT-1), oncostatin-M (OSM), cardiotrophin-like cytokine (plus cytokine-like factor-1 complex) (CLF/CLC) and IL-11, besides IL-6 (Gadient and Otten, 1997; Elson *et al.*, 2000).

In spite of many reports of the variety of biological roles of CNTF *in vitro*, the original question as to what roles CNTF plays in development has been still unsolved as have been reviewed, "During development, expression of CNTF is very low, if indeed it is expressed at all, and the phenotype of mice lacking endogenous CNTF after inactivation of the CNTF gene by homologous recombination suggests that CNTF does not play a crucial role for responsive cells during embryonic development" (Sendtner *et al.*, 1994). CNTF-deficient mice appeared remarkably normal development, although moderate motor neuron deficit was seen during the aging (Masu *et al.*, 1993). In addition, 2.3% of Japanese people have homozygous and 35.8% heterozygous mutations of CNTF genes, without any signs of deficiency including

neurological defect (Takahashi *et al.*, 1994). These data led to a consensus that CNTF may not participate in the development of the nervous system, and might have other functions, for example as a lesion factor, because it seems to be a cytosolic protein and released when a cell is degenerated (Sendtner *et al.*, 1994).

In order to examine whether CNTF is expressed and has a function during the course of embryonic development, I extensively studied the expression pattern of cytokines of the IL-6 family and their receptors during embryonic development. I first found a highly specific expression of CNTF in the pineal gland and eyes during embryonic development of rat. Since the pineal gland is one of the sympathetic target (see the schematic models, Fig. 14A), I next examined the effect of pineal extract containing CNTF on the survival of sympathetic neurons, because there were inconsistent reports on the *in vitro* effect of CNTF on the survival (Barbin *et al.*, 1984; Burnham *et al.*, 1994) or apoptosis (Kessler *et al.*, 1993; Savitz and Kessler, 2000) of sympathetic neurons. There has also been a controversy on whether CNTF is released from cells or not (Kamiguchi *et al.*, 1995; Stockli *et al.*, 1989). Thus, I studied the density of sympathetic neurons that innervated pineal glands in CNTF-deficient and wild-type mice in detail, and the CNTF-inducible gene expressions *in vivo* in these sympathetic neurons in order to clarify the function of CNTF. Finally, I discuss the possibility whether or not CNTF plays a role in photoreceptor development in the pineal gland, in the light of functional differences of CNTF and pineal organs in the rat and chick.

III. RESULTS

Specific expression of CNTF in the rat pineal gland and eyes during embryonic development.

In order to clarify whether CNTF indeed plays any role in embryonic development, I first examined the mRNA expression in the embryonic day 18.5 (E18.5) rat embryos by *in situ* hybridization using antisense CNTF probes prepared for two separate regions (Fig. 1A). I detected the CNTF mRNA expressed specifically in a pineal primordium using both probes (Fig. 1C, right panels). Although there are many reports on the variety of biological spectrum of CNTF *in vitro*, all previous studies *in vivo* have failed to detect CNTF signals in embryonic stages by northern blotting, PCR or *in situ* hybridization technique except for only faint ones (Ip *et al.*, 1993; Stockli *et al.*, 1991). Thus my finding was the first observation that demonstrated the specific expression of CNTF in the development.

I next used monoclonal and polyclonal anti-CNTF antibodies to confirm the expression of CNTF protein in pineal glands. At first I confirmed whether these antibodies recognized CNTF. These antibodies recognized recombinant rat CNTF (rCNTF) and CNTF protein in the rat adult sciatic nerve lysate (known the major site of the CNTF expression in the adult rat) by immunoblot analysis (Fig. 2, upper panels). Note that anti-CNTF polyclonal antibody recognized CNTF in adult rat pineal lysate. In additon, consistent with the results by *in situ* hybridization, these antibodies also detected CNTF signal in E18.5 rat pineal gland, which was diminished when rCNTF was coexisted with the antibodies (Fig. 2, lower panels), confirming the immunoreactivity of the signal as CNTF expressed in the pineal gland. Using serial sections of the entire rat head on E18.5, the CNTF signal was detected only in the pineal gland, and in addition, eyes (lens epithelium) by both antibodies (monoclonal; Fig. 3, and polyclonal; Fig. 4).

Next I observed the developmental changes of the CNTF expression in the embryonic rat pineal gland and eyes. CNTF was detected on E12.5 (before the period of pineal primordia formation) until postnatal day (P) 8.5 when CNTF signals become weak in pineal glands (Fig. 5, middle and lower panels). In embryonic eyes, CNTF was mainly expressed in the lens and retinal (pigment) epithelia from E15.5. In addition, CNTF was expressed in the retina at the newborn stage (Fig. 5 upper right

panels), where CNTF is expressed in the adult as has been already reported (Kirsch *et al.*, 1997), at the newborn stage as well.

The pineal gland is a target organ of superior cervical ganglion (SCG) sympathetic neurons (see the schematic models, Fig. 14A). SCG neurons innervate postnatal pineal glands in the rat. The distribution pattern of nerve fibers in the P8 rat is similar to that in the adult (Machado *et al.*, 1968; Matsuura *et al.*, 1983).

I therefore examined the CNTF expression in the pineal gland at the period of sympathetic innervation by immunoblot using the anti-CNTF polyclonal antibody. Fig. 6B also revealed the existence of CNTF in pineal lysates, and CNTF signals can be detected in the pineal gland from newborn to adult by this method. I estimated the amount of CNTF to be approximately 1 ng per rat newborn pineal gland. This value seems to be very high for the pineal volume [concentration was estimated to be about $\geq$1 μg/ml at a pineal gland because diameters of rat newborn pineal glands were no more than 1 mm (Fig. 6A)], but in fact the concentration is a similar level to that in the adult rat sciatic nerve, where CNTF is expressed in large quantities (Stockli *et al.*, 1989). Expression of large amount of CNTF may be required for exertion of its biological activity, because it is likely to be a cytosolic protein (Stockli *et al.*, 1989). But it may be also noted that cultured astrocytes release CNTF (Kamiguchi *et al.*, 1995), and the degeneration of cells in embryonic, young and aged rat pineal glands are reported (Calvo and Boya, 1981; Humbert and Pevet, 1995). In this case, CNTF may be released in the vicinity of the degenerated cells.

In summary, my results shown above demonstrate for the first time the specific expression of CNTF during the rat embryonic development, revealing the expression of CNTF in the developing pineal gland and eyes. I thus further examined whether CNTF in the pineal glands possess any biological activities *in vitro* and *in vivo*.

Fig. 1

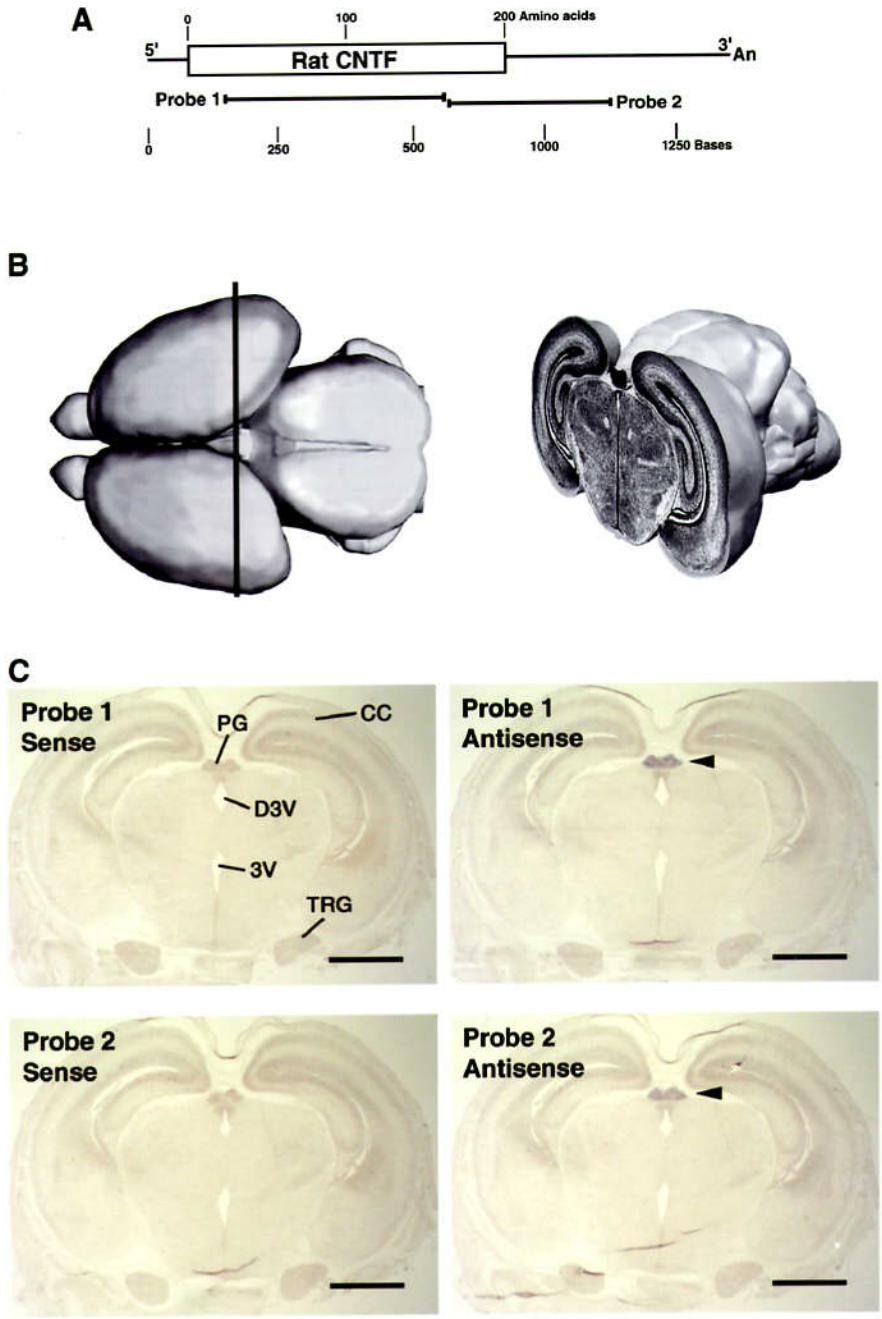

Fig. 1. Expression of CNTF mRNA in rat embryonic brain. (A) A schematic map of cDNA clone and the position of probes used for *in situ* hybridization encoding rat CNTF. (B) Models of the dosal view of the rat brain (left panel) and left-lateral view (right). (C) Coronal sections that showed CNTF signal specifically detected in the pineal gland by *in situ* hybridization (antisense probe 1: upper right panel, and antisense probe 2: lower right panel) among the entire sections of an E18.5 rat head are chosen. Bars = 1 mm. *In situ* hybridization with a sense probe (left panels) are shown as control. Arrowheads indicate sites of CNTF expression. Abbreviations used in the figure: CC, cerebral cortex; D3V, dorsal third ventricle; PG, pineal gland; TRG, trigeminal ganglion; 3V, third ventricle.

Fig. 2

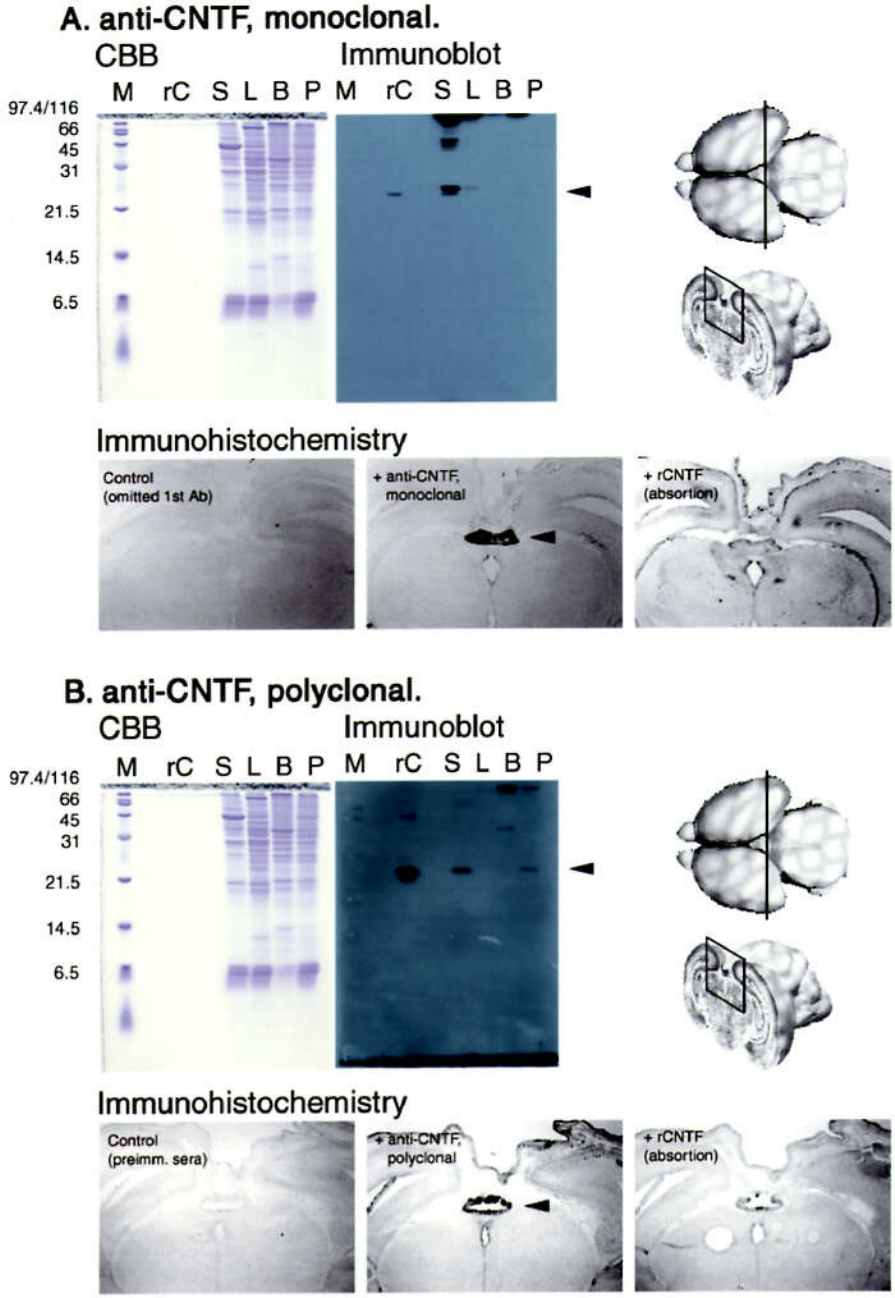

Fig. 2. Specificities of anti-CNTF monoclonal and polyclonal antibodies. (A) and (B) Upper panels: Immunoblotting with the anti-CNTF antibodies detected CNTF signals in lanes of recombinant rat CNTF and adult rat sciatic nerve (rC and S respectively), but not in those of total brain and liver lysate (B and L, respectively). Note that in Fig. 2B, CNTF signal was seen in adult rat pineal lysate (P). Arrowheads indicate the CNTF signal. Lower panels: Immunohistochemistry using anti-CNTF antibodies detected specific CNTF signal in E18.5 rat pineal gland, which confirmed the expression patterns of *in situ* hybridization in Fig. 1. Arrowheads indicate the CNTF signal.

Fig. 3

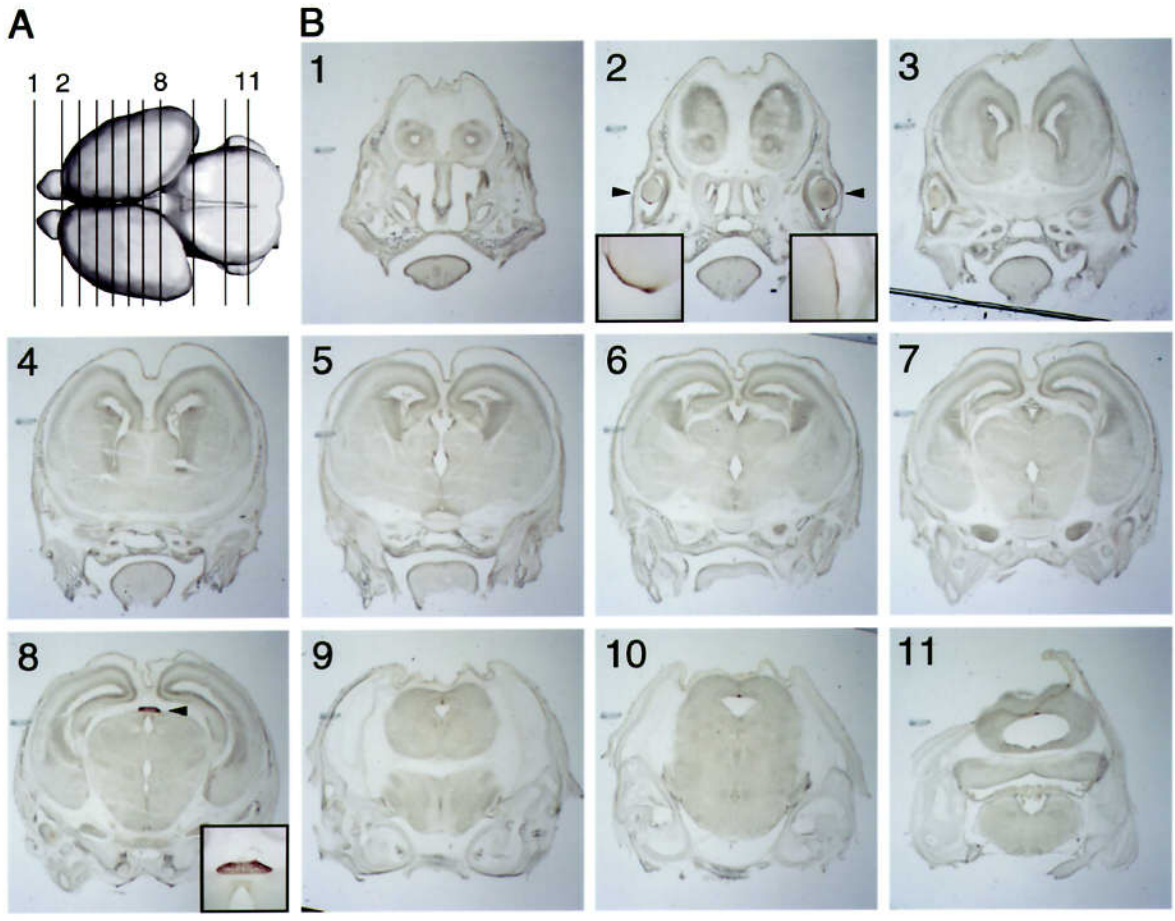

Fig. 3. Immunohistochemistry using the anti-CNTF *monoclonal* antibody in the entire section of an E18.5 rat head. (A) A schematic view of the dorsal rat brain. Vertical bars and numbers indicate the approximate position of the sections used in Fig. 3B. (B) Immunohistochemistry using anti-CNTF *monoclonal* antibody on serial sections of E18.5 rat head. The CNTF signal is seen only in the pineal gland (see the inset of section no.8) and eyes (section no. 2).

Fig. 4

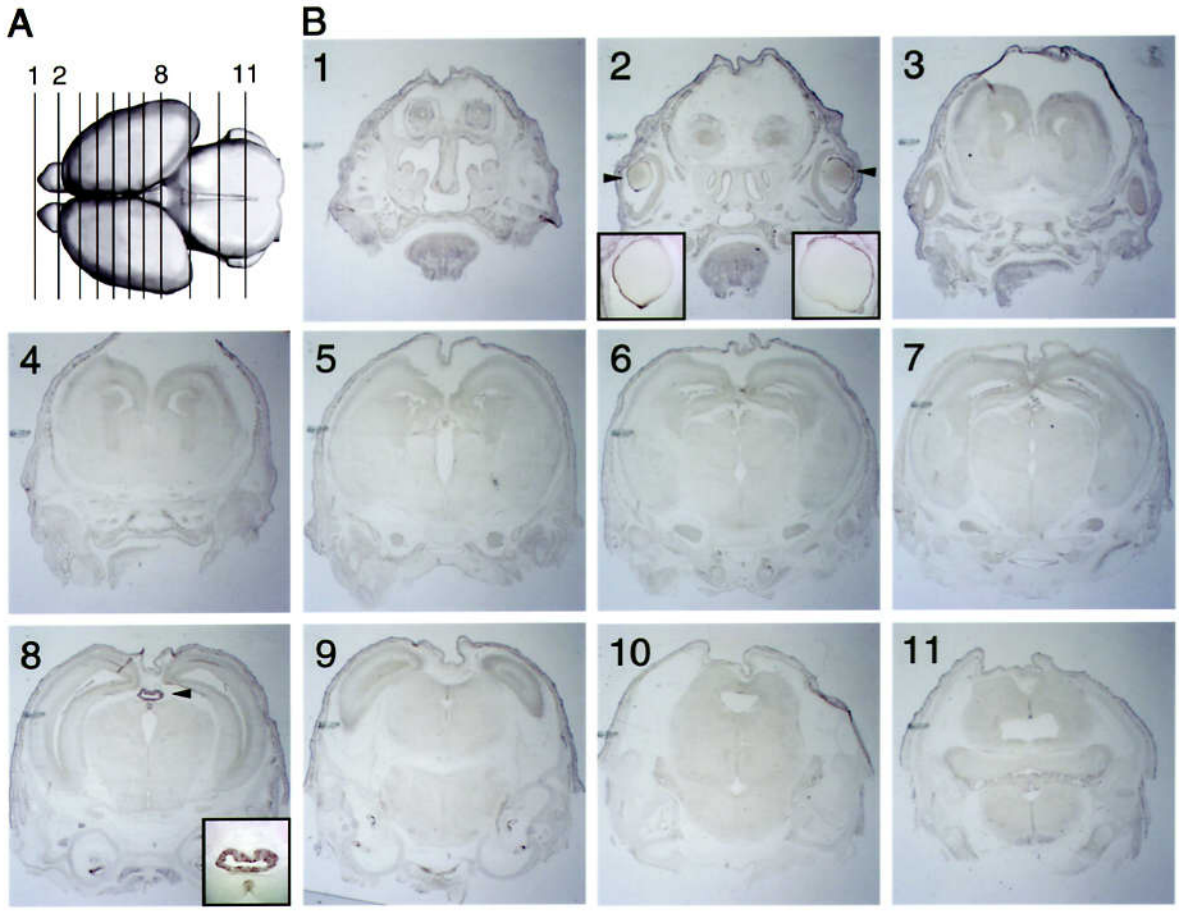

Fig. 4. Immunohistochemistry using the anti-CNTF *polyclonal* antibody in the entire section of an E18.5 rat head. (A) A schematic view of the dorsal rat brain. Vertical bars and numbers indicate the approximate position of the sections used in Fig. 4B. (B) Immunohistochemistry using an anti-CNTF *polyclonal* antibody on serial sections of an E18.5 rat head. CNTF signal is seen only in the pineal gland (see the inset of section no.8) and eyes (section no. 2), The orders of figures are the same as Fig. 3 except that the anti-CNTF polyclonal antibody was used.

Fig. 5

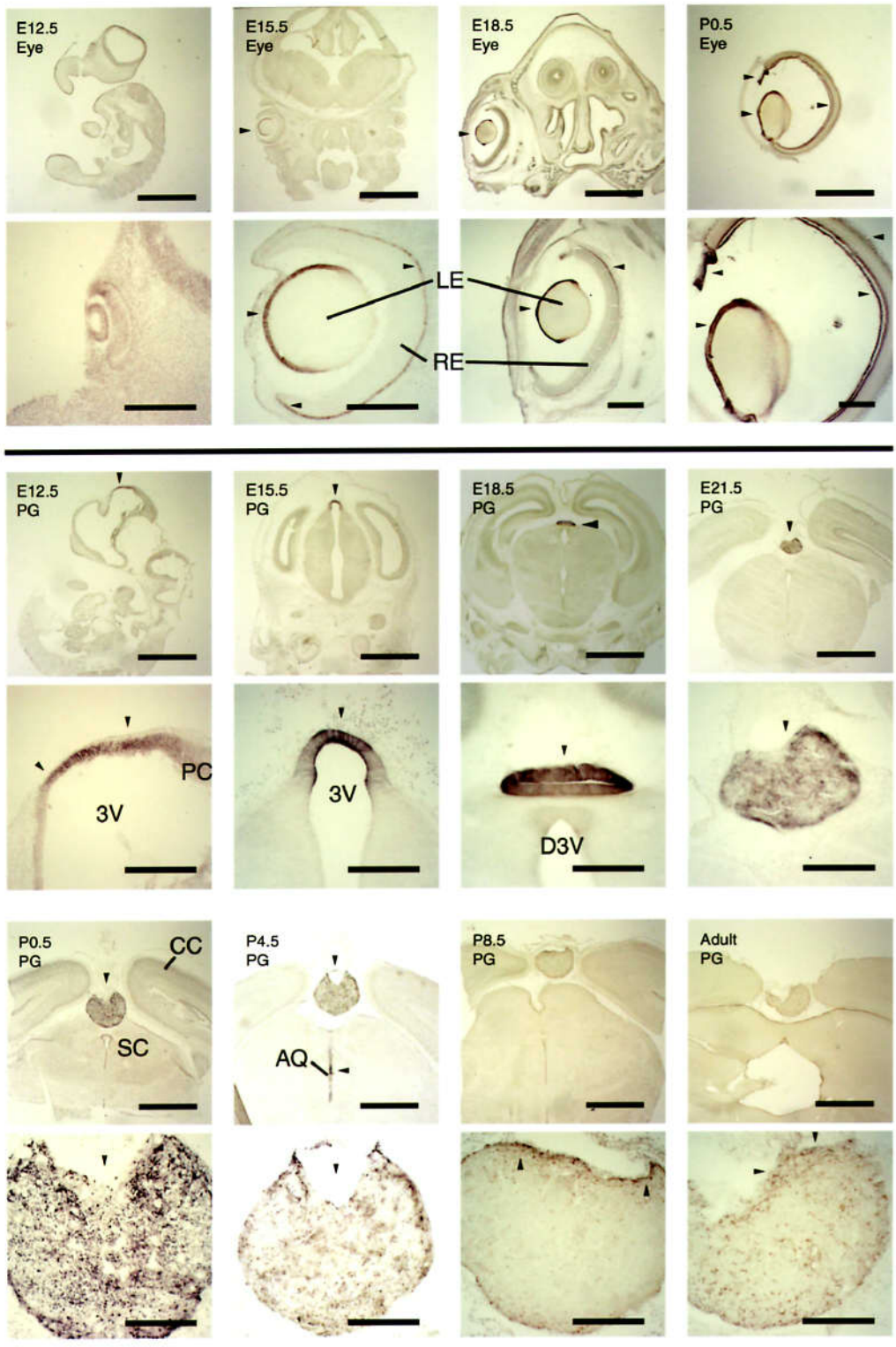

Fig. 5. Photomicrographs at higher magnifications of immunostained sections that show CNTF expression in eyes and pineal glands at different stages. The stage is marked in the upper left of each image. CNTF expression was also transiently seen at ependymal cells around the aqueduct (Sylvius) at P4.5. Bars = 200 μm (each upper panels), 1 mm (lower panels). All arrowheads indicate sites of CNTF expression. Abbreviations used in the figure: AQ, aqueduct (Sylvius); CC, cerebral cortex; D3V, dorsal third ventricle; LE, lens; PC, posterior commissure; RE, retina; SC, superior colliculus; 3V, third ventricle.

Fig. 6

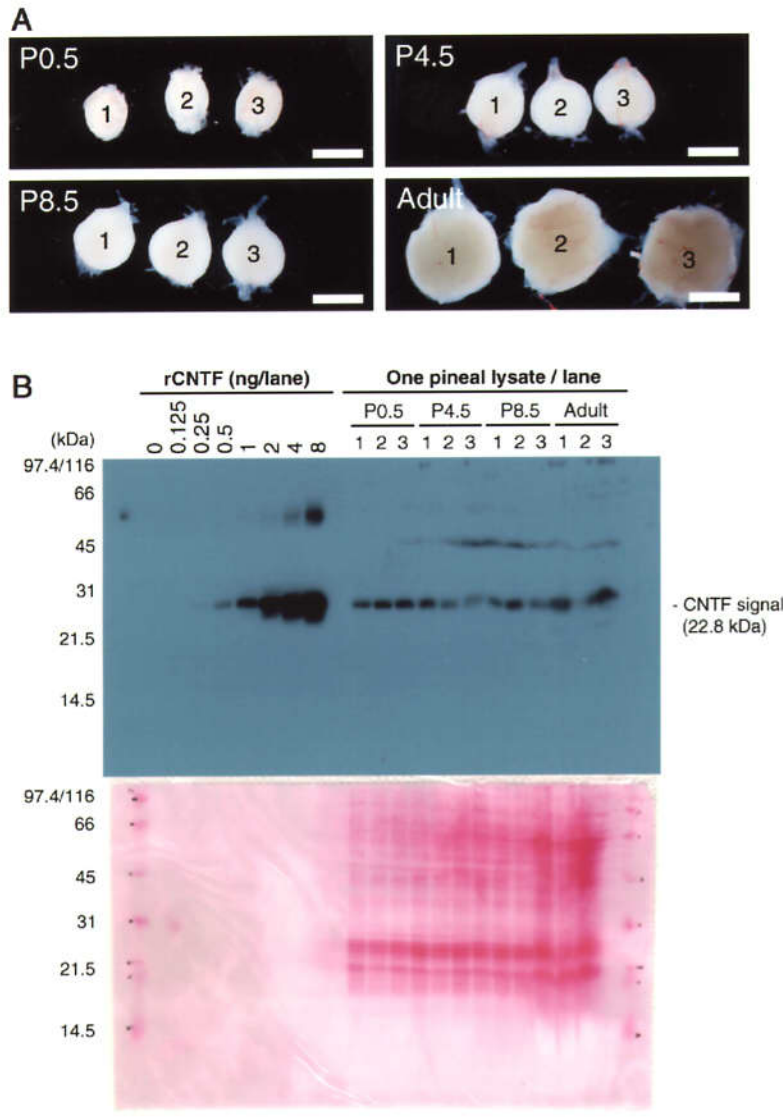

Fig. 6. Immunoblot analysis of newborn-rat pineal lysates using the rabbit anti-CNTF polyclonal antibody. (A) Each of three pineal glands at different stages used in this experiment is shown. Bars = 1 mm. (B) CNTF signal (22.8 kDa) was detected in the pineal gland at the stage when sympathetic neurons innervated. Note that the volumes of adult pineal glands were more than 10-fold larger than those of P0.5 pineal glands. The entire lysate of each gland was loaded in each lane. Embryonic pineal glands which revealed the highest CNTF expression were too small to isolate and thus were not analyzed in this experiment. Left panel: a serial twofold increase in recombinant rat CNTF (rCNTF) (from 0.125 to 8 ng/lane). The experiment for these lanes was performed to serve as the standard to the amount of CNTF immunoreactivity in pineal lysates. Right panel: lysate of each of three pineal glands at different stages was loaded per lane.

Establishment of the biological activity of CNTF in newborn rat pineal extract.

CNTF was first characterized as a neurotrophic factor for embryonic chick ciliary ganglion (CG) neurons from chick eye extract (Nishi and Berg, 1981). The original CNTF assay was performed using chick CG neurons, and rat CNTF were known to have a survival activity on chick CG neurons in spite of the species difference. I prepared extracts from pineal glands of 150 newborn rats to confirm the authentic CNTF biological activity in pineal extract *in vitro* using the same method to prepare the chick eye extract (Fig. 7A).

First, I examined whether pineal extract contains CNTF immunoreactivity by immunoblot using the anti-CNTF polyclonal antibody. Approximately 1 ng of CNTF was detected in one pineal equivalent (8 μg of protein) of pineal extract (Fig. 7B).

Next, ciliary neurotrophic activity in the pineal extract was assayed to examine the survival of chick parasympathetic CG neurons (Barbin *et al.*, 1984). Pineal extract was effective in promoting the survival of these neurons, and the number of surviving neurons significantly decreased following the addition of the anti-CNTF monoclonal and polyclonal antibodies, indicating the presence of CNTF activity in the extract (Fig. 8).

In addition, based on the report that gene expressions of neuropeptides and neurotransmitters such as substance P (SP), vasoactive intestinal polypeptide (VIP), and choline acetyltransferase (ChAT) in rat sympathetic neurons were induced by only cytokines of the IL-6 family (particularly CNTF and LIF) and activin among 33 cytokines examined (Fann and Patterson, 1994), I examined whether or not pineal extract induced these genes in rat sympathetic neurons. Pineal extract was effective in inducing the expression of these genes in rat sympathetic neurons in the presence of NGF, and this effect was suppressed by the addition of the anti-CNTF monoclonal antibody (Fig. 9). This result also indicated the presence of CNTF activity in pineal extract. It should be noted that ChAT mRNA was already expressed in the control sample to some extent due to the serum contained in the cultured medium for SCG neurons (see Fann and Patterson, 1993).

The pineal gland is one of target organs of SCG sympathetic neurons (see Fig. 14A). In the rat, sympathetic neurons do not innervate a pineal gland until the day of birth. A few fibers appear both outside and inside of the gland on day 2. The distribution pattern of nerve fibers on day 8 resembles that of an adult (Machado *et al.*, 1968;

Matsuura *et al.*, 1983).

I tested whether or not pineal extract itself had any effect on the survival of rat SCG neurons. CNTF has a potent short-term effect on the survival of these cells (Barbin *et al.*, 1984; Burnham *et al.*, 1994). On the contrary, several papers reported that CNTF induces the death of cultured sympathetic neurons in the presence of NGF (Kessler *et al.*, 1993; Savitz and Kessler, 2000), which suggest that CNTF is an apoptotic factor that prevents sympathetic neurons from innervating a pineal gland around the day of birth or causes some of the innervating neurons to die. To settle this issue, I examined the survival of newborn-rat SCG neurons in the presence of pineal extract from newborn rats with or without the anti-CNTF antibody. The results showed that the number of surviving SCG neurons was significantly decreased following the addition of the anti-CNTF antibody (Fig. 10A, 10B left panel). Thus CNTF in the extract acts as a positive (survival-supporting) factor but not as an apoptotic factor for rat-newborn SCG neurons. Almost the same results were obtained from P8.5 SCG neuron cultures (Fig. 10B, right panel). These results suggest that CNTF in the pineal gland of a newborn rat may serve as a positive, survival-supporting factor for sympathetic neurons at the period of innervation. Pineal extract was able to support the survival of sympathetic neurons for at least 3 days (Fig. 10C, open triangles), whereas CNTF itself (Fig. 10C, both circles) supported the survival of these neurons only for a short period (Burnham *et al.*, 1994; Fig. 10C). This result suggests that there may be a factor/factors other than CNTF in pineal extract which synergistically supports the survival of sympathetic neurons for at least 3 days *in vitro*.

These results demonstrate that rat newborn pineal extract which includes CNTF activity supports the survival of rat newborn sympathetic neurons prepared from the stage of innervation on the pineal gland, suggesting a role of CNTF in the survival of these neurons *in vivo*.

Fig. 7

A

Newborn rat pineal extract (PE) preparation.

Followed by the method of chick eye extract preparation (*J. Neurosci.* 1(5) 505-13 (1981)).

Collection of 150 newborn rat pineal bodies
↓
Homogenized, 700 rpm, 20 strokes, to 1 ml per 150 pineal bodies in 2 mM sodium phosphate, pH 7.5, with 0.14 M sodium chloride (PBS).
↓
1 hr at 4 degree.
↓
Centrifuged at 27,000 X g for 90 min.
↓
0.22 μm millipore filtered.
↓
Measurement of protein content by Bio-Rad protein assay kit.

B

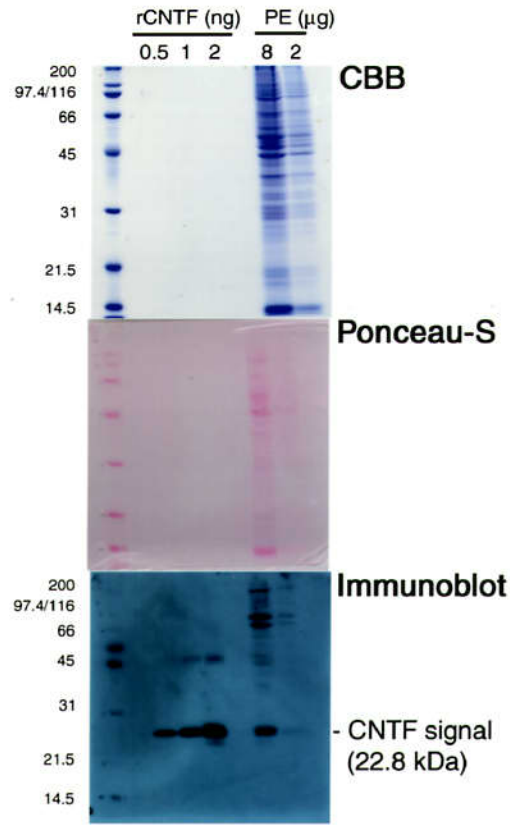

Fig. 7. Method for the preparation of pineal extract (PE) (A) and SDS-PAGE of PE (B). (B) One (about 8 μg of protein) or one-fourth pineal gland equivalent/lane was stained with Coomassie Brilliant Blue (CBB) (the upper panel), and the CNTF signal (22.8 kDa) was detected by immunoblotting using a rabbit anti-CNTF polyclonal antibody (the bottom panel). 8 μg of PE gave a nearly equivalent intensity of the signal derived from 1 ng of rCNTF in this assay.

Fig. 8

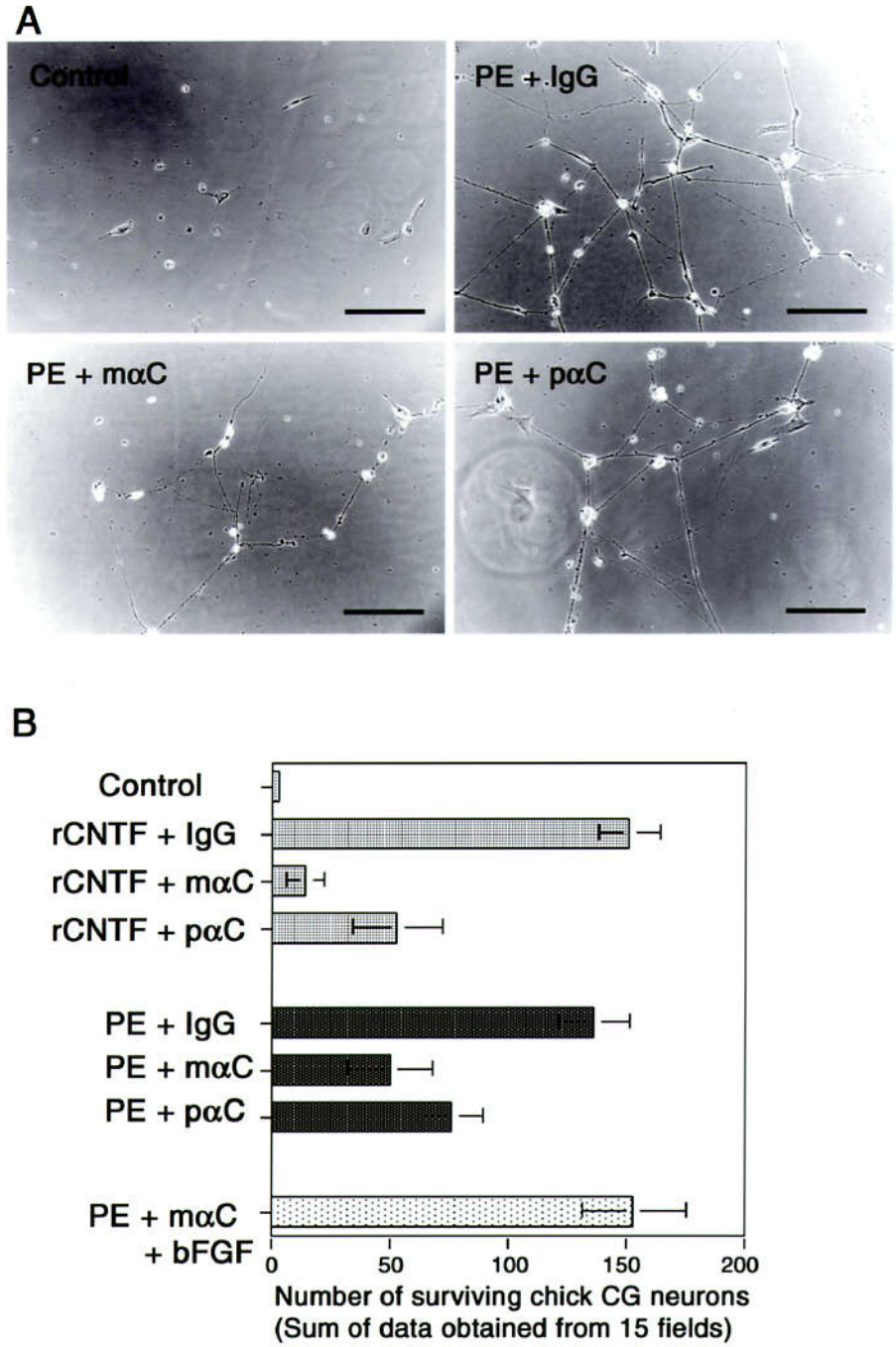

Fig. 8. Effect of PE on survival of E8 chick CG neurons. (A) Photomicrograph of the E8 chick CG neurons cultured for three days in the absence or presence of PE with normal mouse IgG (IgG), anti-CNTF neutralizing monoclonal (mαC), or polyclonal antibody (pαC). Positive controls with additon of recombinant rat CNTF (rCNTF) is not shown. Bars = 200 μm. (B) The surviving neurons were counted by viewing each well at 100X magnification using a phase contrast microscopy. The neurons were counted in 15 fields arbitrarily chosen (area of 2.35% per well) for each culture. Note that bFGF recovered the survival level of chick CG neurons in the presence of mαC to that in the presence of PE. This demonstrated the effect of mαC was not due to a non-specific toxic effect.

Fig. 9

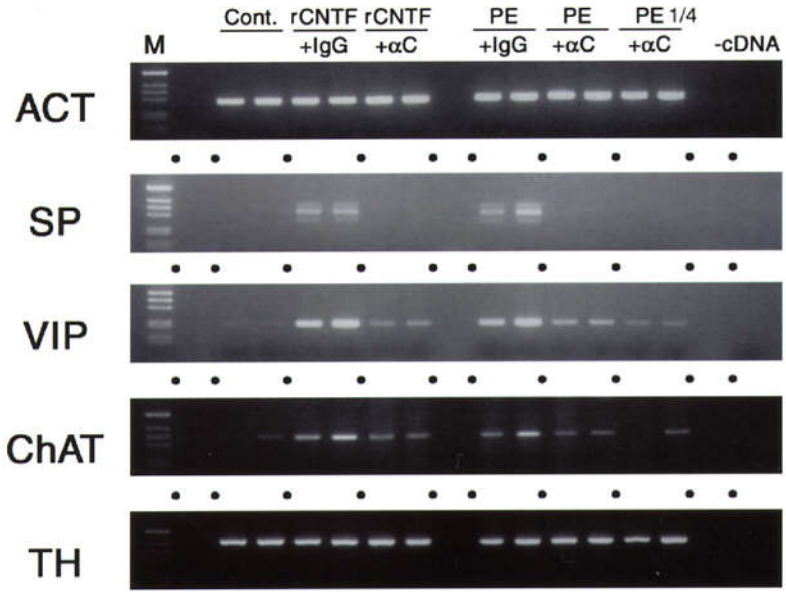

Fig. 9. Expression of several IL-6 family inducible genes in rat SCG neurons in the presence of rCNTF or PE. RT-PCR analysis of genes induced by rCNTF and PE with normal mouse IgG (IgG) or anti-CNTF neutralizing antibody (αC). SCG neurons were cultured in NGF-containing medium. Factors shown at the top of the panel were added every 36 hr for 6 days after plating. In order to compare the expression level of mRNAs of ACT (β-actin), SP (substance P), VIP (vasoactive intestinal polypeptide), ChAT (choline acetyltransferase) and TH (tyrosine hydroxylase) in the SCG neurons treated with different factors, I performed RT-PCR assay. The PCR primers for each RNA species were used as described in another report [Fan and Patterson (1993)]. PCR reaction using the cDNA reverse-transcribed from the same amount of total RNA was performed with the predetermined optimal cycle for each primer set. RT-PCR products obtained with the same primer set for RNAs derived under different conditions of cultured neurons were loaded and separated on the same 3 % agarose gel, and visualized with ethidium bromide. The portions of the gels showing the PCR bands were aligned in rows in the figure. The β-actin (known as an ubiquitously expressed gene) primer set was used as a control and showed no significant difference among different culture conditions, which proved that the amount of the total RNA used in each RT-PCR reaction was equivalent. Note that ChAT mRNA was already expressed in the control sample to some extent, due to the serum contained in the cultured medium for SCG neurons [see Fan and Patterson (1993)]. Concentration of each factor added to the culture medium: rCNTF, 2 ng/ml; PE, 16 μg/ml; anti-CNTF, 5 μg/ml; normal mouse IgG, 5 μg/ml.

Fig. 10

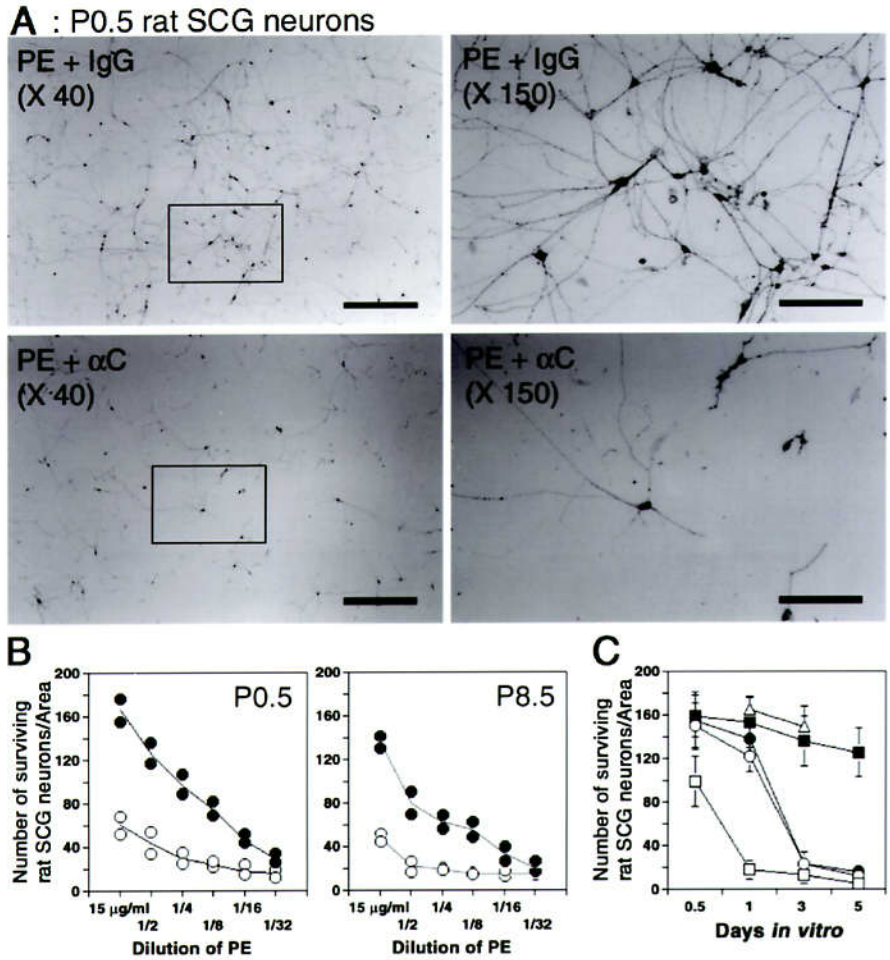

Fig. 10. Effect of PE on survival of rat SCG sympathetic neurons. (A) PE (15 μg/ml) affects the survival of P0.5 rat SCG neurons (top panels) cultured for three days: however, the effect of PE can be neutralized by the anti-CNTF antibody (bottom panels). Bars = 500 μm (left panels), 200 μm (right panels). (B) The number of surviving rat SCG neurons cultured for three days in the serial dilution of 15 mg of PE with or without the anti-CNTF antibody was determined from a total of 15 fields. Closed circles: PE with normal mouse IgG (IgG). Open circles: PE with anti-CNTF antibody (αC). Concentrations of antibodies used: anti-CNTF, 10 μg/ml; normal mouse IgG, 10 μg/ml. Left panel: P0.5 SCG neurons. Right panel: P8.5 SCG neurons. (C) Effects of PE, rCNTF and NGF on rat P0.5 SCG neurons were examined. SCG neurons were cultured for the day as indicated in the abscissa. Open triangle, closed circle, open circle, closed square, and open square indicate cultures with PE (15 μg/ml), rCNTF (1 ng/ml), rCNTF (100 ng/ml), NGF (100 ng/ml), and no factors added (control), respectively.

Analysis of CNTF knockout mice in SCG sympathetic neuronal innervation in the pineal gland.

I demonstrated that CNTF expression and existence of its biological activity in the rat pineal gland. To my knowledge, this is the first report that the site and developmental timing of CNTF production are matched with the projection of CNTF-responsive neurons in mammals, which results support the idea that CNTF in pineal glands may serve as a survival factor for innervating SCG neurons at the postnatal stage *in vivo*. For example, in the rat sciatic nerve, which is the major site of CNTF synthesis in adult rat (Sendtner *et al.*, 1994; and immunoblot in Fig. 2), CNTF is not detectable before postnatal day 4, although the period of naturally occurring cell death of motorneuron ends by the time of birth (Stockli *et al.*, 1989). These data and reports prompted me to examine if CNTF fulfils the requirements of a target-derived neurotrophic factor *in vivo*. Namely, 1) Is CNTF produced in the projection areas of their responsive neurons? 2) If so, is it available for neurons that innervate the target? 3) Does CNTF regulate the survival and differentiation of innervating neurons during embryonic development and maintain the effect at the adulthood in mammalian pineal glands? Chick CNTF is expressed in the target tissues of the avian CG and overexpression of chick CNTF during development rescues CG neurons from the cell death (Finn *et al.*, 1998). These observations suggest that chick CNTF is a true target-derived trophic factor for the chick CG neurons (Finn and Nishi, 1996). In rat SCG neurons *in vitro*, CNTF has a potent short-term effect on the survival (Barbin *et al.*, 1984; Burnham *et al.*, 1994; Fig. 9C). However, it is also reported that high concentration of CNTF induces death of cultured sympathetic neurons in the presence of NGF (Kessler *et al.*, 1993; Savitz and Kessler, 2000), which suggests that CNTF is an apoptotic factor that causes the death of some innervating neurons into pineal glands until birth.

I already answered the first question. To answer the second and third questions, I carried out the analysis of CNTF knockout mice. At first, I conducted seven generations of backcrossing of hetero-CNTF (+/-) male to wild-type CBA/N (or C57BL/6) female mice, because the densities of sympathetic innervations into pineal glands vary between strains of mice but relatively constant within one strain (Brednow and Korf, 1998). Next, I confirmed that CNTF was expressed in the pineal gland and eyes of embryonic wild-type mice by using the anti-CNTF polyclonal antibody. Fig.

11B shows that CNTF was present in E17.5 wild-type murine pineal gland and eyes; but no apparent histological differences were seen between wild and CNTF-knockout litters by hematoxylin/eosin staining (E17.5 in Fig. 11B and adult in Fig. 11C).

I examined the CNTF expression on pineal glands in CNTF knockout and wild-type mice at the period of sympathetic innervation by immunoblot using the anti-CNTF polyclonal antibody. CNTF expression was detected in the wild-type pineal gland, but not in null-CNTF litters (Fig. 12A). I then examined the sympathetic innervation of pineal glands in both wild-type and knockout mice by immunohistochemistry of TH, a sympathetic neuronal marker. In newborn mice, there were very few unbranched TH-positive fibers in both genotypes (Fig. 12B, P0.5). In a total of ten newborn mice of both genotypes that I examined, three pineal glands were innervated and branched in wild-type (+/+) mice, and two of those in -/- mice, but there seemed to be no apparent difference in the innervation pattern in both genotypes (data not shown). These observations suggest that CNTF may not be essential for the onset of sympathetic innervation of the pineal gland. On P6.5 and at the adult stage, there was also no difference in the innervations of pineal glands between the knockout and wild-type litters (Figs. 12B and 12C). A similar pattern of staining for neurofilament, a common neural marker, was also seen (data not shown). In addition to SCG neurons, it was reported that pineal glands of adult gerbil were innervated by calcitonin-gene-relating-peptide (CGRP)- and SP-positive fibers from the trigeminal ganglion, and by VIP-positive neurons from the pterygopalatine ganglion (Shiotani *et al.*, 1986). Pineal glands of CNTF knockout and wild-type litters were faintly innervated by these fibers, but there also seemed to be no difference in the innervation pattern (data not shown).

These results demonstrate that CNTF knockout and wild-type mice seemed no apparent difference in neuronal innervations into the pineal gland, regardless of the CNTF expression on pineal glands at the period of sympathetic neuronal innervation.

Fig. 11

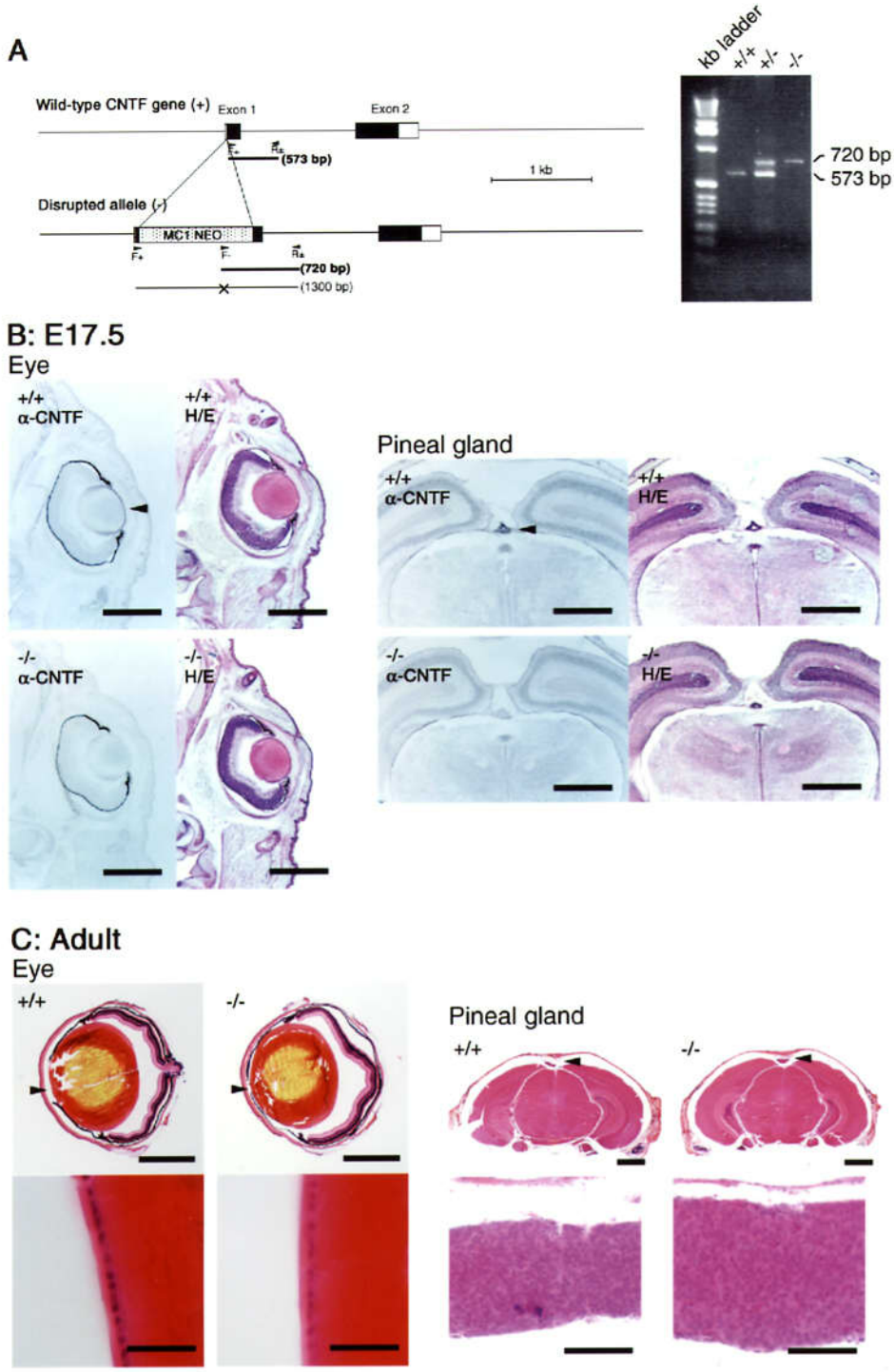

Fig. 11. Histological comparisons of eye and pineal gland between wild-type (+/+) and CNTF-knockout (-/-) mice. (A) Left panel: Maps of the CNTF +/+ gene (upper) and disrupted (-/-) allele (lower). See Masu *et al.* (1993). Arrowheads: positions of primers used for genotyping-PCR. Right panel: Result of genotyping-PCR showed expected bands at 573, 573 and 720, 720 bp in +/+, +/- and -/- mice, respectively. (B) CNTF is expressed at eye (left panels) and pineal gland (right panels) in E17.5 +/+, but not -/- littermates by IHC using anti-CNTF polyclonal antibody (α-CNTF). Arrowheads indicate the site of CNTF expression. Adjacent sections were performed by Hematoxylin/Eosin stains (H/E), respectively. Bars = 1 mm (eyes), 400 μm (pineal glands). (C) H/E stains of eye and pineal gland in adult mice of both genotype showed no apparent histological differences. Arrwoheads indicate the positions showed by higher magnification in the bottom panels. Bars = 1 mm (upper panels), 50 (eye) and 100 (pineal) μm (lower panels).

Fig. 12

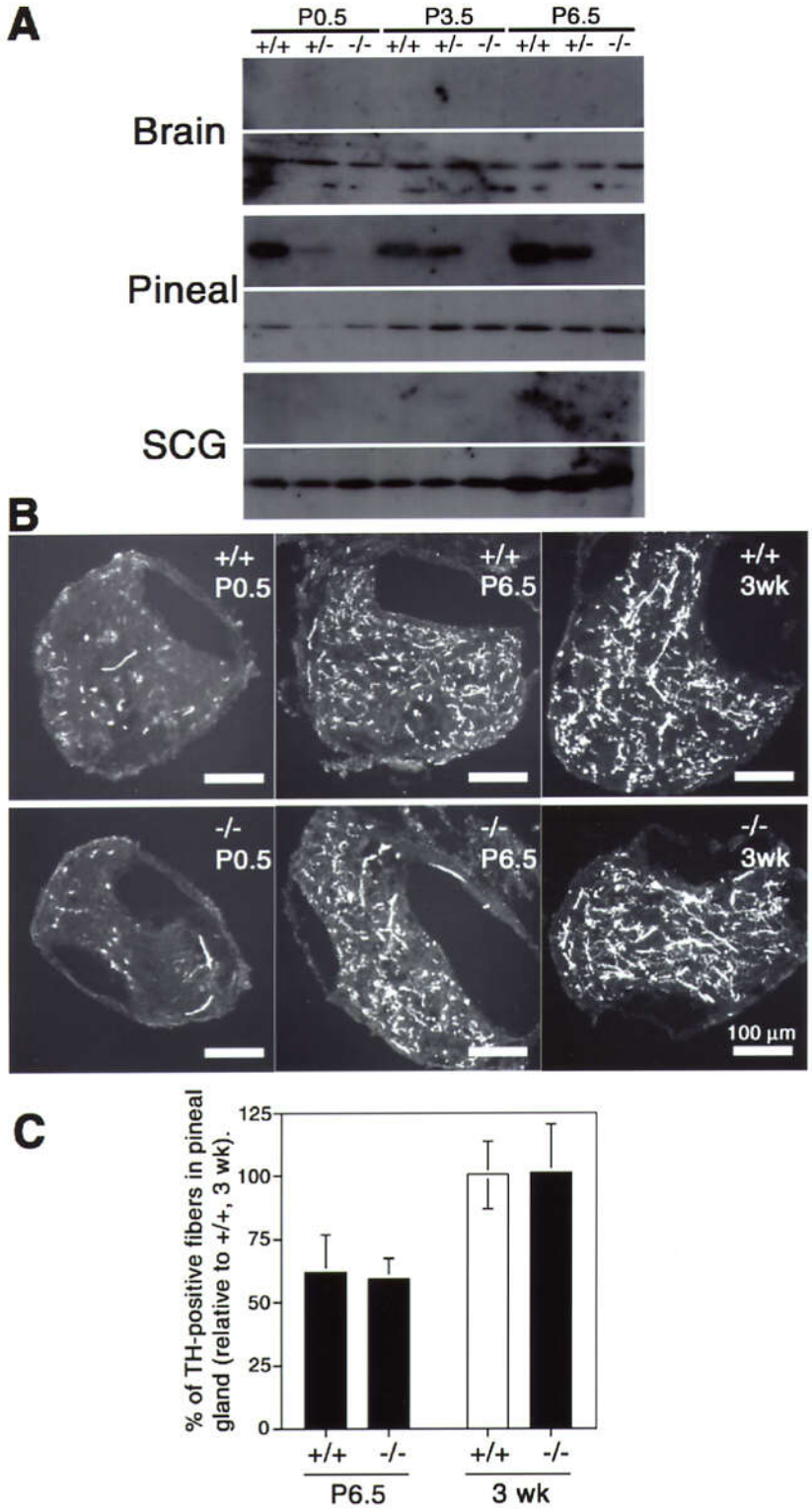

Fig. 12. Sympathetic innervation of pineal glands in CNTF knockout mice. (A) Immunoblot analysis of murine postnatal whole brains, pineal glands and SCG lysates of homo-CNTF (null) (-/-), hetero-CNTF (+/-) and wild-type (+/+) litters. The rabbit anti-CNTF polyclonal antibody (upper panels of each tissue) and anti-ERK1 antibody (lower panels) were used as control. The CNTF signal was detected in pineal glands of the wild-type and hetero-CNTF mice on P0.5, P3.5 and P6.5. (B) Immunohistochemistry of pineal sections from mice of the wild-type and the null genotypes using an anti-TH antibody, a marker for sympathetic neurons. Scale bars = 100 μm. (C) Percent of TH-positive fibers in pineal glands of CNTF knockout mice (black bar) compared to that of the wild type at three weeks (white bar), calculated as described in Materials and Methods.

CNTF in pineal glands may be unavailable for innervating sympathetic neurons.

It has been controversial whether CNTF is released from cells or not (Kamiguchi *et al.*, 1995; Stockli *et al.*, 1989). In order to clarify the function of CNTF *in vivo*, I examined whether or not sympathetic neurons that innervate pineal glands express the genes that are induced by CNTF *in vitro*. In Fig. 9, CNTF in the pineal extract induced neuropeptidergic gene (SP and VIP) expressions on cultured sympathetic neurons. Moreover, it was previously reported that the application of IL-6 family cytokines only on distal neurites was sufficient to induce the cholinergic and neuropeptidergic phenotype in these neurons *in vitro* (Ure *et al.*, 1992) and *in vivo* (Francis and Landis, 1999).

Therefore I examined whether or not cell bodies of the sympathetic neurons that innervated a pineal gland expressed CNTF-inducible neuropeptidergic genes (SP and VIP). DiO crystal, a retrograde tracer, was put into a pineal gland of P2.5 rat to visualize sympathetic cell bodies in SCG neurons whose axons innervate the pineal gland. Two days later, sections of SCG were immunostained with the anti-preprotachykinin-A antibody (PPT: SP precursor protein) or the anti-VIP antibody. Fig. 13A shows that the retrograde tracer DiO was properly transported to neuronal cell bodies of SCG neurons innervating a pineal gland, in agreement with the previous report that the neurons in the rostral region of the ganglion preferably project to a pineal gland (Luebke and Wright, 1992). The results shown in the middle and right panels of Fig. 13B indicate that there were few double-stained sympathetic cell bodies (retrogradely labeled and PPT-positive). Similar results were seen in SCG stained with the anti-VIP antibody two, three and four days after DiO injection (data not shown). I was unable to inject DiO successfully in mouse pineal glands in my hands because they were too small. Thus, I simply counted the number of VIP positive neural cell bodies in SCG in adult knockout and wild-type mice. There were no apparent difference in the numbers of these VIP-positive cells in the adult CNTF knockout and wild-type mice (Fig. 13C).

These results indicate that PPT and VIP expression in sympathetic neurons innervating a pineal gland were seldom induced regardless of the presence of CNTF in the target, suggesting that sympathetic neurons innervating into pineal gland could not receive CNTF signal from the target organ in general, although it is possible that CNTF signal may be received by innervating neurons in a rare case.

Fig. 13

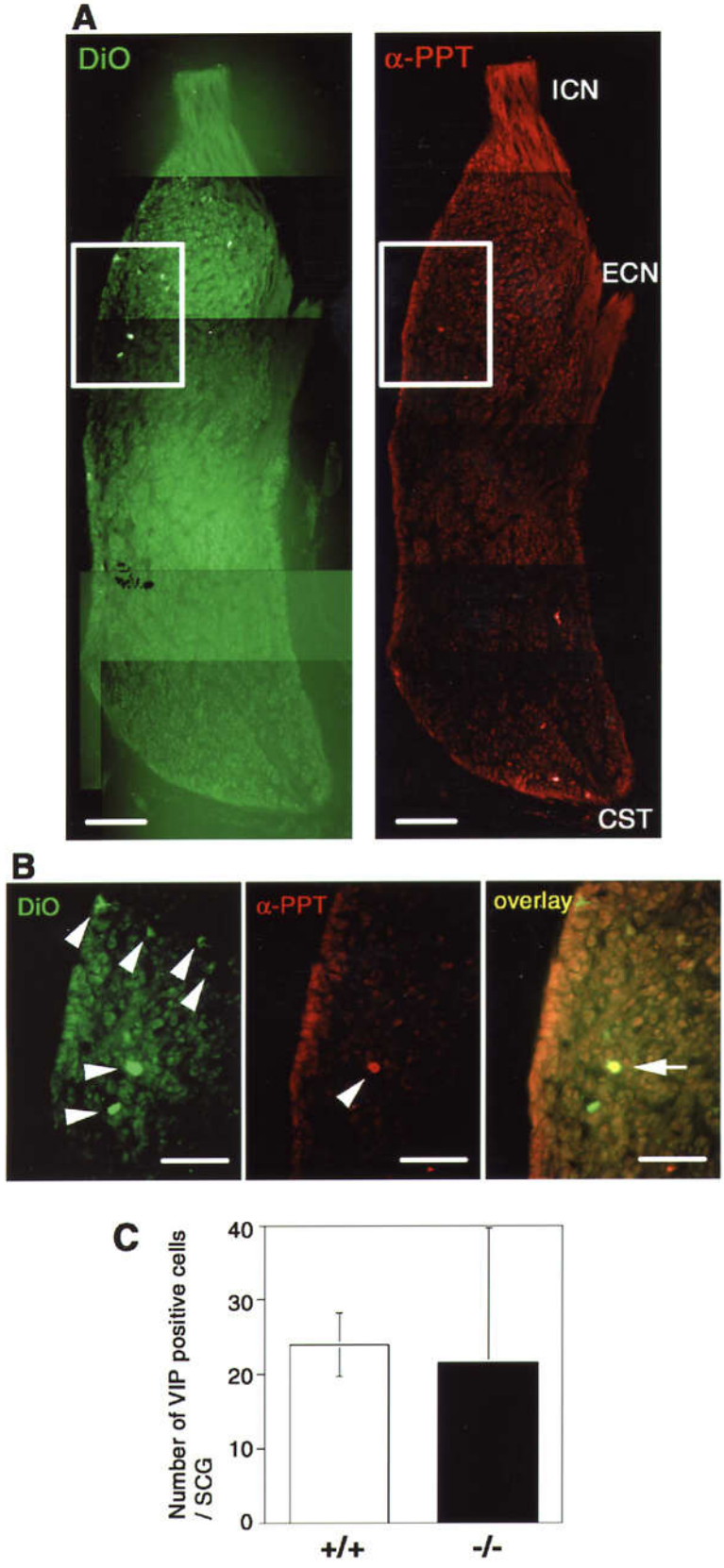

Fig. 13. SCG sympathetic neurons innervated in pineal gland seems not to express effectively CNTF-inducible genes. (A) Double-staining of sympathetic neuronal cell bodies. A rat pineal gland on P2.5 was labeled with a retrograde tracer, DiO (pseudocolor: green). SCGs were removed and sectioned two days later and stained with an anti-preprotachykinin-A antibody (α-PPT: red). ICN, internal carotid nerve; ECN, external carotid nerve; CST, cervical sympathetic trunk. Scale bars = 200 μm. (B) Few DiO-labeled (arrowheads in the left panel) cells were labeled by PPT (middle). Right panel: an overlay image (an arrow indicates a double-labeledcell). Scale bars = 100 μm. (C) There was no statistically difference in VIP positive cells in SCG between CNTF-knockout (-/-) and wild-type litters (+/+).

Effect of CNTF on the development of photoreceptors in vitro rat pineal organ cultures.

I then asked what was the significance that CNTF was only expressed in the developing pineal gland (and eyes)? There are evidences that show that neurotrophic factors may not only function as target-derived survival factors for projection neurons, but also act locally to regulate other developmental processes. I next attended to the function of CNTF not as a neurotrophic factor for neurons that innervate the target, but as a factor for the development of photoreceptors in the photoreceptive organs.

A major pineal function is the rhythmic production of melatonin which phenomenon was observed in all vertebrates investigated thus far. The rhythmic production of melatonin is under the control of photoreceptor cells and endogenous oscillators. In birds (and the lampreys, fish, amphibians, reptile), photoreceptors occur in the pineal complex itself (see the schematic model of Fig. 14A, right panel). In contrast, the mammalian pineal organ lacks functioning photoreceptors, and the melatonin biosynthetis in the mammalian pineal is regulated by photoreceptors located in the retina (Korf, 1994; and see Fig. 14A, left panel). In addition, it is reported that CNTF promotes the development of photoreceptors in chick, while CNTF prevents it in rat retinal cell cultures (Ezzeddine *et al.*, 1997; Fuhrmann *et al.*, 1995). Kirsch *et al.* made an interest report on the effect of CNTF in the photoreceptor development using equally dissociated cultures of chick embryonic and rat newborn retina in order to study the photoreceptor development of opsin. In the presence of CNTF, the number of photoreceptors was increased in chick cultures, but was reduced in rat cultures (Kirsch *et al.*, 1996). These results may infer that CNTF involves in the regulation of photoreceptor development, and that it can have different actions in photoreceptive organs of the two species. I have already described the specific expression of CNTF in the rat developing pineal gland. Taking these reports and my results into account, I asked two questions. First, is CNTF expressed in developing chick pineal glands? Second, similar to the retinal cell culture, do CNTF exert opposite effects on the development of rat and chick photoreceptors in pineal glands, namely, CNTF supports the development of pineal photoreceptors in chick but prevent it in rat?

To answer the first question, I examined the CNTF expression at the developing chick pineal gland by immunoblot with the anti-chick CNTF polyclonal antibody. The developmental expression of chick CNTF in eyes, but not in some tissues such as a

total brain or liver, was already reported using northern blotting technique (Leung *et al.*, 1992). Fig. 14B indicated the existence of CNTF in developing chick pineal lysates at E14.5 and 18.5. These data and Figs. 3 and 4 showed that CNTF protein was expressed at developing pineal gland and eyes, in both species.

Next, I examined the effect of CNTF on the expression of rhodopsin, a marker of photoreceptor, in the newborn rat pineal organ culture. Although the rat pineal is an endocrine organ and has no photoreceptor activity, pineals from neonatal rats contain cells that can differentiate into rod-like cells with rhodopsin immunoreactivity, when cultured *in vitro* (Araki, 1992). And immunocytochemical investigations performed during the last decade have clearly shown that the majority of pineal photoreceptor cells share molecular features with retinal photoreceptors (Korf, 1994). *In vitro* newborn-rat pineal organ cultures, CNTF (and LIF, another IL-6 family cytokine) prevented the expression of rhodopsin immunoreactivity in newborn-rat pineal organ cultures (Fig. 15).

These results (and Figs. 1-9) indicated that CNTF was detected on the developing pineal gland and eyes in the rat and chick, and rat CNTF suppressed the development of rod-like cells in the rat pineal organ culture, which results seems to be a similar effect to that in the rat retinal cell culture. However, whether or not chick CNTF supports the development of photoreceptors in the chick pineal culture remains to be studied. It thus remains for future studies to elucidate the complicated but interesting role of CNTF specifically expressed in developing pineal glands where CNTF may play a new unknown role for the photoreceptor development in the chick and rat.

Fig. 14

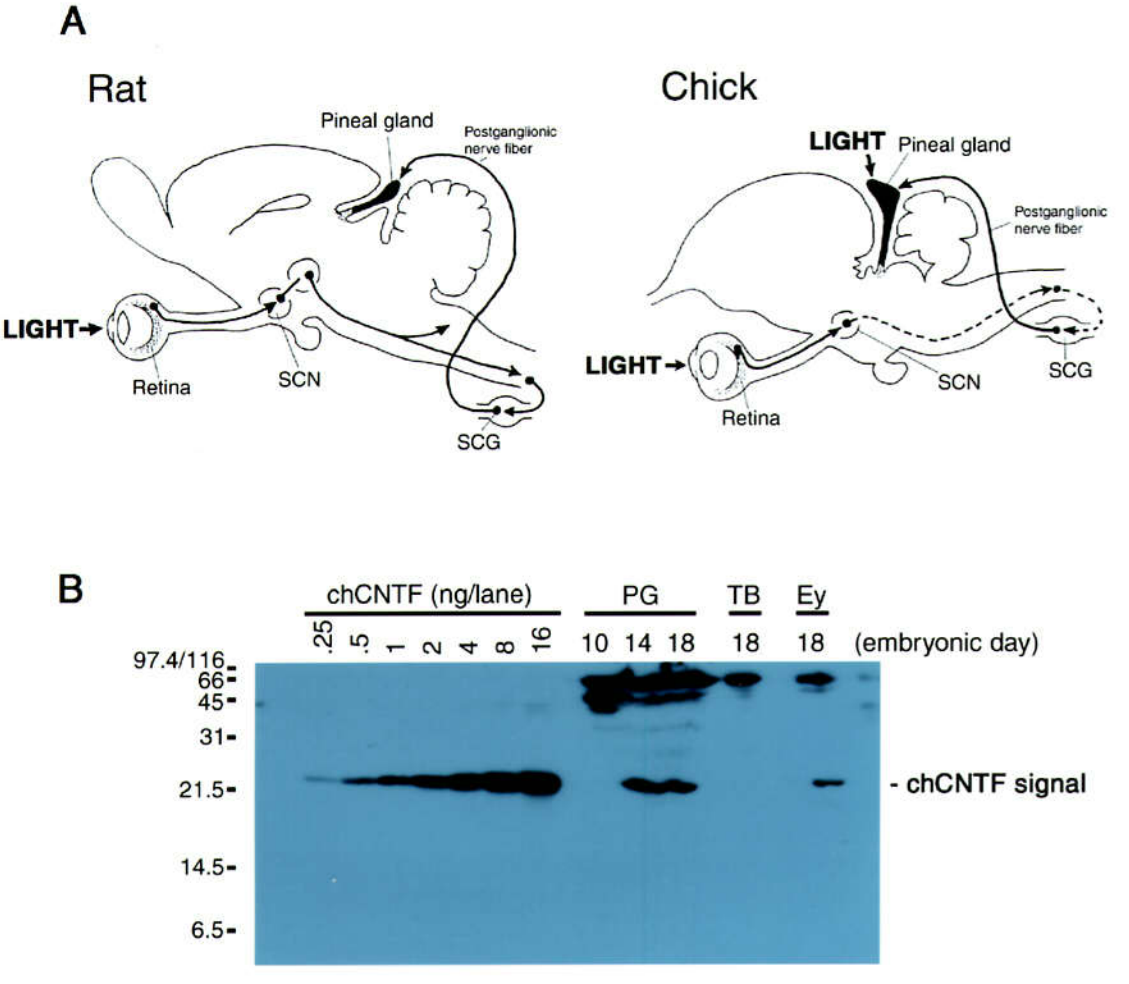

Fig. 14. (A) Schematic models compared between rat and chick in light/nerve inputs to pineal gland. SCN; suprachiasic nucleus. (B) Immunoblot analysis of embryonic chick tissue lysates using rabbit anti-chick CNTF polyclonal antibody. Left panel: a serial twofold increase in the recombinant chick CNTF (chCNTF) (from 0.25 to 16 ng/lane). The experiment for these lanes was performed to serve as the standard of the amount of chCNTF immunoreactivity in the pineal lysate. Right panel: lysate of each of chick E10, 14 and 18 pineal glands (PG) at one pineal lysate per lane, and 20 μg of E18 total brain (TB) and eye (Ey) lysates used for positive or negative control (see Leung *et al.* 1992), respectively. chCNTF signals (21.3 kDa) were detected in the E14 and 18 chick pineal gland.

Fig. 15

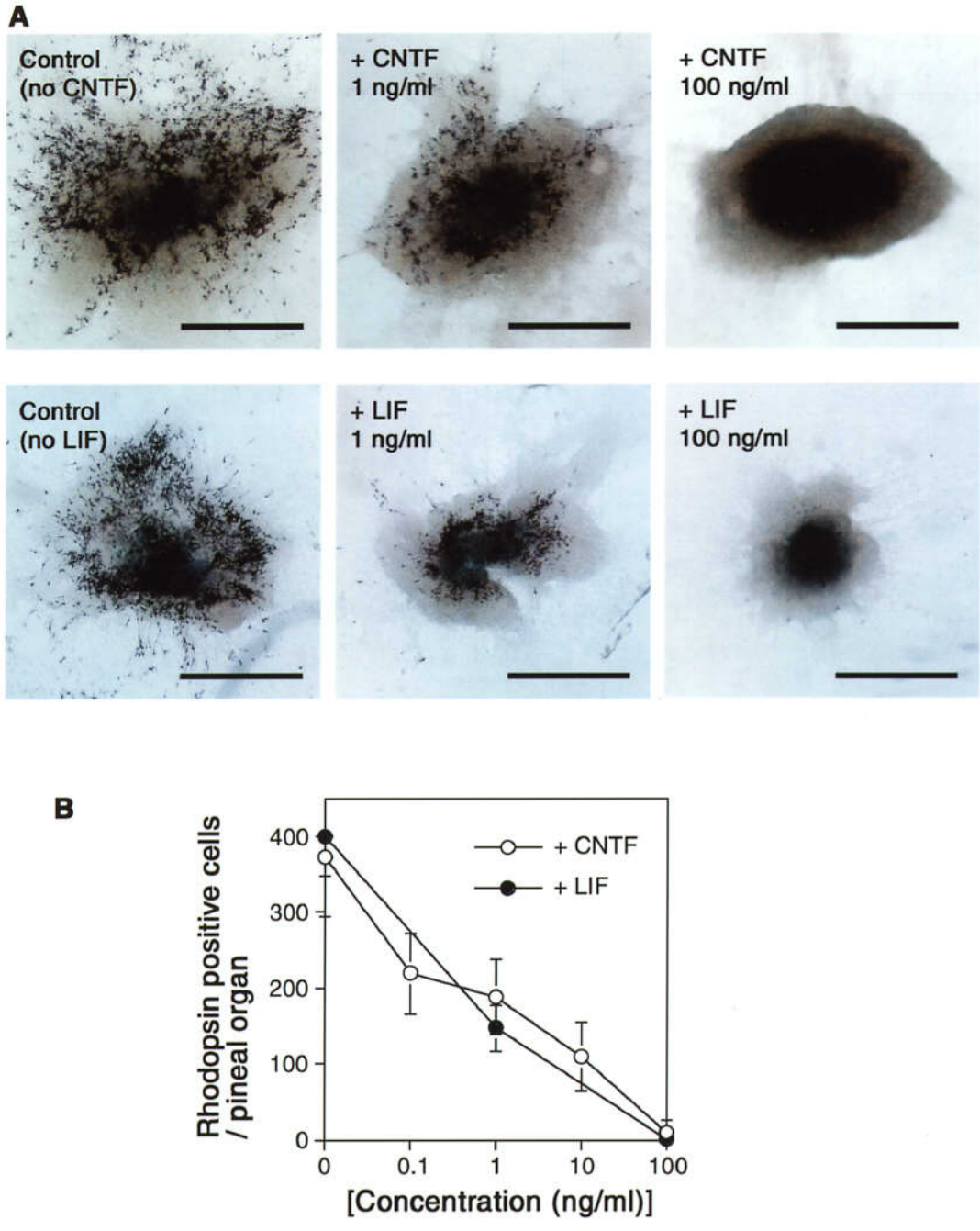

Fig. 15. Effect of CNTF and LIF on differentiation of rhodopsin positive cells in newborn-rat pineal organ cultures. (A) Photomicrograph of pineal organ cultured for 10 days with or without the indicated concentrations of CNTF or LIF. Rhodopsin positive cells were visualized by anti-rhodopsin antibody and ABC-kit as described in Materials and Methods. Bars = 500 μm. (B) Number of rhodopsin positive cells were counted under the light microscopy of three pineal organ cultures in each group.

Analysis of free-running periods and amounts of phase shift in responding to a light in CNTF knockout (-/-) and wild-type mice (+/+).

So far, it has been reported that CNTF-deficient mice appeares to be normal in development, although a moderate motor neuron deficit is seen during the aging (Masu *et al.*, 1993). In addition, 2.3% of Japanese people have homozygous and 35.8% heterozygous mutations of CNTF genes, without any signs of deficiency including neurological defect (Takahashi *et al.*, 1994). The histological examination of pineal gland and sympathetic innervation to that in mice lacking CNTF gene suggests that CNTF does not play a crucial role during the embryonic pineal development (Figs. 11 and 12). These data have led to a supposition that CNTF may not participate in the development, but might have other functions of the pineal gland and eyes.

To find the behavioral phenotype of CNTF-deficient mice relating to the function of the pineal gland and eyes, I examined the difference of free-running rhythms in constant darkness and phase-shift upon exposure to a light pulse between CNTF knockout and wild-type mice in collaboration with Dr. Shin-Ichi T. Inouye and Mr. Takashi Izumi (Department of Physics, Informatics, and Biology, Yamaguchi University, Yamaguchi, Japan). At first, I conducted five generations of backcrossing of hetero-CNTF (+/-) male to wild-type C57BL/6 (+/+) female mice, and obtained genotype of wild-type and knockout mice. Then I examined the free running period and phase-shifting in both genotypes. Free-running periods were not affected by the loss of CNTF gene. But, the phase-shift in responding to a light pulse of 30 minutes at Circadian Time 16 in CNTF knockout mice showed the tendency to somewhat longer than that of wild-type mice, although statistical significance was not seen (Table 1).

This result may imply the effect of the phase-shift was due to the lack of the CNTF gene. However, phenotypic differences among mice with CNTF-disrupted genes and those with wild-type alleles could not be concluded because that the disrupted CNTF gene was maintained on a mixed background including C57BL/6 and 129Sv alleles and the circadian rhythm and phase-shifting effect are known to be varied between strains of mice (Ebihara *et al.*, 1988; Ebihara *et al.*, 1978). I am now conducting further backcrossing of hetero-CNTF (+/-) male to two different strains, wild-type CBA/N and C57BL/6 female mice. It will be necessary to confirm whether or not the effect of the phase-shifts in Table 1 was due to the lack of CNTF gene, by using a significant number of backcrossed mice in these different strains.

Table 1.

Periods and Phase shifts to light in C57BL/6, $CNTF^{+/+}$ and $CNTF^{-/-}$ mice.

	C57BL/6	$CNTF^{+/+}$	$CNTF^{-/-}$
Free running period (hr)	23.67 ± 0.03 (6)	23.61 ± 0.05 (4)	23.65 ± 0.06 (11)
Phase shift (hr)	2.43 ± 0.35 (4)	2.61 ± 0.17 (3)	2.97 ± 0.19 (9)

Note. Free-running periods and amounts of phase shift in responding to a light pulse of 30 minutes at Circadian Time 16 were determined in CNTF knockout (-/-) and wild-type mice (+/+). Animals were housed singly in a cage equipped with a running wheel kept in constant darkness. The experiments were carried out as reported (Pittendrigh and Daan, 1976). The times (hours) of free-running periods and amounts of phase shift are shown as means and standard errors (s. e.). Numbers in each bracket indicate the number of mice used in each experiment.

IV. DISCUSSION

In this research, I observed the specific expression of CNTF in embryonic pineal glands and eyes in rodents. I also observed that CNTF in pineal extract promoted the survival of sympathetic neurons *in vitro*. Since my results show for the first time that CNTF is expressed in the target organ of CNTF-responsible neurons during their innervations, the pineal gland seems to be a good model system to elucidate the function of CNTF *in vivo*. Nevertheless, there were no significant differences between CNTF-deficient mice and wild-type litters in the densities of sympathetic neurons that innervate pineal glands. The retrograde tracing study showed that there were few PPT-neurons among SCG sympathetic neurons that innervate a pineal gland; the gene expression of these peptides is known to be induced by CNTF *in vitro*.

Neutralizing CNTF in pineal extract with the corresponding antibody significantly decreased the survival activity for neonated rat sympathetic neurons (Figs. 10A and 10B), which suggests that CNTF in the pineal gland works as a growth-supporting factor. However, as shown in Fig. 12, CNTF in mouse pineal gland showed no apparent effect of sympathetic innervations in the pineal gland. There may be some explanations for this discrepancy. First, pineal glands produce neurotrophic molecules other than CNTF for innervating sympathetic neurons. Although the anti-CNTF antibody significantly blocked the survival activity in pineal extract of rat SCG and chick CG neurons, about one-third of rat SCG neurons in cultures survived even in the presence of the anti-CNTF monoclonal antibody (Fig. 10B). Although the anti-CNTF monoclonal antibody (mαC) suppresses more than 90% of the survival effect of rCNTF on chick CG neurons, about one-third of these neurons in the presence of pineal extract also survived even when treated with the same antibody (Fig. 8B). It has been reported that the SCG itself is severely shrunken in NGF knockout or its receptor, TrkA knockout mice (Crowley *et al.*, 1994; Smeyne *et al.*, 1994). There is no sympathetic innervation in pineal glands of p75NGFR and NT-3 knockout mice (Lee *et al.*, 1994; ElShamy *et al.*, 1996), and pineal glands in BDNF knockout mice are hyperinnervated by sympathetic neurons (Kohn *et al.*, 1999). Thus, it is conceivable that pineal glands may produce neurotrophic molecules other than CNTF, the gene of which might compensate for the function of CNTF in pineal gland when it is absent in CNTF-deficient mice.

The other possibility may be explained by the fact that CNTF has typical features of a cytosolic molecule, that is, the absence of a signal sequence and glycosylation site (Stockli *et al.*, 1989). Elson *et al.* recently reported that a cardiotrophin-like cytokine (CLC), a novel IL-6 type cytokine, which is not secreted when expressed in COS cells, is secreted forming a stable complex with cytokine-like factor-1 (CLF) when co-expressed with it in COS cells (Elson *et al.*, 2000). But the direct interaction of CNTF with CLF has not been reported yet. CNTF has a strong activity of inducing several neurotransmitter and neuropeptide genes such as SP and VIP in sympathetic neurons *in vitro* (Fann and Patterson, 1994), and CNTF in pineal extract was effective in inducing the expression of these genes in rat sympathetic neurons (Fig. 9). However, in fact, PPT- (or VIP-) positive SCG neurons scarcely existed among neurons innervating a pineal gland regardless of the presence of CNTF in the target (Fig. 13B). It has been reported that cultured astrocytes release CNTF (Kamiguchi *et al.*, 1995). However, if the released CNTF is available by SCG neurons that innervate pineal glands, PPT- (or VIP-) positive SCG neurons might be observed, because IL-6 family cytokines only on distal neurites was sufficient to induce the neuropeptidergic and cholinergic phenotype in sympathetic neurons (Ure *et al.*, 1992; Francis and Landis, 1999).

Therefore, I interpret these results as follows: pineal cells *in vivo* do not constantly release CNTF, in agreement with the previously reported *in vitro*, and CNTF might not be effectively available for innervating sympathetic neurons despite its high level in developing pineal glands. However, the possibility that CNTF is available to innervating sympathetic neurons *in vivo* could not be excluded, because the degeneration of pinealocytes in embryonic, young and aged rat pineal glands has been observed (Calvo and Boya, 1981; Humbert and Pevet, 1995). Although there is no evidence that proves that the expression of PPT in the SCG neurons innervating the pineal gland is due to the effect of CNTF in the pineal gland, such neurons were infrequently seen among neurons innervating a pineal gland (Fig. 13B, overlay panel). Therefore it is possible that only a very limited amount, if any, of CNTF released by these degenerated cells may be available to innervating neurons as a survival-supporting factor.

Then, what is the significance of CNTF expression only in the developing pineal gland and eyes? In order to answer this question, I also investigated the effect of CNTF on pineal glands itself by carrying out organ cultures of developing (postnatal day 0.5)

rat pineal glands. In pineal glands, CNTFRα mRNA was ubiquitously expressed (unpublished observation). Tryptophan hydroxylase (TPOH), one of the serotonergic marker, was also uniformly expressed, but rhodopsin (Rho), one of the photoreceptive cell marker, was expressed by only limitted cells, while microtubule–associated protein-2 (MAP2), a common neuronal marker, was not expressed at all (Besancon *et al.*, 1995; Korf *et al.*, 1985; my unpublished observation). *In vitro*, I confirmed the similar observation in 10 days organ cultures of newborn rat pineal glands (Araki, 1992 and Fig. 15). Interestingly, when CNTF was added, pineal cells completely lost TPOH (serotonergic) and Rho (photoreceptive) signal, and alternatively expressed strong MAP2 (neuronal) signal (Fig. 15 and unpublished observations), although these phenomena were seen only at a very high-concentration of CNTF (>100 ng/ml).

I interpret my results as follows: The expression pattern of CNTF in the chick and rat seems to be closely related to the phylogenetic development of the photoreceptive or its rudimentary organs (see the schematic models, Fig. 14A). CNTF was originally characterized from chick eyes as a survival factor for chick ciliary neurons (Nishi and Berg, 1981). Since CNTF is also expressed at the developing pineal gland in the chick (Fig. 14B), it is possible that CNTF acts to regulate developmental processes, for example the photoreceptor development, on the pineal gland in chick besides it serves as a target-derived neurotrophic factor. In other words, while CNTF might participate in the development of the photoreceptive organs in other animals, expression of CNTF in the eye and pineal gland in mammals seems to reflect on such phylogenetic evolution of the pineal gland as a photoreceptive organ. The major difference of the roles between rat and chick CNTF is that, in contrast to mammalian CNTF, chick CNTF is secreted from cells (Leung *et al.*, 1992; Finn *et al.*, 1998; Reiness *et al.*, 2001). In the presence of CNTF, the number of photoreceptors was increased in chick retinal cell cultures, but was reduced in rat retinal cell cultures (Kirsch *et al.*, 1996). In newborn-rat pineal organ cultures, CNTF prevented the expression of rhodopsin (Fig. 15). Although the biological significance of these results *in vitro* remains to be elucidated, these results and reports may imply the interesting cue that CNTF plays different roles in the photoreceptor development on the rat and chick pineal gland. Whether or not chick CNTF supports the development of photoreceptors in pineal culture remains for the future study.

Since my results in this study show that the site of the production of CNTF and the

target of the innervating neurons that respond to CNTF *in vitro* coincided during development, it could suggest a new function of CNTF in the developing pineal gland and eyes. As I described above, however, at least the majority of CNTF seems to be unlikely available for SCG neurons that innervate the target organ in rat (Figs. 12 and 13). It thus remains for future studies to elucidate the biological significance of CNTF that is specifically expressed in the developing pineal gland and eyes where CNTF may play a new unknown role in mammals.

V. MATERIALS AND METHODS

Animals.

Wistar/ST rats and C57BL/6 or CBA/N mice were obtained from SLC (Shizuoka, Japan). CNTF-deficient mice (Masu *et al.*, 1993) were obtained from RCC Ltd., Biotechnology and Animal Breeding (Switzerland). Chick eggs were obtained from a local chick yard (in Mie, Japan). All procedure for animal care and use in this study followed the guidelines for animal experiments of Okazaki National Research Institute and NIH.

Sectioning, in situ hybridization and immunohistochemistry. Animals were transcardially perfused with physiological saline followed by 4 % paraformaldehyde (PFA) in 0.1M phosphate buffer and immersed for 4 hr in the same buffer, placed through 15% to 30% sucrose in PBS overnight at 4°C and then embedded. Each section of 10 μm for *in situ* hybridization, and 12 and 20 μm for immunohistochemistry of mice and rats was cut with cryostat and then attached on a 3'-aminopropyltriethoxysilane (APS)-coated slide.

Probes for *in situ* hybridization was prepared as follows. Two fragments of rat CNTF cDNA clones, positions of 142-551, (409 bp, aa 23-159) and 553-855 (302 bp, aa 160-3'-untranslated region), were amplified by polymerase chain reaction (PCR) using two sets of primers (5'-TAGCAAGGAAGATTCGTTCAGA-3' and 5'-AAGCCCCATAGCTTCTTCTCAAA-3' for probe 1 or (5'-AAGGTCCTTCAAGAGCTCTCACAG -3' and 5'-CACATTTGCATAGCCAGAATAACC-3' for probe 2), subcloned into pBluescript II phargemid vector (Stratagene). Antisense and sense RNA probes were synthesized by the DIG-RNA labeling kit (Boehringer Mannheim) as described by the supplier.

In situ hybridization was performed as follows. Slides were fixed for 10 min in PBS containing 4% PFA, acetylated for 10 min (triethanolamine hydrochloride/0.25% acetic anhydride). Prehybridization was carried out for 2 hr at room temperature in a hybridization buffer consisting of 50% foramide, 5X SSC, 5X Denhardt's solution, 250 μg/ml baker's yeast tRNA, and 500 μg/ml sermon-sperm DNA. For hybridization, 500 ng/ml of DIG-labeled cRNA probe in hybridization solution was denatured for 5 min at 80°C. Hybridization was done for overnight (approximately 16 hr) at 72°C in a

humid chamber. Slides were then dipped in 5X SSC followed by three times of high-stringency wash for 60 min at 72°C in 0.2X SSC. After three times of each 20 min equilibration in TS7.5 (0.1 M Tris-HCl/0.15 M NaCl, pH 7.5) and 60 min blocking with TS7.5 containing 1 % blocking reagent (Boehringer Mannheim), slides were incubated overnight at 4°C with anti-DIG antibody conjugated to alkaline phosphatase (dilution 1:2000) in a humid chamber. Slides were rinsed three times with TS7.5 for 20 min and equilibrated for 20 min in TS9.5 (0.1 M Tris-HCl/0.15 M NaCl/5 mM $MgCl_2$, pH 9.5). For the color reaction, slides were incubated with a substrate solution [200 µl of 50X NBT/BCIP stock solution (Boehringer Mannheim), and 2.4 mg levamisole per 10 ml of TS9.5] for 2 days in a humid chamber protected from light. This reaction was stopped by TE (10 mM Tris-HCl/1 mM EDTA, pH 8.0).

Immunohistochemistry was performed essentially as follows. For DAB detection, slides were fixed for 10 min in 4% PFA in PBS, quenching for 10 min in 0.3% H_2O_2/methanol. Blocking was carried out for 2 hr at room temperature in a blocking buffer consisting of 5% skimmed milk and 0.2% Triton-X 100 in PBS. Slides were incubated with primary antibodies (dilutions of primary antibodies are described below) in the blocking buffer overnight at 4°C. After washed with PBS, the slides were incubated with appropriate secondary antibodies conjugated to biotin-SP for 2 hr at room temperature. After washing with PBS, avidin-biotin complex (ABC) method (Vectastain Elite, Vector Labs) was used for visualization. For fluorescent detection, slides were fixed for 10 min in 4% PFA in PBS. Blocking was carried out for 2 hr at room temperature in blocking buffer consisting of 5% skimmed milk and 0.2% Triton-X 100 in PBS. The slides were incubated with primary antibodies (dilutions of primary antibodies used are described in the next section) in blocking buffer for 2 hr at room temperature. After washed with PBS, the slides were incubated with appropriate secondary antibodies conjugated to Cy3 for 2 hr at room temperature.

Antibodies for immunohistochemistry.

Antibodies, shown the sources and the catalogue numbers (in parenthesis), were used with the following dilutions: anti-CNTF monoclonal, Chemicon (cat. No. MAB338), 1:100; anti-mouse IgG-biotin, Jackson immunoresearch (cat. No. 715-065-151), 1:500; anti-tyrosine hydroxylase, Chemicon (cat. No. AB152), 1:400; anti-rhodopsin (1D4), kindly gifted by Dr. R. S. Molday, 1/50; anti-VIP, chemicon (cat. No.

AB982), 1/1000; anti-PPT, kindly gifted by Dr. T. Kaneko, 1:100; anti-rabbit IgG-Cy3, Jackson immunoresearch (cat. No. 111-165-003), 1:100. Since the monoclonal antibody was not useable for immunoblotting, I prepared the anti-rat and chick CNTF polyclonal antibody for immunoblotting as follows. Full-length cDNA of rat and chick CNTF was subcloned into pET-19b plasmid (Novagen). This plasmid was transfected in *E. coli* BL21(DE3) (Novagen), and chloramphenicol-induced histidine-tagged recombinant rat and chick CNTF (CNTF-Histag) was collected. 1 mg of CNTF-Histags were immunized with Freund's complete adjuvant (priming) and with Freund's imcomplete adjuvant (boosting : 2 times) in a Japanese white rabbit, and rabbit serums of anti-CNTF-Histag were obtained. Immunohistochemistry and immunoblotting for CNTF were performed using the affinity-purified antibodies, prepared using the same method described previously (Ritter, 1991), and neutralizing antibody for Fig. 8B were obtained by Protein-A column purification.

Immunoblotting.

Pineal glands, SCG, total brains and eyes of rats, mice or chick were lysed and briefly sonicated in 0.1 M phosphate buffer contatining 150 mM NaCl, 1% Triton-X 100, 0.5 mM PMSF, 1 μg/ml pepstatin A. Proteins were separated by sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred polyvinylidene-difluoride (PVDF) membranes. Membranes were blocked with 5% skimmed milk for 30 min, incubated with an anti-CNTF polyclonal antibody or anti-ERK1 antibody in the same buffer overnight at 4 °C, treated with a peroxidase-labelled goat anti-rabbit antibody for 2 hr, and developed by chemiluminescence (ECL, Amersham Parmacia Biotech). In Fig. 12A, I used the anti-ERK1 antibody as a control, purchased from Chemicon (cat. No. AB8917), 1:1000; and the anti-rabbit IgG-HRP, Zymed (cat. No. 65-6120), 1:1000.

Cytokines and other factors.

Pineal extract was obtained from a pool of 150 (lot 1: used for Fig. 8 and Fig. 9) or 25 (lot 2: used for Fig. 10) pineal glands of newborn rats by the same method for the extraction of chick CNTF as described previously (Nishi and Berg, 1981). Measurement of the protein content in the extract using a Bio-Rad protein assay kit (cat. No. 500-0006) was performed with BSA as a control. The monoclonal anti-rat

CNTF antibody was purchased from Genzyme/Techne (cat. No. 43557), recombinant rat CNTF (rCNTF) from Peprotech (cat. No. 450-50), NGF from Wako (cat. No. 143-04861), recombinant bFGF from Biomedical Technologies Inc. (cat. No. BT-108) and normal mouse IgG from Santa Cruz (cat. No. sc2025). The concentration of each factor, which was added to the culture medium as shown is as follows: rCNTF, 2 ng/ml; pineal extract (lot 1), 16 μg/ml; anti-CNTF, 5 μg/ml; normal mouse IgG, 5 μg/ml in Figs. 8 and 9: rCNTF, 1 or 100 ng/ml; pineal extract (lot 2), 15 μg/ml; NGF, 100 ng/ml; anti-CNTF, 10 μg/ml; normal mouse IgG, 10 μg/ml in Fig. 10. One-half of the medium was changed every 36 hr to ensure that cytokines maintain their activity.

Primary culture of rat SCG and chick CG neurons.

Primary cultures of rat SCG neurons and chick CG neurons were performed as previously reported (Fann and Patterson, 1993; Nishi and Berg, 1981). Briefly, for the culture of rat SCG neurons, SCG from neonatal Wistar/ST rats were dissected and decapsulated with forceps. Ganglia were first dissociated with 5 mg Dispase I/0.1 % collagenase in HBSS for 50 min at 37°C. Cells were dispased by gentry triturating through a fine-bore glass pipette, washed 2 times with DMEM, and dissociated neurons were seeded in 96-well plates at a density of one ganglion equivalent per well and cultured for six days (Fig. 9), or at a density of one-third ganglion equivalent per well in 24-well plates and cultured for 3 days (Figs. 10A and 10B) or 0.5, 1, 3 and 5 days (Fig. 10C) in DMEM containing 10% fetal bovine serum, 0.006% kanamycin, 0.005% sodium pyruvate, 0.6% glucose, 2 mM L-glutamine and 0.14% $NaHCO_3$. Cytosine arabinonucleoside (Ara-C) was added at 1 μM to eliminate nonneuronal cells. For the culture of chick CG neurons, E8 chick CG were first dissociated with 0.1 % collagenase in HBSS for 30 min at 37°C. Cells were dispased by gentry triturating through a fine-bore glass pipette, washed 2 times with EMEM, and plated in poly-lysine/laminin-coated 24-well plates in EMEM containing 10% horse serum and 0.006% kanamycin at a density of one ganglion equivalent per well, and cultured for 3 days. In Fig. 10A, cultured SCG neurons were treated with the anti-tyrosine hydroxylase (TH) antibody and the signal was visualized using an ABC-kit. The surviving neurons were counted by viewing each well in 15 fields arbitrarily chosen (4.14 mm^2: area of 2.35% per well) for each culture at 100X magnification using phase contrast or light microscopy.

RT-PCR.

Neuronal culture, preparation of RNA and cDNA, RT-PCR and primer sequences followed those previously reported (Fann and Patterson, 1993), to minor modifications, using ExTaq DNA polymerase (TaKaRa) in PCR amplification, and PCR conditions described as follows: β-actin, annealing temperature (AT)=58°C, 30 cycles; Substance-P (SP), AT=55°C, 35 cycles; vasoactive intestinal peptide (VIP), AT=58°C, 35 cycles; Choline acetyltransferase (ChAT), AT=55°C, 35 cycles; Tyrosine hydroxylase (TH), AT=58°C, 30 cycles. Dissociated sympathetic neurons were seeded in 96-well plates in medium containing NGF (100 ng/ml). Ara-C (1 μM) was added to eliminate nonneuronal cells. Recombinant rat CNTF, normal mouse IgG, pineal extract and anti-CNTF neutralizing antibody were added 36 hr after starting culture in duplicate wells used for each condition. Cultures were maintained for 6 days. Data were reproduced in two separate experiments. PCR products were analyzed on 3% agarose gels, being visualized with ethidium bromide staining and UV illumination.

Genotyping and analysis of CNTF-deficient mice.

I conducted cntf (-/-) transgene incorporation (Masu *et al.*, 1993) into the CBA/N strains by at least seven generations of backcross for the experiments in Figs. 11, 12 and 13C or into the C57BL/6 by five generations of backcross for the experiment in Table 1. Heterozygous offspring were crossed to obtain homozygous individuals. For genotyping of mice, template DNA extracted from the tail or ear was analyzed using PCR. I used a common primer of 5'-AAACAAGCCCAGAACTGTGG-3' (Fig. 11A, R±), and two other primers of 5'-CTCTGTAGCCGCTCTATCTG-3' (Fig. 11A, F-) and 5'-GCGGACCGCTATCAGGACAT-3' (Fig. 11A, F+) for the mutant CNTF allele and wild-type CNTF allele, respectively, which amplify 573 bp, 573 bp plus 720 bp, and 720 bp in wild-type, hetero-, and null-CNTF mice, respectively (Fig. 11A, right panel). PCR conditions using ExTaq DNA polymerase were: 95 °C for 10 min and 30 cycles each of 94 °C for 30 sec, 60 °C for 15 sec, and 72 °C for 30 sec, followed by the final reaction of 72 °C for 10 min.

To quantitate the density of sympathetic innervation, I adopted a method previously described for immunostaining with the secondary Cy3-conjugated anti-rabbit IgG following the treatment with the primary anti-TH antibody (Kohn *et al.*, 1999). One-

third sections of entire pineal glands of each genotype were analyzed, after immunostaining for TH, to obtain a quantitative measure of innervation density. Briefly, images were captured and analyzed using the IPLab Spectrum Ver. 3.0 (Signal Analytics Corporation). Then the average of signal intensity of TH positive signals per area in adult +/+ mice was taken "100" as the standard (Fig. 12C, white bar) and the value of each section compared to the standard was determined. The relative intensity of the signal per area stained for TH signal was measured for each section. Comparisons were only made between the slides that were processed at the same time. The mean ± s.e. were calculated and are shown in the Fig. 12C.

Retrograde tracing studies.

For retrograde tracing, P2.5 rat pineal glands were injected with the lipophilic dye DiO (1,1-dioctadecyloxacarbocyanine perchlorate; Molecular Probes, cat. D275) under hypothermic anesthesia. To avoid possible artifact due to CNTF release following degeneration of pineal cells by the retrograde tracer suspended in organic solvents, a DiO crystal without suspension was directly applied to the tip of a fine tungsten needle, put into pineal glands under a dissection microscope, enclosed with the head skin using an adhesive material, and kept in the rats for 2 days. The rats were then perfused with 4% paraformaldehyde to collect SCGs, which were cut into sections (30 μm thick) and immunostained with appropriate secondary antibodies conjugated to Cy3, followed with the anti-preprotachykinin-A antibody (PPT: SP precursor protein) (Lee *et al.*, 1997), or anti-VIP antibody. Tween-20 was used instead of Triton X-100 for permeation (Lukas *et al.*, 1998).

Newborn-rat pineal gland organ culture.

Newborn rat pineal glands for culture were obtained from neonatal Wistar/ST rats within 24 hr after birth. The method for culture was almost the same as reported in the previous paper (Araki, 1992). Briefly, isolated pineal glands were treated with 0.1 % collagenase solution (C-9407; Sigma) at 37 °C for 30 min and placed in a culture dish (Corning, 35 mm in diameter) which had been previously coated with collagen (type I; Nitta Gelatin Co.). The medium was DMEM supplemented with 5% fetal bovine serum, 0.006% kanamycin, 0.005% sodium pyruvate, 0.6% glucose, 2 mM L-glutamine and 0.14% $NaHCO_3$. Cultures were maintained for 10 days. For

immunochemical analysis of rhodopsin immunoreactivity, cultures were fixed for 10 min in 4% PFA in PBS, quenching for 10 min in 0.3% H_2O_2/methanol. Blocking was carried out for 2 hr at room temperature in blocking buffer consisting of 5% skimmed milk and 0.2% Triton-X 100 in PBS, and subsequently incubated with rho-1D4 (1:50 in dilution) in blocking buffer overnight at 4°C. After washed with PBST, the samples were incubated with anti-mouse IgG conjugated to biotin-SP for 2 hr at room temperature. After washing with PBST, avidin-biotin complex (ABC) method (Vectastain Elite, Vector Labs) was used for visualization.

Analysis of free-running periods and amounts of phase shift in responding to a light in CNTF knockout (-/-) and wild-type mice (+/+).

Free-running periods and amounts of phase shift in response to a 30 min light pulse at Circadian Time 16 were determined in CNTF knockout (-/-) mice and their wild littermates (+/+) described previously (Pittendrigh and Daan, 1976). Animals were housed singly in a cage equipped with a running wheel kept in constant darkness. The results are represented as mean ± s.e.

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