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TGF-ß1 and IL-6 expression in rat pineal gland is regulated by norepinephrine and interleukin-1ß

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Summary. The pineal gland is part of the neuroendocrine system that modulates immune functions. Because the gland is outside the blood-brain barrier, it is accessible to direct feedback from circulating cytokines that affect the synthesis and secretion of melatonin. Recent studies have suggested that intrinsic immunoregulatory cytokines mediate these neuro-immune interactions under the control of sympathetic innervation to the pineal. This study focused on the expression of transforming growth factor-ß1 (TGF-ß1) and interleukin-6 (IL-6), two cytokines that have important regulatory functions on both neurons and immune cells. Northern blot RNA analysis showed that TGF-ß1, but not IL-6, was expressed in freshly dissected rat pineal glands from neonatal age (1-day-old) into adults. Immunocytochemistry for TGF-ß1 in adult glands revealed localization of this protein in astrocyte-like cells. The sympathetic neurotransmitter norepinephrine (NE) increased transcript levels for both TGF-ß1 and IL-6 in adult pineal organ cultures. The effect of NE on IL-6 expression was not found in dispersed cell cultures established from neonatal pineal glands. The immunoregulatory molecule interleukin-1ß (IL-1ß) up-regulated the expression of both IL-6 and TGF-ß1 in adult pineal organ cultures, but not in neonate pineal organ cultures. These findings suggest that TGF-ß1 and IL-6 have intrinsic regulatory roles in the pineal gland and that both neural and immune factors are important mechanisms of regulation.

Key words: Cytokine, IL-1ß, IL-6, Norepinephrine, Pineal gland, TGF-ß1

Introduction

There is now a convincing body of evidence supporting the concept that immunoregulatory cytokines affect neuronal functions, and *vice versa*. The pineal gland is one of several circumventricular organs in the brain that plays an important role in these neuro-immune interactions. On the one hand, the principal pineal gland hormone melatonin influences a variety of immune functions (Guerrero and Reiter, 1992). On the other hand, because the pineal gland is outside the blood-brain barrier, it is accessible to circulating cytokines that modulate the synthesis of pineal indoles. For example, IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF) as well as granulocyte-colony stimulating factor (G-CSF) increased, and IL-1ß decreased melatonin production in vitro as well as in vivo following peripheral injection (Withyachumnarnkul et al., 1990, 1991; Mucha et al., 1994; Zylinska et al., 1995). These feedback interactions have led to a working hypothesis that the pineal gland is an important "gateway" for neuroimmune communications (Guerrero and Reiter, 1992; Tsai and McNulty, 1997).

Currently, little information is available on the expression and regulation of cytokines produced endogenously in the pineal gland. We reported previously that IL-1ß was expressed in the adult pineal gland and was up regulated by the neurotransmitter norepinephrine (NE) as well as the cytokine IFN-γ plus lipopolysaccharide (LPS) (Tsai and McNulty, 1999). This present study examined two additional cytokines, TGF-ß1 and IL-6, which are expressed by glia in other parts of the brain (Constam et al., 1992; Sawada et al., 1992). Previous studies also demonstrated that both TGF-ß1 and IL-6 affect the differentiation and survival of neuronal cells (Krieglstein and Unsicker, 1994; Maeda et al., 1994; Mehler and Kessler, 1997).

We specifically addressed the hypothesis that cytokine expression in the pineal gland is regulated by both neural and immunological mechanisms. Neural regulation is particularly important because the principal innervation to the pineal gland is from postganglionic sympathetic neurons from the superior cervical ganglia. The circadian metabolic functions of the pineal gland are mediated by adrenergic mechanisms following the release of NE from the sympathetic nerve terminals (Vollrath, 1981). The density of sympathetic fibers innervating the pineal gland gradually increases from

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postnatal day 1 to postnatal day 20 (Machado, 1971) providing an opportunity to investigate the relationship of endogenous levels of NE with cytokine expression *in vivo*. Adrenergic regulation of cytokine expression would be consistent with homeostatic functions of these immunoregulatory cytokines in this part of the brain. Toward this aim, we examined cytokine expression using Northern blot RNA analysis during the postnatal development of sympathetic innervation to the gland both *in vivo* and *in vitro*.

A second aim of the study was to investigate the effects of IL-1ß on TGF-ß1 and IL-6 expression in the pineal gland. The effect of IL-1ß was studied because it is a strong inducer of glial TGF-ß1 and IL-6 (da Cunha and Vitkovic, 1992; Lee et al., 1993; da Cunha et al., 1997). It is well documented that IL-1ß is found in high concentrations in the circulation during systemic infection and IL-1ß is known to affect other neural centers, such as hypothalamus-pituitary-adrenal axis and hippocampus (Cunningham and De Souza, 1993). Furthermore, IL-1ß protein is constitutively detected in glia of the adult pineal gland and the levels of expression are regulated by neural and immunological mechanisms (Tsai and McNulty, 1999).

Materials and methods

Animals

Sprague-Dawley rats (Zivic-Miller, IN) of both sexes were used in this study. The animals were housed in fully accredited animal care facilities under controlled lighting conditions (12:12 light:dark). Food and water were *ad libitum*. Principles of laboratory animal care and specific national laws were followed. All animals were killed by decapitation following anesthesia by hypothermia (neonates) or CO_2 inhalation (adults).

Tissue preparation

Freshly isolated glands

The pineal glands were removed from rats of different ages (1, 5, 15, 21 days, and 3-4 months) 4 hours after lights on. The glands were kept in cold Dulbecco's modified Eagle's medium (DMEM) containing penicillin (PCN, 200 U/ml) and streptomycin (SM, 200 mg/ml), carefully cleaned of meninges, frozen immediately on dry ice and stored at -70 °C for further RNA isolation.

Whole pineal gland organ cultures

Whole pineal gland organ cultures were prepared as reported (Tsai and McNulty, 1999). Pineal glands collected from different ages (1, 5, 10, 21 days, and 3-4 months) were immersion cultured in serum-free DMEM containing antibiotics (PCN, 100 U/ml and SM, 100 μg/ml) in 5% CO_2, humidified air at 37 °C. Tissues were placed in medium for 3 h followed by replacing with fresh medium before further treatment to reduce the effects from residual NE in the glands.

Pineal organ cultures were stimulated with NE (10^{-5} M dissolved in 10^{-5} M ascorbic acid) after medium change. Control cultures were treated with vehicle (10^{-5} M ascorbic acid). In a separate experiment, pineal glands collected from one-day-old and 3-4 month old animals were cultured as described above and treated with recombinant human IL-1ß (Genzyme, 100U/ml) after medium change. Glands were collected and further processed for RNA analysis.

Dispersed pineal cell cultures

Dispersed pineal cell cultures were prepared as reported (Tsai and McNulty, 1999). One-day old pineal glands were dissociated and plated on 35 mm Petri dishes at a density of $5x10^5$ pineal cells per dish. Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml PCN and 100mg/ml SM in 5% CO_2 humidified air at 37 °C for 7 days. The culture medium was changed after the 4th day and immediately before treatment. Cultures were stimulated with NE (10^{-5} M dissolved in 10^{-5} M ascorbic acid) for 4 h in freshly changed medium after 7 days of culturing. Control cultures were treated with vehicle (10^{-5} M ascorbic acid).

RNA blot analysis

Total cellular RNA was prepared and analyzed as reported (Tsai and McNulty, 1999). Pineal glands and cell cultures were washed in phosphate buffer and lyzed in GITC. Total cellular RNA was extracted by RNeasy Midi Kits (Qiagen Inc. Valencia, CA) or pelleted by ultra-centrifugation through CsCl solution as described (Schluns et al., 1995). Total cellular RNA (8μg) was electrophoresed in 1.2% formaldehyde-denaturing agarose gels in MOPS buffer (40 mM, pH 7.0). The gels were stained with acridine orange (33 mg/ml H_2O), destained in ddH_2O, and exposed under UV light to visualize 18S and 28S ribosomal RNA. RNA was transferred overnight onto nylon membranes (Gene Screen, Du Pont) followed by UV cross-linking. Prehybridization was carried out at 42 °C overnight. Hybridization was performed at 42 °C overnight with $1x10^6$ cpm/ml of ^{32}P-dCTP labeled specific cytokine cDNA probes at 42 °C. The prehybridization and hybridization buffer was composed of 50% (v/v) de-ionized formamide, 5X Denhardt's solution, 1% SDS in 5X SSPE containing denatured salmon sperm DNA (10μg/ml). The following cytokine cDNA were labeled using random primer methods (Klenow fill-in kit, Stratagene, La Jolla, CA): TGF-$ß_1$ (mouse, 0.97 Kb, SmaI fragment) courtesy of Dr. H. Moses, and IL-6 (rat, 0.74 Kb, Pst fragment) courtesy of Dr. J. Gauldie. The membranes were washed (2X SSC containing 2mM EDTA, and 0.1% SDS) at room temperature followed by

stringency washes (0.1X SSC containing 2mM EDTA and 0.1% SDS) at 60 °C. Membranes were exposed to X-ray films at -70 °C overnight, developed and checked for signal strength. Blots were exposed for an additional 6 days.

The optical densities of blots were analyzed using NIH Image software. Each blot was standardized to the ribosomal RNA.

Immunocytochemistry and confocal microscopy

Immunocytochemical methods were as reported previously (Tsai and McNulty, 1999). Pineal sections were blocked with PBS (10% normal goat serum and 0.5% Triton-X) for 1 h at room temperature followed by primary antibody incubation (1:100 in PBS with 10% NGS and 0.5% Triton-X, monoclonal mouse anti-TGF-ß1 (Celtrix Pharmaceuticals Inc., Santa Clara, CA) overnight at 4 °C. Tissues were rinsed with PBS and further incubated in FITC-conjugated goat anti-mouse antibody (1:200 in PBS with 10% NGS and 0.5% Triton-X, DAKO, DK) at room temperature for 1 h. Biotinylated tomato lectin (*Lycopersicon esculentum*, Sigma) was applied (18μg/ml, in TBS with 0.5% Triton-X overnight at 4 °C after several rinses of TBS. Tissues were further incubated with PE-conjugated streptavidin (1:70 in TBS with 0.5% Triton-X, BD PharMingen, San Diego, CA) after TBS rinse. Tissues were rinsed in TBS followed by distilled H_2O and mounted.

Sections double labeled with FITC (anti-TGF-ß1) and PE (tomato lectin) were viewed with a Zeiss LSM 510 laser scanning microscope. Excitation of the FITC and PE fluorescence was accomplished with Ar-Kr lasers (488 nm and 565 nm respectively) using emission filters of 520 nm and 578 nm respectively.

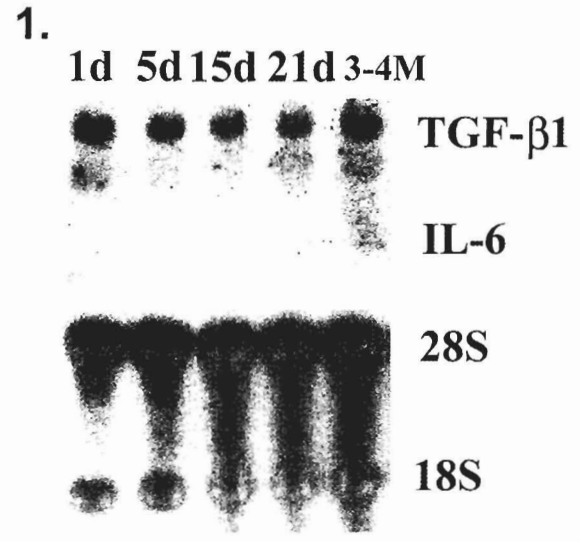

Fig. 1. Northern RNA analysis of total cellular RNA hybridized to radio-labeled cDNA probes for TGF-ß1 and IL-6. Pineal glands were freshly isolated from 1d, 5d, 15d, 21d, and 3-4 months (3-4M) animals.

Results

Expression of cytokine mRNA in freshly isolated pineal glands during development (Fig. 1, Tables 1, 2)

The first experiment provided baseline data on cytokine expression during the developmental period spanning the sympathetic innervation of the gland (1-20 days of age). Our results showed that expression of TGF-ß1 transcripts in pineal glands freshly isolated from 1-day to 3-4 month old animals were relatively constant. IL-6 transcripts were not detected in pineal glands of rats at any age.

Immunocytochemical studies revealed cells labeled positively for TGF-ß1 in the pineal gland of adults, but not 1 day-old pups. TGF-ß1-positive cells frequently adjoined capillaries and exhibited fusiform cell bodies with numerous processes surrounding capillaries (Fig. 2). Double labeling experiments showed that TGF-ß1 positive cells did not co-localize tomato lectin, which is a marker for microglia and endothelial cells (Castellano et al., 1991). Immunocytochemical studies for IL-6 were not conducted because no mRNA for this protein was detected.

Adrenergic regulation of cytokine mRNA expression in pineal explant cultures (Figs. 3, 4, Tables 1, 2).

TGF-ß1 mRNA, but not IL-6, was constitutively expressed in control glands consistent with the previous experiment, although the level of transcript was reduced after 7 hours in culture (Table 1). Adult pineal gland cultures treated with NE (10^{-5} M) showed increased

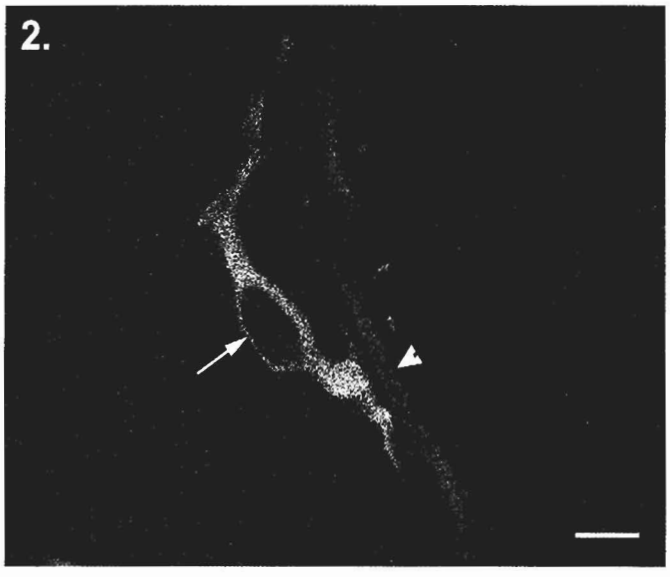

Fig. 2. Confocal laser scanning micrograph showing double labeling of a TGF-ß1 positive astrocyte-like cell (arrow) and tomato lectin positive endothelial cells (arrowhead) of a capillary in an adult pineal gland. Bar: 10 μm.

expression of TGF-ß1 and IL-6 mRNA levels between 2 and 4 hours of stimulation as compared to vehicle treated controls (Fig. 3, Tables 1, 2).

We next sought to determine if there was an effect of age on adrenergic stimulation of cytokine expression. As observed in the first experiment (Fig. 1), TGF-ß1 was expressed constitutively in pineal glands isolated from different ages. In contrast to freshly isolated glands, IL-6 transcripts were detected in cultured glands from 10 and 21-day old animals (Fig. 4). As in the first experiment, NE elevated expression of both TGF-ß1 and IL-6 in adults, but had no effects in animals 21 days and younger. NE showed no effect on TGF-ß1 and IL-6 mRNA levels of animals 21 days and younger (Fig. 4).

Regulation of cytokine expression in dispersed cell cultures by NE (Fig. 5).

This study tested the effects of NE on TGF-ß1 and IL-6 transcripts expression in dispersed pineal cell cultures because of important difference in glial proliferation in dispersed cell vs. organ cultures. The

Table 1. Ratio of TGF-ß1 bands to ribosomal RNA in each of the figures. Numbers of animals are given in parentheses.

	1 day			5-7 day		15 day		21 day		Adult			
	C	NE +4h	IL1 +4h	C	NE +4h	C	NE +4h	C	NE +4h	C	NE +2h	NE +4h	IL1 +4h
Fig. 1	2.2 (30)			1.7 (20)		1.2 (15)		1.0 (10)		1.1 (4)			
Fig. 3										0.4 (4)	1.8 (4)	1.0 (4)	
Fig. 4	1.9 (30)	2.4 (30)		1.2 (20)	1.6 (20)	1.3 (15)	1.1 (15)	0.8 (10)	0.7 (10)	0.5 (4)		1.5 (4)	
Fig. 5	1.4 (10)	2.0 (10)											
Fig. 6	1.7 (30)		1.7 (30)							0.8 (4)			2.2 (4)

Table 2. Ratio of IL-6 bands to ribosomal RNA in each of the figures. Numbers of animals are given in parentheses.

	1 day			5-7 day		15 day		21 day		Adult			
	C	NE +4h	IL1 +4h	C	NE +4h	C	NE +4h	C	NE +4h	C	NE +2h	NE +4h	IL1 +4h
Fig. 1	0 (30)			0 (20)		0 (15)		0 (10)		0 (4)			
Fig. 3										0 (4)	3.4 (4)	2.0 (4)	
Fig. 4	0 (30)	0 (30)		0 (20)	0 (20)	0.1 (15)	0.2 (15)	0.4 (10)	0.5 (10)	0 (4)	2.6 (4)		
Fig. 5	0 (10)	0 (10)											
Fig. 6	0 (30)		0 (30)							0.2 (4)		3.2 (4)	

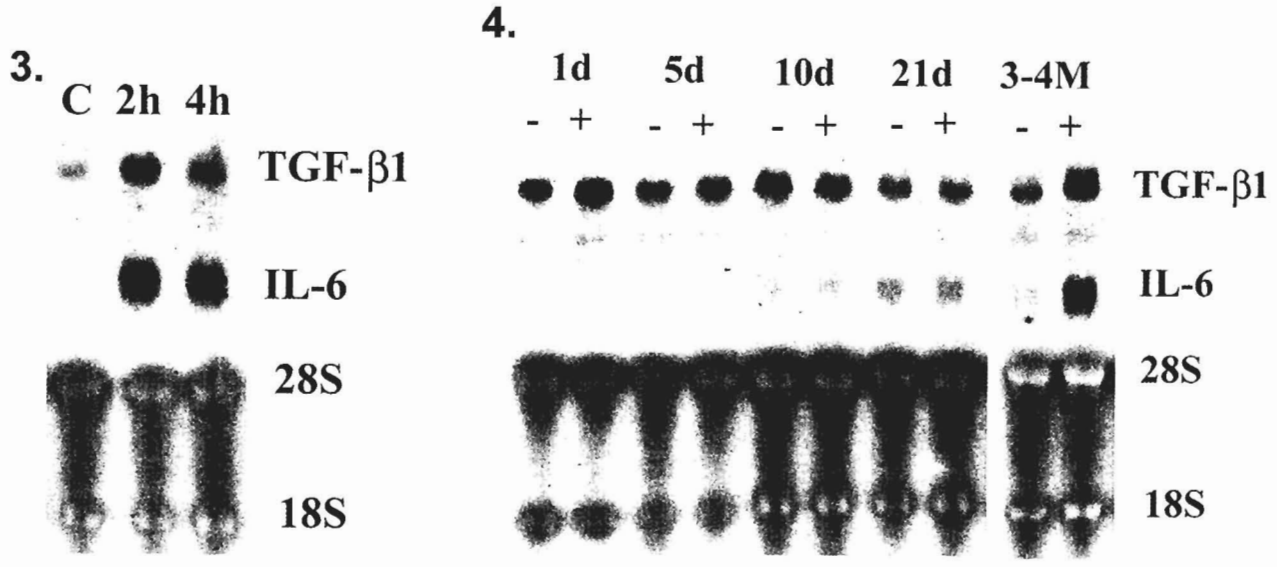

Fig. 3. Northern RNA analysis of cultured whole adult pineal gland explants for TGF-ß1 and IL-6 mRNA expression following treatment with norepinephrine (NE). Control glands (C) were treated with vehicle.

Fig. 4. Northern RNA analysis of total cellular RNA isolated from cultured whole pineal explants collected from rats age 1d to 3-4 months. Pineal glands were placed in organ cultures and stimulated with vehicle (-: 10^{-5} M ascorbic acid) and NE (+: 10^{-5} M) for 4 h.

constitutive expression of TGF-ß1 transcripts in 7-day pineal cell cultures was slightly elevated by treatment with NE (10^{-5} M, 4 h). IL-6 mRNA was not detectable in any of the dispersed cell cultures tested.

IL-1ß regulation of cytokine expression in pineal organ cultures (Fig. 6).

The pro-inflammatory cytokine IL-1ß (100 U/ml, 4 h) enhanced TGF-ß1 and IL-6 mRNA expression in adult pineal gland explants, but had no effect on 1-day-old neonate glands. The levels of TGF-ß1 transcripts tended to be greater in 1-day old glands, a trend that was also observed in the previous experiments.

Discussion

Three general conclusions can be drawn from the present study. First, pineal TGF-ß1 was constitutively expressed at all ages of postnatal development of the gland. Second, TGF-ß1 and IL-6 transcript levels were up-regulated by adrenergic mechanisms. Finally, IL-1ß enhanced the expression of TGF-ß1 and IL-6 in the pineal gland. The latter observation suggests that multiple cytokines are involved in neuro-immune interactions in this part of the brain.

IL-1ß is one of the more abundant pro-inflammatory cytokines released into the circulation during immunological insults and it is an immuno-mediator of neural functions. Several observations suggest that IL-1ß also modulates neuroendocrine functions of the pineal gland. First, the constitutive expression of IL-1ß transcript in the pineal gland was shown to fluctuate over the light:dark cycle (Tsai and McNulty, 1999). Second, IL-1ß mRNA was greatly enhanced in the pineal gland after induction of systemic inflammatory responses by LPS treatment (Wong et al., 1997). Finally, the nocturnal rise in serum melatonin was reduced in rats given peripheral injections of IL-1ß (Mucha et al., 1994). In this context, the observation that IL-1ß up-regulates expression of TGF-ß1 and IL-6 is noteworthy because it suggests that the effects of IL-1ß on pineal gland neuroendocrine functions may, in part, be mediated by these other cytokines.

The pineal gland is extensively innervated by sympathetic fibers from the superior cervical ganglia which synchronize the rhythmic functions of the gland to the light:dark cycle via adrenergic receptor mechanisms. Our finding that the neurotransmitter NE regulates expression of both TGF-ß1 and IL-6 is important because it suggests a homeostatic role for these cytokines in the cyclical activities of the gland, as previously demonstrated for IL-1ß (Tsai and McNulty, 1999). The concentration of NE used for the present studies was selected to maximally stimulate the gland as shown previously (Heiter et al., 1991; Maimone et al., 1993). This concentration is an order of magnitude less than the stimulated concentration at the intercellular junction (Bergquist et al., 1998). Additional studies are needed to determine whether mRNA and protein levels of the cytokines in question fluctuate over the light:dark cycle and if so, whether these rhythms can be dampened following denervation of the gland by superior cervical ganglionectomy in the adult.

Both the degree of sympathetic innervation and the adrenergic receptor subtypes exhibit age-related differences in the pineal gland (Auerbach, 1981; Pfeffer et al., 1998). The lack of effect of NE on cytokine expression in neonatal glands is probably not related to development of the innervation to the gland because we

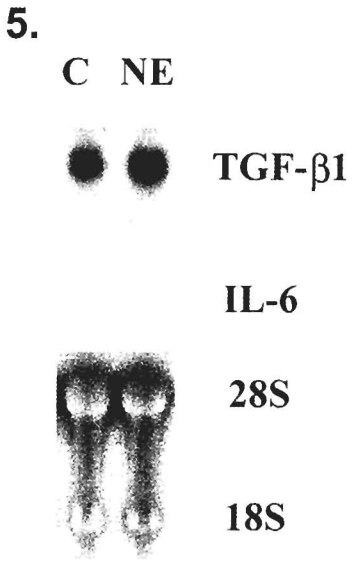

Fig. 5. Northern RNA analysis showed expression of TGF-ß1 mRNA in 7-day dispersed pineal cell cultures from 1 day neonatal rat pineal glands following treatment with NE (10^{-5} M, 4h).

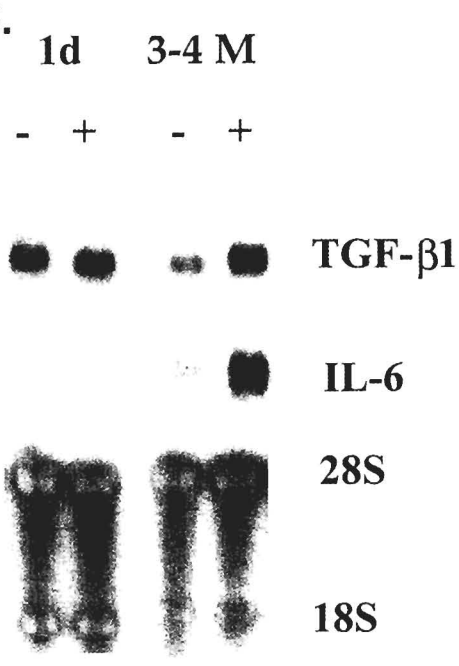

Fig. 6. Northern RNA analysis of TGF-ß1 and IL-6 expression in cultured 1-day old and adult pineal glands following treatment with vehicle (-) and IL-1ß (+; 100 U/ml, 4h).

were unable to detect NE effects in animals as old as 21 days when the sympathetic innervation is complete.

Alternatively, maturational changes in the cell type(s) (glia?) expressing the cytokines may be responsible for the NE effects observed in older animals. For instance, it has been shown that astrocytes exhibit increased levels of GFAP expression with pineal gland development (Lopez-Munoz et al., 1992; Borregon et al., 1993). Neonatal glands and dispersed cell cultures contain astrocytes that are S-100 protein positive, vimentin-positive, and GFAP-negative in addition to very few GFAP-positive astrocytes (Borregon et al., 1993; McNulty et al., 1995). It was suggested that GFAP-negative, vimentin-positive astrocytes in the pineal gland are immature astrocytes (Schachner et al., 1984). Moreover, cultured astrocytes collected from animals of different ages exhibited different responses to IL-1ß treatment (Colasanti et al., 1991), which is consistent with the developmental differences in this study (see Fig. 6).

The immunocytochemical studies indicate that glia are the principal cells involved in the production of the cytokines in question. Both astrocytes and microglia are common in the pineal gland (Vollrath, 1981; Pedersen et al., 1993) and several studies have reported that IL-6 expression was increased in cultured brain astrocytes treated with NE and IL-1ß (Maimone et al., 1993; Norris and Benveniste, 1993). However, cultured microglia are relatively unresponsive to NE treatment in IL-6 mRNA expression (Maimone et al., 1993; Norris and Benveniste, 1993). The expression of IL-6 in pineal explants, but not in freshly isolated glands of the same age, is likely due to central necrosis and subsequent activation of glia in the explants. This response is comparable to that reported following ischemia of the brain and subsequent activation of both astroctyes and microglia (Kim, 1996; Saito et al., 1996; Sharma and Kumar, 1998).

In summary, our results provide the first evidence that important immunoregulatory molecules are expressed in the pineal gland and are regulated by adrenergic neural mechanisms that are known to synchronize the pineal neuroendocrine functions to the 24-hour light:dark cycle. This observation suggests that both TGF-ß1 and IL-6 may play important roles in the homeostatic, circadian properties of the pineal gland. Our findings further suggest that both of these cytokines may have intrinsic roles in the pineal neuroendocrine responses to other circulating cytokines such as IL-1ß.

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