# Endothelin $ET_A$ and $ET_B$ receptor expression in the human trigeminal ganglion

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## Abstract

**OBJECTIVES**: Endothelin is a potent peptide mediator that is synthesized by a number of cells. Previous studies have revealed the occurrence of endothelin in nerve cell bodies of some peripheral ganglia. Endothelin mediates its effects via two distinct receptor subtypes $ET_A$ and $ET_B$. The present study was designed to investigate the presence of these two receptors in the human trigeminal ganglion.
**METHODS**: Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to show the presence of mRNA encoding $ET_A$ and $ET_B$ receptors in the human trigeminal ganglion. To localize the protein immunocytochemistry with antibodies against the endothelin receptors was used.
**RESULTS**: Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed mRNA for both receptor subtypes in the human trigeminal ganglion. Immunocytochemistry revealed numerous cell bodies containing the $ET_A$ and the $ET_B$ receptor proteins.
**CONCLUSIONS**: The expression of $ET_A$ and $ET_B$ receptors in the human trigeminal ganglion suggests a role for endothelin in autonomic and sensory neural transmission.

## Introduction

The endothelins (ET-1, ET-2, ET-3) form a group of regulatory peptides that contains 21 amino acid residues [18,21,22]. The endothelin isopeptides transmit their effects via two distinct receptors, termed ETA and ETB [2,17]. Besides their profound effects in the vascular and respiratory systems, where both ETA and ETB receptors may contribute to the maintenance of tone, it has been demonstrated that ET-1, in addition, takes part in a variety of biological activities within the peripheral and the central nervous systems [23].

Immunocytochemical studies have shown that endothelin is synthesized and secreted from postganglionic sympathetic neurons in the dog, in neurons of the human spinal cord and in the dorsal root ganglia [3,5,6,9]. ET-1 may facilitate parasympathetic



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neurotransmission via the release of acetylcholine and there is evidence for effects of ET-1 on both cholinergic and adrenergic neurons [20,24]. Intracerebroventricular injection of ET-1 in rats induces profound peripheral pressor and vasoconstrictor responses suggestive of receptor occurrence in nerve cells [12,16,18]. These data suggest that the endothelin family of peptides play an important role in both sympathetic and parasympathetic neurotransmission.

In the present study, we have examined the molecular presence of mRNA for ETA and ETB receptors in the human trigeminal ganglion by RT-PCR and immunocytochemistry.

## Material and methods

*Materials*

Trigeminal ganglia were obtained at autopsy from adult subjects with an average age of 74.8 years (range 51–85 years). The ganglia were collected within 24 hours of death from 7 subjects (5-male; 2-female). Cause of death was related to cardiac disease and none of the subjects were suffering from central nervous system diseases. The collection of tissue samples was done in accordance with the Albert Szent-Györgyi University Medical School guidelines for ethics in human tissue experiments.

Isolation of total RNA. Total cellular RNA was extracted from 6 trigeminal ganglia using the TRIzol reagent (GIBCO BRL, Life Technologies, Sweden) following the suppliers protocol. Using a polytron homogenizer (Polytron Kinematica AG, Model PT 1200, Labora, Sweden) frozen tissue was homogenized in 1 ml of TRIzol Reagent at room temperature until completely disrupted. The homogenates were mixed with chloroform and centrifuged at 12000 × g for 15 min at 4° C. The aqueous phase, containing RNA, was transferred to a fresh tube and the RNA was precipitated by the addition of isopropanol. Samples were incubated at room temperature for 10 min and centrifuged at 12000 × g for 10 min at 4° C.

The RNA pellet was finally washed with 70% ice-cold ethanol, air-dried, dissolved in 20 µL of diethylpyrocarbonate-treated water and stored at –20° C until use. The purity and yield of total RNA was determined spectrophotometrically at 260 nm and 280 nm, using a DU-65 spectrophotometer (Beckman Instruments, Stockholm, Sweden). The ratio of absorption (260:280) of all preparations was between 1.6 and 1.8. Finally, the integrity of the samples was assessed by gel electrophoresis and examination of 18 and 28 S ribosomal RNAs.

*Reverse transcriptase-polymerase chain reaction (RT-PCR)*

Synthesis of first strand cDNA and subsequent PCR amplification was carried out using the GeneAmp RNA PCR kit reagents (Perkin-Elmer AB, Sweden) in a DNA thermal cycler (Perkin-Elmer AB, Sweden).

DNase-treated RNA samples were reverse transcribed to cDNA in a 20 µL reaction volume in the presence of PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 5 mM $MgCl_2$, 1mM of each nucleotide (dNTP), 50 pmol of oligo (dT) primers, 50 units of M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase. The samples were incubated at room temperature for 10 min, at 42° C for 15 min, heated to 99° C for 5 min and chilled to 5° C for 5 min.

The PCR reaction was performed using specific oligonucleotide primers according to published sequences of the human $ET_A$- and $ET_B$- receptors, (Scandinavian Gene Synthesis, Köping, Sweden):

$ET_A$-receptor forward:
5′-TGG CCT TTT GAT CAC AAT GAC TTT -3′
$ET_A$-receptor reverse:
5′- TTT GAT GTG GCA TTG AGC ATA CAG GTT-3′
$ET_B$-receptor, forward:
5′-ACT GGC CAT TTG GAG CTG AGA TGT-3′
$ET_B$-receptor reverse:
5′- CTG CAT GCC ACT TTT CTT TCT CAA-3′

The primers were designed to span one or more introns in order to exclude signals from genomic DNA contaminations [2,7].

The PCR amplification reaction was composed of 5 µL of the first strand cDNA reaction mixture and 45 µL master mix containing PCR buffer, 1 mM $MgCl_2$, 25pM of each sense and antisense specific primers, and 2.25 units of AmpliTaq Gold DNA Polymerase (Perkin-Elmer AB, Sweden). The samples were overlaid with mineral oil and then subjected to PCR amplification using the following profile. Initial denaturation step at 94° C for 2 min, followed by 35 cycles of denaturation for 1 min at 94° C and annealing for 1 min at 60° C. After the final cycle, the temperature was maintained at 72° C for 7 min to allow completion of synthesis of amplified products. To ensure that the amplification products came exclusively from the RNA, a reverse transcriptase negative reaction was run in all experiments where the enzyme was replaced by RNA-se free water. The optimal number of cycles for the RT-PCR assay was determined previously (Moller et al., 1997). For the qualitative purpose of our study 35 cycles appeared to be most suitable. The PCR products were separated on a 1.5% agarose gel in 0.5x TBE, stained with ethidiumbromide and photographed using an UV transilluminator.

*DNA-sequencing*

The identity of the PCR-products was verified by sequencing using the Amplitaq FS cycle sequencing kit (Perkin-Elmer AB) on a DNA thermocycler (Perkin-Elmer AB). Approximately 80 ng of gel-purified PCR-product was directly sequenced with both forward and reverse primers according to the protocol of the manufacturer. The samples were purified after the reaction and run on an ABI Prism 310 DNA sequencer. The resulting sequences were finally compared to the published sequences of $ET_A$ and $ET_B$ receptors using the BLAST algorithm [1].

*Immunocytochemistry*

For immunocytochemistry trigeminal ganglia (n=3) were immersed overnight in a fixative consisting of 2% paraformaldehyde and 0.2% picric acid in 0.1 mol/l phosphate buffer, pH 7.2. After fixation the specimens were rinsed in sucrose-enriched (10%) Tyrode solution. Sections were cut at 10 μm thickness in a cryostat and mounted on chrome-alum coated slides.

For the immunocytochemical demonstration of $ET_A$ and $ET_B$ indirect immunofluorescence was used. Briefly, the cryostat sections were rinsed for 15 min in PBS with pH 7.2. They were then incubated with 5% swine serum in PBS for 30 min, followed by incubation with $ET_A$ and $ET_B$ (1:400, antihuman $ET_A$ and $ET_B$ receptor raised in rabbit, IBL Co, LTD, Japan). The site of the antigen-antibody complex was revealed by application of a secondary antibody (1:80, swine anti-rabbit IgG, DAKO, Copenhagen, Denmark) conjugated to fluorescein isothiocyanate (FITC). In the control experiments the primary antibody was omitted.

## Results

Agarose gel electrophoresis of the RT-PCR products from the human trigeminal ganglion demonstrated products of the expected sizes, corresponding to mRNA encoding both the human $ET_A$ receptor (302 bp) and the human $ET_B$ receptor (428 bp) in all specimens (Fig. 1). No signal was observed when the enzyme was omitted in the RT-reaction. Sequence analysis of the amplified products showed no dissimilarities with the published sequences of $ET_A$- and $ET_B$-receptor

$ET_A$ and $ET_B$ immunoreactivity occurred in numerous cell bodies distributed homogenously throughout the trigeminal ganglion. The immunoreactivity was seen in medium sized to large cell bodies (Fig. 2). Autofluorescent lipofuchsins, emitting orange-yellow fluorescence, were present within perikarya as a characteristic of adult human nervous tissue.

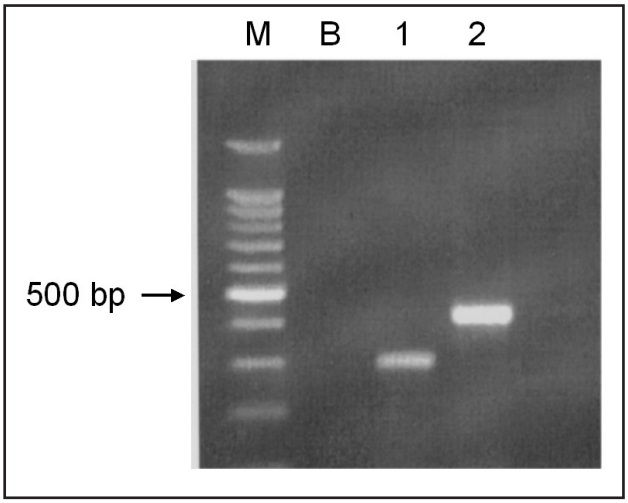


**Fig. 1.** Representative gel electrophoresis of RT- PCR products. Single bands of the expected sizes (302 bp for $ET_A$; 428 bp for $ET_B$) were observed, no signal was visible in blanks (lane B). A 100 bp DNA ladder (Promega) was run to confirm molecular size of the amplification products (lane M). The presence of $ET_A$- and $ET_B$-receptor transcripts was detected in the human trigeminal ganglion (lanes 1 and 2).

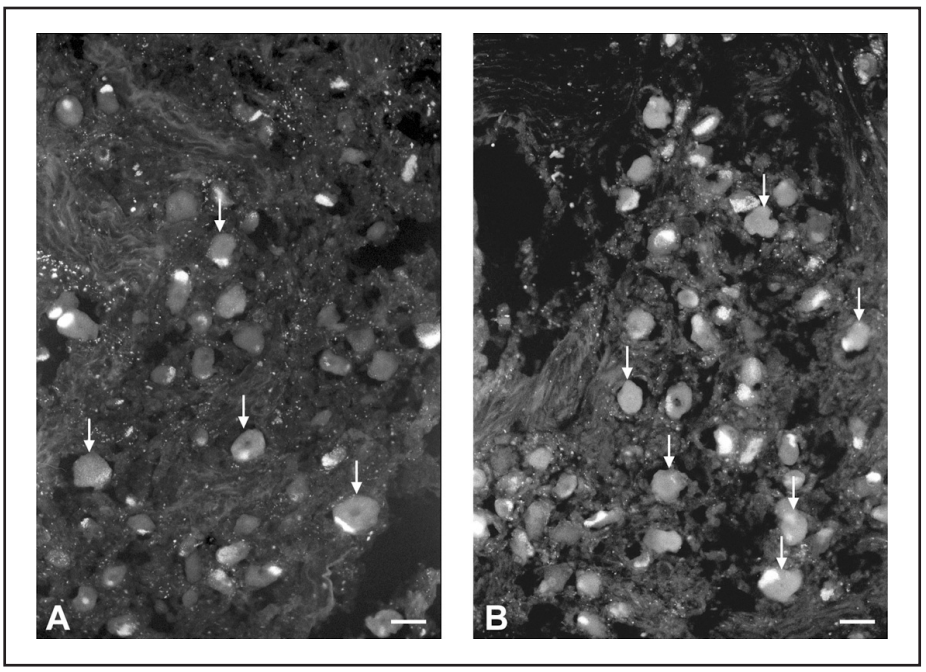


**Fig. 2.** Immunofluorescence for $ET_A$ and $ET_B$ receptors in the human trigeminal ganglion. (A) Large to medium sized cell bodies exhibit $ET_A$ receptor immunofluorescence (arrows). (B) Numerous cell bodies exhibit strong $ET_B$ receptor immunofluorescence (arrows). Scale bar: 30μm.

## Discussion

In the present study we have shown that the human trigeminal ganglion contains receptor $ET_A$ and $ET_B$ mRNA and that the neuronal cell bodies in the ganglion were seen to express $ET_A$ and $ET_B$ protein using immunocytochemistry. Only few studies have been carried out on human cranial ganglia with regard to their expression of neuropeptides and their corresponding receptors. Previous studies have shown that the human trigeminal ganglion harbors nerve cell bodies containing substance P, calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase activating polypeptide (PACAP) and nitric oxide synthase (NOS) [4,15,19]. Concerning ET-1 expression in human cranial ganglia less is known. ET-1 mRNA and endothelin-like immunoreactivity have been detected in sections of the human brain, spinal cord and dorsal root ganglia using in situ hybridization and immunocytochemistry [6,9]. In the dorsal root ganglia, ET-1 transcripts were localized to nerve cell bodies expressing β-preprotachykinin and CGRP mRNAs suggestive of a sensory role. This is in part supported by studies showing that stimulation of the trigeminal ganglion results in an inflammatory reaction in the dura mater [10]. This can be blocked by the $ET_A/ET_B$ antagonist bosentan, suggesting the involvement of ET and ET receptors in craniovascular neurogenic inflammation.

Using RT-PCR, we observed that mRNA encoding $ET_A$ and $ET_B$ receptors were present in the human trigeminal ganglion. This may however derive from blood vessels, satellite cells or be due to contamination with adjacent tissue. Gel electrophoresis of the PCR products demonstrated single products of the expected sizes. The identity of the products was verified by sequencing, which showed no dissimilarities with the published nucleotide sequences. Immunocytochemistry revealed numerous cell bodies in the trigeminal ganglion containing the $ET_A$ and $ET_B$ receptors, suggestive of the formed receptor protein. This localization of the $ET_B$ and the $ET_A$ receptor raises the question of the physiological role of endothelins and their receptors in the nervous system. Yoneyama et al. [24] observed that ET-1, via the release of acetylcholine, augmented electrically induced contractions of rabbit trachea, suggestive of a role of ET-1 as a modulator of classical neurotransmitters. Even autocrine/paracrine effects of endothelins on growth and differentiation of nervous cells are possible. This is further illustrated by the fact that knockout mice lacking the $ET_B$ receptor or ET-3 show a failure in the development of the enteric nervous system, resulting in the aganglionic megacolon of Hirschsprung´s disease [14]. Although a critical role in the development of cranial ganglia was not evident in the $ET_B$ or ET-3 knockout mice a modulatory role of ETs in ganglionic transmission is not inconceivable.

The present results demonstrate the presence of mRNA encoding both $ET_A$ and $ET_B$ receptors in the human trigeminal ganglion, which was further confirmed by the immunocytochemical finding of the $ET_A$ and $ET_B$ receptor proteins in medium sized and large cell bodies. The finding suggests that endothelins may be of importance as a regulator of sensory processing. It is obvious that further analysis is needed to unravel the functional role of the endothelins and their receptors in the peripheral nervous system.

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