The Vesicular Acetylcholine Transporter Is Present in Melanocytes and Keratinocytes in the Human Epidermis

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The human epidermis holds the full machinery for cholinergic signal transduction. However, the presence of the vesicular transporter (vesicular acetylcholine (ACh) transporter (VAChT)) for both choline and ACh has never been shown in this compartment. The results of this study confirm the presence of VAChT in cutaneous nerves and in both epidermal melanocytes and keratinocytes as well as in their nuclei using immunofluorescence labelling *in situ* and *in vitro*, Western blot analysis of cellular and nuclear extracts and reverse transcription-PCR. These results underline that ACh/choline transport in the non-neuronal epidermis is no different from the neuronal pathway. However, the function of VAChT in the nucleus remains to be shown.

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INTRODUCTION

It has been demonstrated *in situ* and *in vitro* by several investigators that the human epidermis holds the full capacity for autocrine cholinergic signal transduction (Grando, 1997; Grando and Horton, 1997; Grando *et al.*, 2003). The neurotransmitter acetylcholine (ACh) is synthesized in a one-step reaction from choline and acetyl-coenzyme A by ACh-*O*-transferase (EC 2.3.1.6, chAT) and hydrolysed by acetylcholinesterase (EC 3.1.1.7). In cholinergic nerve endings ACh is produced in the cytoplasm, then transported and stored in synaptic vesicles (Israel, 1970). The vesicular ACh transporter (VAChT) is responsible for the transport of ACh and choline using a proton electrochemical gradient generated by a vacuolar type $H+$ ATPase with the exchange of two luminal protons for one cytoplasmic ACh or choline (Parsons *et al.*, 1993; Usdin *et al.*, 1995; Bravo *et al.*, 2004). ACh transport requires a transmembrane pH gradient with an internal pH between 5 and 6 causing the protonation of two internal sites (Nguyen and Parsons, 1995).

Interestingly, the VAChT gene is located on chromosome 10 (10q11.2) which has been assigned also to the chAT gene (Erickson *et al.*, 1994). The VAChT gene is contained entirely within the first intron of the chAT gene (Cervini *et al.*, 1995). VAChT and chAT are transcribed together in the same direction. This unique nested gene arrangement allows a tight coordinated regulation of both systems of ACh and choline transport (Berrard *et al.*, 1995; Usdin *et al.*, 1995). Only recently it was shown that the facilitation of VAChT trafficking occurs through the *trans*-Golgi network-associated AP-1 clathrin complex and the plasma membrane AP-2 complex (Kim and Hersh, 2004). Expression of this transporter was demonstrated in nerve fibers, at sweat glands and in motor endplates (Haberberger *et al.*, 2002). However, the presence of VAChT was never documented in human epidermal cells. Since these cells hold the entire cholinergic machinery, it was tempting to look for the presence of this important transporter.

RESULTS

In situ VAChT expression throughout the epidermis

Nowadays it is established that chAT is expressed in the human epidermis (Grando *et al.*, 1993) and that human keratinocytes and melanocytes synthesize, secrete, and degrade ACh (Grando *et al.*, 1993). Since both chAT and VAChT are sitting in the first intron of the same gene, it was tempting to look for the expression of VAChT (Cervini *et al.*, 1995). For this purpose, we used full skin biopsies from healthy controls and stained for VAChT protein expression using immunofluorescence tetramethyl rhodamine isothiocyanate/FITC labelling. Moreover, the specificity of the antibody was confirmed by utilizing a specific blocking peptide.

As seen in Figure 1a VAChT is expressed throughout the entire epidermis with the strongest positivity in the stratum granulosum compared to basal and suprabasal layers. VAChT is also expressed in nerves at the epidermal/dermal junction (Figure 1e). This expression is significantly abrogated in the presence of the blocking peptide indicating that the antibody specifically detects VAChT (Figure 1b and f).

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Abbreviations: ACh, acetylcholine; chAT, ACh-O-transferase MC, melanocyte cell; VAChT, vesicular acetylcholine transporter

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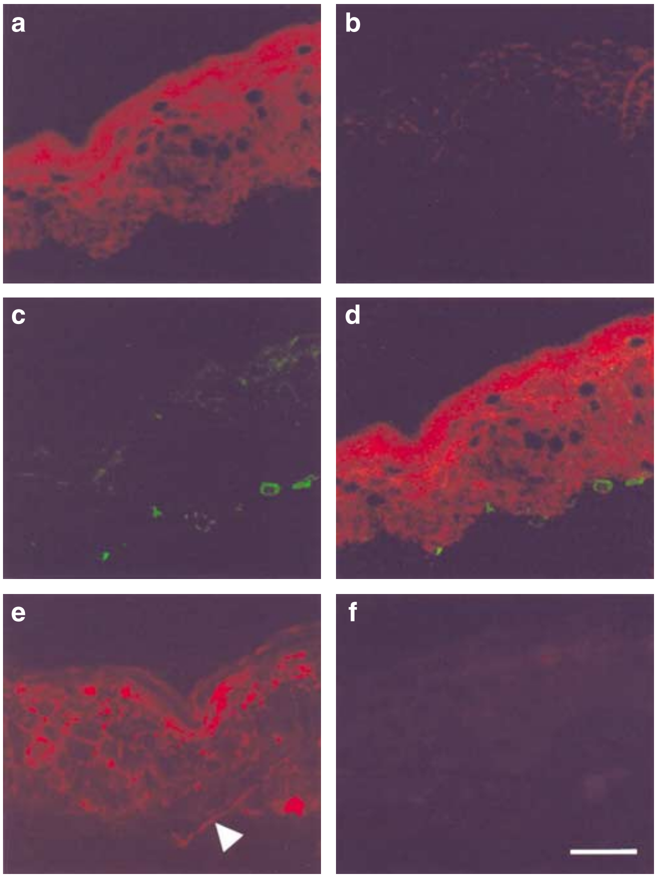

Figure 1. ***In situ* immunofluorescence labelling demonstrates VAChT expression in human epidermal keratinocytes, melanocytes and nerves.** (**a**) *In situ* expression. (**b**) Abrogation of immunoreactivity in the presence of blocking peptide. (**c**) *In situ* expression of gp100 (NKI/beteb) in melanocytes. (**d**) Overlay of the images showing no colocalization. (**e**) Positive immunoreactivity in nerves at the epidermal/dermal junction (▲). (**f**) Abrogation of immunoreactivity in the presence of blocking peptide (original magnification ×400, bar = 50 μm).

In order to identify whether melanocytes do express VAChT *in situ*, we utilized double immunofluorescence with VAChT and the melanocyte-specific gp100 (NKI/beteb) protein (Figure 1c). The overlay shows that melanocytes fail to express VAChT *in situ* (Figure 1d).

Epidermal keratinocytes and melanocytes express VAChT *in vitro*

Both undifferentiated and differentiated keratinocytes express VAChT in a perinuclear distribution, with some immunopositivity in the nucleus (Figure 2).

Under *in vitro* conditions VAChT is expressed throughout the melanocyte including the nucleus and the dendrites (Figure 3a). The expression of VAChT colocalizes strongly with NKI/beteb in a subpopulation of cells while some other cells do not colocalize (Figure 3b and c).

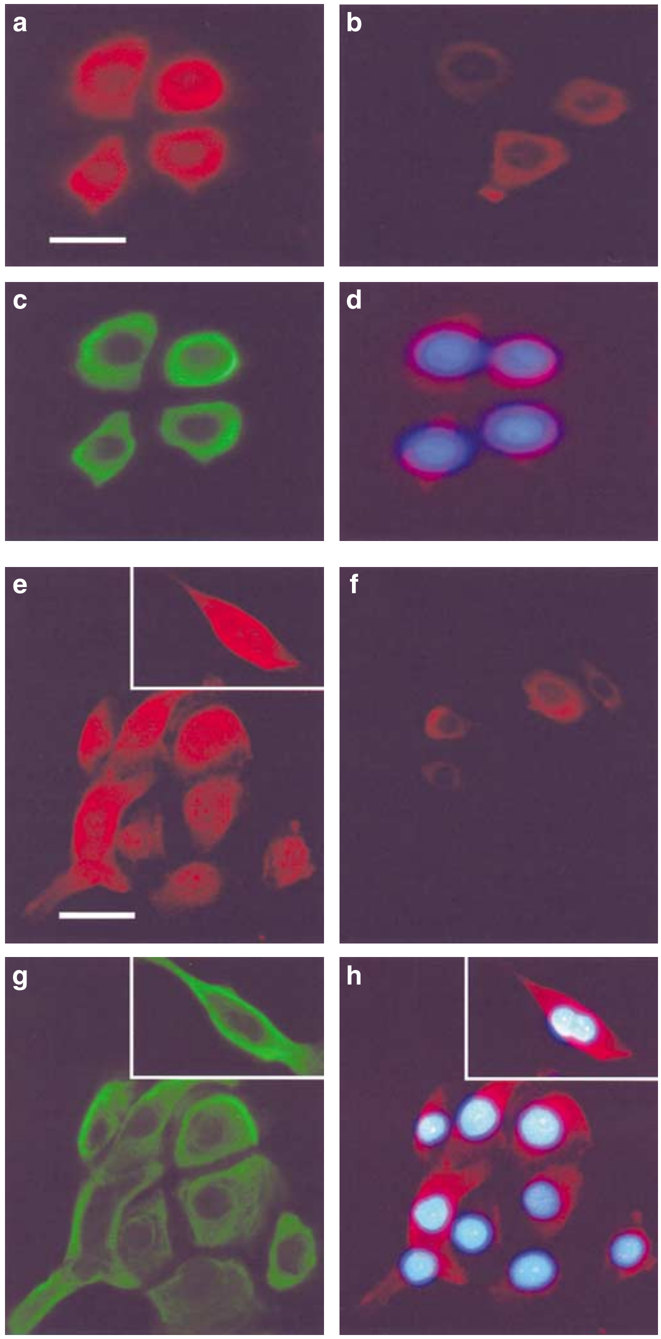

Figure 2. ***In vitro* expression of VAChT in epidermal keratinocytes.** (**a–d**) Undifferentiated keratinocytes. (**a**) VAChT. (**b**) VAChT in the presence of blocking peptide. (**c**) CK5 expression confirming undifferentiated cells. (**d**) VAChT and 4′,6-diamino-phenylindole colocalization showing little nuclear VAChT expression (original magnification ×400, bar = 50 μm). (**e–h**) Differentiated keratinocytes. (**e**) VAChT. (**f**) VAChT in the presence of blocking peptide. (**g**) CK10 expression confirming differentiated cells. (**h**) VAChT and 4′,6-diamino-phenylindole colocalization suggesting nuclear VAChT expression. The inset shows a close up of a single cell (original magnification ×400, bar = 50 μm).

The presence of VAChT was confirmed in keratinocytes and melanocytes as well as in their nuclei by Western blotting

In order to confirm VAChT expression as observed *in situ* and *in vitro* in keratinocytes and in melanocytes, Western blotting was performed in cellular and nuclear extracts of these cells and in spinal cord extract (positive control). The extracts were protected against possible proteolysis. The results showed one band at ∼70 kDa in all extracts tested which was absent

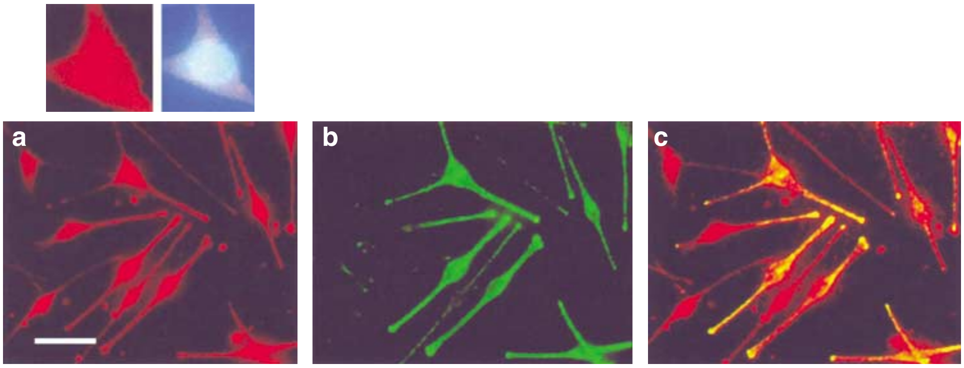

Figure 3. *In vitro* expression of VAChT in epidermal melanocytes. (**a**) VAChT. (**b**) Expression of gp100 (NKI/beteb) in melanocytes. (**c**) Overlay of the images showing colocalization. The inset shows VAChT and 4′,6-diamino-phenylindole colocalization indicating the presence of VAChT in the nucleus (original magnification $\times 400$, bar = 50 μm).

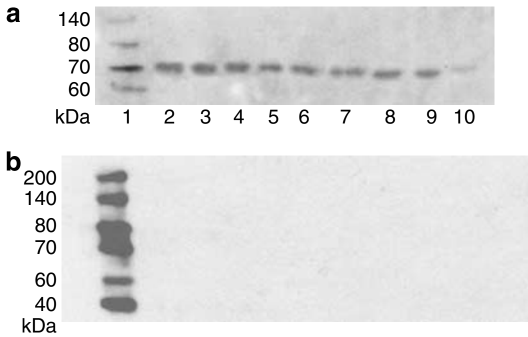

Figure 4. Immunoblotting confirms the presence of VAChT in epidermal keratinocytes, melanocytes, and in the nuclei of both cell types together with positive control (spinal cord extract). (**a**) Western blot analysis: VAChT is expressed in total, cytosolic and nuclear extracts of melanocytes and keratinocytes and in spinal cord extract (positive control) with one band at approx. 70 kDa. (Lane 1: MW Ladder, lane 2: total keratinocyte cells, lane 3: cytosolic keratinocyte cells, lane 4: nuclear keratinocyte cells, lane 5: total MC, lane 6: cytosolic MC, lane 7: nuclear MC, lanes 8 and 9: spinal cord extract (10 μl), and lane 10 spinal cord extract (2 μl). (**b**) Western blot analysis after pre-absorption with the specific VAChT-blocking peptide.

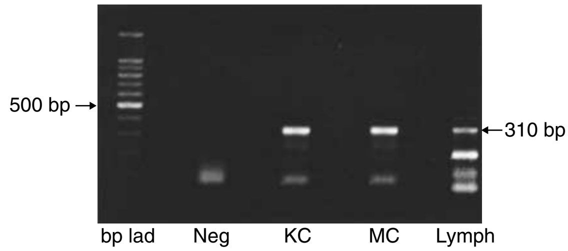

Figure 5. Reverse transcription-PCR confirms the presence of VAChT mRNA in epidermal melanocytes and keratinocytes. The 310 bp band is in agreement with the expected size. Lymphocytes served as positive control. VAChT mRNA is expressed in MC and keratinocyte cells. The product was confirmed by sequence analysis (data not shown).

when the extracts were blocked before analysis (Figure 4a and b). The result is in agreement with the published size of VAChT (Varoqui and Erickson, 1996; Tayebati *et al.*, 2002; Oda *et al.*, 2004). Based on these data we can conclude that epidermal melanocytes and keratinocyte and their nuclei express VAChT.

Presence of mRNA for VAChT in epidermal keratinocytes and melanocytes

To further support the evidence for VAChT in keratinocytes and melanocytes we employed reverse transcription-PCR. As positive control we used peripheral lymphocytes (Tayebati *et al.*, 2002). The product was calculated based on the GenBank accession number: U10554 and the product size was predicted as 310 bp. The band corresponded to the expected size of VAChT in lymphocytes and in both melanocytes and keratinocytes (Figure 5). The PCR product confirmed the correct sequence (data not shown).

DISCUSSION

The presence of VAChT expression in epidermal keratinocytes and melanocytes has never been explored previously. Our findings show that this transporter is present with homogeneous distribution throughout the epidermis. The positivity of this expression was increased upon differentiation as seen both *in situ* (Figure 1a) and *in vitro* (Figure 2a–d and e–h). This result would be in agreement with higher ACh levels in the suprabasal layers supporting that more ACh is needed towards differentiation (Grando *et al.*, 1993).

Even though the *in situ* results showed no detectable expression of VAChT in melanocytes (Figure 1d), the expression was very pronounced in a subpopulation of melanocytes cultured under *in vitro* conditions (Figure 3a and c). The presence of subpopulations deserves further investigation. Western blotting analysis confirmed the presence of VAChT in both keratinocytes and melanocytes as well as in their nuclei. The role for the transporter in the nucleus has to be established. Transcription of the important transporter was confirmed by reverse transcription-PCR showing the presence of its mRNA in both keratinocytes and melanocytes.

Since, VAChT is present at the periphery of melanocytes, keeping ACh close to the myosin Va fibers, which reside in the same area, we propose that ACh transport by VAChT may facilitate transfer of melanosomes. This hypothesis would be in agreement with dispersion, pigment redistribution melanosomal movement by ACh as observed in the skin of lower vertebrates (Hayashi and Fujii, 1994; Ovais, 1994; Ali *et al.*, 1995).

In summary, we here provide early evidence for the presence of VAChT in human epidermal melanocytes and keratinocytes *in situ* and *in vitro*. Our results add this ACh/choline transporter as an important missing piece to the well-documented cholinergic signal transduction system in the epidermal compartment.

MATERIALS AND METHODS

Full skin biopsies from Caucasian healthy controls (skin phototype III, Fitzpatrick classification)

Punch biopsies (3 mm) were obtained under local anesthesia and embedded in OCT™ medium (Sakura, Newbury, Bershire, UK) followed by snap freezing in liquid nitrogen. The samples were stored at −80°C until required or cut into 5–7 μm thick sections using a cryostat (Leica CM 1800, Wetzlar, Germany), directly affixed onto prepared poly-L-lysine-coated slides (SIGMA, Poole, UK), and stored at −80°C until further use. The local ethics committee approved this study. The study was conducted according to the Declaration of Helsinki Principles.

Human epidermal melanocytes and keratinocytes cell cultures

Epidermal melanocytes were grown from epidermal suction blister roof tissue in MCDB 153 medium using the method of Pittelkow and Shipley (1989).

Keratinocytes were established from breast reduction skin or epidermal suction blister roofs in MCDB 153 medium using the method of Wille *et al.* (1984). Suction blisters were obtained from the distal inner forearm using the method of Kiistala (1968).

Immunofluorescence labelling of full skin sections, cultured melanocytes, and keratinocytes

Cryo-cut sections (5–7 μm) of normal human full skin biopsies and slides containing keratinocyte or melanocyte cell (MC) cultures were fixed in acetone for 15 minutes at −20°C. Sections/cells were blocked with 10% normal serum for 90 minutes followed by a 3 × wash in phosphate-buffered saline. Subsequently, sections/cells were incubated overnight at 4°C with the primary antibody(s), washed 3 × in phosphate-buffered saline followed by incubation for 90 minutes at room temperature with FITC/tetramethyl rhodamine isothiocyanate-labelled secondary antibody(s). Immunoreactivity was viewed and captured using a Leica DM-IRB inverted microscope (Leica Microsystems, Wetzlar, Germany) coupled to a digital camera together with Neotech ''Image Grabber PCI'' imaging software. In order to compare intensity of protein expression, staining was carried out in one set. Sources and dilutions of the antibodies used are summarized in Table 1.

Western blot analysis

In order to confirm the presence of the VAChT protein observed *in situ* and *in vitro*, we used Western blot analysis with specific blocking peptide as negative controls. Table 2 summarizes the antibody, blocking peptide, and dilutions used for this purpose.

Table 1. Sources and dilutions of primary and secondary antibodies

Ab name/clone type	Host	Antigen	Dilution	Antibody source
Melanoma-associated Ag premelanosomal Ab gp100, clone NKI/beteb	Mouse	100 kDa, 7 kDa	1:20 in all sources	Monosan, Bradsure Biologicals Ltd, Loughborough, UK
FITC-conjugated anti-mouse IgG (for double labelling of MC)	Donkey	Mouse IgG	1:100 in all sources	Jackson Immunoresearch Laboratories, West Grove, PA
Anti-VAChT, polyclonal	Goat	Synthetic peptide from cloned rat VAChT carboxy terminal	*In situ* 1:500 KC 1:100 MC 1:5	CHEMICON International, Temecula, CA
VAChT-blocking peptide for anti-VAChT	Synthetic peptide	Synthetic peptide	*In situ* 1:500 KC 1:100 MC 1:5	CHEMICON International, Temecula, CA
TRITC-conjugated anti-goat IgG	Donkey	Goat IgG	1:100 in all sources	Jackson Immunoresearch Laboratories, PA
Anti-cytokeratin 5, monoclonal	Mouse	Prokaryotic recombinant protein corresponding to 103 amino-acid portion of the C-terminal region of the cytokeratin 5 molecule (human)	KC 1:150	Abcam Ltd, Cambridge, UK
Anti-cytokeratin 10, monoclonal	Mouse	Tissue/cell preparation (human). Cytoskelaton preparation (extracted from human epidermis by detergent/light salt extraction)	KC 1:150	Abcam Ltd, Cambridge, UK

FITC emits a green fluorescence (λ_{ex} 495 nm/λ_{em} 515 nm). Tetramethyl rhodamine isothiocyanate emits a red fluorescence (λ_{ex} 575 nm/λ_{em} 600 nm).

Table 2. Antibody and the corresponding blocking peptide as negative controls used in Western blotting analysis

Ab/blocking peptide	Host	Antigen	Dilution	Antibody/blocking peptide source
Anti-VAChT Ab, polyclonal IgG, Sc-7716	Goat	A peptide mapping at the C-terminus of (VAChT) of human origin	1:500	Santa Cruz Biotechnology, Inc., Santa Cruz, CA
Blocking peptide (sc-7716 P)	Synthetic peptide	Synthetic peptide	1:500	Santa Cruz Biotechnology, Inc.
HRP-conjugated anti-goat, polyclonal (ab 6885-1)	Donkey	Goat IgG	1:6,000	Abcam Ltd, Cambridge, UK

Human spinal cord tissue lysate (Abcam Ltd, Cambridge, UK) was used as positive control for Western blotting.

Preparation of total keratinocyte and melanocyte cellular lysates

The flasks are rinsed 2× with ice-cold phosphate-buffered saline keeping the flask on ice at all times followed by the addition of 2 ml cold phosphate-buffered saline/EDTA solution and 10 μl Pi (protease inhibitor) (SIGMA, poole, UK). Cells were harvested by gentle scraping followed by centrifugation for 5 minutes at 750 × *g*. The pellet is resuspended in 150 μl sterile distilled water followed by 6 × repeat freezing-thawing cycles and a final centrifugation for 5 minutes at 750 × *g*. The determination of the protein concentration was based on the $OD_{280\,nm}$ measurement using the method of Kalb and Bernlohr (1977). The samples were mixed with sample buffer and used for SDS-PAGE electrophoresis.

Preparation of cytoplasmic and nuclear keratinocyte cell and MC extracts

Cytoplasmic and nuclear extracts were obtained by following the manufacturer's instructions (Active Motif, Rixensart, Belgium). Briefly, cells were cultured until near confluency followed by treatment with phosphate-buffered saline/phosphatase inhibitors and then gently scraped and pelleted. The pellet was resuspended in hypotonic buffer and the obtained supernatant contained the cytoplasmic extract. The remaining pellet was resuspended in lysis buffer and the supernatant provided the nuclear fraction. In order to ensure nuclear purity, samples were tested for cross-contamination via the lactate dehydrogenase assay (Stockland and San Clemente, 1968).

Peripheral blood lymphocyte preparation

Peripheral blood lymphocytes were obtained from healthy volunteers after written and signed consent. They were prepared using Lymphoprep™ (Axis-shield poc AS, Oslo, Norway).

Total RNA preparation

Total RNA was isolated from epidermal primary keratinocyte and MC cultures as well as lymphocyte preparation using TRI REAGENT™ (SIGMA, Poole, UK) following manufacturers instructions. cDNA was synthesized using the reverse transcription system (Promega, Southampton, UK). The reaction mix contained about 1 μg total RNA and 500 pmol of oligo dT primer in a final volume of 20 μl. The negative control contained RNA but no reverse transcriptase.

Reverse transcription-PCR for the detection of VAChT-mRNA

PCR amplification of 2 μl cDNA was used in a final volume of 50 μl containing 10× PCR reaction buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl; Life Technologies, Paisley, UK), 1 μmole dNTP (10 mM each of the four nucleotides; Promega, Southampton, UK), 100 nmole $MgCl_2$ (Life Technologies, Paisley, UK), 1 pmole of each primer and 2.5 U of recombinant *Taq* DNA polymerase (Life Technologies, Paisley, UK). The mixture was incubated initially for 3 minutes at 94°C followed by 40 cycles with 1 minute at 94°C, 1 minute at 56°C (reduced by 0.1°C per cycle), and 30 seconds at 72°C. The primer pair (forward: 5′-ACTACTACACCCGCAGCTAG-3′ and reverse: 5′-ACAGATGCAGGCTCTACACA-3′) was designed using the mRNA sequence of human VAChT (GenBank accession number: U10554). As positive control we utilized mRNA from peripheral blood lymphocytes.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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