

Cloning and sequence of full-length cDNAs encoding the human neuronal nicotinic acetylcholine receptor (nAChR) subunits $\beta 3$ and $\beta 4$ and expression of seven nAChR subunits in the human neuroblastoma cell line SH-SY5Y and/or IMR-32

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Abstract Using PCR-based techniques, we have isolated and sequenced the full-length cDNAs that encode the human neuronal nAChR subunits $\alpha 3,4,5,7$ and $\beta 2,3,4$ in the neuroblastoma cell lines SH-SY5Y and/or IMR-32. The predicted nAChR $\beta 3$ - and $\beta 4$ -subunit proteins contain 458 and 498 amino acids, respectively. Except for the $\beta 2$, all the other cloned cDNAs showed differences with the published sequences. Northern blots show expression of the nAChR subunits $\alpha 3,7$ and $\beta 2,4$ in the human neuroblastoma cell line SH-SY5Y, with intensity of the hybridisation signal decreasing in the order $\alpha 3 > \beta 4 > \beta 2 > \alpha 7$.

Key words: Neuroblastoma; Nicotinic receptor; Polymerase chain reaction; Gene expression; Human

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are members of a superfamily of ligand-gated ion channels that mediate fast signal transmission at synapses ([1] review). The nAChRs are presumed to be (hetero)pentamers of homologous subunits. The subunits that constitute muscle and neuronal forms of vertebrate nAChRs are the product of separate genes and differ in primary structure [2]. The neuronal nAChRs are divided into several subtypes that are formed through varying combinations of homologous subunits arranged around a central channel [3]. A variety of nAChR subunits have been identified in the nervous system in recent years ([4,5] reviews). They are classified as α -subunits ($\alpha 2$ – $\alpha 9$) if they share, as part of the presumed acetylcholine binding site, a pair of adjacent cysteines with the $\alpha 1$ -subunit of the muscle-type nAChR. Subunits lacking these cysteine residues are called β -subunits ($\beta 2$ –

$\beta 4$). To date, cDNAs encoding seven gene products ($\alpha 3,4,5,7$ and $\beta 2,3,4$) have been isolated from different human tissues. For two subunits, $\beta 3$ and $\beta 4$, the 5' coding region remains unknown. In this report we describe the cloning, using PCR-based techniques, and sequence of the full-length coding region of the nAChR subunits $\alpha 3,4,5,7$ and $\beta 2,3,4$ from the human neuroblastoma cell lines SH-SY5Y and/or IMR-32. Furthermore, we show the expression, with Northern blots, of the nAChR subunits $\alpha 3,7$ and $\beta 2,4$ in the SH-SY5Y cell line.

2. Material and methods

2.1. Materials

Dimethylsulphoxide (DMSO) was from Sigma, St. Louis, MO, USA. Guanidinium thiocyanate and glyoxal were from Fluka Chemie, Bornem, Belgium. Takara *ExTaq* polymerase was from Intec Diagnostics, Antwerp, Belgium. *Pfu* polymerase was from Stratagene, Heidelberg, Germany. $MgCl_2$, dNTPs, and 10 \times PCR buffer II were from Perkin Elmer Cetus, Zaventem, Belgium. The [$\alpha^{32}P$]dCTP was from NEN, Brussels, Belgium. Caesium chloride, restriction endonucleases and herring sperm DNA were from Boehringer, Mannheim, Germany. Low melting point agarose was from FMC Bioproducts, Rockland, ME, USA. The RNA molecular size standard and all media for cell culture were from Gibco BRL, Merelbeke, Belgium. The pUC18 vector was from Pharmacia, Roosendaal, The Netherlands. Multi-prime DNA labeling system was from Amersham, Gent, Belgium. Marathon cDNA amplification kit, 1st-strand cDNA synthesis kit and *Taq*-start antibody were from Clontech, Leusden, The Netherlands. PolyATtract mRNA Isolation System 1, and Wizard Clean-Up system were from Promega, Leiden, The Netherlands. Zeta Probe nylon membrane, ion exchange mixed bed resin (AG 501-X8) and SDS were from Biorad, Brussels, Belgium. Formamide was from Janssen, Beerse, Belgium. Human pons and human frontal cortex tissue were a generous gift of Dr. P. Cras, University of Antwerp, Belgium. All other chemicals were of analytical grade and were obtained from Merck, Darmstadt, Germany. Primers for PCR and DNA sequencing were designed with the Genetics Computer Group (GCG) sequence analysis package version 8 (GCG, Madison, WI, USA). All primers have three additional non-complementary nucleotides at the 5' end in case the *Pfu* DNA polymerase does not copy a DNA strand all the way to the end. All oligonucleotides (Table 1) were synthesised by MWG-Biotech (Ebersberg, Germany), HPLC-purified and checked for homogeneity by capillary electrophoresis.

2.2. Mammalian cell culture

Cells were cultured in a 95% air/5% CO₂ humidified incubator at 37°C. The human neuroblastoma cell line IMR-32 was obtained from the American Type Culture Collection (ATCC, CCL-127, batch F-11534). Cells were cultured in modified Eagle's medium (MEM) supplemented with 10% (v:v) heat-inactivated fetal calf serum, 100 U/ml penicillin G, 100 μ g/ml streptomycin sulphate, 1 mM pyruvate and 2 mM L-glutamine.

The human neuroblastoma cell line SH-SY5Y (thrice cloned sub-

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Abbreviations: nAChR, nicotinic acetylcholine receptor; TD-PCR, touch-down polymerase chain reaction; RACE, rapid amplification of cDNA ends; T_m , melting temperature; nt, nucleotide; bp, base pair; kb, kilobase; dNTP, deoxynucleotide triphosphate; UTR, untranslated region; GSP, gene-specific primer; AP, adapter primer; MEM, modified Eagle's medium; DMEM, Dulbecco MEM; DMSO, dimethylsulphoxide; SDS, sodium dodecyl sulphate; SSC, standard sodium chloride/sodium citrate buffer; SSPE, standard sodium chloride/sodium phosphate/ethylene diamine tetraacetate buffer

The nucleotide sequence data for nAChR subunits $\alpha 3,4,5,7$ and $\beta 2,3,4$ were deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Y08415 to Y08421.

line of SK-N-SH) was initially provided by June Biedler and Barbara Spengler of the Sloan Kettering Institute for Cancer Research [6]. Cultures were grown in a 1:1 mixture of Ham's F12 medium and Dulbecco modified Eagle's medium (DMEM) supplemented with 15% (v:v) heat-inactivated fetal calf serum, 100 U/ml penicillin G, 100 µg/ml streptomycin sulphate, 1 mM pyruvate and 2 mM L-glutamine.

2.3. Preparation of polyA⁺ mRNA

Total RNA was extracted by dissolving the cells in guanidinium thiocyanate buffer followed by centrifugation on a discontinuous caesium chloride gradient as described [7]. PolyA⁺ mRNA was isolated with the PolyAtract mRNA Isolation System I according to the manufacturer's instructions.

2.4. 5' RACE PCR amplification of cDNAs encoding nAChR β3- and β4-subunits

Three gene-specific primers, GSP1, 2 and 3 (Table 1, Fig. 1), were designed for each subunit based on the published sequences [8,9]. Adapter-ligated oligo(dT)-primed cDNA and adapter-ligated internally primed cDNA batches were prepared from the IMR-32 cell line and human pons tissue with the Marathon cDNA amplification kit according to the manufacturer's instructions. To obtain oligo(dT)-primed cDNAs the oligo(dT) cDNA synthesis primer (Marathon kit) was used. For preparation of the internally primed cDNAs, the GSP1 (β3-GSP1 and β4-GSP1) primers were used. The cDNA batches were prepared in a final volume of 250 µl. Of each cDNA batch, 5 µl was used in a 50 µl first-round TD-PCR with the adapter primer 1 (AP1, Marathon kit) and the GSP2 for 40 cycles each consisting of (a) 1 min at 94°C, (b) 1 min at 72°C, gradually (0.3°C/cycle) decreasing to 65°C, and (c) 3 min at 72°C. In the reaction mixture 2.5 units of Takara *Ex-Taq* polymerase with corresponding buffer, 0.55 µg *Taq*-start antibody, 0.2 µM of each primer, 5% (v:v) DMSO and 0.2 mM dNTPs were used. A second-round nested PCR was performed with 0.5 µl of the first-round PCR sample, the AP2 (Marathon kit) and the GSP3. The composition of the PCR reaction was the same as in the first-round PCR.

2.5. PCR amplification of full-length cDNAs encoding nAChR α3,4,5,7- and β2,3,4-subunits

Gene-specific primers (Table 1) were designed for the α3,4,5,7- and β2-subunit based on published sequences [10–14]. For the β3- and β4-subunits, gene-specific anti-sense (based on the published sequence [8,9]) and sense (based on the 5' RACE sequence) primers were designed. Both oligo(dT)- and random-primed cDNA batches were prepared from polyA⁺ mRNA of the IMR-32 and SH-SY5Y cell lines, and of human frontal cortex tissue, with the 1st-strand cDNA synthesis kit according to the manufacturer's instructions in a final volume of 100 µl. Of each cDNA batch 5 µl was used in a 50 µl PCR. First-round TD-PCR was performed with the 5A/3A primer combination (Table 1) for 30 cycles consisting of (a) 1 min at 94°C, (b) 1 min at 5°C above the calculated *T_m* value, gradually (0.5°C/cycle) decreasing to 10°C below the calculated *T_m* value and (c) 3 min at 75°C. This was followed by 0, 5, 10 or 15 additional cycles of (a) 1 min at 94°C, (b) 1 min at 10°C below the calculated *T_m* value and (c) 3 min at 75°C. The reaction mixture contained 1.25 units of *Pfu* polymerase, 0.5 µM of each primer, 5% (v:v) DMSO, 1×PCR buffer II, 0.2 mM dNTPs, and MgCl₂ at concentrations ranging from 0.5 to 3.0 mM. PCR samples were separated on a 1% low melting point agarose gel, bands of the correct size were cut out, purified with the Wizard Clean-Up system and dissolved in 50 µl MilliQ-water (Millipore). With 0.5 µl of the purified first-round PCR fragment and the 5/3 primer combination a second-round nested PCR was performed for 15, 20 or 25 cycles ((a) 1 min at 94°C, (b) 1 min at 5°C below the calculated *T_m* value, and (c) 3 min at 75°C). The composition of the PCR reaction was the same as in the TD-PCR.

2.6. Cloning PCR products in pUC18 and DNA sequencing

The PCR fragments were size-fractionated on a 1% low melting point agarose gel, bands of the correct size were cut out, purified with the Wizard Clean-Up system, and ligated into the dephosphorylated *Sma*I site of the pUC18 vector. Sequencing was done by Dye Terminator cycle sequencing (Perkin Elmer, ABI Division, Foster, CA, USA) with custom primers. All sequencing gels were run on an

Applied Biosystems 373A or 377 sequencing apparatus. Each insert was sequenced fully on both strands.

2.7. Northern blot analysis

De-ionised glyoxal/DMSO, Denhardt's solution, SSPE, and SSC were prepared as described [7]. Approximately 3 µg of polyA⁺ RNA from the SH-SY5Y cell line was denatured with de-ionised glyoxal/DMSO, and size-fractionated on a 1% agarose gel as described [7]. The marker lane containing the 0.24–9.5 kb RNA ladder was cut off, stained with ethidium bromide (0.5 µg/ml) for 30 min and – aligned with a fluorescent ruler – photographed under ultraviolet trans-illumination. The log₁₀ of the size of the RNA standards was plotted against the distance migrated. The resulting regression line was used to calculate the size of the RNA transcripts on autoradiographs. The RNA gel containing the polyA⁺ samples was vacuum-blotted with 0.1 N NaOH onto a Zeta probe nylon membrane. Full-length cDNA probes were labelled with [α -³²P]dCTP using the Multi-prime DNA labelling system according to the manufacturer's instructions. Hybridisation was performed overnight at 42°C in a solution containing 50% (v:v) formamide, 5×SSPE, 5×Denhardt's solution, 0.1% (w:v) SDS and 100 µg/ml denatured herring sperm DNA. Blots were finally washed at 60°C in 0.1×SSC containing 0.1% SDS for 16 h and exposed to Kodak X-omat AR film at –70°C in cassettes containing two Dupont Cronex lightning plus-T intensifying screens.

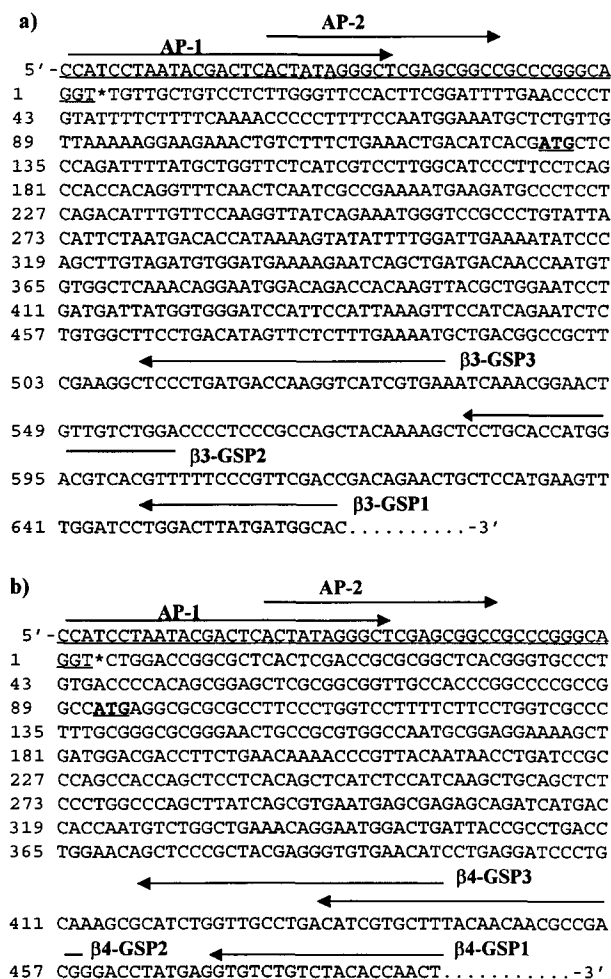


Fig. 1. The 5' end sequences obtained with the 5' RACE PCR of the human nAChR subunits β3 (a) and β4 (b). The position of the gene-specific primers (GSP) and the adapter primers (AP) are indicated. The nucleotide sequence of the adapter and the putative start codon are underlined.

Table 1
Primers used for the amplification of the 5' end (GSPs) and the full-length coding region of the $\alpha 3,4,5,7$ - and $\beta 2,3,4$ -subunits

Primer	5'- sequence -3'
$\beta 3$ -GSP1	GAAGTGCATCATCAAGTCCAG
$\beta 3$ -GSP2	GTTAACGTGACGTCCATGGTGCAGG
$\beta 3$ -GSP3	GAATCAGGATGACCTTGGTCATCAGGG
$\beta 4$ -GSP1	GTTAGTTGGTGTAGACAGACACC
$\beta 4$ -GSP2	GAAGTCGGCGTGTGTAAAGCACGATG
$\beta 4$ -GSP3	CATAAGCACGATGTCAAGCAACCAGATG
$\alpha 3$ -5A	CAACCACGAGCATGGCTCTG
$\alpha 3$ -5	GTAGCCACCATTGGCTCTGGCCGTCTCG
$\alpha 3$ -3A	GAACAGGCGAGGCACACAGCTTAG
$\alpha 3$ -3	GTGTTATGCATCTTCCCTGGCCATCAGG
$\alpha 4$ -5A	GAACACAGGAGAACCAACCGGG
$\alpha 4$ -5	CGAGCCACCATTGGAGCTAGGGGGCCC
$\alpha 4$ -3A	GTTGCTCCCGTCCCTTCCTAGATC
$\alpha 4$ -3	CCACTAGATCATGCCAGCCAGCCAGG
$\alpha 5$ -5A	GAAGCTCCATTCCCAAGAGTTCG
$\alpha 5$ -5	CAAGCCACCATTGGCGCGCGGGG
$\alpha 5$ -3A	GTTATGTATACTTCACTCCCTTGGGAGGC
$\alpha 5$ -3	CGTGCTTCACTTATTTCGATTTCCAATATGAAGTGG
$\alpha 7$ -5	CAAGGATCCGCGCCAGTGGCTGCTCGCC
$\alpha 7$ -3	GAATTACGCAAGTCTTTGGACACG
$\beta 2$ -5A	GATTAGCGGAGGCAGCGAGCTATG
$\beta 2$ -5	GAAGCCACCATTGGCCCGGCGCTG
$\beta 2$ -3A	GTTAGGTGGCTGGTGAAAGAGC
$\beta 2$ -3	CTAGGATCCTCACTTGGAGCTGGGGGGC
$\beta 3$ -5A	GGTGAATCTGTGTGCTTCCTTTGGGTTCC
$\beta 3$ -5	GGTGAATTCGCCACCATTGCTCCAGATTTTATGCTG
$\beta 3$ -3A	GCATCTAGACGTAGAACAGCACATGCATTTGG
$\beta 3$ -3	GGTTCTAGACTAATGGTAACTATGTAGCCACATCTTC
$\beta 4$ -5A	GATCGACTCACTATAGGGCTCGAGCGG
$\beta 4$ -5	GAAGCCACCATTGAGCGCGCGCC
$\beta 4$ -3	GTTTCAGTCACGCTGGGCAGCGTAG

3. Results

3.1. Cloning and sequence of the 5' end of the human nAChR $\beta 3$ - and $\beta 4$ -subunit cDNAs

To obtain the 5' end of the human nAChR $\beta 3$ - and $\beta 4$ -subunits we chose the 5' RACE method [15]. Adapter-ligated oligo(dT)-primed cDNA and adapter-ligated internally primed cDNA were prepared from 1 μ g of IMR-32 polyA⁺ mRNA. After a first round of 5' RACE PCR a ~500 bp reaction product was obtained with the $\beta 4$ -GSP2/AP1 primer combination (Fig. 1), starting from either internally primed or oligo(dT)-primed cDNA preparations. After a second nested round of PCR, with the $\beta 4$ -GSP3/AP2 primer combination (Fig. 1), the expected ~470 bp PCR product could be detected for both cDNA preparations. No specific PCR product could be detected for the $\beta 3$ -subunit. To obtain the 5' end of the $\beta 3$ -subunit mRNA we decided to use human pons because it has been shown that the $\beta 3$ -subunit is expressed in this brain region [8]. From 1 μ g of human pons polyA⁺ mRNA, adapter-ligated oligo(dT)- and adapter-ligated internally primed cDNA were prepared. After a first round of 5' RACE PCR with the $\beta 3$ -GSP2/AP1 primer combination (Fig. 1), no specific PCR products for either cDNA preparations could be detected. However, a second nested round of PCR with the $\beta 3$ -GSP3/AP2 primer combination (Fig. 1) yielded a PCR product of ~570 bp starting from the internally primed cDNA preparation. The two ~500 bp $\beta 4$ and the 570 bp $\beta 3$ PCR products were cloned into the dephosphorylated *Sma*I site of the pUC18 vector and the nucleotide

sequence was determined (Fig. 1). Compared to the published incomplete sequences [8,9], 240 nt of additional 5' end $\beta 3$ sequence and 231 nt (internally primed cDNA preparation) or 226 nt (oligo(dT)-primed cDNA preparation) of additional 5' end $\beta 4$ sequence were obtained. Combining these sequence data with the published sequences, an open reading frame of 1374 nt for the $\beta 3$ -subunit and of 1494 nt for the $\beta 4$ -subunit could be identified.

3.2. Cloning and sequence of the full-length human nAChR $\alpha 3,4,5,7$ - and $\beta 2,3,4$ -subunits

To obtain full-length clones of $\alpha 3,4,5,7$ - and $\beta 2,3,4$ -subunits we designed primers to amplify by TD-PCR their coding region exactly from the start to the stop codon (included) and adding the Kozak consensus sequence (GCCACC) upstream of the start codon. With this strategy we were able to amplify the human nAChR $\alpha 7$ -subunit from the neuroblastoma cell lines SH-SY5Y and IMR-32 (Table 2). None of the other subunits could be amplified using the same strategy, notwithstanding numerous attempts to optimise PCR conditions (different concentrations of MgCl₂, DMSO, glycerol, formamide, cDNA and primers, different annealing temperatures and number of PCR cycles).

Therefore, a different strategy to amplify the other subunits was chosen: nested PCR. This permits the choice of optimum PCR primers in the first round, combined with amplification in the second round of the exact coding region with primers that may be less than ideal due to the strict constraints on their 5' ends. In the first round of TD-PCR, primers directed to the sequence upstream of the start codon (5A sense, Table 1) and downstream of the stop codon (3A anti-sense, Table 1) were used. The primers were designed as ideal as possible (length: 20 nt, GC content: 50%, same T_m value (~70°C) for both sense and anti-sense primers). Six MgCl₂ concentrations ranging from 0.5 to 3.0 mM (in 0.5 mM increments) were used and the number of PCR rounds kept to a minimum. The DNA fragment of the expected size was isolated and used in a second nested PCR with the primers amplifying

Table 2
PCR amplification conditions for the coding region of the human nAChR $\alpha 3,4,5,7$ - and $\beta 2,3,4$ -subunits

Subunit	Source	[MgCl ₂]	First TD-PCR	Nested PCR
$\alpha 3$	SH-SY5Y	1.0 mM	1550 bp, 0	1521 bp, 15
	IMR-32	1.0 mM	1550 bp, 10	1521 bp, 20
$\alpha 4$	SH-SY5Y	all	X	X
	IMR-32	1.0 mM	2043 bp, 15	1896 bp, 15
$\alpha 5$	SH-SY5Y	1.5 mM	1519 bp, 0	1419 bp, 15
	IMR-32	1.5 mM	1519 bp, 0	1419 bp, 20
$\alpha 7$	SH-SY5Y	2.0 mM	1527 bp, 15	
	IMR-32	2.0 mM	1527 bp, 15	
$\beta 2$	SH-SY5Y	1.5 mM	1584 bp, 0	1527 bp, 15
	IMR-32	1.5 mM	1584 bp, 0	1527 bp, 25
	H.F.C.	1.5 mM	1584 bp, 15	1527 bp, 20
$\beta 3$	SH-SY5Y	2.5 mM	1613 bp, 15	1400 bp, 25
	IMR-32	all	X	X
$\beta 4$	Human pons	2.5 mM	1613 bp, 15	1400 bp, 25
	SH-SY5Y	1.0 mM	1566 bp, 0	1507 bp, 20
	IMR-32	1.0 mM	1566 bp, 0	1507 bp, 20

For each RNA source, the optimal [MgCl₂] is listed as well as the predicted DNA fragment length followed by the number of additional PCR cycles after the initial 30 cycles in the first TD-PCR or the total number of cycles in a second (nested PCR) round (bp, x). X = no specific PCR product obtained; all = all [MgCl₂] tested gave similar results; H.F.C. = human frontal cortex tissue.



Fig. 2. Amino acid sequence alignment of the human nAChR α 3,4,5,7- and β 2,3,4-subunits. Indicated are the positions of the predicted signal peptide, the conserved cysteine residues, the four putative transmembrane domains (TMD I–IV) and the cytoplasmic region. Underlined are the amino acids which differ from the published versions [8–14]. Alignment and predicted motifs were obtained with the GCG program (Genetics Computer Group, Madison, WI, USA).

the subunit coding region exactly from the start to the stop codon. A minimum number of PCR cycles was used to obtain enough material for cloning the PCR fragment in pUC18 for subsequent determination of its nucleotide sequence. The cDNA prepared from the neuroblastoma cell lines SH-SY5Y and IMR-32 was the starting material for the PCR reactions. For the β 2-subunit we also used cDNA prepared from human frontal cortex tissue and for the β 3-subunit we also used adapter-ligated oligo(dT)-primed cDNA prepared from human pons tissue. Using this nested PCR approach we were able to clone the human nAChR subunits α 3,4,5 and β 2,3,4 from different sources (Table 2).

The human nAChR subunit β 3 clones (isolated from human pons and the SH-SY5Y cell line) contained an open reading frame of 1374 nt, coding for a protein of 458 amino acids (Fig. 2). The human nAChR subunit β 4 clones (isolated from both neuroblastoma cell lines) contained an open read-

ing frame of 1494 bp, coding for a protein of 498 amino acids (Fig. 2).

Compared to the published sequences [8–14], we found several differences for the α 3,4,5,7- and β 3,4-subunits (all nucleotide sequences have been submitted to the Genbank database: accession numbers Y08415–Y08421). At the amino acid level this led to the following changes (Fig. 2; all residues are indicated in one-letter code and numbered independently in the published as well as in our sequence counting the predicted initiator methionine as 1 in either case): for the α 3-subunit [10], CRA (11–13)→LSPP (11–14), G (99)→D (100), TT (131,132)→DD (132,133), S (234)→I (235), V (429)→L (430); for the α 5-subunit [12], ALRSSRARRAAR→RCGLAGAAGGAQ (23–34), S→V (128), S→C (365), T→R (405); for the α 7-subunit [13], G→A (11); for the β 3-subunit [8], EWK (1–3)→LFQ (36–38); for the β 4-subunit [9], DE (25,26)→EQ (73,74). For the α 4-subunit no changes were

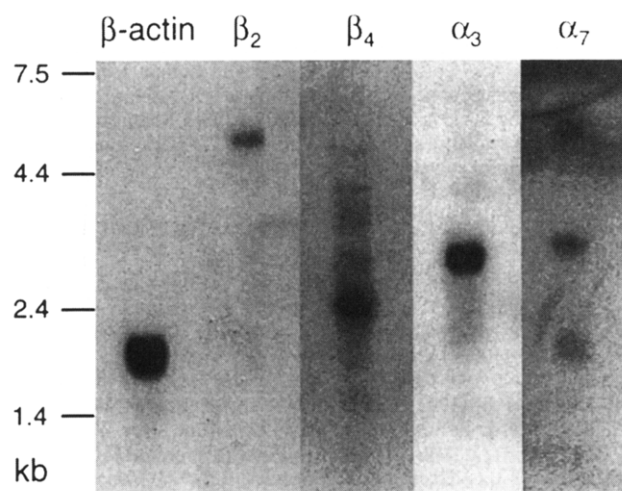


Fig. 3. Northern blots of the SH-SY5Y cell line showing mouse β -actin (2 h exposure) and the human neuronal nAChR subunits β_2 , β_4 , α_3 (16 h exposure) and α_7 (88 h exposure).

predicted at the amino acid level. All the differences were found in both the clones isolated from the SH-SY5Y and the IMR-32 cell line (for β_3 the SH-SY5Y cell line and human pons).

3.3. Northern blot analysis

Full-length clones of the human nAChR $\alpha_3,4,5,7$ - and $\beta_2,3,4$ -subunits were used to probe Northern blots containing 3 μ g polyA⁺ mRNA isolated from the SH-SY5Y neuroblastoma cell line. As a positive control probe, a full-length mouse β -actin (92% identical to the homologous human sequence) cDNA was used. Blots were washed for 16 h at a final stringency of $0.1 \times \text{SSC}$ and 0.1% (w:v) SDS. After a 2 h exposure, a strong signal of a 1.8 kb β -actin transcript (Fig. 3) was observed as expected [16]. After a 16 h exposure, expression of the 3.0 kb α_3 , the 2.4 kb β_4 and the 5.6 kb β_2 transcripts can be detected in the SH-SY5Y cell line. After an 88 h exposure three α_7 transcripts of 5.7 kb, 3.1 kb and 1.9 kb can be detected in the SH-SY5Y cell line (Fig. 3). No specific transcripts could be detected for the α_4 -, α_5 - and β_3 -subunits in the SH-SY5Y cell line.

4. Discussion

With the 5' RACE method we obtained further 5' sequences of the human neuronal nAChR β_3 - and β_4 -subunits. Two adapter-ligated cDNA preparations were used: oligo(dT)-primed and internally primed (with gene-specific primers). Specific β_3 -subunit 5' sequences were obtained only when starting from cDNA prepared with the β_3 gene-specific primer. Therefore it seems that preparing cDNA with gene-specific primers enhances the chance of obtaining 5' end sequences by RACE.

Willoughby et al. [8] showed on a Northern blot a 1.7 kb β_3 transcript in human pons. Their sequence showed a duplication of 60 nt at the 3' end which we were not able to detect. Discounting this duplication, their partial sequence combined with our 5' RACE data yield a total of 1595 nt. Based on the Northern blot (Fig. 3), the presumably incomplete 3' end, and the typical length of a polyA tail, it is likely that the 5' end of

our β_3 5' RACE clone is close to the 5' cap site of the β_3 mRNA.

For the β_4 -subunit sequence we obtained in total 1588 nt. Based on the Northern blot (Fig. 3) there is still ~ 800 nt unaccounted for, at least part of which corresponds to 3' UTR since the published sequence extends only to the stop codon. Because the two clones isolated have different 5' end sequences it is not likely that we reached the 5' cap site of the β_4 mRNA.

The neuroblastoma cell lines SH-SY5Y and IMR-32 proved to be an excellent source for isolation of human neuronal nAChR subunit cDNAs. From the SH-SY5Y cell line we isolated the $\alpha_3,5,7$ - and $\beta_2,3,4$ - subunit cDNAs. From the IMR-32 cell line we isolated the $\alpha_3,4,5,7$ - and $\beta_2,4$ -subunit cDNAs. Furthermore, we isolated the β_3 -subunit cDNA from human pons tissue and the β_2 -subunit cDNA from human frontal cortex.

These neuronal nAChR subunit cDNAs were isolated with two different PCR approaches: TD-PCR and nested TD-PCR. In the first approach the primers were designed to amplify the coding region exactly from start to stop codon and adding immediately upstream of the start codon a Kozak consensus sequence to improve translation in subsequent heterologous expression studies. This leads to cDNAs without any 5' or 3' UTRs, which are known to have occasionally a negative effect on heterologous expression [17]. With this approach only one subunit (α_7) could be amplified. The problem with this approach is that the sequence of the primers is fixed and to obtain desired T_m values only the length of the primers can be changed. In the case of the nAChR subunits this led to short GC-rich sense and long AT-rich anti-sense primers. Therefore, in the nested TD-PCR approach, PCR primers with properties as close as possible to the ideal (for criteria used, see Section 3) were designed upstream of the start and downstream of the stop codon. With these primers the other six nAChR subunits were amplified. To remove the 5' and 3' UTR sequences, the PCR products were used in a second nested PCR with the same primers as in the first PCR approach. Because in this reaction only a single template is available, and in large amounts, the amplification of the exact coding sequence of the nAChR subunits was possible.

Whenever subunit clones from two different sources were obtained, their sequence was identical. Compared to published sequences [8–14], only the β_2 -subunit DNA sequence gave an 100% match [14]. Furthermore, the predicted α_4 -subunit amino acid sequence is identical to the published version [12]. For all other subunit sequences, differences were found with published DNA and deduced amino acid sequences. These differences could be due to allelic variation, cDNA synthesis artefacts or PCR artefacts, or sequencing errors. Because our clones were isolated from two different sources, the first and second explanation seem unlikely. PCR conditions to minimise error rates (e.g. DNA polymerases with proof-reading ability such as *Pfu*) were used. Also at least two independent (from different tissue or cell source) PCR clones were isolated for each subunit (except α_4 , where both clones were obtained from the IMR-32 cell line). Therefore, PCR artefacts are also an unlikely explanation. Furthermore, all clones were fully sequenced on both strands, virtually excluding sequencing errors.

The human neuronal nAChR β_3 - and β_4 -subunits show 94% and 89% amino acid sequence identity compared to the

rat neuronal nAChR β 3- [18] and β 4- [19] subunits, respectively. Stop codons were found upstream of the AUG codon that opens the longest open reading frame, as well as a purine 3 nt upstream of this predicted start codon (found in 97% of vertebrate mRNAs [20]). The length of the corresponding predicted rat and human amino acid sequences is approximately the same. Therefore, we conclude that we have cloned the full-length coding region of the human neuronal nAChR β 3- and β 4-subunits.

In the SH-SY5Y cell line the 3.0 kb α 3 transcript appears most abundant, followed by the 2.4 kb β 4 transcript and the 5.6 kb β 2 transcript. For the α 7-subunit, at high stringency, we observe moderate expression of three transcripts (5.7 kb, 3.1 kb and 1.9 kb). Peng et al. [13] also reported in the SH-SY5Y cell line three α 7 transcripts of approximately the same size: 5.9, 2.6, and 1.3 kb. The three transcripts could result from alternative splicing, or the use of different transcription start sites or polyadenylation sites. No mRNA transcripts could be detected on Northern blots for the α 4-, α 5- and β 3-subunits although the more sensitive PCR method shows that at least the α 5- and β 3-subunits are also expressed in the SH-SY5Y cell line. This means that the SH-SY5Y cell line expresses at least six neuronal nAChR subunits. Based on the PCR results the IMR-32 cell line expresses also at least six subunits: α 3,4,5,7 and β 2,4.

The question remains whether all these subunits form part of functional receptors. Expression of an α 7 nAChR has been demonstrated in the SH-SY5Y cell line, but this expression is not particularly high [13]. According to Wang et al. [21], most of the functional receptors in the SH-SY5Y cell line consist of an α 3/ β 2 or an α 3/ β 4 combination. These data correspond well with the expression pattern of the nAChR subunits on our Northern blot. Wang et al. [21] also showed that α 5 can form a functional receptor with the α 3/ β 2 or the α 3/ β 4 combination expressed in oocytes and in the SH-SY5Y cell line. To demonstrate this, they made use of mouse monoclonal antibody 210 which recognises the main immunogenic region amino acids NQIVTTNVRL [21] of the α 5-subunit (amino acids 90–99, Fig. 2). Exactly the same sequence can be found in the β 3-subunit (amino acids 72–81, Fig. 2). Combining this information with our PCR data showing that the β 3-subunit is expressed in the SH-SY5Y cell line, it cannot be excluded that the β 3-subunit forms part of the nAChRs found in the SH-SY5Y cell line. Whether the β 3-subunit can indeed be part of a functional nAChR in the SH-SY5Y cell line still remains to be proven.

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