Cloning and sequence of full-length cDNAs encoding the human neuronal nicotinic acetylcholine receptor (nAChR) subunits β3 and β4 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 α3,4,5,7 and β2,3,4 in the neuroblastoma cell lines SH-SY5Y and/or IMR-32. The predicted nAChR β3- and β4-subunit proteins contain 458 and 498 amino acids, respectively. Except for the β2, all the other cloned cDNAs showed differences with the published sequences. Northern blots show expression of the nAChR subunits α3,7 and β2,4 in the human neuroblastoma cell line SH-SY5Y, with intensity of the hybridisation signal decreasing in the order α3 > β4 > β2 > α7.

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 (α2–α9) if they share, as part of the presumed acetylcholine binding site, a pair of adjacent cysteines with the α_1-subunit of the muscle-type nAChR. Subunits lacking these cysteine residues are called β-subunits (β2–β4). To date, cDNAs encoding seven gene products (α3,4,5,7 and β2,3,4) have been isolated from different human tissues. For two subunits, β3 and β4, 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 α3,4,5,7 and β2,3,4 from the human neuroblastoma cell lines SH-SY5Y and/or IMR-32. Furthermore, we show the expression, with Northern blots, of the nAChR subunits α3,7 and β2,4 in the SH-SY5Y cell line.

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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 α3,4,5,7 and β2,3,4 were deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Y08415 to Y08421.

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 Imtec Diagnostics, Antwerp, Belgium. *Pfu* polymerase was from Stratagene, Heidelberg, Germany. $MgCl_2$, dNTPs, and 10×PCR buffer II were from Perkin Elmer Cetus, Zaventem, Belgium. The [α^{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. Multiprime 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 I, 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% CO_2 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 μg/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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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 PolyATtract 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 $MgCl_2$ 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 $\log_{10}$ 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-^{32}P]dCTP using the Multiprime 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.

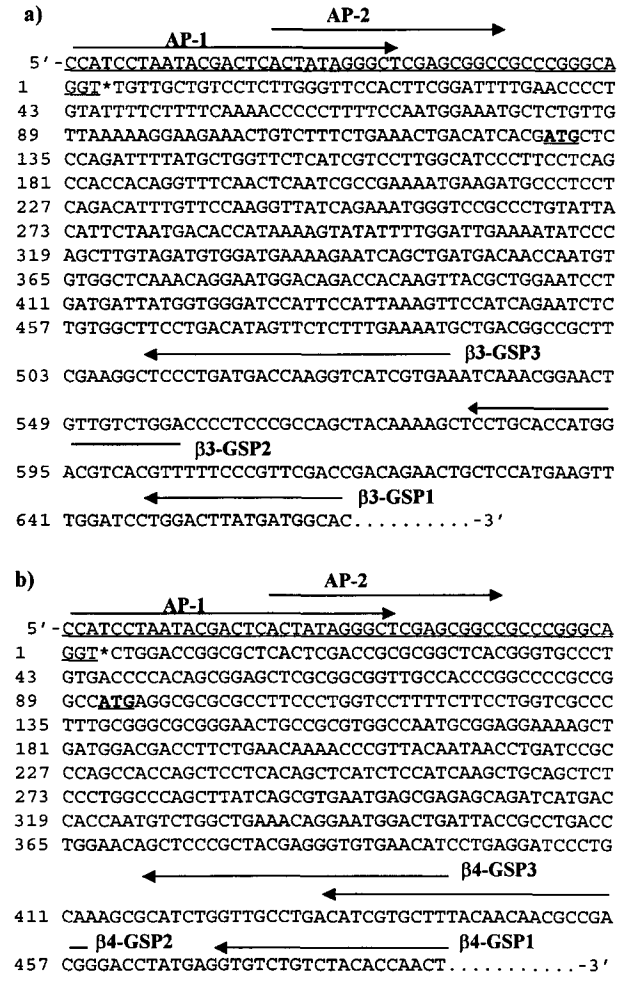

Fig. 1. The 5′ end sequences obtained with the 5′ RACE PCR of the human nAChR subunits β3 (a) and β4 (b). The position of the gene-specific primers (GSP) and the adapter primers (AP) are indicated. The nucleotide sequence of the adapter and the putative start codon are underlined.

Table 1
Primers used for the amplification of the 5′ end (GSPs) and the full-length coding region of the α3,4,5,7- and β2,3,4-subunits

Primer	5′- sequence -3′
β3-GSP1	GAAGTGCCATCATAAGTCCAG
β3-GSP2	GTTAACGTGACGTCCATGGTGCAGG
β3-GSP3	GAATCACGATGACCTTGGTCATCAGGG
β4-GSP1	GTTAGTTGGTGTAGACAGACACC
β4-GSP2	GAAGTCGGCGTTGTTGTAAAGCACGATG
β4-GSP3	CATAAGCACGATGTCAGGCAACCAGATG
α3-5A	CAACCACCAGCCATGGCTCTG
α3-5	GTAGCCACCATGGCTCTGGCCGTCTCG
α3-3A	GAACAGGCAGGCACACAGCTTAG
α3-3	GTGTTATGCATCTTCCCTGGCCATCAGG
α4-5A	GAACACAGGAGAAGACGAACCGGG
α4-5	CGAGCCACCATGGAGCTAGGGGGCCC
α4-3A	GTTGCTCCCGGTCCCTTCCTAGATC
α4-3	CCACTAGATCATGCCAGCCAGCCAGG
α5-5A	GAAGCTCCATTCCCCAAGAGTTCG
α5-5	CAAGCCACCATGGCGGCGCGGGG
α5-3A	GTTATGTATACTTCAGTCCCTTGGGAGGC
α5-3	CGTGCTTCACTTATTTGCATTTCCAATATGAACTGG
α7-5	CAAGGATCCGCCACCATGCGCTGCTCGCC
α7-3	GAATTACGCAAAGTCTTTGGACACG
β2-5A	GATTAGGCGAGGCAGCGAGCTATG
β2-5	GAAGCCACCATGGCCCGGCGCTG
β2-3A	GTTAGGGTGGCTGGGTGAAAGAGC
β2-3	CTAGGATCCTCACTTGGAGCTGGGGGC
β3-5A	GGTGAATTCTGTTGCTGTCCTCTTGGGTTCC
β3-5	GGTGAATTCGCCACCATGCTCCCAGATTTTATGCTG
β3-3A	GCATCTAGACGTAGAACAAGCACATGCATTTGG
β3-3	GGTTCTAGACTAATGGTAACTATGTAGCCACATCTTC
β4-5A	GATCGACTCACTATAGGGCTCGAGCGG
β4-5	GAAGCCACCATGAGGCGCGCGCC
β4-3	GTTTCAGTCACGCTGGGCAGCGTAG

3. Results

3.1. Cloning and sequence of the 5′ end of the human nAChR β3- and β4-subunit cDNAs

To obtain the 5′ end of the human nAChR β3- and β4-subunits we chose the 5′ RACE method [15]. Adapter-ligated oligo(dT)-primed cDNA and adapter-ligated internally primed cDNA were prepared from 1 μg of IMR-32 polyA$^+$ mRNA. After a first round of 5′ RACE PCR a ~500 bp reaction product was obtained with the β4-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 β4-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 β3-subunit. To obtain the 5′ end of the β3-subunit mRNA we decided to use human pons because it has been shown that the β3-subunit is expressed in this brain region [8]. From 1 μg 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 β3-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 β3-GSP3/AP2 primer combination (Fig. 1) yielded a PCR product of ~570 bp starting from the internally primed cDNA preparation. The two ~500 bp β4 and the 570 bp β3 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 β3 sequence and 231 nt (internally primed cDNA preparation) or 226 nt (oligo(dT)-primed cDNA preparation) of additional 5′ end β4 sequence were obtained. Combining these sequence data with the published sequences, an open reading frame of 1374 nt for the β3-subunit and of 1494 nt for the β4-subunit could be identified.

3.2. Cloning and sequence of the full-length human nAChR α3,4,5,7- and β2,3,4-subunits

To obtain full-length clones of α3,4,5,7- and β2,3,4-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 α7-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 $MgCl_2$, 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 $MgCl_2$ 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 α3,4,5,7- and β2,3,4-subunits

Subunit	Source	[$MgCl_2$]	First TD-PCR	Nested PCR
α3	SH-SY5Y	1.0 mM	1550 bp, 0	1521 bp, 15
	IMR-32	1.0 mM	1550 bp, 10	1521 bp, 20
α4	SH-SY5Y	all	X	X
	IMR-32	1.0 mM	2043 bp, 15	1896 bp, 15
α5	SH-SY5Y	1.5 mM	1519 bp, 0	1419 bp, 15
	IMR-32	1.5 mM	1519 bp, 0	1419 bp, 20
α7	SH-SY5Y	2.0 mM	1527 bp, 15	
	IMR-32	2.0 mM	1527 bp, 15	
β2	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
β3	SH-SY5Y	2.5 mM	1613 bp, 15	1400 bp, 25
	IMR-32	all	X	X
	Human pons	2.5 mM	1613 bp, 15	1400 bp, 25
β4	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 [$MgCl_2$] 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 [$MgCl_2$] tested gave similar results; H.F.C. = human frontal cortex tissue.

Fig. 2. Amino acid sequence alignment of the human nAChR α3,4,5,7- and β2,3,4-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 β2-subunit we also used cDNA prepared from human frontal cortex tissue and for the β3-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 α3,4,5 and β2,3,4 from different sources (Table 2).

The human nAChR subunit β3 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 β4 clones (isolated from both neuroblastoma cell lines) contained an open reading 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 α3,4,5,7- and β3,4-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 α3-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 α5-subunit [12], ALRSSRARRAAR→RCGLAGAAGGAQ (23–34), S→V (128), S→C (365), T→R (405); for the α7-subunit [13], G→A (11); for the β3-subunit [8], EWK (1–3)→LFQ (36–38); for the β4-subunit [9], DE (25,26)→EQ (73,74). For the α4-subunit no changes were

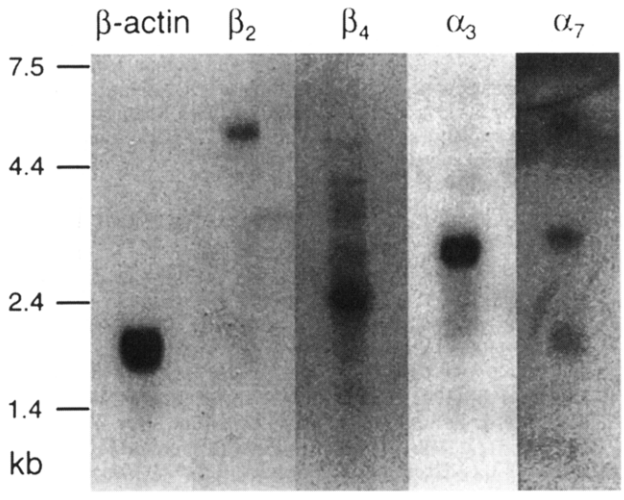

Fig. 3. Northern blots of the SH-SY5Y cell line showing mouse β-actin (2 h exposure) and the human neuronal nAChR subunits β2, β4, α3 (16 h exposure) and α7 (88 h exposure).

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 β3 the SH-SY5Y cell line and human pons).

3.3. Northern blot analysis

Full-length clones of the human nAChR α3,4,5,7- and β2,3,4-subunits were used to probe Northern blots containing 3 μg 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×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 α3, the 2.4 kb β4 and the 5.6 kb β2 transcripts can be detected in the SH-SY5Y cell line. After an 88 h exposure three α7 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 α4-, α5- and β3-subunits in the SH-SY5Y cell line.

4. Discussion

With the 5′ RACE method we obtained further 5′ sequences of the human neuronal nAChR β3- and β4-subunits. Two adapter-ligated cDNA preparations were used: oligo(dT)-primed and internally primed (with gene-specific primers). Specific β3-subunit 5′ sequences were obtained only when starting from cDNA prepared with the β3 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 β3 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 β3 5′ RACE clone is close to the 5′ cap site of the β3 mRNA.

For the β4-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 β4 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 α3,5,7- and β2,3,4- subunit cDNAs. From the IMR-32 cell line we isolated the α3,4,5,7- and β2,4-subunit cDNAs. Furthermore, we isolated the β3-subunit cDNA from human pons tissue and the β2-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 (α7) 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 β2-subunit DNA sequence gave an 100% match [14]. Furthermore, the predicted α4-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 α4, 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 β3- and β4-subunits show 94% and 89% amino acid sequence identity compared to the

rat neuronal nAChR β3- [18] and β4- [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 β3- and β4-subunits.

In the SH-SY5Y cell line the 3.0 kb α3 transcript appears most abundant, followed by the 2.4 kb β4 transcript and the 5.6 kb β2 transcript. For the α7-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 α7 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 α4-, α5- and β3-subunits although the more sensitive PCR method shows that at least the α5- and β3-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: α3,4,5,7 and β2,4.

The question remains whether all these subunits form part of functional receptors. Expression of an α7 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 α3/β2 or an α3/β4 combination. These data correspond well with the expression pattern of the nAChR subunits on our Northern blot. Wang et al. [21] also showed that α5 can form a functional receptor with the α3/β2 or the α3/β4 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 α5-subunit (amino acids 90–99, Fig. 2). Exactly the same sequence can be found in the β3-subunit (amino acids 72–81, Fig. 2). Combining this information with our PCR data showing that the β3-subunit is expressed in the SH-SY5Y cell line, it cannot be excluded that the β3-subunit forms part of the nAChRs found in the SH-SY5Y cell line. Whether the β3-subunit can indeed be part of a functional nAChR in the SH-SY5Y cell line still remains to be proven.

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