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Efrat Lev-Lehman · Dani Bercovich · Wei Xu
David W. Stockton · Arthur L. Beaudet

Characterization of the human β4 nAChR gene and polymorphisms in *CHRNA3* and *CHRNB4*

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Abstract Most neuronal nicotinic acetylcholine receptors are heteropentamers, composed of α and β subunits. Mice lacking the α3 subunit and mice lacking both the β2 and β4 subunits, but not mice lacking the β2 or β4 subunits alone, have a severe phenotype characterized by megacystis, failure of bladder strips to contract in response to nicotine, widely dilated ocular pupils, growth failure, and perinatal mortality. The deficit in bladder contraction was also found in mice lacking only the β4 subunit, although they did not develop megacystis. The major bladder phenotype resembles the human autosomal recessive disorder of megacystis-microcolon-hypoperistalsis syndrome (MMIHS). Based on the similarity of the mouse and human phenotypes, we initiated mutation analyses in the α3 and β4 genes in MMIHS families. The human gene encoding the β4 subunit was fully characterized, including refinement of its mapping. Analysis of disease families and controls identified numerous genetic variants, including high-frequency polymorphisms in both *CHRNA3* and *CHRNB4*. Although no loss-of-function mutations have been identified to date, these genes remain strong candidates for involvement in MMIHS, because various mutations might be obscured within the complex cluster of genes. Some of the markers presented here are valuable tools for analysis of the role of genetic variation in responses to nicotine and for characterization of various dysautonomic abnormalities.

Key words Nicotine · Smoking · Megacystis · Pseudo-obstruction · SNPs · DHPLC · Sequencing

E. Lev-Lehman · D. Bercovich · W. Xu · D.W. Stockton · A.L. Beaudet (✉)
Department of Human and Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
Tel. +1-713-7984795; Fax +1-713-7987773
e-mail: abeaudet@bcm.tmc.edu

Introduction

The neuronal nicotinic acetylcholine receptor (nAChR) subunits are widely expressed throughout the central nervous system (CNS) and the peripheral nervous system, including its autonomic components. The neuronal nAChR are pentamers made up of α and β subunits (Anand et al. 1991; Karlin 1993). There are at least eight α subunits (α2–α9) and three β subunits (β2–β4) encoded by 11 distinct genes. The subunit composition of the native, usually heteropentameric, receptors as they relate to specific functions is generally unknown.

To resolve the composition and functions of the different subunits, our laboratory had previously prepared null mice for several neuronal nicotinic acetylcholine receptors. Mice lacking the α3 subunit, although born alive in the expected Mendelian ratios, have impaired growth and increased perinatal mortality (Xu et al. 1999a). Multiorgan dysfunction, reflecting effects on the autonomic nervous system, is the major phenotypic finding. The null mice demonstrate bladder distension, urinary retention with secondary infection and bladder stones, dilated ocular pupils, and, possibly, hypoperistalsis (Xu et al. 1999a). Similar phenotypic traits are found in mice homozygous for null mutations in both the β2 and β4 subunits (Xu et al. 1999b). These findings in the α3 and the β2/β4 null mice resemble the human autosomal recessive disorder known as megacystis-microcolon-hypoperistalsis syndrome (MMIHS, OMIM 249210) and perhaps the related phenotype of intestinal pseudo-obstruction (OMIM 243180), isolated or in association with megacystis. Further analysis of bladder function in the α3 and β2/β4 null mice suggested that neuronal nAChRs containing α3 and β4 subunits are most prominently involved in bladder contractility, with the β2 subunits able to participate, but less significantly (De Biasi et al. 2000; Xu et al. 1999a; Xu et al. 1999b). This is based on the finding that bladder strips from β2 null mice contract in response to nicotine, while those from α3 or β4 null mice do not, although neither the β2 or β4 single homozygotes develop megacystis, while the β2/β4 double null and α3 single null

mice have megacystis. These findings led to the hypothesis that altered α3 or β4 subunits underlie the MMIHS condition in humans. We therefore initiated mutational analyses in patients with this rare autosomal recessive condition.

Materials and methods

Patients and research subjects

We have studied 13 families, including seven individuals affected with MMIHS and their family members, three pairs of parents of deceased MMIHS-affected children, two individuals and their family members with the possibly related condition of intestinal pseudo-obstruction without megacystis, and one pair of parents of a deceased child with intestinal pseudo-obstruction. Most of the patients were referred from distant cities through other physicians, or they contacted the investigators through the American Society of Pseudo-obstruction and Hirschsprung's Disease (APHS). All blood samples were collected after the signing of an informed consent form as part of an approved Baylor College of Medicine Institutional Review Board (IRB) protocol. Control DNA samples were obtained through the Baylor Polymorphism Resource developed by Dr. John Belmont (jbelmont@bcm.tmc.edu), with the support of the Kleberg Foundation, or from unrelated Centre d'Etude du Polymorphisme Humain (CEPH) individuals (www.cephb.fr).

Closing gaps within the *CHRNB4* and *CHRNA3* genes

Since this study was completed, the sequence of the *CHRNB4/A3/A5* gene cluster has appeared in the database (AC067863) as ten unordered contigs, with gaps in introns 1 and 4 in the *CHRNB4* and *CHRNA3* genes, respectively. Based on our cloning efforts, the size of *CHRNB4* IVS1 was predicted to be between 3.7 kb and 7.5 kb. To close the gap in IVS1 of *CHRNB4*, we designed the following primers, based on the available intronic sequences from contigs V and VII: forward, 5′-CGCCTTCCCTGGTCCTT-3′ and reverse 5′-AGGGAGACTCAGAGGAGACT-3′, and amplified bacterial artificial chromosome (BAC)s 252H1 or 136A3 at an annealing temperature of 62°C. Previous work on the *CHRNA3* gene (Rempel et al. 1998) did not define the size of IVS4. To close the gap in IVS4 of *CHRNA3*, we designed the following primers according to contigs VII and IX: forward 5′-GGCTGGACTGTGGGACA-3′ and reverse 5′-ACCAGGTTCAAGCGATTCT and amplified BACs 192B14 or 136A3 at an annealing temperature of 62°C.

Screening for mutations

Genomic DNA was amplified using primers flanking the intron-exon boundaries of both the *CHRNA3* gene (polymerase chain reaction [PCR] conditions and primers are available as electronic supplementary material) and the *CHRNB4* gene (Table 1). For the *CHRNB4* gene, PCR reactions were performed using a standard protocol in the presence of 5% dimethylsulfoxide (DMSO). PCR fragments were either analyzed by denaturing high-performance liquid chromatography (DHPLC) or by direct sequencing, using the ABI PRISM big dye primer or big dye terminator ready reaction kits on the ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Establishing conditions for DHPLC

DHPLC analysis (Underhill et al. 1996) was run on a WAVE apparatus from Transgenomics (Omaha, NE, USA). DNA was eluted within a linear acetonitrile gradient consisting of buffer A (0.1 M triethylammonium acetate

Table 1. Primers for amplification of *CHRNB4* exons

Exon	Size		Primer sequence (5′ → 3′)	Annealing temp.
1	626 bp	F	TTGCGAGGCCCGAAGAGATAG	67°C
		R	GCAAAGCCGGTGTCATCCAG	
2	428 bp	F	CAGGCCTGCCAGCACCTTCG	68°C
		R	CCCTTCTGCAGCCTCCCTGACAC	
3	270 bp	F	UT-GTGACATGCCTTTGGGCCAT	68°C
		R	GTCCACTGCCACCAAAGGG	
4	409 bp	F	TGAGCCCCAGAGATCTTTTCTTTAG	63°C
		R	AGACAAGGTCCTTCCCTTCCTTCAG	
5 Up	588 bp	F	GAACAGGCCAGGTTTTGTGTATGGGTTTC	65°C 5 cycles
		R	RT-AGGGAGGTGGGTGGCACGAT	68°C 30 cycles
5 Middle	562 bp	F	TCCCAGGGAGAAGGACAGTG	60°C
		R	GCGGGGTTCACAAAGTACATG	
5 Down	326 bp	F	CCAGCCCCTCCAACTTCTATGGGAAC	60°C
		R[a]	GCCCGCAGAGTTTGAGAGTCCTTGTC	
6	248 bp	F	CCAGTACTTGGCACCTGTGACCAAC	64°C
		R	CCCACCCGGCCACTCACATCCTCTC	
6/3′UTR	744 bp	F	UT-ACAGCCCAAGTTCCCAGTG	65°C 5 cycles
		R	RT-AGTAGGTGCTGCTACGAAGTCATC	68°C 30 cycles

F, Forward primer; R, reverse primer; UT, universal tail; RT, reverse tail; UTR, untranslated region
[a] Primer with a GC clamp (GCCCG)

[TEAA]) / buffer B (0.1M TEAA, 25% acetonitrile). DHPLC gradients for analysis of the *CHRNB4* gene were all run over 5min as follows: exon 1, buffer B from 56%–70% at 67°C; exons 2 and 4, buffer B from 54%–68% at 64°C; exon 3, buffer B from 51%–65% at 61°C; exon 5 upstream and downstream, buffer B from 54%–68% at 63°C; exon 5 middle, buffer B from 56%–70% at 63°C; exon 6, buffer B from 48%–62% at 64°C.

Mapping of the *CHRNB4* gene

The GeneBridge 4 radiation hybrid panel (Research Genetics, Huntsville, AL, USA) (Walter et al. 1994) was used for PCR-based mapping of *CHRNB4* exon 3 (primers listed in Table 1). Results were scored using the WICGR program (http://carbon.wi.mit.edu:8000/cgi-bin/contig/rhmapper.pl).

Results

Mutation analysis in the α3 nAChR gene (*CHRNA3*)

Based on the previous findings of an MMIHS-like phenotype in mice lacking the α3 neuronal nAChR (Xu et al. 1999a), we conducted a search for mutations in patients with MMIHS. Genomic DNA was screened for mutations by PCR, followed by direct sequencing of all coding exons of the *CHRNA3* gene. Although disease-causing mutations were not identified, a previously reported (Rempel et al. 1998) single amino-acid insertion in the signal peptide region (L17insL) and multiple previously unreported single-nucleotide polymorphisms (SNPs) were identified. We found three silent changes in the coding sequence: 345A → G, 477G → A, and 831C → T, that are base pair substitutions in Val52, Lys96, and Tyr214, respectively. In addition, we found six changes within the introns and the 3′untranslated region (UTR) sequences: IVS1 + 11A → G, IVS1 + 112C → T, IVS2-34insG, IVS3-64A → C, IVS3-5T → C, and 1857insA (Table 2, left panels). All of the silent and intronic changes were found both in patient families and in controls. Interestingly, the IVS3-64A → C, IVS3-5T → C, and 477G → A were found in strict linkage disequilibrium and in high frequency in normal chromosomes (46%; Table 2, left panels).

Characterization of the human β4 subunit of the nicotinic acetylcholine receptor gene (*CHRNB4*)

Because no loss-of-function mutations were identified in the *CHRNA3* gene, we began a mutation screen in the second candidate gene, *CHRNB4*. We determined the exon-intron boundaries of *CHRNB4* by PCR-based BAC screening and subcloning of restriction digest fragments hybridizing to exon-specific oligonucleotides (data not shown GenBank AF306325, AF306326, AF306327, AF306328, AF306329). A summary of the genomic structure with intron/exon junctions is presented in Table 3, including the sizes of introns and exons. By the time of completion of this study, the sequence of the BAC containing the *CHRNB4/A3/A5* gene cluster had appeared in the database (AC067863) however, there were gaps within the relevant genes. To close the gap in IVS1 of *CHRNB4*, we PCR-amplified and cloned a 3.5-kb fragment and further sequenced it from both cloning ends. Sequencing of this band closed the gaps between three different contigs in AC067863 and revealed the complete IVS 1, which spans over 5492 bp (GenBank AY027915, AY027916). To close

Table 2. Genetic variations within the *CHRNA3* and *CHRNB4* genes

CHRNA3			*CHRNB4*		
Coding	Nucleotide[a]	Allele frequency	Coding	Nucleotide[a]	Allele frequency
L17insL	238insCTG	5/26	IVS1	IVS1+53G → T	ND[c]
IVS1	IVS1+11A → G	6/96	IVS1	IVS1-91T → G	10/30[A], 0/30[Cd]
IVS1	IVS1+112C → T	ND[b]	IVS2	IVS2-47G → C	2/104
V52V	345A → G	20/88	T81I	392C → T	6/374
IVS2	IVS2-34insG	2/30	R136W	526C → T	1/500
IVS3	IVS3-64A → C	14/30	S140G	538A → G	3/500
IVS3	IVS3-5T → C	14/30	S151S	573C → T	1/500
L96L	477G → A	14/30	I240I	840C → T	2/166
Y214Y	831C → T	12/30	M467V	1519A → G	13/590
3′UTR	1857insA	13/22	3′UTR	1693C → T	15/30
			3′UTR	1775G → A	ND[b,c]

[a] Numbering for the cDNA sequences is based on GenBank U62432 and NM_000750 for *CHRNA3* and *CHRNB4*, respectively. For *CHRNA3*, all changes were detected by sequencing, except the IVS1+11A → G, which was detected by *Bsi*EI digestion, and 345A → G, which was detected by *Msc*I digestion. For *CHRNB4*, all screening of multiple individuals was performed by denaturing high-performance liquid chromatography (DHPLC) Frequencies are expressed as variant chromosomes out of total control chromosomes

[b] Observed in more than one unrelated individual in megacystis-microcolon-hypoperistalsis (MMIHS) families, but was not tested in controls

[c] Observed only in the unaffected sib of a patient, but was not found in controls

[d] [A]Asian controls; [C]Caucasian controls

Table 3. Exon/intron junctions in the *CHRNB4* gene (sequences are 5′ → 3′)

Exon/intron	Acceptor site	5′ of exon	Exon size (bp)	3′ of exon	Donor site	Intron size (kb)
1	CCCGCCGGCC	ATG AGG CGC	55	TGC GGG CGC G	gtgagttctt	5.49
2	gttttctcag	GG AAC TGC	149	ATC AGC GTG	gtaggtgcag	4.10
3	ccctccccag	AAT GAG CGA	45	CTG AAA CAG	gtaagtgccc	0.10
4	gtctgtccag	GAA TGG ACT	110	TAC AAC AA	gtgagtggcag	1.13
5	ccctgcccag	C GCC GAC	979	GAC CAG AGT	gtaagttgcta	3.67
6	ctgcctgcag	GTC GTT GAG	159	CGT GAC TGA	GGGCCCCCTG	

Splice sites are underlined, noncoding mRNA sequence is in upper case, and introns are in lower case. ATG codon for initiator methionine and TGA codon for termination are underlined

the gap in *CHRNA3* IVS4, we PCR-amplified the 192B14 and 252HI BACs with primers designed according to contigs VII and IX, and subcloned the products. We found two groups of subclones: one with a total length of 452 bp that was sequenced successfully and deposited in GenBank (AY027912), and the other with a total length of approximately 650 bp that could not be completely sequenced from either end (GenBank AY027913, AY027914). Comparison of the sequence of both types of clones suggested that the shorter clones may arise due to an inverted repeat sequence in the region. Therefore, we predict that the correct size of this gap is 650 bp, which indicates that the size of intron 4 of *CHRNA3* is 14.59 kb.

Refinement of the *CHRNB4* mapping

The *CHRNB4* locus was originally mapped to chromosome 15q24 (Raimondi et al. 1992) in a cluster with *CHRNA3* and *CHRNA5*. Recently, Bonati et al. (2000) genetically mapped this cluster between D15S1027 and D15S1005 upstream of the region involved in autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). Using the genomic sequence of *CHRNB4*, we now refined its mapping by direct amplification from intronic sequences adjacent to exon 3 and scoring on a radiation hybrid panel. By this analysis we localized the *CHRNB4* gene 3.1 cR downstream from WI-7183, which lies on the distal end of 15q24.3, and 14.3 cR upstream of WI-6335, which lies in 15q25.1–15q25.3.

Polymorphisms in the *CHRNB4* gene

All coding exons of the *CHRNB4* gene were amplified with the primers shown in Table 1 located in the adjacent introns. As with the *CHRNA3* gene, multiple variations were found in both coding and noncoding sequences of *CHRNB4*. Most of the variations found in the patient families were either intronic changes (IVS1+53G → T, IVS1-91T → G and IVS2-47G → C) or changes within the 3′UTR sequence. The IVS1+53G → T, although not found in controls, appeared only in an unaffected sibling and her mother, and therefore is not implicated in MMIHS. The 3′UTR changes included the 1693C → T, which was present on 50% of 30 Caucasians; chromosomes, and the more rare change 1775G → A, which was seen in several unrelated MMIHS families and in one family only in an unaffected sibling (Table 2, right panels). All the other changes listed in Table 2 were observed in controls and are therefore interpreted as not causing MMIHS. Two amino acid substitutions, T81I and M467V, were found originally in two MMIHS families, but subsequently in controls, suggesting that these are benign polymorphisms. We identified additional variants, using DHPLC to screen control samples for the previously identified variants. In effect, we screened between 190 and 295 normal unrelated individuals for alterations in exons 4, 5, or 6, using DHPLC. The additional changes found initially in the normal samples include S140G, 840C → T, and the more rare variants R136W and 573C → T.

Possible alternative exon in *CHRNB4*

Although the *CHRNB4* gene is expressed primarily in the nervous system, we attempted to detect its mRNA in lymphoblastoid cells from MMIHS families. By this method, using nested reverse transcription (RT)-PCR, we identified an alternatively spliced mRNA from the healthy sibling of a patient. This spliced mRNA included a cryptic exon of 34 bases (designated 4a GenBank AF306327, not shown) between exon 4 and exon 5. The genomic sequence of exon 4a lies in intron 4 and includes the conserved AG/GT splice sites. This mRNA, if translated, is predicted to produce a shorter protein that reads through exon 4a to exon 5, with a stop codon at nucleotide 494, which is between amino acids Y115 and E116. However, because no genomic alterations were found in the sequence adjacent to exon 4a in this family, this transcript may represent either a splicing error or, potentially, a biologically functional isoform.

Discussion

In this report, we describe the characterization of the human gene for the β4 subunit of nAChR and the refinement of its chromosomal localization. We also report the finding of multiple polymorphic variants within the *CHRNA3* and *CHRNB4* genes that may be useful for studies of responses to nicotine and of disorders that involve the nervous system, perhaps, especially, the autonomic components.

The functional β4 nAChR subunit cDNA is formed by splicing of the six coding exons of the *CHRNB4* gene. The additional exon found in this study (designated as exon 4a)

is spliced between exon 4 and 5 and could represent a splicing error, or an exon of unknown biological significance. The complex genomic structure of the *CHRNB4/A3/A5* gene cluster (Raimondi et al. 1992) may predispose to rearrangement events between the *CHRNB4* and/or the related *CHRNA3* and/or *CHRNA5* genes.

In this study we have found multiple polymorphic markers in *CHRNA3* and *CHRNB4*. These changes included the insertion of an amino acid, amino acid substitutions, intronic changes, and many silent changes within the coding sequence**.** The more frequent variations in *CHRNA3* included the three polymorphisms in tight linkage disequilibrium in exon 4 (IVS3-64A → C, IVS3-5T → C, and 477G → A), the 831C → T, and the insertion of an adenosine (1857insA) in a stretch of ten adenosine nucleotides. The most frequent variation in *CHRNB4* is a substitution of thymidine for cytosine (1693C → T). These polymorphic changes would be informative tools for future association or linkage studies for either the effects of nicotine on smokers or for other diseases involving the autonomic nervous system.

Although we did not find any loss-of-function mutations in either *CHRNA3* or *CHRNB4* genes in DNA from patients with MMIHS, further studies are warranted, given the strong similarity of the phenotypes in MMIHS and mutant mice.

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References

Anand R, Conroy W, Schoepfer R, Whiting P, Lindstrom J (1991) Neuronal nicotinic acetylcholine receptors expressed in Xenopus oocytes have a pentameric quaternary structure. J Biol Chem 266:11192–11198

Bonati MT, Asselta R, Duga S, Ferini-Strambi L, Oldani A, Zucconi M, Malcovati M, Dalpra L, Tenchini ML (2000) Refined mapping of *CHRNA3/A5/B4* gene cluster and its implications in ADNFLE. NeuroReport 11:2097–2101

De Biasi M, Nigro F, Xu W (2000) Nicotinic acetylcholine receptors in the autonomic control of bladder function. Eur J Pharmacol 393:137–140

Karlin A (1993) Structure of nicotinic acetylcholine receptors. Curr Opin Neurobiol 3:299–309

Raimondi E, Rubboli F, Moralli D, Chini B, Fornasari D, Tarroni P, De Carli L, Clementi F (1992) Chromosomal localization and physical linkage of the genes encoding the human alpha-3, alpha-5 and beta-4 neuronal nicotinic receptor subunits. Genomics 12:849–850

Rempel N, Heyers S, Engels H, Sleegers E, Steinlein OK (1998) The structures of the human neuronal nicotinic acetylcholine receptor beta2- and alpha3-subunit genes (*CHRNB2* and *CHRNA3*). Hum Genet 103:645–653

Underhill PA, Jin L, Zemans R, Oefner PJ, Cavalli-Sforza LL (1996) A pre-Columbian Y chromosome-specific transition and its implications for human evolutionary history. Proc Natl Acad Sci USA 93:196–200

Walter MA, Spillett DJ, Thomas P, Weissenbach J, Goodfellow PN (1994) A method for constructing radiation hybrid maps of whole genomes. Nat Gen 7:22–28

Xu W, Gelber S, Orr-Urtreger A, Armstrong D, Lewis RA, Ou CN, Patrick J, Role L, De Biasi M, Beaudet AL (1999a) Megacystis, mydriasis, and ion channel defect in mice lacking the alpha3 neuronal nicotinic acetylcholine receptor. Proc Natl Acad Sci USA 96:5746–5751

Xu W, Orr-Urtreger A, Nigro F, Gelber S, Sutcliffe CB, Armstrong D, Patrick JW, Role LW, Beaudet AL, De Biasi M (1999b) Multiorgan autonomic dysfunction in mice lacking the beta2 and the beta4 subunits of neuronal nicotinic acetylcholine receptors. J Neurosci 19:9298–9305