Biochemical Pharmacology 82 (2011) 904–914

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The chimeric gene *CHRFAM7A*, a partial duplication of the *CHRNA7* gene, is a dominant negative regulator of α7*nAChR function

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ARTICLE INFO

Article history:
Received 30 March 2011
Accepted 13 June 2011
Available online 28 June 2011

Keywords:
CHRNA7
Schizophrenia
Nicotinic receptor
CHRFAM7A

ABSTRACT

The human α7 neuronal nicotinic acetylcholine receptor gene (*CHRNA7*) is a candidate gene for schizophrenia and an important drug target for cognitive deficits in the disorder. Activation of the α7*nAChR, results in opening of the channel and entry of mono- and divalent cations, including Ca^{2+}, that presynaptically participates to neurotransmitter release and postsynaptically to down-stream changes in gene expression. Schizophrenic patients have low levels of α7*nAChR, as measured by binding of the ligand [^{125}I]-α-bungarotoxin (I-BTX). The structure of the gene, *CHRNA7*, is complex. During evolution, *CHRNA7* was partially duplicated as a chimeric gene (*CHRFAM7A*), which is expressed in the human brain and elsewhere in the body. The association between a 2 bp deletion in *CHRFAM7A* and schizophrenia suggested that this duplicate gene might contribute to cognitive impairment. To examine the putative contribution of *CHRFAM7A* on receptor function, co-expression of α7 and the duplicate genes was carried out in cell lines and *Xenopus* oocytes. Expression of the duplicate alone yielded protein expression but no functional receptor and co-expression with α7 caused a significant reduction of the amplitude of the ACh-evoked currents. Reduced current amplitude was not correlated with a reduction of I-BTX binding, suggesting the presence of non-functional (ACh-silent) receptors. This hypothesis is supported by a larger increase of the ACh-evoked current by the allosteric modulator 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea (PNU-120596) in cells expressing the duplicate than in the control. These results suggest that *CHRFAM7A* acts as a dominant negative modulator of *CHRNA7* function and is critical for receptor regulation in humans.

1. Introduction

The human α7 neuronal nicotinic receptor gene (*CHRNA7*) is one of approximately 8 genes consistently reported to be associated with schizophrenia [1]. Expression of α*7nAChRs in schizophrenic postmortem brain is decreased [2], but the mechanism for this reduction has not been definitively determined. In the current report, we show that a chimeric gene, *CHRFAM7A* formed as a partial duplication of *CHRNA7*, is a dominant negative regulator of function when coexpressed with the *CHRNA7* gene product.

Genetic linkage to the *CHRNA7* gene at 15q13.3 was first found to an endophenotype in schizophrenia, the P50 deficit (LOD = 5.30) [3] and then to the disease itself. Replicated linkage has been found in multiple ethnic populations suggesting that this candidate gene is associated with schizophrenia in most all populations [4–12]. Genetic data also implicates the *CHRNA7* gene in smoking in the disorder. A specific dinucleotide repeat allele (D15S1360) in intron 2 of the *CHRNA7* gene was found to be associated with smoking in schizophrenia [13]. Recent genetic linkage studies showed that three nicotinic receptors are associated with smoking in schizophrenia, the α2 nicotinic receptor subunit gene (*CHRNA2*; 8p21), the β2 nicotinic receptor subunit gene (*CHRNB2*; 1q21), and the α7 nicotinic receptor subunit gene (*CHRNA7*; 15q14) [14].

Behaviorally, smoking normalizes auditory sensory deficits, found in schizophrenics and 50% of their first-degree relatives [15], and improves cognition in human subjects [16]. It has been

Abbreviations: *CHRNA7*, human α7 nicotinic acetylcholine receptor gene; *Chrna7*, mouse α7 nicotinic acetylcholine receptor gene; α7, α7 receptor subunit protein; α7*nAChR, α7 assembled surface pentameric receptor; *CHRFAM7A*, duplicated *CHRNA7* gene; *CHRFAM7AΔ2 bp*, duplicated gene containing a 2 bp deletion in exon 6; I-BTX, [^{125}I]-α-bungarotoxin; α-BTX, α-bungarotoxin; PNU-120596, 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea.

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doi:10.1016/j.bcp.2011.06.018

suggested that smoking may be a form of self-medication for these patients [17,18]. Smoking cessation in control subjects shows improvement in visuospatial working memory tasks, but in schizophrenics, withdrawal from smoking leads to decreased cognitive performance [19]. These results may be due to differences in neuroadaptive responses to nicotine resulting from decreased levels of nicotinic receptors [20].

The α7 receptor subunit gene, *CHRNA7*, is a member of a large gene family coding for neuronal nicotinic acetylcholine receptor subunits. Unlike high-affinity nicotinic receptors in which multiple subunits assemble to form α4β2*nAChRs, the α7*nAChR is homomeric in most tissues and binds nicotine with low affinity [21]. The antagonist, α-bungarotoxin, usually used as the iodinated product [^{125}I]-α-bungarotoxin specifically labels α7*nAChRs showing very broad expression throughout the brain [22–24]. Recently, [^{3}H]-CHIBA-1001 a molecule derived from the specific α7 agonist SSR–180711 was used for α7 labeling in humans, confirming the high level and general expression of this receptor subtype [25,26]. Such promising ligands are expected to advance our understanding of the cholinergic system in brain impairment. α7*nAChRs are located both pre- and postsynaptically [27,28]. Presynaptically, they are involved in neurotransmitter release, including release of GABA, glutamate, and dopamine from specific terminals [28–33]. Postsynaptically, α7*nAChRs are localized in or near the postsynaptic density (PSD) [34], where the Ca^{2+} flux increases phosphorylation and affects gene expression [27,35]. The α7*nAChR is also found in the periphery where it is involved in neuropeptide release and protects against inflammatory processes [36,37].

The low-affinity nicotine binding site in the α7*nAChR is specifically measured by binding of [I^{125}]-α-bungarotoxin (I-BTX) [22,24]. In postmortem hippocampus, frontal cortex, dorsolateral prefrontal cortex, cingulate gyrus, and in the reticular nucleus of the thalamus of schizophrenic subjects, α7*nAChR levels are decreased, as determined by I-BTX binding and western blot [2,38–40], suggesting that functional surface receptors are decreased in all brain regions examined to date. I-BTX, which binds to the low-affinity nicotine site, can bind to a partially assembled muscle receptor of three subunits [41]. It is assumed, but not known whether this is also true of the α7*nAChR. Thus, schizophrenic subjects with low levels of I-BTX binding, may have defects early in the assembly process. The structure and regulation of α7*nAChR expression is, however, complex. Decreased receptor expression can occur at one of many levels, including transcription [42], translation [43], or copy number variation [44].

1.1. Structure of the CHRNA7 gene

The *CHRNA7* gene on chromosome 15q13.3 has 10 exons, differing from other nicotinic receptor subunits, which only have six. We cloned the human gene from postmortem brain (Genbank U40583) and discovered that the gene is partially duplicated [45]. Exons 5–10 are duplicated, along with a large cassette of DNA (~300 kb). The duplication interrupted a second partial duplication

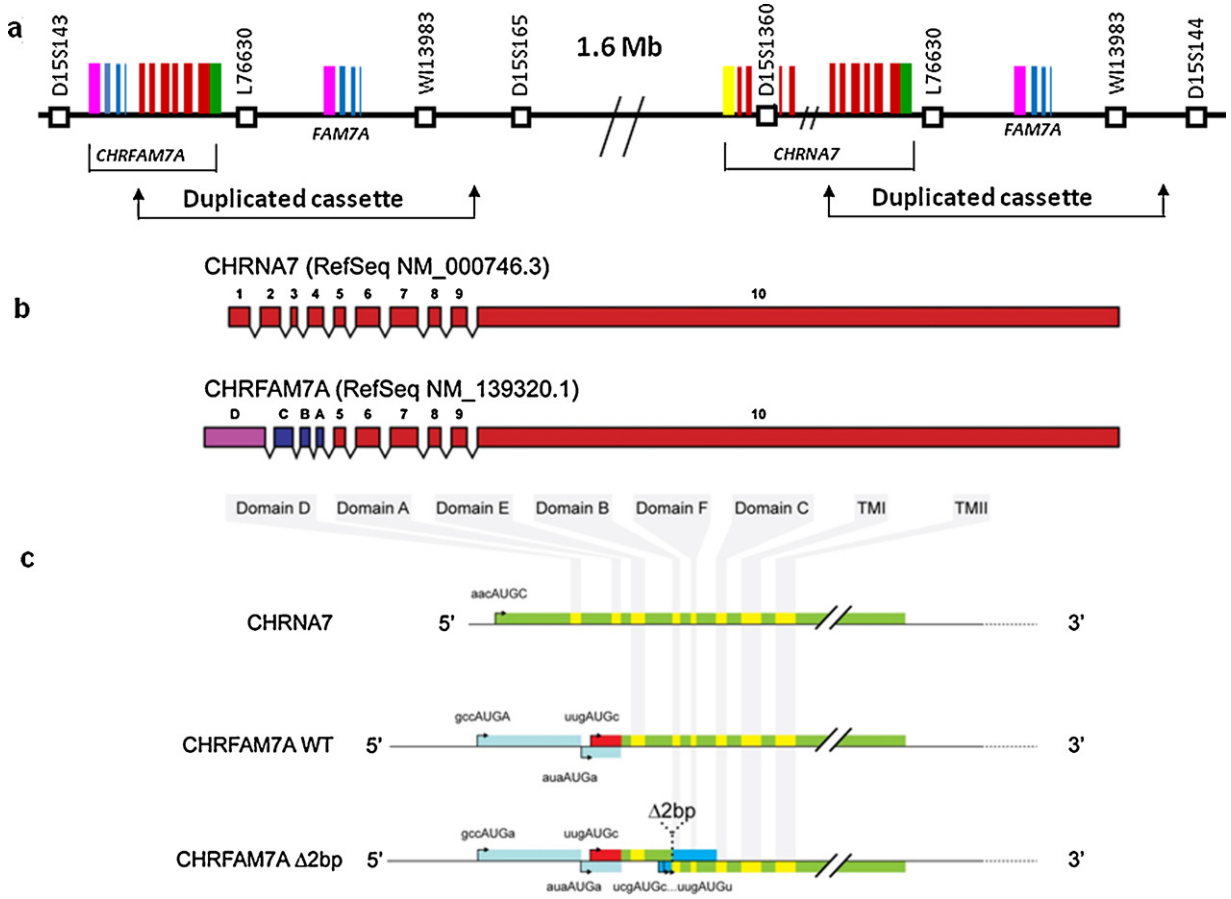

Fig. 1. Structure of the *CHRNA7/CHRFAM7A* gene cluster on chromosome 15q13.3. (a) Map of the partial duplication of *CHRNA7* on 15q13.3. Exons 5–10 of *CHRNA7* were duplicated in a duplicon of ~300 kb, mapping centromeric by 1.6 Mb. The duplicon interrupted a partial duplication of a second gene, *ULK4*. *CHRNA7* exons, red; *ULK4* exons, blue; exon D, pink. (b) Schematic representation of the exon organization of the transcripts coding for *CHRNA7*, *CHRFAM7A*, *CHRFAM7A* based on the RefSeq NM_000746.3 and NM_139320.1. (c) Putative translation products from *CHRNA7*, *CHRFAM7A*, and *CHRFAM7AΔ2 bp* mRNAs. Amino acid sequence of α7 is represented in green and yellow (for the different domains). Alternative amino acids from *CHRFAM7A* are indicated in red and alternative amino acids from *CHRFAM7AΔ2 bp* in blue. The start codons in Kozak context are indicated, as are the stop codons.

of another gene, and maps centromeric to *CHRNA7* by 1.6 Mb [45–47]. The chimeric gene *CHRFAM7A* is complex (Fig. 1). Upstream of Exons 5–10 of *CHRNA7* (red), are three exons from a partial duplication of *ULK4* (C,B,A; blue), a serine/threonine kinase gene mapping to 3p22.1. 5′ of these three exons is an additional exon of unknown provenance (D; pink). *CHRNA7* DNA sequence in *CHRFAM7A* is 99.9% conserved [45]. *CHRFAM7A* is expressed in human brain at low levels (approximately one order of magnitude less than *CHRNA7* in hippocampus), but is expressed abundantly in peripheral lymphocytic cells and in multiple other tissues. While a full phylogenetic study has not yet been done, *CHRFAM7A* is not found in closely related primates [48] and does not appear to be found in rodents (unpublished data). Thus, the duplication is recent. This may not be surprising as *CHRNA7* is one of the oldest ligand-gated ion channel genes and phylogenetic duplication may have contributed to the origin of this large gene family [49–51]. *CHRFAM7A* was recently shown to have a regulatory role that, thus, is unique to humans [52].

1.2. Copy number variation in the chromosome 15q13.3 region

Reports of large copy number variations (CNV) on 15q13.3 that include the *CHRNA7* gene suggest that CNV in this gene cluster is associated with schizophrenia [44,53]. Examination of these reports of duplications and deletions at 15q13.3 show that in many cases *CHRNA7* has been deleted, but the partial duplication, *CHRFAM7A* is present. It has not been known whether copy number of *CHRFAM7A* could be important. There are also smaller insertions and deletions within the *CHRNA7/CHRFAM7A* gene cluster. Approximately 10% of individuals have only one copy of *CHRFAM7A*. This CNV has an allele frequency of 0.08 in Caucasians and 0.12 in African Americans (not significantly different). Rare individuals are missing both copies [45,54]. Again, a common CNV for *CHRFAM7A* would not be unusual if it is a new duplication.

1.3. Mutations in the CHRNA7 and CHRFAM7A genes in schizophrenia

Mutation screening in exons and splice junctions of the *CHRNA7* gene and its partial duplication, *CHRFAM7A* revealed no mutations in the coding region of the full-length gene, *CHRNA7*, that were associated with schizophrenia [45,55]. Therefore, the receptors from *CHRNA7* that are expressed in the disorder have a normal amino acid sequence. However, we did find mutations in the proximal promoter region of the *CHRNA7* gene that functionally reduce transcription and are associated with both schizophrenia and the P50 deficit [42]. The promoter mutations are more prevalent in schizophrenic non-smokers and may account for the low levels of *CHRNA7* mRNA in that group [56]. One of these mutations, rs3087454, has recently been associated with a pharmacogenomic response to DMXB–A, an α7*nAChR partial agonist [57].

We also found a 2 bp deletion in exon 6 that is found only in the *CHRFAM7A* gene [55]. The overall allele frequency of this mutation, *CHRFAM7AΔ2 bp* is 0.22. To evaluate the 2 bp deletion in exon 6 of *CHRFAM7A*, the CNV must be measured in addition to screening for the polymorphism, since ~10% of individuals have only one copy. We have recently completed such a study and find that the 2 bp deletion in exon 6 of *CHRFAM7A* is associated with schizophrenia [54]. Others have found association of the 2 bp deletion to schizophrenia [58] or to sensory processing deficits such as the P50 deficit and antisaccade performance [59–61].

The 2 bp deletion in *CHRFAM7A* is also associated with a gene inversion [47]. The wild type allele is in a head-to-head orientation with respect to the full-length *CHRNA7* gene, but *CHRFAM7AΔ2 bp* is oriented in the same direction. *CHRFAM7AΔ2 bp* is more common in Caucasians than in African Americans, suggesting that the inversion of the gene occurred simultaneously or shortly prior to the 2 bp deletion [54]. The functions of these two versions of the gene are not known.

There is a common, but synonymous, mutation in *CHRFAM7A* at position 654 bp [55]. Of the Caucasian individuals that have been genotyped for both the 2 bp deletion and the SNP at 654 bp, the two mutations appear to be in linkage disequilibrium. However, more subjects need to be genotyped to confirm this finding. An additional common mutation is non-synonymous, 1466 bp C→T, resulting in an amino acid change of a serine to a leucine at aa 489. This amino acid would be in the cytosolic tail. Although a function for this S489L mutation has not yet been defined, a similar mutation that changed a glycine to a serine in *CHRNA7* resulted in phosphorylation differences and alterations in receptor function [62].

1.4. The CHRNA7 gene is regulated differently in schizophrenic smokers, compared to control smokers

A conundrum concerning the expression of the *CHRNA7* gene product, α7*nAChR, exists in schizophrenia. In a microarray comparison of gene expression in postmortem hippocampus of control and schizophrenic smokers and non-smokers, we found that the *CHRNA7* gene is differentially regulated at the mRNA and protein level in schizophrenic smokers [35,56]. Schizophrenic non-smokers have low levels of *CHRNA7* mRNA and protein, compared to controls, but schizophrenic smokers have mRNA and protein levels for *CHRNA7* similar to control smokers. Thus, in schizophrenic smokers there is adequate mRNA and protein [56]. These results are not consistent with the low levels of [^{125}I]-α-bungarotoxin and [^{3}H]-methyllycaconitine binding seen in human postmortem brain of schizophrenic subjects [2,39,40]. The data are consistent, however, with an assembly, trafficking, or binding site defect. In the current report we investigated the possibility that the gene duplication, *CHRFAM7A* regulates the expression and function of *CHRNA7*.

2. Materials and methods

2.1. Cloning of CHRFAM7A and CHRFAM7AΔ2 bp

Generation of a full-length cDNA clone of the duplicated gene, *CHRFAM7A*, was performed by PCR cloning. The exon D–3′ UTR cDNA was generated by PCR amplification of reverse transcribed mRNA derived from human primary lymphocytes, which abundantly express *CHRFAM7A*. Primer sequences were 5′-CTCGGTGCCCCTTGCCATTT-3′ (Sense) and 5′-CCTTGCCCATCTGTGAGTTTTCCAC-3′ (antisense) and were located at position 140 and 1850 from the start of exon D. PCR was performed using a *taq/pfu* polymerase mix with supplied buffers and reagents (Stratagene, La Jolla, CA). After TA cloning, the fragment was moved to pcDNA3.1 (Invitrogen, Carlsbad, CA), a mammalian expression vector.

The 2 bp deletion in exon 6 of *CHRFAM7A* was introduced into pcDNA3.1*CHRFAM7A* by PCR with the QuikChange II XL Site-Directed Mutagenesis Kit, according to the manufacturer's protocols (Stratagene, La Jolly, CA). All clones were sequenced bidirectionally using internal primers and dRhodamine dye terminators on an ABI 3100 Avant DNA sequencer (Applied Biosytems, Inc., Foster City, CA) for confirmation of correct sequence. Clones were designated as pcDNA3.1-CHRFAM7A and pcDNA3.1-CHRFAM7AΔ2 bp.

An α7 full-length clone was also constructed as a control. Primer sequences were 5′-CGCTGCAGCTCCGGGACTCAACATG-3′ (sense) and 5-TGCCCATCTGTGAGTTTTCCACATG-3′ (anti-sense) and were located at position (from start ATG) (−22) and +1638, respectively. PCR fragments were gel purified and TA cloned into

the pcDNA3.1mammalian expression vector (Invitrogen) (pcDNA3.1-CHRNA7).

From the original exon D-10/pcDNA3.1 and α7/pcDNA3.1 cDNA constructs, PCR primers were designed to amplify α7-in frame sequences for expression with a V5 epitope; the 5′ end of the antisense primer terminated just prior to the normal stop codon in exon 10. PCR fragments were purified from 1% agarose and TA cloned into the pcDNA3 Topo/V5 vector (Invitrogen, Carlsbad, CA), transformed, isolated, prepped and sequenced as described above. The clone is designated as pcDNA3.1-CHRFAM7A-V5.

2.2. Cell culture and transfections

SH-EP1 cells were used for heterologous expression studies. SH-EP1 are a non-neuronal epithelial cell line derived from the neuroblastoma cell line SH-SY5Y [63]. Wild-type SH-EP1 cells do not express endogenously detectable α7 mRNA or protein. Cells were cultured in DMEM media supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 1 mM sodium pyruvate, 4 mM L-glutamine, and 100 U/ml penicillin/streptomycin (Gibco Life Technologies/Invitrogen, Rockville, MD) in 5% CO_2 at 37 °C.

Optimal transfection conditions were determined empirically using Green Fluorescent Protein (Invitrogen). Cells (150,000) were seeded in 12-well plates containing 12 mm poly-L-lysine-coated glass coverslips (Becton-Dickinson, Franklin Lakes, NJ). Transfections were performed 24 h after plating using Superfect reagent (Qiagen, Valencia, CA). Briefly, 5–10 μg of DNA was added to an unsupplemented D-MEM in final volume of 150 μl. Superfect (60 μl) was then added and the mixture was allowed to incubate for 10 min at room temperature before the addition of 1 ml of complete media. During DNA complex formation, cells were washed once with phosphate-buffered saline (PBS) and the DNA/Superfect mix was added to the cells. Cells were then incubated for 2 h before the transfection reagent was removed. Cells were washed twice with PBS and returned to the incubator with complete media for 48 h. Forty-eight hours post-transfection, cells were fixed in 4% paraformaldehyde, washed with PBS and permeabilized in successive washes of 10, 95 and 100% ethanol. After washing, coverslips were blocked for 20 min in complete media. Cells were then blocked using 4% goat serum (containing 2% bovine gamma globulin and 0.3% Triton-X100) at room temperature for 1 h. Primary antibodies were mouse anti-V5 (Invitrogen) diluted 1:100 and rabbit anti-α7 (kind gift of Cecilia Gotti, University of Milan) diluted 1:1000. Cells were incubated for 1 h, washed 3× with PBS and secondary antibodies applied. Secondary antibodies were donkey-anti-mouse Cy2 (1:1000) and donkey anti-rabbit tetramethylrhodamine (TRITC) (1:1000) (Jackson Immunochemicals, West Grove, PA) and were incubated for 1 h at room temperature. All coverslips were then washed 3× with PBS, mounted and visualized using appropriate filters on a Microphot-FXA fluorescence microscope (Nikon, Melville, NY).

2.3. Oocyte expression

Oocytes were isolated and selected as previously described [64]. Briefly, oocytes were harvested from mature *Xenopus* laevisfemales, anesthetized with tricaine. Frogs were sacrificed following ethical animal protocolsin Geneva, Switzerland. Oocytes were isolated by mechanical and enzymatic action using type I collagenase (Sigma, Basel Switzerland) at 0.2% in a medium deprived of calcium. Stage V and VI oocytes were placed in 96 wells microtiter plates (NUNC) in BARTH medium that contained 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4{\cdot}7H_2O$, 0.33 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.41 mM $CaCl_2{\cdot}6H_2O$, adjusted to pH 7.4 with NaOH and supplemented with kanamycin (20 mg/ml), penicillin (100 U/ml) and streptomycin (100 U/ml) (Sigma). Nuclear injections of 2 ng of cDNA expression vectors, containing equal molar ratios of the genes of interest were performed with an automatic injection system [65]. Cells were subsequently maintained in Barth's medium at 18 °C.

2.4. Recording of α7* receptor properties

Electrophysiological properties of oocytes were determined 2–5 days after injection with an automated system equipped with a two electrode voltage clamp (Geneclamp Axon Instrument, Molecular Devices, Sunnyvale, CA). Oocytes were continuously superfused with OR2 (control medium) that contained 82.5 mM NaCl, 2.5 mMKCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, adjusted to pH 7.4 with NaOH. Current was generated by 5 s pulses of 200 μM acetylcholine (ACh). Data were filtered at 10 Hz and digitized at 100 Hz with an analog to digital converter (National Instruments, Austin, TX) and stored on a personal computer. Data acquisition, treatment and statistics were performed using Matlab (MathWorks Inc., Natick, MA).[I^{125}]-α-Bungarotoxin assay

Injected oocytes generating a current were selected and were pre-incubated in a solution of OR2 with 2%BSA for 10–20 min. Oocytes were then incubated in 50 μl of OR2-2%BSA with 10 nM [I^{125}]-α-bungarotoxin (Institute of Isotopes Co., Budapest, Hungary) for 1 h at 18 °C. After 3 washes with OR2-2%BSA, the radioactivity of each oocyte was measured with a scintillation counter and normalized to non-injected oocytes.

2.6. RNA isolation from injected oocytes and QRT-PCR quantification of CHRFAM7A and CHRNA7

Oocytes were placed in 1 ml RNAlater (Ambion, Austin, TX) at 4 °C for >24 h. Ten eggs were homogenized for 30 s in 0.5 ml of Trizol (Invitrogen, Carlsbad, CA) using a Pestle Grinder (Fisher Scientific, Pittsburgh, PA). The homogenate was placed on ice for 1 min, then room temperature for 5 min before extraction with chloroform. Following centrifugation at 12,000 rpm for 15 min at 4 °C, the aqueous layer was removed to a new tube. Purification of RNA was done using an RNeasy mini column (Qiagen, Valencia, CA) using manufacturer's protocol. Yield was 0.5–1 μg total RNA per oocyte.

cDNA was synthesized using Superscript 3 (Invitrogen) according to manufacturer's directions. Real-time PCR was performed using human *CHRNA7* primer sets (Forward 5′-TTTACAGTGGAATGTGTCAGA-3′, Reverse 5′-TGTGGAATGTGGCGTCAAG-3′), *CHRFAM7A* (Forward 5′-TGGATAGCTGCAAACTGCGA-3′, Reverse 5′-TACTGGCAATGCCCAGAAGA-3′) and GAPDH (5′-Forward GGTATCGTGGAAGGACTC-3′, Reverse 5′-GGATGATGTTCTGGAGAGC-3′), using iQ SYBR Green (Biorad, Hercules, CA). All three real-time assays were run for 40 cycles; 94 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s.

3. Results

3.1. Structure of the CHRNA7/CHRFAM7A gene cluster

The partial duplication of the *CHRNA7* gene is recent, not being found in primates or rodents [48]. The relationship of the two genes is shown in Fig. 1a. They are approximately 1.6 Mb apart. The region between the *CHRFAM7A* and *CHRNA7* contains two other genes *TRPM1* and *KLF13*. A detailed study, utilizing tagged SNPs across the linkage region at 15q13.3 shows that there is broad association with schizophrenia at this locus [66].

The chimeric gene, *CHRFAM7A*, contains exons 5–10 of the full-length *CHRNA7* gene, exons A, B, and C duplicated from ULK4, and exon D of unknown provenance (Fig. 1a). Exon sizes were

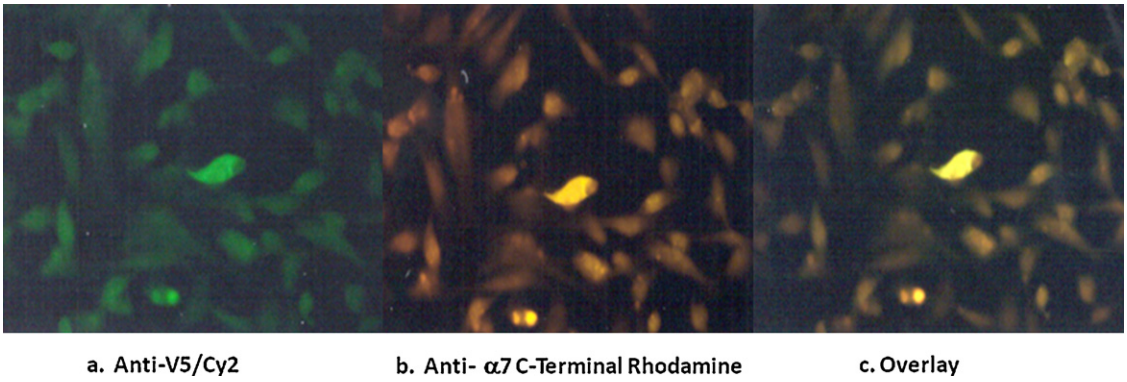

Fig. 2. Translation of *CHRFAM7A*. Cloned *CHRFAM7A-V5* was transfected into SHEP cells. A V5 antibody labeled with Cy2 (green), in a indicates that *CHRFAM7A-V5* protein is present. The anti-α7 antibody and the overlay (b and c) confirm that the peptide has amino acid sequence in-frame with α7 amino acid sequence.

determined: A, 47 bp; B, 63 bp; C, 125 bp; D, 297 bp [45] and the gene was registered in GenBank (AF029837). Mutation screening of the coding regions and exon/intron borders in both *CHRNA7* and *CHRFAM7A* was previously reported [55]. Non-synonymous mutations were rare in *CHRNA7* and were not associated with schizophrenia. Therefore, the receptors from *CHRNA7* that are expressed in the disorder have a normal amino acid sequence.

Several mutations were mapped specifically to *CHRFAM7A* (Gault et al., 2003). A 2 bp deletion in exon six of *CHRFAM7A* (*CHRFAM7AΔ2 bp*) was found. The mutation is common; the overall allele frequency of this mutation is 0.22, and it is more prevalent in Caucasian individuals than in African Americans [55].The *CHRFAM7A* gene also varies in copy number. Approximately 30% of individuals have only one copy and 5% have no copies. Thus to evaluate the 2 bp deletion in exon 6 of *CHRFAM7A*, copy number variation must be measured in addition to screening for the polymorphism. We have recently completed such a study and find that the 2 bp deletion in exon 6 of *CHRFAM7A* is associated with schizophrenia in both Caucasians and African Americans [54]. Others have found association of the 2 bp deletion to schizophrenia [58] or to sensory processing deficits such as the P50 deficit and antisaccade performance[59,60].The 2 bp deletion may change the structure of the protein translated from the mRNA.

3.2. CHRFAM7A is expressed in transfected cells

A cDNA clone of *CHRFAM7A* with a V5 peptide tag (pcDNA3.1CHRFAM7A-V5) was transfected into SH-EP cells, plated on cover slips. An anti-V5 antibody (Invitrogen) was utilized to detect protein product. Fig. 2a shows a positive V5 signal, indicating that the peptide product was transcribed and translated, although it is not abundantly expressed in the transfected cells. An α7 polyclonal antibody to the large cytoplasmic loop of the protein was used in Fig. 2b, labeled with rhodamine. A positive signal and overlap in Fig. 2c shows that the translated peptide contains sequence for the α7 subunit.

3.3. Translation of CHRFAM7A mRNA

Several initiator methionines in exon D could result in truncated transcripts that do not contain *CHRNA7* coding sequence. Use of an initiator methionine in exon B of *CHRFAM7A* would produce a protein including amino acids coded by part of exon B, exon A, and exons 5–10 of *CHRNA7*. The peptide would include the disulfide bridge and vicinal cysteines contained in the amino terminus of the α7 subunit. A putative glycosylation site would be lost and part of the agonist binding site (Fig. 1c). If the 2 bp deletion in exon 6 is present, use of the same initiating methionine would code for a peptide with exon 6 amino acid sequence to the deletion. At that point a frame shift would result in 40 unique amino acids before a stop codon was reached. There are, however, two ATG codons in exon 6. A translation start at one of these would terminate within a few amino acids unless the 2 bp deletion was present. Deletion of these two base pairs would allow translation of a peptide with considerable α7 sequence. The peptide would be out of frame until the 2 bp were reached, coding for either 6 or 13 amino acids, depending on which ATG was used. At that point, the frame shift caused by the 2 bp deletion would return the amino acid sequence to that of the α7 subunit. The peptide resulting from translation of mRNA without the 2 bp, initiating in exon 6, would be missing the signal peptide and nearly the entire binding site, but would contain all of the *CHRNA7* membrane-spanning regions.

3.4. The CHRFAM7A gene product is a dominant negative regulator of α7*nAChR function

The *CHRFAM7A* gene is expressed in both human brain and in the periphery. To study function of this chimeric gene product and effects of the 2 bp deletion in exon 6, we expressed clones of the genes alone and together with a full length *CHRNA7* cDNA in oocytes. *Xenopus laevis* oocytes were isolated and injected as described. A total of 2 ng of cDNA, containing molar equivalent amounts for each gene were injected. Three injection groups were utilized: pcDNA3.1-CHRNA7 + vector, pcDNA3.1-CHRNA7 + pcDNA3.1-CHRFAM7A, and pcDNA3.1-CHRNA7 + pcDNA3.1-CHRFAM7AΔ2 bp. Two to five days after injection, oocytes were superfused with control medium and current, generated by 5 s pulses of 200 μM acetylcholine (ACh), was measured.

Determination of the ACh-evoked current in a large population of oocytes obtained from different batches revealed that expression of the duplicate gene caused a reduction of the response amplitude (Fig. 3a). Currents were reduced by 53% when pcDNA3.1-CHRFAM7A was included. The presence of the 2 bp deletion in exon 6, pcDNA3.1-CHRFAM7AΔ2 bp, further reduced the current amplitude of the full-length gene product by an additional 10%. The difference in current amplitude is best evidenced by plotting the histogram of current distribution for pcDNA3.1-CHRNA7 + pcDNA3.1, pcDNA3.1-CHRNA7 + pcDNA3.1-CHRFAM7A, and pcDNA3.1-CHRNA7 + pcDNA3.1-CHRFAM7AΔ2 bp as shown in Fig. 3b–d, respectively.

3.5. Coexpression of CHRNA7 and CHRFAM7A does not alter transcription from either gene

Total RNA was isolated from oocytes injected with cDNA coding for CHRNA7 and vector or cDNA for *CHRNA7* and either *CHRFAM7A* or *CHRFAM7AΔ2 bp*. Real-time QRT-PCR was utilized to quantify message for *CHRNA7* and *CHRFAM7A* in RNA isolated from each

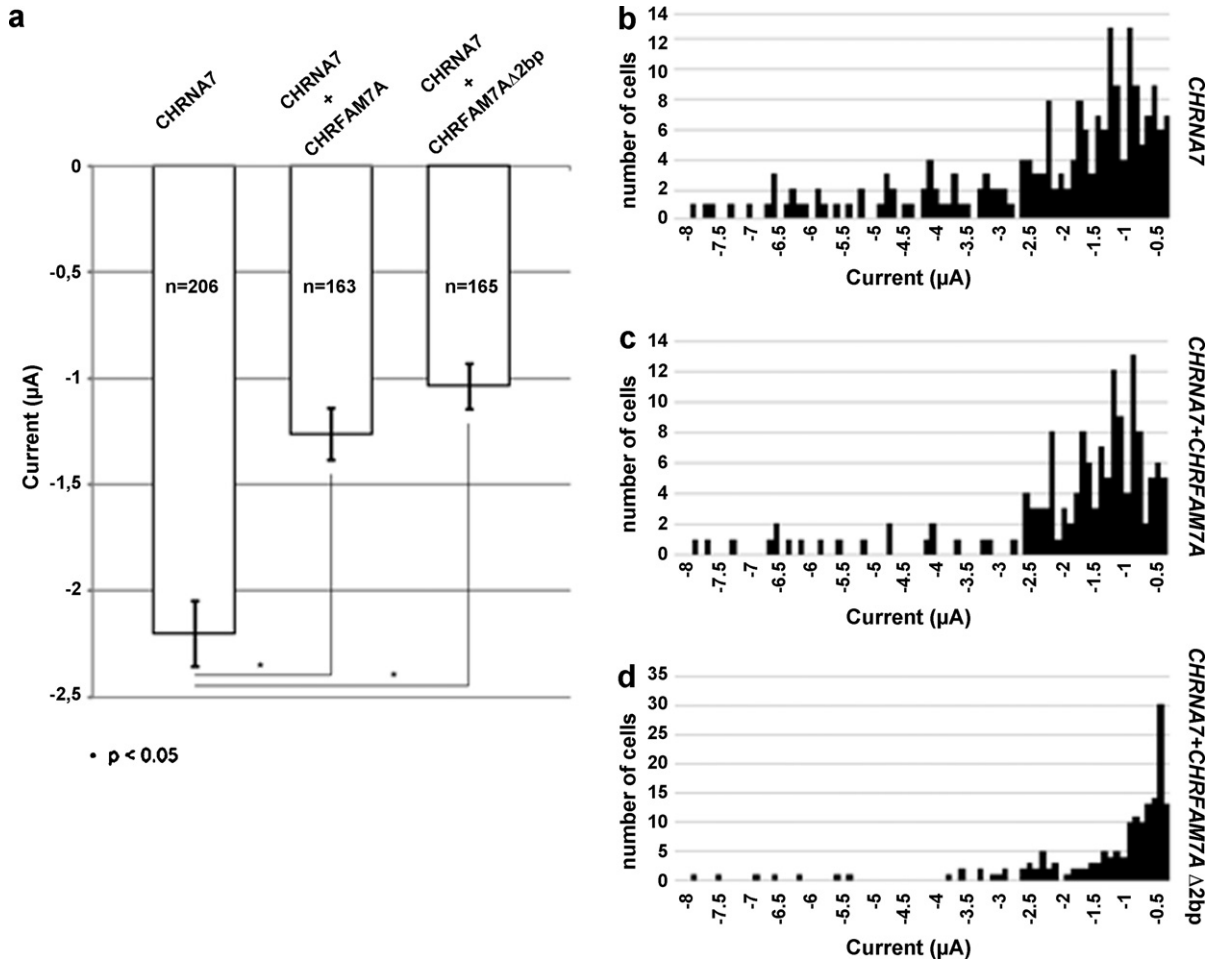

Fig. 3. The *CHRFAM7A* gene product is a dominant-negative regulator of α7*nAChR function. Oocytes were harvested from mature Xenopus females and isolated by mechanical and enzymatic action. Stage V and VI oocytes were placed in 96 wells microtiter plates (NUNC) in Barth medium. Nuclear injections of 1 ng *CHRNA7* and 1 ng of *pcDNA3* or *CHRFAM7A* or *CHRFAM7AΔ2 bp* were made and cells maintained at 18 °C. Electrophysiological properties of oocytes were determined 3 days later with an automated two electrode voltage clamp. Oocytes were continuously superfused with OR2 (control medium) and tested by the application of 200 mM of ACh. (a) Mean of the currents obtained for pcDNA3.1-CHRNA7 + pcDNA3.1, pcDNA3.1-CHRNA7 + pcDNA3.1-CHRFAM7A, or pcDNA3.1-CHRNA7 + pcDNA3.1-CHRFAM7AΔ2 bp; (b) current distribution for pcDNA3.1-CHRNA7 + pcDNA3.1; (c) for pcDNA3.1-CHRNA7 + pcDNA3.1-CHRFAM7A, and (d) for pcDNA3.1-CHRNA7 + pcDNA3.1-CHRFAM7AΔ2 bp.

preparation, with primers specific for each gene. Mean normalized expression was calculated from the control values for the gene of interest and the housekeeping gene, GAPDH, and the amplification efficiencies [67]. As shown in Table 1, expression of the gene duplication, *CHRFAM7A* did not alter the transcription of *CHRNA7*.

3.6. Presence of the duplicated gene product from CHRFAM7A, changes the [^{125}I]-α-bungarotoxin binding properties of α7*nAChR

Oocytes were injected with cDNA for *CHRNA7* + *pcDNA3*.1, *CHRNA7* + *CHRFAM7A*, or *CHRNA7* + *CHRFAM7AΔ2 bp* at a final concentration of 2 ng/μl. After 5–7 days, the amplitude of the current elicited by brief pulses (5 s) of ACh (200 μM) was measured as described in Section 2. Oocytes responding to the ACh test pulse were incubated in OR2-BSA with 10 nM [^{125}I]-α-bungarotoxin (I-BTX) for 1 h. Cells were then thoroughly washed to remove unspecific binding and the radioactivity in each oocyte was measured in a scintillation counter. Non-specific binding was determined by measuring the average I-BTX binding on non-injected oocytes. The histograms of I-BTX binding obtained after subtraction of the non-specific binding obtained for *CHRNA7*, *CHRNA7* + *CHRFAM7A* and *CHRNA7* + *CHRFAM7AΔ2 bp* are shown in Fig. 4a and suggest that cells expressing the duplicate might have a smaller number of binding sites at the cell surface. As there was less current for equivalent binding this is consistent with the hypothesis that the assembly of a truncated subunit, such as the chimeric *CHRFAM7A* or *CHRFAM7AΔ2 bp* with the *CHRNA7* gene product produces a receptor that is less functional. A plot of the peak current versus counts (CPM) yielded linear relationships that were readily fitted by linear regression with high correlation coefficients (*CHRNA7* + *pcDNA3.1*, $R^2 = 0.995$; *CHRNA7* + *CHRFAM7A*, $R^2 = 0.867$; *CHRNA7* + *CHRFAM7AΔ2 bp*, $R^2 = 0.915$ (Fig. 4b).

Table 1
No effect of *CHRFAM7A* on transcription of *CHRNA7*. RNA was isolated from oocytes injected with molar equivalents of either *CHRNA7* and empty vector, or *CHRNA7* and *CHRFAM7A* with or without the 2bp deletion. MNE, mean normalized expression to *GAPDH*.

OOCYTE	MNE	Fold change
CHRNA7 + pcDNA3.1	243.9	1.0
CHRNA7 + CHRFAM7A	245.6	1.0
CHRNA7 + CHRFAM7AΔ2 bp	261.4	1.1

3.7. Potentiation of CHRNA7 and CHRFAM7A by the allosteric modulator PNU-120596

PNU-120596 is a potent allosteric modulator of the α7*nAChR causing an increase of the maximal amplitude of the ACh-evoked current, reduction of the EC_{50} and reduced desensitization [68].

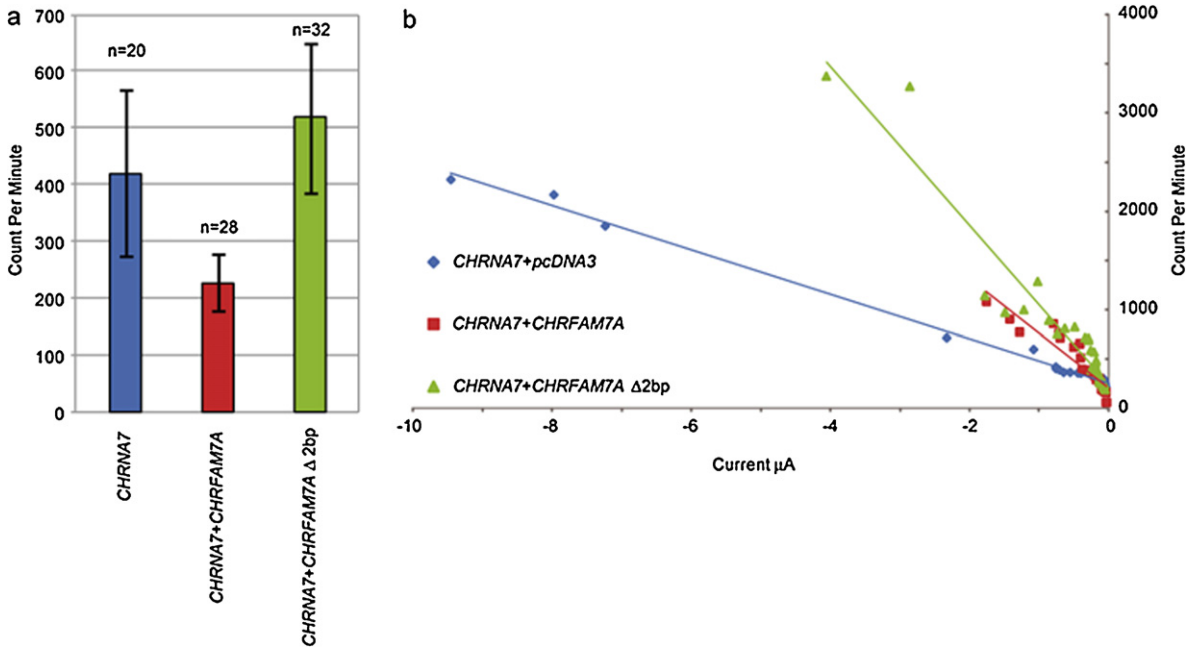

Fig. 4. Plot of the current registered versus the [^{125}I]-α-bungarotoxin binding activity for oocytes injected with plasmid expressing *CHRNA7* with different variants of *CHRFAM7A*. Oocytes were injected with pcDNA3.1-CHRNA7 + pcDNA3.1, pcDNA3.1-CHRNA7 + pcDNA3.1-CHRFAM7A, or pcDNA3.1-CHRNA7 + pcDNA3.1-CHRFAM7AΔ2 bp at a final concentration of 2 ng/μl. On 5 to 7 days after the injection, the current generated by an application of 200 μM of ACh for 5 s was registered by an homemade automated two electrode voltage clamp. Oocytes generating a current were selected and incubated into OR2-BSA with 10 nM [^{125}I]-α-bungarotoxin for 1 h. After 3 washes with OR2-BSA, the radioactivity of each oocyte was measured with a scintillation counter and normalized to non-injected oocytes. (a) Means of binding in each group. (b) Lines represent a linear regression. *CHRNA7 + pcDNA3* ($Y = -225.93X + 249.89$ $R^2 = 0.995$), *CHRNA7 + CHRFAM7A* ($Y = -555.45X + 207.02$ $R^2 = 0.867$), *CHRNA7 + CHRFAM7A*Δ2 bp ($Y = -805.79X + 244.72$ $R^2 = 0.915$).

Previous work suggested that co-expression of the duplicate gene could alter the allosteric modulation of the receptor [52]. To understand the possible relevance of such an observation it must be recalled that the absence of potentiation by PNU-120596 observed in the α7-5HT3 chimera suggested that this molecule must interact with the transmembrane domain [69]. As the duplicate is thought to have a different N-terminal domain from the α7 subunit, but has the same amino acid sequence for the transmembrane domains, PNU-120596 potentiation in the presence of the duplicate in the receptor complex might be expected. To assess the hypothesis, PNU-120596 effects were examined at *CHRNA7*, *CHRNA7 + CHRFAM7A* and *CHRNA7 + CHRFAM7AΔ2 bp*, injected oocytes. Cells injected with an equivalent concentration of the cDNAs encoding the two genes showed non-significant differences in the magnitude of potentiation measured as the ratio of the ACh-evoked current observed after PNU-120596 versus the current evoked by the same ACh test pulse (1280 μM, 5 s) in control (Fig. 5b). As shown in Fig. 5a, exposure to 10 μM PNU-120596 for 30 s causes a dramatic increase in the amplitude of the ACh-evoked current that is accompanied by a significant increase in the response duration that was interpreted as a reduction of desensitization [68]. It could be argued that only receptors composed of *CHRNA7* are potentiated by PNU-120596 and this effect might mask the fraction of receptors that include the duplicate. To address this hypothesis additional experiments were designed in which the ratio of *CHRNA7* versus the duplicate gene was reduced to 1:10. Currents evoked by a brief pulse of 1280 μM ACh for 5 s were measured in control and following 30 s exposure to 10 μM PNU-12056. Average currents measured on the same day in sibling oocytes were respectively 5.8 ± 0.66 μA for *CHRNA7*, 0.9 ± 0.33 μA for *CHRNA7 + CHRFAM7A* and 1.1 ± 0.19 μA for cells injected with *CHRNA7 + CHRFAM7AΔ2 bp*. These data are in agreement with previous experiments indicating that expression of the duplicate gene causes a reduction of the average ACh-evoked current [52]. However, a different picture emerged when examining the amplitude of the ACh-evoked current after PNU-120596 exposure. Peak inward currents were of 28.0 ± 3.3 μA for *CHRNA7*, 12.8 ± 1.23 μA for *CHRNA7 + CHRFAM7A* and 22.0 ± 1.77 μA for cells injected with *CHRNA7 + CHRFAM7AΔ2 bp*. The potentiation caused by PNU-120596 is best observed when plotting the ratio of these values as shown in Fig. 5c.

Determination of the concentration activation curves in control and following PNU-120596 exposure revealed no major difference in sensitivity between the α7 cells and those expressing the duplicate gene. In control conditions the EC_{50}s and Hill Coefficients were: 38 ± 3 μM, nH = 1 for *CHRNA7*; 105 ± 18 μM, nH = 0.8 for*CHRNA7 + CHRFAM7A*; 47 ± 8 μM, nH = 0.9 for *CHRNA7 + CHRFAM7AΔ2 bp*. Following exposure to PNU-120596 the values were: 3.6 ± 0.1 μM, nH = 5 for *CHRNA7*; 5 ± 0.9 μM, nH = 3 for*CHRNA7 + CHRFAM7A*; 3.6 ± 0.3 μM, nH = 3 for *CHRNA7 + CHRFAM7AΔ2 bp*. As expected, exposure to the positive allosteric modulator caused a shift to the left of the ACh sensitivity accompanied by an increase in the Hill coefficient. This suggests that while expression of the duplicate gene causes a reduction of the ACh current amplitude it does not significantly affect the sensitivity of the receptor to its natural ligand.

4. Discussion

The α7*nAChR gene, *CHRNA7*, has been consistently associated with schizophrenia [1,4]. Expression of α7*nAChR, as measured by [^{125}I]-α-bungarotoxin binding, is decreased in postmortem hippocampus, cortex, and the reticular nucleus of the thalamus in schizophrenic subjects [2,39,40]. We recently found that *CHRNA7* mRNA and protein are differentially regulated in postmortem hippocampus of schizophrenics [56]. In schizophrenic non-smokers, the *CHRNA7* mRNA and protein are both decreased compared to controls. However, in schizophrenic smokers, both

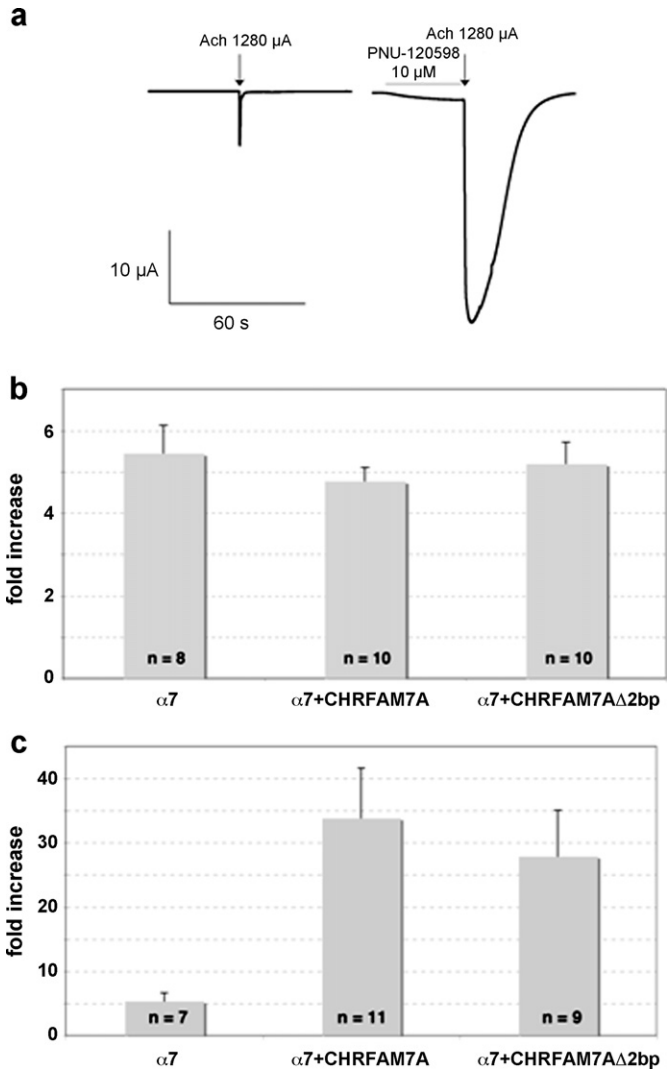

Fig. 5. Potentiation of acetylcholine stimulated currents by PNU-120596. (a)Typical current recorded in an oocyte injected with CHRNA7 + CHRFAM7A (in a 1:1 ratio) evoked by 1280 μM ACh in control and following exposure to 10 μM PNU-120596. Note the large increase in current amplitude and reduction of desensitization. (b) Histogram of the ratio of currents measured following exposure to the positive allosteric modulator versus control (1:1 ratio). (c) Potentiation by PNU-120596 in cells injected with a 1:10 ratio in favor of the duplicate gene. Histogram of the ratio of currents measured following exposure to the positive allosteric modulator versus control. Recordings were performed on the same day in sibling oocytes.

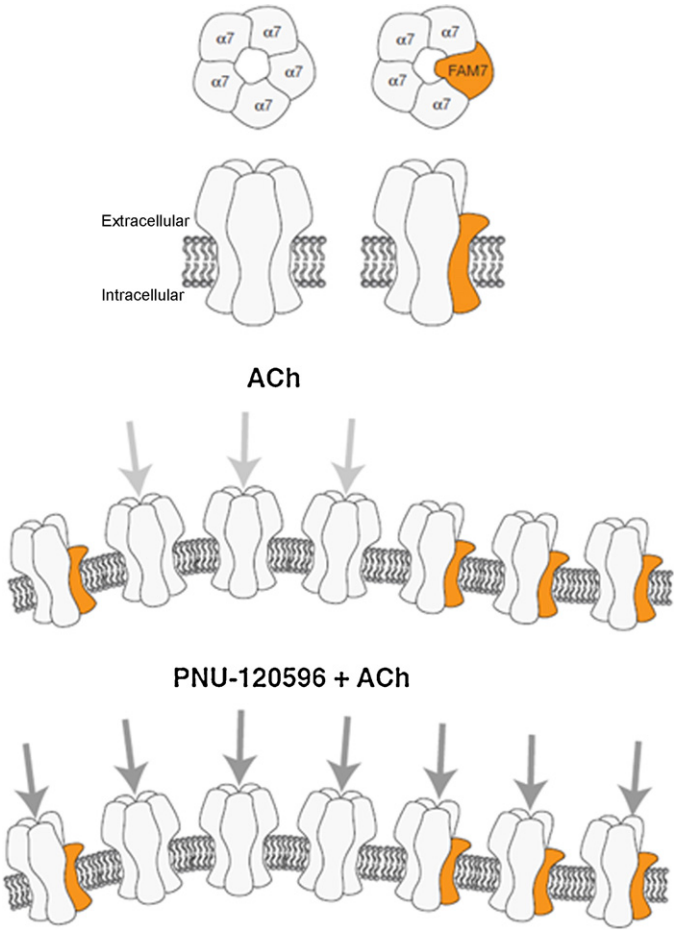

Fig. 6. Putative incorporation of the *CHRFAM7A* gene product into α7*nAChR. The gene product from *CHRFAM7A* would be missing the signal peptide and part of the amino terminal binding site for ligands. A *CHRFAM7AΔ2 bp* product would be missing the entire binding site. As suggested in this figure, there may be steric effects on the other subunits that would alter the ligand binding site. Assuming that a fraction of the receptors comprise α7, whereas another fraction includes the duplicate protein, it can be proposed that ACh activates only the α7 containing receptors. Exposure to PNU-120596 might facilitate the opening of the receptor causing larger inward currents and increase in the PNU-120596 potentiation.

mRNA and protein return to control levels. Thus, in schizophrenic smokers, there is adequate α7 protein, but low bungarotoxin binding, suggesting a problem with assembly or trafficking of the receptor.

There are multiple mechanisms for regulation of α7*nAChR expression. Mutations in the proximal promoter decrease transcription of *CHRNA7* and are associated with both schizophrenia and with the P50 deficit in the disorder [42]. Another mutation 1831 bp from exon 1 is associated with schizophrenia [70], and with an improvement in the fMRI default network after treatment with DMXB-A, an α7*nAChR agonist [57]. Additional complexity is introduced by the partial duplication of *CHRNA7*, which results in the formation of a chimeric gene, *CHRFAM7A* [45–47]. Large deletions of 15q13.3, the map locus of both *CHRNA7* and *CHRFAM7A* have been associated with schizophrenia [44,53]. In many of these patients, the *CHRNA7* gene is deleted, but the *CHRFAM7A* gene remains. We also found that a 2 bp deletion in exon 6 of the duplication *CHRFAM7A*, but not in the full-length gene, *CHRNA7*, is associated with schizophrenia [54]. This deletion is associated with the P50 deficit [60] and with a chromosomal inversion of the *CHRFAM7A* gene [47]. There is a copy number variation for *CHRFAM7A*; approximately 10% of individuals have only one copy and 5% have no copies [45,54]. A recent study suggested that the *CHRFAM7A* gene may be a dominant-negative regulator of α7*nAChR function [52]. These investigators found that co-expression of α7 subunits and the *CHRFAM7A* gene product dupα7 in oocytes resulted in decreased ACh stimulated current.

In the current study, we have examined the effects of both the *CHRFAM7A* gene and the gene copy with the 2 bp deletion in exon 6, *CHRFAM7AΔ2 bp*, on function of the full-length gene product of *CHRNA7* in an oocyte expression system. Our results confirm the de Lucas-Cerrillo et al. study [52] and show that the 2 bp deletion in exon 6 further decreases function when co-expressed with the α7 subunit. Thus copy number variation in *CHRFAM7A* and presence of the 2 bp deletion are likely to be factors when considering the overall activity of α7*nAChR in human subjects. Rodents and primates apparently do not have such a chimeric gene, suggesting that it is a relatively new duplication [47,54,71].

The mechanism by which *CHRFAM7A* acts as a dominant negative regulator of α7*nAChR function is not clear. *CHRFAM7A* is missing *CHRNA7* exons 1–4, including the signal peptide, and does not appear to assemble alone (not shown). Leukocytes express a

protein, identified with an α7 antibody, but are not labeled by I-BTX, suggesting that the peptide expressed is from *CHRFAM7A* and that functional receptors are not assembled nor do they migrate to the surface [72]. Thus, co-expression of the *CHRFAM7A* and *CHRNA7* gene products could result in receptor sequestration in the endoplasmic reticulum. Further, *CHRFAM7A* mRNA is down regulated by nicotine [52] and by bacterial infection [73].

Our [^{125}I]-α-bungarotoxin experiments also suggest another alternative; the binding sites for bungarotoxin and other ligands may be altered. When only the α7 gene product was expressed, current increased commensurate with bungarotoxin binding, a measure of receptor number. Moreover, since labeling was carried out on intact oocytes, labeling corresponds to receptors expressed in the plasma membrane. When the *CHRFAM7A* gene product was present, bungarotoxin binding increases showed a significantly steeper correlation slope. The increase in bungarotoxin binding for an equivalent current suggests the presence in the membrane of receptors that cannot be activated by ACh. Putative translation start sites for *CHRFAM7A* and *CHRFAM7AΔ2 bp* and possible structures of the assembled receptor are illustrated in Fig. 1 and Fig. 6. Translation of *CHRFAM7A* mRNA likely starts in exon B and is in frame through the *CHRNA7* sequence. The peptide is missing the signal peptide and two of the glycosylation sites, retaining the cysteine bridge and vicinal cysteines. It is, thus, missing domains A and D of the binding site [74]. The 2 bp deletion in exon 6 would result in a truncated receptor, not likely to assemble with α7 subunits, if translation of *CHRFAM7AΔ2 bp* mRNA begins in exon B. A translation start from either of the two AUG codons in exon 6 would result in an open reading frame with *CHRNA7* sequence after the 2 bp deletion through exon 10, but the amino terminus would be missing domains E and B in addition to domains A and D. The vicinal cysteines and the cysteine bridge would be absent. We speculate that this could result in an assembled receptor resembling that in Fig. 6. The lack of steric association in the upstream amino termini of the assembled subunits might lead to some instability, changing the receptor properties.

Interestingly, the de Lucas-Cerrillo et al. study [52] indicates that expression of the duplicate does not modify the receptor sensitivity to ACh. As it is known that the ligand binding site lies at the interface between two adjacent subunits, reviewed in [74,75], the incorporation of the duplicate should not contribute to the formation of distinct ACh binding sites. The stoichiometry of wild type α7 versus the duplicate in a single receptor is unknown; some receptor complexes might, thus, incorporate more than one duplicate subunit. Such receptors may display distinct properties and be less activatable by ACh. To evaluate this hypothesis, experiments were carried out using the positive allosteric modulator PNU-120596 [68]. Evaluation of the potentiation caused by brief exposure to 10 μM PNU carried out in sibling oocytes injected with *CHRNA7* and *CHRFAM7A* genes showed no detectable differences when genes were injected in a 1:1 ratio. However, a marked difference was observed when the gene ratio was changed to 1:10 in favor of the duplicate. Cells expressing this gene ratio showed a significantly higher potentiation by PNU-120596 suggesting the presence of otherwise silent receptors. Although our observations differ from those reported by de Lucas-Cerrillo et al. [52], these authors restricted their study at 1 μM PNU-120596 which, as shown in Fig. 1 of the original work published by Hurst and collaborators [68], is in the lowest part of the concentration activity curve for this compound.

Experimental evidence indicates that PNU-120596 interacts with transmembrane domains of the receptors [69]. As the duplicate gene encodes an equivalent amino acid sequence in the transmembrane segments, it can be postulated that receptors containing the duplicate must have an equivalent pore formation and transmembrane domain organization. It is therefore likely that an allosteric modulator interacting through the transmembrane domain must remain efficacious in receptors incorporating the duplicate gene product.

The use of an allosteric modulator interacting with the transmembrane domains presents several advantages versus orthosteric ligands that are known to bind at the interfaces between N-terminal domains of the subunits. Namely, as it was shown that expression of α7 with the duplicate causes no changes in the ACh sensitivity, it can be speculated that some α7-α7 interfaces are conserved with complexes comprising the duplicate. This hypothesis is further supported by the fact that these receptors are still able to bind α-bungarotoxin and, in view of the marked difference observed in the amino-acid sequence of the duplicate N-terminal domain, it is unlikely that this subunit can contribute an identical principal or complementary binding site. The most likely explanation is therefore that receptors incorporating the duplicate maintain enough α7-α7 interfaces to keep most of the pharmacological properties of the receptors. Our transfection results in Fig. 2 suggest that *CHRFAM7A* is not abundantly expressed, which may result in more α7-α7 interfaces than α7-duplicate interfaces. Furthermore, it is important to recall some of the unique features of the α7*nAChR and their modulation by endogenous proteins such as Lynx-1 or SLURP-1 [76–79]. Although it can be speculated that such modulation is likely to remain present in receptors containing the duplicate, further investigation into the pharmacological properties of *CHRFAM7A* is needed. It is not known whether *CHRFAM7A* expression is altered in schizophrenia or by the use of tobacco products in these patients. Based on the data presented here, one hypothesis for the decreased I-BTX binding in schizophrenic postmortem brain could be overexpression of *CHRFAM7A* or *CHRFAM7AΔ2 bp*.

As a strong regulator of α7*nAChR function, *CHRFAM7A* holds some promise as a drug target. The *CHRNA7* gene is not only associated with mental illness, but plays a role in inflammation where it is protective and seems to prevent cytokine expression following an inflammatory event [37,80–82]. Maternal infection is a known risk factor for development of neuropsychiatric disorders, including schizophrenia [83–85]. Agonists, specific for α7*nAChR protect against inflammation [86]. Recently lipopolysaccharide, a surrogate for bacterial inflammation was shown to down regulate *CHRFAM7A* [73]. Nicotine also down regulates *CHRFAM7A* expression [52]. Since the *CHRFAM7A* gene product is a dominant negative regulator, decreasing its expression may be beneficial for treatment of both mental illness and inflammation. Additionally, the development of new Type II positive allosteric modulators for the α7*nAChR might open new therapeutic avenues.

Acknowledgements

An antibody for the α7 subunit was kindly provided by Dr. Cecilia Gotti, University of Milan. The authors thank Margaret Short for technical help with this project. The work was supported by National Institutes of Health grants MH81174, DA09457, and the Veterans Affairs Medical Research Service to S.L., and EEC grant Neurocypres to D.B.

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