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CHRFAM7A alters Binding to the Neuronal alpha-7 Nicotinic Acetylcholine Receptor

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Abstract

Introduction: CHRFAM7A is a uniquely-human gene that encodes a human-specific variant of the alpha-7 nicotinic acetylcholine receptor ($\alpha 7nAChR$). While the homopentameric $\alpha 7nAChR$ consists of 5 equal subunits, previous studies demonstrated that CHRFAM7A expression disrupts the formation of $\alpha 7nAChR$ homopentamers. Here we use a rat neuronal cell line expressing CHRFAM7A and a transgenic mouse expressing CHRFAM7A to define the alpha-bungarotoxin (α -BTX) binding in vitro and in vivo.

Methods: Rat PC12 cells were stably transfected with human CHRFAM7A. α -BTX, a protein that irreversibly binds the $\alpha 7nAChR$, was utilized to assess the capacity for CHRFAM7A to interfere with $\alpha 7AChR$ subunits using immunohistochemistry and flow cytometry. To evaluate the effects of CHRFAM7A on $\alpha 7nAChR$ at the neuromuscular junction in vivo, transgenic mice were engineered to express the uniquely human gene CHRFAM7A under the control of the EF1- α promoter. Using this model, muscle was harvested and CHRFAM7A and CHRNA7 gene expression evaluated by PCR. Binding of α -BTX to the $\alpha 7nAChR$ in muscle was compared in sibling-matched wild-type C57 mice by immunostaining the neuromuscular junction using α -BTX and neurofilament antibodies.

Results: Expression of CHRFAM7A in transfected, but not vector cells, was confirmed by PCR and by immunoblotting using an antibody we raised to a peptide sequence unique to CHRFAM7A. CHRFAM7A decreased α -BTX binding as detected by immunohistochemistry and flow cytometry. In vivo, α -BTX co-stained with neurofilament at the neuromuscular junction in wild-type mice, however, α -BTX staining was decreased at the neuromuscular junction of CHRFAM7A transgenic mice.

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Conclusion: CHRFA7A expression interferes with the binding of $\alpha 7$ nAChR to α -BTX.

Understanding the contribution of this uniquely human gene to human disease will be important in the identification of potential therapeutic targets.

Keywords

duplicated nicotinic acetylcholine receptor; CHRFA7A; human-specific genes; α -bungarotoxin; transgenic mouse; neural $\alpha 7$ nAChR

Introduction:

First discovered in the 1990s, the $\alpha 7$ subunit of the nicotinic Acetylcholine receptor ($\alpha 7$ nAChR), encoded by the CHRNA7 gene on chromosome 15, is a homopentamer with both transmembrane and cytoplasmic domains [6]. The $\alpha 7$ nAChR is widely expressed in the human nervous system and has been linked to neurocognitive function including memory and attention as well as neuropsychiatric disorders such as schizophrenia [5]. Therefore, the $\alpha 7$ nAChR has long been considered a potential therapeutic target for neurologic disease.

Interestingly, humans express a variant of CHRNA7 called CHRFA7A located 1.6Mb upstream of the CHRNA7 gene. Uniquely human CHRFA7A is a partial duplication and rearrangement of CHRNA7 consisting of a duplication of exons 5–10 fused to 4 new exons duplicated from chromosome 3 and encoding 27 amino acids [13]. We have previously shown that CHRFA7A is widely expressed and has its own promoter regulating expression of a functional open reading frame that produces a protein product [9]. Although CHRFA7A expression has been associated with neurocognitive disorders including schizophrenia, psychosis, bipolar disorder, autism, and dementia [1, 3, 15, 21], the effects of CHRFA7A expression on $\alpha 7$ nAChR binding in the nervous system remains largely unknown.

Based on its similarity to the CHRNA7 gene, several investigators have demonstrated that CHRFA7A alters the assembly and function of the $\alpha 7$ nAChR complex by forming a heteropentamer of $\alpha 7$ nAChR/CHRFA7A subunits that alter ligand binding to the receptor [8, 19]. Here, we hypothesize that the expression of CHRFA7A would alter binding of α BTX to the $\alpha 7$ nAChR in a neural cell line *in vitro* and at a neuromuscular junction *in vivo*. Based on the human variability in CHRFA7A expression [9], understanding the effects of CHRFA7A expression on binding to the $\alpha 7$ nAChR may have important effects on the response to therapeutics targeting the $\alpha 7$ nAChR.

Material and Methods:

PC12 culture

PC12 cells were obtained from ATCC (Manassas, VA) and maintained at 37°C (5% CO₂) in RPMI-1640 medium supplemented with 5% fetal bovine serum, 10% horse serum, 1% penicillin/streptomycin (Gibco). Medium was changed every 2–3 days.

PC12 transfection with CHRFA7A vector

PC12 cell lines stably expressing CHRFA7A gene or vector were created using Lenti-X Packaging Single Shots system according manufacture (Clontech). Briefly, 7 μ g of pLVX-IRES-ZsGreen1 lentiviral vector encoding CHRFA7A gene or vector only was mixed with Lenti-X Packaging plasmids to transfect Lenti-X 293T cells in 10 cm plate. After 48 hours, lentiviral particles were collected and used immediately to infect PC12 cells for stable expression with ratio of 1 part of lentiviral medium to 3 parts of fresh culture medium. Three to four days after infection, the cells were sorted by fluorescence-activated cell sorting (FACS) for ZsGreen positive population (GFP+), and expanded in regular PC12 culture medium.

PCR for CHRNA7 and CHRFA7A

RNA was prepared using Direct-zol RNA miniprep plus kit (Zymo Research, Irvine, CA) including DNase treatment. Total of 2 μ g of total RNA was submitted for cDNA generation using the BioRad Reverse Transcriptase Kit according to manufacturer's directions (Hercules, CA); and 1 μ l of cDNA reaction product was used per reaction for PCR. Expression levels of CHRNA7 and CHRFA7A mRNAs were measured by PCR (Bio-Rad iQ5, Hercules, CA); using the SYBR Green gene expression assays (OriGene, Rockville, MD). PCR rat *CHRNA7* and rat *GAPDH* primers were obtained from Quantitect; *CHRFA7A* specific primers were Forward 5'-ATAGCTGCAAACCTGCGATA-3', Reverse 5'-CAGCGTACATCGATGTAGCAG-3'. For PCR experiments, Platinum blue PCR super mix was used for 35 cycles; 94°C for 20 sec, 56.5°C for 30 sec, 72°C for 1 min. PCR of muscle for CHRNA7 and CHRFA7A was performed as described above.

Immunoblotting

Total protein extracts were prepared using 10×10^6 cells. Briefly, PC12 cells were collected by centrifugation for 5 minutes at 1000 rpm in 4°C. The pellets were resuspended in 300 μ l of Triton X-100-containing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, protease and phosphatase inhibitors (Sigma-Aldrich Co.), 5 mM DTT and 0.2 mM PMSF) and sonicated at moderate speed on ice (three times for 20 seconds with 30 second intervals). The protein was extracted after 30 minutes incubation on ice by centrifugation at 13000 rpm for 15 minutes. Cell protein concentrations were measured using the BCA method. Fifty μ g of total PC12 protein extracts, and a standard molecular weight marker (New England Biolabs) were size-fractionated on 4–12% SDS-PAGE mini-gel and transferred to a PVDF membrane (Invitrogen, #LC2005). The membranes were incubated for 1 hour with blocking buffer (5% BSA in TBS with 0.1% Tween - TBST), following incubation with anti-CHRFA7A (1 μ g/ml). For creation of a specific antibody for CHRFA7A, antibodies to the 27 amino acid peptide that differentiates CHRFA7A from CHRNA7 were raised in rabbits by contract with GenWay (San Diego, CA), affinity purified by the supplier, and titers measured by ELISA with the synthetic peptide. Specificity of the antibody for CHRFA7A and its inability to cross-react with the α 7nAChR was established by immunoblotting transfected cells, respectively. Anti- β -Actin was used as a loading control (dilution 1:1000, Cell Signaling Technology #8457). After being washed three times in TBST, the membranes were incubated for 1 hour using a secondary with a corresponding

HRP-linked antibody. After three further washes in TBST and two washes in H₂O, signals were developed using Super Signal West Femto (Pierce Biotechnology Inc) on CL-XPosure film (Thermo, Rockford, IL).

Immunohistochemistry

PC12 cells expressing CHRFA7A or parental PC12 cells were plated on collagen 1 coated coverslips in 6 well at 5×10^4 cells per well. Following day live cells were directly stained with α -Bungarotoxin (α -BTX)-tetramethylrhodamine (Sigma, conc: $1 \mu\text{g}/\text{ml}$) in PBS for 30 minutes at RT in dark. After three washes in PBS for 5 minutes, exposure matched images (40x, 1/3 second exposure) were taken using FSX100 inverted microscope equipped with contrast optics (Olympus).

Measurement of Fluorescence

Changes in α -BTX binding levels were also confirmed by measuring fluorescence on a Fluostar Omega Fluorimeter using 544/ 590 excitation and emission filters. In this instance, 2×10^5 PC12 stably expressing vector GFP or CHRFA7A cells were live-stained with 0 or $1 \mu\text{g}/\text{ml}$ of α -BTX-tetramethylrhodamine (Sigma) in PBS for 30 minutes at RT in dark. After two washes in PBS, cells were transferred onto 96 well plate (Corning, CLS3915) for fluorescence measurement. Final fluorescence intensity was normalized by individual cell count.

Flow cytometry

For flow cytometry analysis, 5×10^5 of PC12-CHRFA7A or vector GFP expressing cells were collected and washed once with PBS. Live cells were directly stained with Alexa Fluor® 647 α -BTX (ThermoFisher $1 \mu\text{g}/\text{ml}$) in PBS for 30 minutes at RT in dark. In some instances, cells were also co-treated with 0 or $50 \mu\text{M}$ nicotine in four replicates per condition for 30 minutes in incubator. The cells were washed three times with PBS and analyzed by flow cytometry. α -BTX binding intensity was quantified by using Flowjo software (TreeStar, Oregon) on the GFP positive cell population.

α -bungarotoxin binding at the neuromuscular junction

Transgenic mice were produced by contract with Cyagen Biosciences (Santa Clara, CA) with CHRFA7A regulated by the EF1 α promoter in a C57BL/6 mouse background. Each mouse was genotyped to confirm the presence of CHRFA7A by the contractor and colonies established with breeding pairs at UC San Diego. Animal housing and experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee. Control mice used for experimentation were sibling-matched wild-type (WT) C57BL/6 mice.

Gastrocnemius muscle neuromuscular junction staining for α -BTX binding was performed as previously described[14, 23]. Briefly, mice were anesthetized and euthanized by cervical dislocation. Gastrocnemius muscle was gently dissected over ice immediately after autopsy and the tissue fixed in 4% paraformaldehyde for 20 minutes. The muscle tissue was then incubated with α -BTX conjugated to tetramethylrhodamine (TMR) (Sigma-Aldrich T0195 $1 \mu\text{g}/\text{mL}$). Tissues were then submerged in methanol at -20°C for 5 min and subsequently

blocked with 1 mg/mL BSA, 0.1% triton in PBS for 1 hour. To help localize neuromuscular junctions, an anti-neurofilament primary (Rabbit; Sigma-Aldrich N4142 1:500) was used in an overnight incubation at room temperature. Tissues were then exposed to an AlexaFluor 488 conjugated secondary antibody (Rabbit; Invitrogen A11008 1:400) for 4 hours. All gastrocnemius muscle samples were mounted and affixed with SlowFade (Invitrogen S36936) for confocal imaging. All WT and CHRFAM7A images were exposure-matched. Confocal images were captured with an Olympus FluoView 1000 (ASW 1.7b) laser scanning confocal microscope equipped with a 20x/0.7NA dry objective lens on a BX61 microscope (Olympus).

Statistical Analysis

Values are expressed as the mean and standard deviation where n represents the number of animals in each experimental group. The statistical significance between groups was determined using a two-tailed student t test. A *p* value < 0.05 was considered statistically significant.

Results:

To evaluate the effects of CHRFAM7A on $\alpha 7$ nAChR binding, we performed stable transfection of CHRFAM7A in rat neural cells that have no endogenous expression of this gene as it is unique to humans. Next, we used PCR to confirm that CHRFAM7A gene expression was present in transfected cells, with no CHRFAM7A expression seen in vector cells (Fig. 1A). Both vector and CHRFAM7A cells express endogenous CHRNA7, the gene that produces the $\alpha 7$ nAChR (Fig 1B). We verified that insertion of the CHRFAM7A gene resulted in protein production by immunoblotting with an antibody raised to a peptide sequence unique to CHRFAM7A (Fig. 1C). As expected, no CHRFAM7A protein expression was seen in vector cells.

To visualize binding to the $\alpha 7$ nAChR, cells were stained with α -BTX that binds $\alpha 7$ nAChR with high specificity [12], and exposure-matched images were viewed by microscopy. Cells were also stained with DAPI to visualize the cell nucleus. Vector cells demonstrated uniform staining with α -BTX showing binding to $\alpha 7$ nAChR (Fig. 1D, top row). Cells expressing CHRFAM7A, however, demonstrated decreased α -BTX staining compared to vector cells (Fig. 1D, bottom row). Fluorescence of cells stained with α -BTX was also directly measured in transfected cells to demonstrate that introduction of CHRFAM7A decreases α -BTX binding to the $\alpha 7$ nAChR compared to vector cells (Fig. 1E).

To further quantify the effects of CHRFAM7A expression on $\alpha 7$ nAChR subunits, α -BTX binding was measured using flow cytometry (Fig. 2A). Transfection of cells with CHRFAM7A decreased α -BTX binding compared to vector (Fig. 2B). Next, cells were pre-incubated with nicotine to compete for binding to the $\alpha 7$ nAChR to assess the specificity of α -BTX binding in CHRFAM7A transduced cells. As expected, pre-incubation with nicotine prevented α -BTX binding in vector PC-12 cells. Nicotine also decreased α -BTX binding in CHRFAM7A transduced cells (Fig. 2C). This suggests that the expression of CHRFAM7A decreased the number of functional $\alpha 7$ nACh receptors but did not completely abrogate

$\alpha 7$ nAChR ligand binding (Fig. 2D). These findings demonstrate that CHRFAM7A decreases ligand binding to the neural $\alpha 7$ nAChR *in vitro*.

To evaluate the effects of CHRFAM7A expression on $\alpha 7$ nAChR ligand binding *in vivo*, we created transgenic mice expressing uniquely human CHRFAM7A. Muscle was harvested from CHRFAM7A transgenic mice and WT siblings to confirm CHRFAM7A gene expression in transgenic but not WT animals (Fig. 3A). CHRNA7 gene expression was seen in both WT and CHRFAM7A transgenic animals (Fig. 3B). To evaluate binding to the $\alpha 7$ nAChR at the neuromuscular junction, muscle was dissected and stained with α -BTX. Exposure matched images were viewed using confocal microscopy (Fig. 3C, red). Muscle tissue was also costained with a neurofilament specific antibody to visualize neurons and confirm their colocalization with α -BTX at the neuromuscular junction (Fig 3C, green). Sections from WT animals demonstrated staining with α -BTX that co-stained with neurofilament at the motor endplate. Muscle from CHRFAM7A transgenic mice, conversely, demonstrated decreased α -BTX binding at the neuromuscular junction compared to WT. These findings confirm that CHRFAM7A expression decreased $\alpha 7$ nAChR ligand binding *in vivo*.

Discussion:

In humans, the $\alpha 7$ nAChR is expressed both pre- and post-synaptically in neuronal and non-neuronal cells where it has been implicated in diverse physiologic functions ranging from cognitive function to the regulation of the inflammation response [4, 8, 10]. Uniquely human CHRFAM7A is a partial duplication and rearrangement of the CHRNA7 gene that encodes the $\alpha 7$ nAChR. Previous data suggests that CHRFAM7A expression may alter the structure of the $\alpha 7$ nAChR [16]. CHRFAM7A expression is highly variable across individuals suggesting that $\alpha 7$ nAChR function may be variable as well [9]. Currently, the effects of CHRFAM7A on $\alpha 7$ nAChR ligand binding are poorly understood.

Previous studies have considered the potential of CHRFAM7A to alter $\alpha 7$ nAChR function in heterologous systems [22], however, the effects of CHRFAM7A on $\alpha 7$ nAChR ligand binding in a mammalian system that endogenously expresses CHRNA7 have previously been undefined. Here, we demonstrate that CHRFAM7A expression decreases α -BTX binding using both *in vitro* and *in vivo* models. These are the first studies to utilize a transgenic animal model to evaluate the effects of human CHRFAM7A when expressed in mouse.

Araud, et al. previously evaluated the ability of CHRFAM7A expression to alter $\alpha 7$ nAChR channel function using a heterologous system utilizing oocytes harvested from *Xenopus* that underwent nuclear injection of both CHRNA7 and CHRFAM7A[2]. They performed voltage clamp experiments and found that calcium evoked current was decreased in cells injected with both CHRFAM7A and CHRNA7 compared to cells injected with CHRNA7 alone. Based on these results, the authors concluded that if CHRFAM7A is not a pseudogene, its expression could have dominant negative effects on $\alpha 7$ nAChR function.

This study was followed shortly thereafter by de Lucas-Cerrilo, et al. who used a similar *Xenopus* oocyte model to evaluate α -BTX binding [11]. After injection of oocytes with α 7nAChR mRNA alone or in combination with CHRFAM7A mRNA they found that α -BTX staining was decreased in cells expressing both α 7nAChR and CHRFAM7A compared to α 7nAChR alone and concluded that the presence of CHRFAM7A decreases the number of functional α 7nAChR. More recently, studies evaluating the effect of CHRFAM7A on α 7nAChR binding have demonstrated that while both CHRFAM7A and α 7nAChR are expressed simultaneously, the physical interaction between the CHRFAM7A and α 7nAChR subunits results in a heteropentamer that can be trapped in the endoplasmic reticulum and decreases the expression of functional α 7nAChR homopentamers that can bind their ligand [18].

Lasala, et al. also demonstrated that CHRFAM7A expression alters the assembly of the α 7nAChR homopentamer in mammalian BOSC-23 kidney cells [16]. Utilizing a human kidney cell line, they showed that CHRFAM7A expression resulted in heteromeric receptors containing combinations of α 7 and CHRFAM7A subunits. Using patch clamp analysis to measure single channel function, they showed that at least two α 7 subunits are required for channel function and that CHRFAM7A co-expression could decrease ligand binding in a subunit dependent fashion. Here, we demonstrate that CHRFAM7A expression alters binding to the α 7nAChR in neuronal cells. α -BTX binding is decreased in cells expressing CHRFAM7A, however the ability to further limit α -BTX binding by pre-incubation with the α 7nAChR ligand nicotine demonstrates that some α 7nAChR binding in the presence of CHRFAM7A remains functional.

The α 7nAChR is widely expressed in the brain in areas ranging from the cerebral cortex to the hippocampus to the dorsal motor nucleus of the vagus nerve [7]. Impaired function of the α 7nAChR in the brain has also been linked to neurocognitive disorders including schizophrenia, depression, and autism spectrum disorders [17]. The ability of CHRFAM7A, which is also widely expressed in the brain to potentially alter α 7nAChR function and contribute to these neurocognitive disorders is critical to understand the pathogenesis of these diseases [20]. Similarly, these findings could be critical in the development of therapeutics targeting the α 7nAChR.

Conclusions:

We demonstrate that CHRFAM7A decreases ligand binding to the neuronal α 7nAChR *in vivo* but further studies will be required to determine the biological activity of CHRFAM7A and its potential as a target for therapeutic interventions to modulate α 7nAChR-mediated signaling. The availability of a transgenic mouse that expresses a gene that is normally found in humans but is absent from all other species, opens the possibility of studying its contribution to human mental health in ways that were not previously possible.

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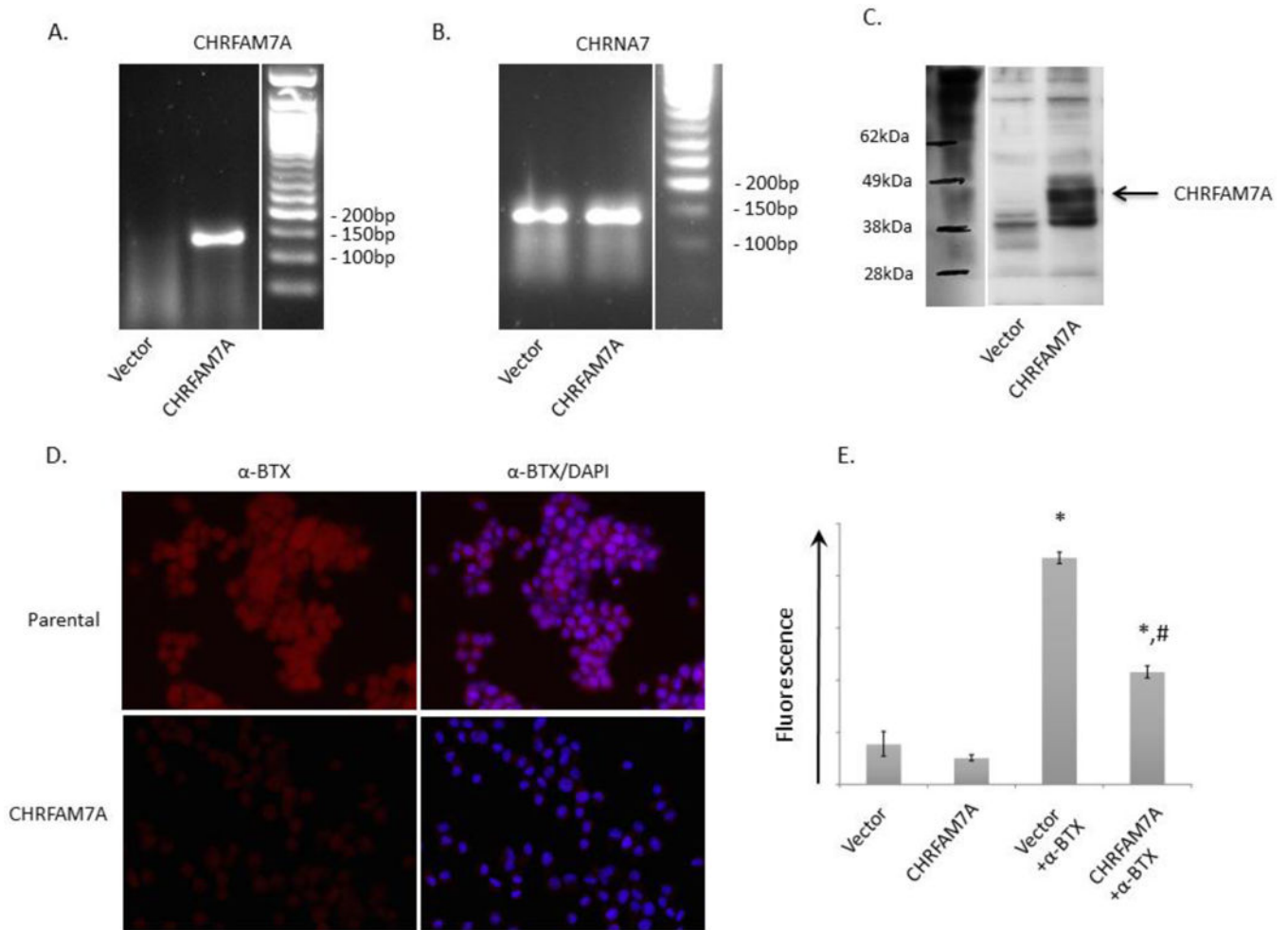
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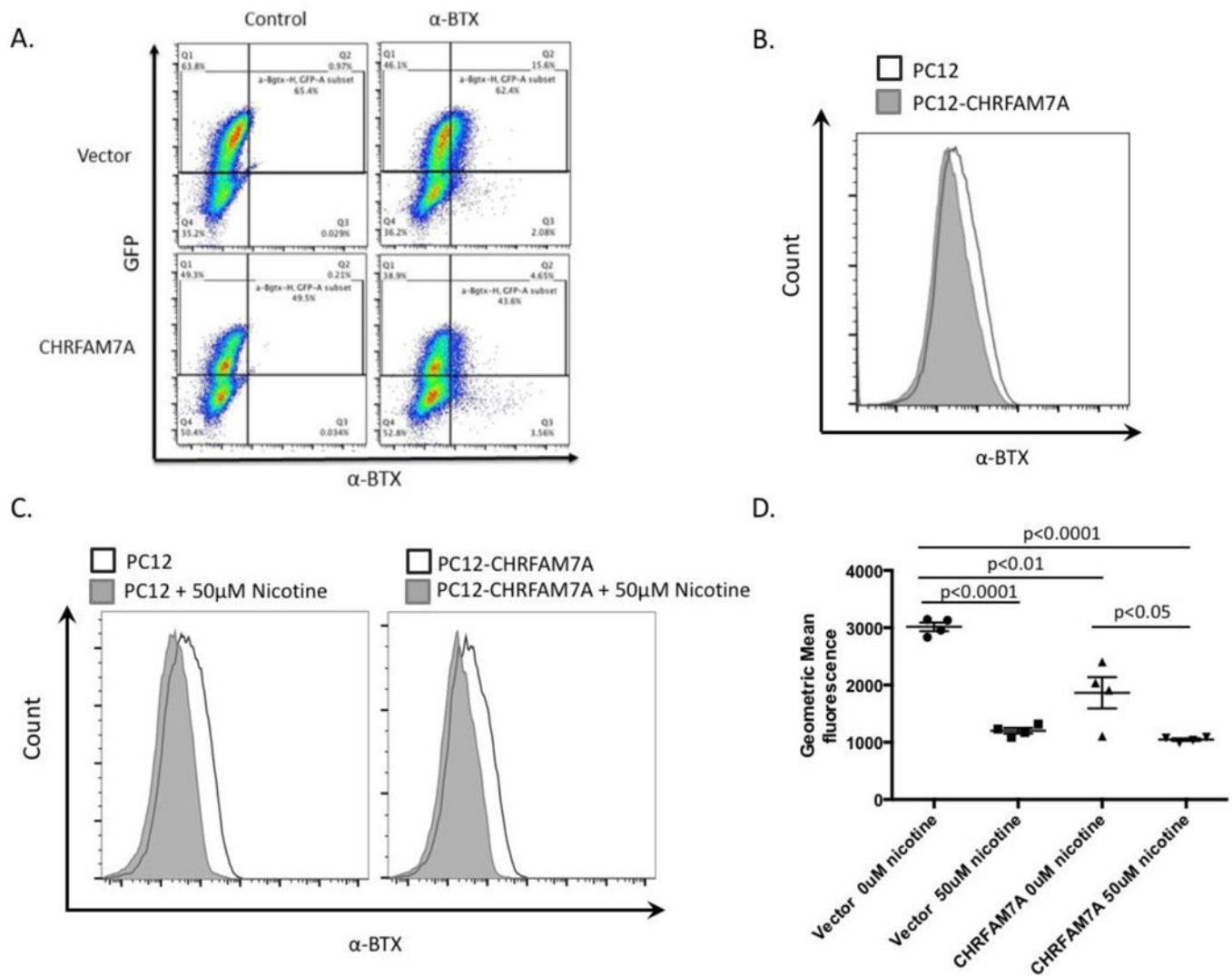
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Key Highlights:

- CHRFBM7A is a uniquely-human gene that encodes a human-specific variant of the alpha-7 nicotinic acetylcholine receptor ($\alpha 7nAChR$).
- Forced expression of CHRFBM7A in an *in vitro* neuronal cell line decreases binding to the $\alpha 7nAChR$.
- Alpha-bungarotoxin staining was decreased at the neuromuscular junction of CHRFBM7A transgenic mice, demonstrating that CHRFBM7A alters binding to the $\alpha 7nAChR$ *in vivo*.

**Figure 1:**

Forced expression of CHR FAM7A alters α -bungarotoxin (α -BTX) binding. Rat PC12 cells were transduced with CHR FAM7A to assess the effect of the uniquely human gene on ligand binding to the α 7nAChR. **A.** PCR confirmed the presence of CHR FAM7A in transduced cells with no expression seen in vector PC12 cells. **B.** PCR demonstrated that CHR NA7, the α 7nAChR gene, was expressed in both vector and CHR FAM7A transduced cells. **C.** Immunoblotting demonstrated that CHR FAM7A transduced PC12 cells were functional and produced a protein with molecular weight consistent with CHR FAM7A. **D.** PC12 cells were stained with α -BTX (red) to assess binding to the α 7nAChR. Cells were co-stained with DAPI (blue) to identify the cell nucleus. **E.** Fluorescence was measured using an Omega Fluorimeter to quantify changes in α -BTX binding between vector and CHR FAM7A transduced cells. Decreased staining for α -BTX was seen in CHR FAM7A transduced cells compared to vector. * p<0.0001 vs Vector, CHR FAM7A; # p<0.01 vs Vector + α -BTX.

**Figure 2:**

ChRFAM7A expression decreases ligand binding to the α 7nAChR in PC12 cells. Rat PC12 cells were transduced with human ChRFAM7A to quantify changes in α -BTX binding using flow cytometry. **A.** Gating strategy to identify the GFP⁺ChRFAM7A⁺ population in vector and ChRFAM7A-transduced cells. **B.** Representative histogram demonstrating decreased α -BTX binding in ChRFAM7A transduced PC12 cells compared to vector. **C.** Cell preincubated with the α 7nAChR ligand nicotine (50 μ M) to prevent α -BTX binding to functional α 7nACh receptors. **D.** Quantification of all flow cytometry experiments showing that the introduction of uniquely human ChRFAM7A decreases α -BTX binding to PC12 cells. * $p < 0.05$.

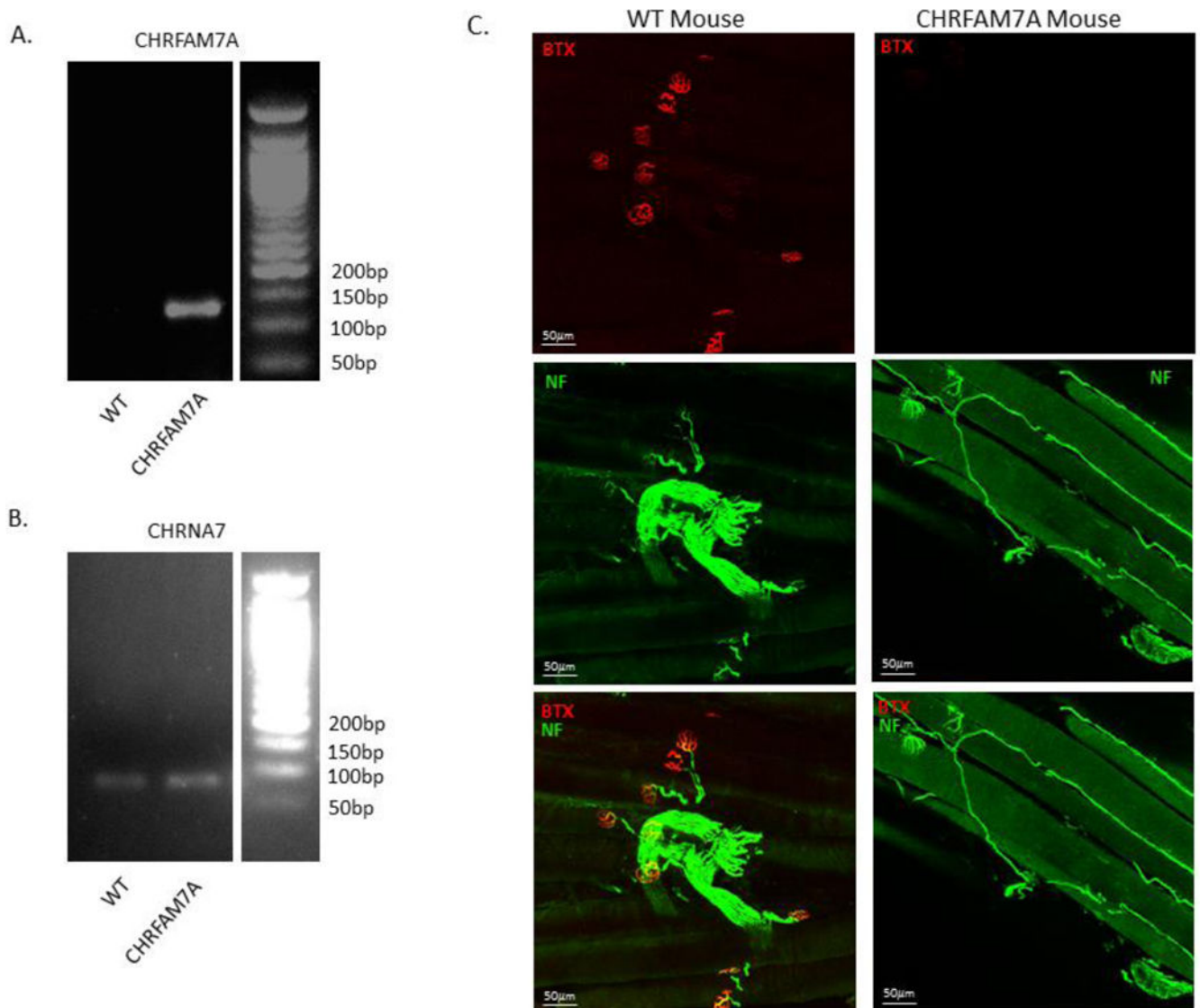


Figure 3: Binding to the neural $\alpha 7$ nAChR is decreased in CHR FAM7A transgenic animals. Transgenic mice expressing the uniquely human gene CHR FAM7A were utilized to assess binding to the $\alpha 7$ nAChR at the neuromuscular junction in muscle. **A.** PCR of muscle tissue demonstrates CHR FAM7A gene expression in transgenic mice. There was no expression of CHR FAM7A in WT mice. **B.** PCR of muscle tissue demonstrates CHR NA7, the $\alpha 7$ nAChR gene, is present in the muscle of both WT and CHR FAM7A transgenic mice. **C.** Exposure matched confocal microscopy of muscle tissue stained for α -BTX (red) to identify staining to the $\alpha 7$ nAChR and Neurofilament (green) to identify the neuromuscular junction. There is decreased α -BTX staining at the neuromuscular junction in CHR FAM7A transgenic mice compared to sham. Size bar = 50μm.