Mice Deficient in CHRNA7, a Subunit of the Nicotinic Acetylcholine Receptor, Produce Sperm with Impaired Motility

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ABSTRACT

In this study we investigate the role of the CHRNA7 subunit (also known as the alpha7 subunit) of the nicotinic acetylcholine receptor in mouse sperm function. We confirm by reverse-transcription-polymerase chain reaction the expression in adult mouse testis of Chrna7 mRNA and demonstrate the subunit's presence in mouse sperm by immunoblot. Alpha-bungarotoxin binds a range of nicotinic acetylcholine receptor subunits, including the CHRNA7 subunit. Localization studies using a fluorescent alpha-bungarotoxin-tetramethyl-rhodamine conjugate revealed specific binding sites on the midpiece of mouse sperm with fainter alpha-bungarotoxin binding on the remainder of the flagellum. Mice engineered with a double-null disruption of the Chrna7 gene displayed only faint fluorescence on the midpiece, suggesting that the CHRNA7 contributed the majority of the observed alpha-bungarotoxin binding sites. The location of alpha-bungarotoxin binding suggested that nicotinic acetylcholine receptors may play an ionotropic role in sperm motility. Sperm from Chrna7−/− mice displayed no difference in number, morphology, viability or spontaneous acrosome reaction rate compared with Chrna7+/+ sperm. Studies using computer-assisted sperm analysis indicate the motility of Chrna7−/− sperm is significantly impaired. This impairment is characterized by significantly reduced swimming velocities, failure to maintain vigorous swimming, and lower levels of hyperactivated swimming patterns in Chrna7−/− sperm compared with Chrna7+/+ sperm. This is the first genetic evidence that sperm nicotinic acetylcholine receptors are important for maintenance of normal sperm motility.

INTRODUCTION

Following spermatogenesis and transit through the epididymis, mammalian sperm begin to swim progressively upon being deposited into the female reproductive tract. Within this milieu, sperm undergo a complex series of biochemical changes leading to functional maturity, collectively termed capacitation [1, 2]. Capacitation has also been used to describe the collective molecular events occurring in sperm in vivo or in vitro that allow the sperm to respond to initiators of the acrosome reaction (AR) [1, 3]. Hyperactivation, a form of motility essential to fertilization [2], to initiators of the acrosome reaction (AR) [1, 3]. Hyperactivation, a form of motility essential to fertilization [2], is the first genetic evidence that sperm nicotinic acetylcholine receptors are important for maintenance of normal sperm motility.

Sperm are small, short-lived, highly motile, terminally differentiated cells. These characteristics, in conjunction with the heterogeneous activation states of individual cells within a population and the inherent difficulty in quantifying their complex swimming behaviors has presented a considerable barrier to our understanding of the mechanisms controlling sperm function. One strategy that has proved particularly successful in identifying novel protein components regulating sperm function has been the use of molecular biology to screen the testsis for expression of promising targets, followed by confirmation of the presence on mature sperm using specific antibodies. Subsequent generation of mice engineered with a targeted disruption of the gene of interest and comparison of their reproductive phenotype to wild-type animals has led to the identification of several novel components of the signaling pathways governing sperm behavior and function [7].

Many neuronal receptors, including nicotinic acetylcholine (ACh) receptors, have been implicated in sperm function [8]. In vertebrates, 10 CHRNA; 4 CHRNB; and single CHRND, CHRNE, and CHRNG subunits have been identified to date [9, 10]. Unlike muscle-type nicotinic ACh receptors, neuronal-type nicotinic ACh receptors are composed solely of CHRNA and CHRNB subunits, and their subunit association is less constrained than the muscle type. The promiscuous nature of subunit assembly in neuronal-type nicotinic ACh receptors generates a high level of functional diversity in terms of pharmacological specificities, channel permeability, and kinetics [9]. From in vitro expression studies in mammalian or amphibian cells, neuronal nicotinic ACh receptors can be categorized into two types: heteromeric pentamers and homomeric pentamers. Homomeric pentamers consist of a combination of CHRNA2-10 and CHRNB2-4 subunits in an (CHRNA)2 (CHRNB)3 stoichiometry. Homomeric pentamers consist exclusively of CHRNA7, CHRNA8 (expressed in avian species only), or CHRNA9 subunits, with the CHRNA7 subunit-containing...
nicotinic ACh receptor being the most abundant example of this subgroup. Homomeric nicotinic AC receptors consisting solely of CHRNA7 subunits have been demonstrated to preferentially form in vitro and to exist in vivo [11, 12]. Additional complexity governing the coassociation of different subunits may exist in vivo [9, 13, 14]. Both CHRNA7 and CHRNA9 nicotinic ACh receptors have been demonstrated as being unusually permeable to Ca²⁺ ions when compared to other nicotinic ACh receptors, with flux through either channel alone being enough to elevate intracellular calcium levels in vivo [15, 16].

Previously, Baccetti and coworkers [17] localized binding sites for alpha-bungarotoxin (α-BTX; a nicotinic ACh receptor antagonist binding to the CHRNA1, CHRNA7, CHRNA8, and CHRNA9 subunits) on sperm from a broad range of species, including sea urchin, rabbit, pig, and human. Binding of a fluorophore-conjugated α-BTX in those mammalian species studied (sheep, pig, rabbit, and human sperm) was predominately detected on the postacrosomal and midpiece regions. A range of nicotinic ACh receptor subunits are expressed on human sperm, and are localized to the postacrosomal, neck, and midpiece regions [18]. In this study, we investigated the presence of the CHRNA7 subunit (also known as the α7 subunit) of the nicotinic ACh receptor in mouse sperm by immunoblotting and α-BTX binding. We also used computer-assisted sperm analysis (CASA) to examine the motility of sperm from mice engineered with a double-null disruption for the Chrna7 gene encoding the CHRNA7 subunit [19].

MATERIALS AND METHODS

Materials

Salts, metabolites, and antibiotics used in media were of reagent grade and purchased from Fisher Scientific (Pittsburgh, PA), Irvine Scientific (Irvine, CA), Mallinckrodt (Paris, KY), or Sigma (St. Louis, MO). Fraction V bovine albumin (Pentex 81-066 lot 59) was purchased from Serologicals Corp. (Kankakee, IL). Monoclonal antibody (mAb) 306 directed against the nicotinic ACh receptors CHRNA7 subunit; α-BTX; Coomassie Brilliant blue G 250; and the protease inhibitors leupeptin, aprotinin, AEBSF, benzamidine HCl, pepstatin A, and E-64 were purchased from Sigma; nitrocellulose membranes, Tween-20, and broad-range molecular weight standards were from Bio-Rad Laboratories (Hercules, CA); precast NuPAGE gels and NuPAGE LDS sample buffer was from Invitrogen (Carlsbad, CA); Vectashield was from Vector Laboratories (Burlingame, CA), and α-Conotoxin IMI was purchased from Peptide International (Louisville, KY). Triton X-100 was purchased from Pierce Biotechnology (Rockford, IL); an enhanced chemiluminescence (ECL) kit, Percoll, and N-hydroxy-succinimide-activated Sepharose were from Amersham Biosciences Corp. (Piscataway, NJ); the Qiagen RNaseasy kit was from Qiagen Inc. (Valencia, CA); the Titan One Tube RT-PCR kit. The primer sequences (forward, 5′-GCA CCT CAT GCA TGG TAC AC; reverse, 5′-GGA CAC AGC CTC CAC AAA GT3′) were designed to amplify a sequence corresponding to exons 8–10 of the Chrna7 gene and were expected to yield a 241-base pair (bp) product [21]. The amplified product was checked by sequencing (Davis Sequencing, Davis, CA).

α-Conotoxin IMI-Sepharose Batch Affinity Purification of the CHRNA7 Subunit

Mouse sperm preparations were obtained from Percol-gradient centrifugation and washed with medium containing 1 mg/ml polyvinyl alcohol [18]. The final pellet was resuspended in medium A (10 mM Tris pH 7.4, 150 mM NaCl, plus protease inhibitors [1 mM EDTA, 20 μM leupeptin, 1 μg/ml aprotinin, 1 mM AEBSF, 1 mM benzamidine HCl, 1 μM pepstatin A, 1 mM E-64]) plus 1% Triton X-100 at a sperm concentration of 250 × 10⁶ cells/ml. Sperm suspensions were incubated with ice for 1 h and centrifuged at 10 000 × g for 30 min. NHS-α-Conotoxin IMI-Sepharose slurry was prepared by linking 2 μM of α-conotoxin IMI/ml of NHS-activated Sepharose [18]. The supernatant obtained after Triton X-100 extraction of sperm was incubated with NHS-α-Conotoxin IMI slurry for 6 h at 4°C and spun at 500 × g. The pellet was washed three times with medium A and resuspended in LDS-sample buffer. The sample was boiled for 3 min and loaded onto a 7% precast NuPAGE Tris-acetate gel, and Western blot analysis was performed as described below.

Western Blot Analysis

For immunoblotting, the proteins were transferred onto nitrocellulose membrane at 100 mA constant current for 2 h. The membrane was blocked for 2 h with 3% BSA in TBST (10 mM Tris pH 7.4, 150 mM NaCl, and 0.05% Tween-20) antibody and incubated overnight at 4°C with a 1:5000 dilution of the mAb 306, the CHRNA7 subunit antibody diluted in blocking buffer. The membrane was washed three times with TBST and incubated with a 1:2000 dilution of sheep anti-mouse antibody conjugated to horseradish peroxidase for 1 h at room temperature. The membrane was subsequently washed extensively with TBST and developed using the Amersham Biosciences ECL kit.

Localization of α-BTX Binding Sites on Mouse Sperm

Sperm were fixed with 4% paraformaldehyde (10 min; 22°C), pelleted (850 × g for 5 min), washed with PBS four times, and dried onto microscope slides. To demonstrate the specific nature of α-BTX binding, sperm were incubated with PBS or α-BTX (20 μg/ml) for 4 h at 22°C. Following washing, sperm were incubated with 5 μg/ml of α-BTX-RH for 2 h at 22°C. Excess α-BTX-RH was washed off with PBS and Vectashield was added to limit photobleaching. The α-BTX-RH binding patterns were examined using an epifluorescence microscope (Nikon E800 eclipse) fitted with a rhodamine filter set. Images were captured with a charge-coupled device (CCD) camera (Evolution MP5.0; Media Cybernetics, Silver Spring, MD) and were processed in NIH Image (National Institutes of Health, Bethesda, MD). The patterns of α-BTX-RH binding were confirmed with sperm collected from three different animals of each type (Chrna7+/− and Chrna7−/−).

Assessment of Sperm Viability

To ensure experimental comparability between Chrna7+/− and Chrna7−/− sperm populations, each sample was assessed using a LIVE/DEAD sperm viability kit (L-7011) and a LIVE/dead®-BTX-RH was washed off with PBS and Vectashield was added to limit photobleaching. The α-BTX-RH binding patterns were examined using an epifluorescence microscope (Nikon E800 eclipse) fitted with a rhodamine filter set. Images were captured with a charge-coupled device (CCD) camera (Evolution MP5.0; Media Cybernetics, Silver Spring, MD) and were processed in NIH Image (National Institutes of Health, Bethesda, MD). The patterns of α-BTX-RH binding were confirmed with sperm collected from three different animals of each type (Chrna7+/− and Chrna7−/−).
DEAD sperm viability kit. Following staining, sperm were examined using an epifluorescence microscope fitted with a fluorescein isothiocyanate filter set (Nikon E800 eclipse) and a CCD digital camera (Evolution MP5.0; Media Cybernetics). The number of viable cells was assessed (≥200 sperm per sample) in a blind fashion.

Assessment of Acrosomal Status

To gain a measure of spontaneous acrosome reactions within each sperm sample, aliquots (50 μl) of sperm were removed immediately following collection and after the capacitation period, and fixed in a 4% paraformaldehyde solution. Acrosomal status was assessed by light microscopy using Coomassie Brilliant blue 250, with ≥200 sperm per treatment examined in a blind fashion [20].

Sperm Motility Analyses

Sperm motility parameters were quantified using a Hamilton Thorne (Beverly, MA) computer-assisted semen analysis system (CASA) running CEVOS (version 10.8). A 10-μl aliquot was placed in a prewarmed counting chamber (37°C; depth 20 μm) and ≥400 cells per sample analyzed. Parameters used were as follows: negative phase-contrast; recording at 60 frames/sec; minimum contrast, 60; minimum cell size, 7 pixels; average path velocity (VAP) cutoff, 25 μm/sec; straight line velocity (VSL) cutoff, 30 μm/sec; slow cells were included as motile; minimum static contrast, 30; head intensity, 120; static head size, 1.0–2.9; static head intensity, 0.6–1.4; and static elongation, 20–80. In pilot experiments, more than 90% of sperm were correctly identified as assessed by the internal quality control screens within the IVOS software.

For assessment of hyperactivated motility, fields of Chrna7−/− and Chrna7+/− sperm were recorded onto mini-DV tape. Hyperactivated motility was defined within the SORT function of the IVOS software by the Boolean function curvilinear velocity (VCL) ≥180 μm/sec, amplitude of lateral head displacement (ALH) ≥9.5 μm, and linearity (LIN) ≥38%; for a sperm to be classified as hyperactivated with all three conditions within this equation needed to be met. The kinematic terms in this equation were selected as increases in VCL and ALH, and decreases in LIN are indicative of hyperactivated swimming patterns in a number of species [22]. The threshold value for each term within the sort equation was determined as follows: 200 sperm from the wild-type population (four animals) were visually identified as displaying hyperactivated motility. Hyperactivated motility was defined by distinctive rapid star spin, figure-eight, and saw-tooth-circle swimming patterns [22, 23]. Track data from each hyperactivated sperm were collected and imported into Excel (Microsoft, Seattle, WA). Distributions of values for each condition of the sort function (VCL, ALH, and LIN) within the hyperactivated data set were examined, and thresholds for each were set such that 90% of the hyperactivated population could satisfy all three conditions when the equation was applied. To validate this method, the sort function was applied to additional tapered fields of sperm. Cells classified as hyperactivated were reexamined by eye to confirm the associated swimming patterns were displayed. Due to the strict selection criteria, the measure of hyperactivated sperm in a population estimated by this methodology is an underestimate when compared to assessment by eye, but serves as a useful relative measure. The tapes of sperm were analyzed using the SORT function to gain a relative measure of hyperactivated cells within the Chrna7−/− and Chrna7+/− populations, with ≥200 cells assessed per sample.

Statistical Analysis

An unpaired Student t-test or Tukey multiple range test was applied appropriately for the comparison of group means. All data expressed as percentages were transformed to the arcsine of their square roots. In all studies, statistical significance was determined at P ≤ 0.05.

RESULTS

CHRNA7 Subunits Are Expressed in Mouse Testis and on Sperm

Extraction of total RNA and RT-PCR using specific primers confirmed the expression of Chrna7 mRNA in the testis of Chrna7−/− mice, but the subunit was absent in the testis of Chrna7−/+ mice (Fig. 1a). RT-PCR yielded a 241-bp product, and sequencing confirmed the amplified sequence shared 100% identity with the corresponding sequence in the GenBank database (accession number 31982868). The presence of CHRNA7 subunits on mouse sperm was confirmed by α-Conotoxin IMI-Sepharose batch affinity purification followed by Western blotting (Fig. 1b). The size of the detected protein band (57 kDa) was the same size as that reported in mouse brain [24].

Localization of α-BTX Binding Sites on Mouse Sperm

The α-BTX binding sites of mouse sperm were localized to the midpiece (Fig. 2, A and B) with fainter fluorescence detected along the remaining length of the flagellum. The specific nature of the binding was confirmed by preincubation with unlabeled α-BTX before the addition of α-BTX-RH (Fig. 2, C and D); α-BTX preincubation eliminated all fluorescence due to binding of BTX-RH.

α-BTX is able to bind to CHRNA1, CHRNA7, CHRNA8, and CHRNA9 nicotinic ACh receptor subunits; however, the CHRNA1 subunit has been found only in muscle, and the CHRNA8 subunit is not a mammalian subunit [10, 14]. To address the possibility that CHRNA9 subunits contributed to the observed pattern of fluorescence, we also examined the pattern of α-BTX-RH binding on sperm from Chrna7+/− mice (Fig. 2, E and F). The detected fluorescence on the midpiece was greatly reduced and was absent in the rest of the flagellum of Chrna7+/− sperm, suggesting that the CHRNA7 subunit contributed the majority of the observed α-BTX-RH binding sites in Chrna7+/− sperm. The residual fluorescence on the midpiece of sperm from Chrna7+/− mice may be due to the presence of CHRNA9 subunits. The specific nature of the α-BTX-RH binding was confirmed by preincubation of Chrna7+/− sperm with unlabeled α-BTX before the addition of α-BTX-RH (Fig. 2, G and H). This preincubation eliminated fluorescence due to BTX-RH binding.

Comparison of Body and Testis Weight, Sperm Number, and Morphology for Chrna7−/− and Chrna7+/− Mice

We found no significant difference in the overall body weights, or that of the testis, or the total number of sperm collected from Chrna7+/− and Chrna7−/− mice (Table 1). The morphology of the Chrna7−/− sperm appeared normal.

Comparison of Sperm Viability and Spontaneous Acrosome Reaction for Chrna7+/− and Chrna7−/− Mice

To ensure experimental comparability, the viability of Chrna7+/− and Chrna7−/− sperm was assessed. Before and after capacitation, the viability of Chrna7−/− sperm was not significantly different from that of Chrna7+/− (Table 2). The rate of senescence over 2 h being 14.1% (± 3.99 SEM) for Chrna7+/− compared with 10% (± 2.22 SEM) for the Chrna7−/− (n = 6). The rate of spontaneous acrosome reaction (AR) within Chrna7+/− and Chrna7−/− sperm were also comparable.

Chrna7−/− Sperm Display Impaired Motility

Initial assessment of motility was based upon the velocity distributions within both uncapacitated and capacitated sperm populations. Rapid cells were defined as cells with a VAP ≥25 μm/sec. Medium cells were defined as possessing a VAP of between 10 μm/sec and 25 μm/sec. Slow cells defined as cells with a VAP ≤10 μm/sec, or a VSL ≤10 μm/sec, or both. Sperm displaying no motility were defined as static. Uncapacitated Chrna7−/− sperm displayed significant reductions in medium and rapid cells compared...
with Chrna7+/− sperm (Fig. 3). This disparity increased following capacitation, with a large relative reduction of rapid sperm in Chrna7+/− populations. Concurrent to this was a significant reciprocal rise in static and slow sperm (Fig. 3). When normalized against the initial percentage of rapid cells in the uncapacitated population, the rate of loss of rapid cells from the ChRNA7-/- sperm was twice that of ChRNA7+/− following the capacitation period.

We compared the motility of the ChRNA7+/− and ChRNA7−/− sperm populations by examining kinematic values generated by CASA. The VAP, VSL, and VCL were significantly lower in uncapacitated and capacitated ChRNA7−/− sperm populations compared with ChRNA7+/− populations, indicating the mean swimming speed of the population was reduced (Fig. 4). Uncapacitated ChRNA7−/− sperm demonstrated reductions in VAP, VSL, and VCL of 36.5%, 34.7%, and 42% respectively. These reductions were maintained in capacitated sperm (33.3%, 39.4%, and 29.25% respectively to those of the wild type).

To establish whether the reduced swimming speeds in the ChRNA7−/− population were due to the sperm swimming at slower speeds per se or due to a shift in a numerical distribution across the range of velocities, by using VCL as the primary measure of motility we examined the range of swimming speeds found in uncapacitated sperm populations. Uncapacitated sperm displayed vigorous motility, the widest range of velocities, and a lower percentage of cells displaying hyperactivated swimming patterns. Plotted as a histogram (60-μm/sec increments), the distribution of ChRNA7−/− sperm was even across the entire range of velocities, whereas the majority of ChRNA7+/− sperm were found in the lower cohorts (Fig. 5). A small subpopulation of sperm with maximal VCL values equivalent to those found in animals of the wild type was present, but as a significantly reduced percentage of the total population.

Of the additional measurements produced by CASA, maximal ALH was significantly different. The ALH value derived for uncapacitated ChRNA7+/− sperm (7.71 μm ± 0.37 SEM; n = 8) was significantly greater than that observed in the ChRNA7−/− sperm (5.6 μm ± 0.59 SEM; n = 6). This increased in magnitude following capacitation (8.59 μm ± 2.11 SEM; n = 8), whereas the ALH of the ChRNA7−/− remained essentially unchanged (5.8 μm ± 0.35 SEM; n = 8). Of the remaining measurements of sperm motility generated by the CASA analysis, none were significantly different between the ChRNA7+/− and ChRNA7−/−.

Straightness, linearity, and beat frequency in uncapacitated sperm were 71% (± 5.9 SEM; n = 8) for ChRNA7+/− compared with the ChRNA7−/− (Fig. 6) (n = 4 experimental animals of each type; a total of ChRNA7+/− 815 cells vs. ChRNA7−/− 1000 cells examined; P < 0.05) (ChRNA7+/− 23.75% ± 5.9 SEM; ChRNA7−/− 6.4% ± 1.5 SEM).

**DISCUSSION**

The CHRNA7 subunits expressed on mouse sperm are localized predominantly to the midpiece, but are present on the entire length of flagella. Sperm from mice lacking the CHRNA7 subunit exhibit motility defects and reduced hyperactivation. The ability of sperm to swim and respond to environmental cues within the female reproductive tract is of fundamental importance if fertilization is to be achieved. Previous in vitro studies have reported that nanomolar and low micromolar concentrations of ACh stimulate sperm motility in a number of species, including sea urchin, bull, chimp, mouse, and human [25–28]. Reported effects of ACh on sperm include increases in swimming speed and flagella beat frequency, and changes in swimming patterns [26]. ACh has been reported to stimulate chemotaxis in mouse sperm in vitro [28]. Nicotine has been reported to stimulate sperm motility in a number of species [25, 26]. We demonstrated that nicotinic ACh receptor antagonists can inhibit AR initiation by both ACh and ZP or recombinant human ZP in mouse and human sperm, respectively, but did not observe any influence upon sperm motility [20, 29]. However, high levels of α-BTX (mM) can abolish sea urchin and human sperm motility and inhibit stimulation by ACh or nicotine, suggesting mediation via nicotinic ACh receptors [30, 31]. The antagonist concentrations used in our previous studies may have been sufficient to inhibit the AR but not high enough to cause readily observable alterations in sperm motility. Additionally, we used a visual assessment as a subjective measure of sperm motility; CASA allows detection of differences in motility not readily discernable by our previous methods.

The swimming speed of the ChRNA7−/− sperm population is significantly reduced compared with a ChRNA7+/− population when examined by CASA. Following an incubation
creased senescence or spontaneous AR does not accompany paired ability to maintain progressive forward motility. In a separate study to this, sperm from alive.

levels of capacitated sperm in the measure of capacitation; we observed no difference in the capacitation state. We used the chlortetracycline assay as a measure of Chrna7.

The ability of a sperm to undergo AR is dependent on its failure to undergo AR initiated by either ACh or ZP [32].

period to allow in vitro capacitation, fewer sperm displaying hyperactivated swimming patterns were observed in the Chrna7−/− population. The Chrna7−/− sperm possess an impaired ability to maintain progressive forward motility. Increased senescence or spontaneous AR does not accompany these defects. When examined by eye, many nontwist Chrna7−/− sperm displayed a quivering head and midpiece but no forward movement, confirming the cells were still alive.

We have suggested a role for nicotinic ACh receptors containing the CHRNA7 subunit in the ZP-initiated AR [20, 29]. In a separate study to this, sperm from Chrna7−/− mice failed to undergo AR initiated by either ACh or ZP [32]. The ability of a sperm to undergo AR is dependent on its capacitation state. We used the chlortetracycline assay as a measure of capacitation; we observed no difference in the levels of capacitated sperm in the Chrna7−/− and the Chrna7−/− samples [32].

How do sperm nicotinic ACh receptors exert their influence on sperm swimming behavior? The sperm flagellar beat develops starting from the neck and propagates through the midpiece to the rest of the tail [33]. While cAMP and ATP are sufficient to stimulate motility, Ca2+ is the most important factor in the initiation and maintenance of motility and is a key factor in the development of hyperactivation [5]. Studies using in vitro expression systems have demonstrated that nicotinic ACh receptors consisting solely of CHRNA7 subunits are unusually permeable to calcium ions compared to heteromeric nicotinic ACh receptors [15]. The ability of ACh to induce Ca2+ uptake has previously been observed in ram sperm by histochemical means [34]. Cytochemical studies have shown that nicotine enhances Ca2+ entry into the midpiece and tail of bovine sperm cells [35]. Our live single-cell imaging studies have confirmed that micromolar ACh stimulates calcium entry into the midpiece and tail of bovine sperm, with the majority of the influx mediated via nicotinic ACh receptors [5]. Studies using in vitro expression systems have demonstrated that nicotinic ACh receptors consisting solely of CHRNA7 subunits are unusually permeable to calcium ions compared to heteromeric nicotinic ACh receptors [15]. The ability of ACh to induce Ca2+ uptake has previously been observed in ram sperm by histochemical means [34]. Cytochemical studies have shown that nicotine enhances Ca2+ entry into the midpiece and tail of bovine sperm cells [35]. Our live single-cell imaging studies have confirmed that micromolar ACh stimulates calcium entry into the midpiece and tail of bovine sperm, with the majority of the influx mediated via nicotinic ACh receptors containing the CHRNA7 subunit (unpublished results). We suggest that sperm nicotinic ACh receptors play a central role in the control of motility and associated calcium influx mechanisms. In neurons, Na+ and Ca2+ influx through nicotinic ACh receptors results in membrane depolarization. Whether the rise in sperm [Ca2+], due to Ca2+ flux is solely through nicotinic ACh receptors themselves or the recruit-ment of voltage-operated calcium channels following membrane depolarization or release from intracellular stores remains unclear. Increases in sperm intracellular calcium concentration may also activate the unique soluble Ca2+-sensitive adenyl cyclase present in sperm to produce cAMP [36]. Such an increase in cAMP may in turn influence sperm motility via the activation of protein kinase A, triggering a cascade of protein tyrosine phosphorylation of flagellar proteins or produce further increases in sperm [Ca2+]i through the activation of cyclic nucleotide-gated channels [37, 38].

For a receptor to exert its effect on cellular function it must be activated by an appropriate ligand. ACh is a labile molecule produced by the enzyme choline acetyltransferase (ChAT; EC 2.3.1.6) from acetyl coenzyme A and choline. While ACh is generally perceived as a neurotransmitter within the central and peripheral nervous systems, the pres-

TABLE 1. Comparison of body weight (g), testis weight (g), total sperm count of wild-type (Chrna7+/−) mice and mice lacking the CHRNA7 subunit (Chrna7−/−).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body weighta,c</th>
<th>Testis weighta,c</th>
<th>Sperm numbera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrna7+/−</td>
<td>34.60 (± 3.70)</td>
<td>0.21 (± 0.02)</td>
<td>34.3 (± 4.9)</td>
</tr>
<tr>
<td>Chrna7−/−</td>
<td>29.80 (± 0.77)</td>
<td>0.22 (± 0.02)</td>
<td>29.7 (± 1.8)</td>
</tr>
</tbody>
</table>

a Values are presented as mean ± SEM.

b Values are presented as mean ×10⁶ ± SEM.

c No statistically significant differences between the Chrna7+/− and Chrna7−/− sperm were observed (P ≥ 0.05).

TABLE 2. Viability and spontaneous AR of capacitated (Cap) and uncapsulated (Uncap) wild-type (Chrna7+/−) mice and mice lacking the CHRNA7 subunit (Chrna7−/−).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Viability Uncapa</th>
<th>Viability Capa</th>
<th>Spontaneous AR Uncapb</th>
<th>Spontaneous AR Capb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrna7+/−</td>
<td>78.7% (± 2.6)</td>
<td>64.3% (± 2.8)</td>
<td>23.5% (± 3.1)</td>
<td>33.7% (± 7.6)</td>
</tr>
<tr>
<td>Chrna7−/−</td>
<td>70.1% (± 1.5)</td>
<td>59.2% (± 1.9)</td>
<td>25.4% (± 2.8)</td>
<td>34.5% (± 5.4)</td>
</tr>
</tbody>
</table>

a No statistically significant differences between the Chrna7+/− and Chrna7−/− sperm were observed (mean % ± SEM, P ≥ 0.05).
ence of cholinergic systems on many nonneuronal tissues and cells demonstrate that ACh also acts as a local signaling molecule, contributing to the regulation of many basic cell functions. Nonneuronal ACh signaling has been demonstrated in diverse systems [39], such as the activation of lymphocytes [40] and the chemokinetic and chemotactic pathways governing the migration of keratinocyte cells in vitro [41]. A notable feature of nonneuronal ACh is the apparent ubiquitous nature of its secretion from epithelial systems, including the airways, oral mucosa, gastrointestinal tract, and skin [42]. The presence of ChAT has also been reported in human, bovine, and ram sperm [43, 44]. ChAT immunoreactivity has been demonstrated on the postacrosomal region, midpiece, and principal piece of human sperm [45]. ACh synthesis by both ChAT and carnitine acetyltransferase has been reported to occur in rat and human sperm [46, 47]. Moreover, specific inhibitors of ChAT inhibit human sperm motility [48].

Acetylcholinesterase (AChE; EC 3.1.1.7), the main enzyme responsible for ACh degradation in vivo, is expressed in the neck, midpiece, and principal piece of human sperm [49]. Physostigmine, an AChE inhibitor (and thus an indirect agonist of AChRs), has previously been reported to stimulate sperm motility [26].

**FIG. 3.** Velocity distributions of uncapacitated (Uncap) and capacitated (Cap) sperm from Chrna7<sup>+/+</sup> mice and from mice lacking the CHRNA7 subunit (Chrna7<sup>−/−</sup>) as determined by CASA. Rapid cells were defined as cells with a VAP >25 μm/sec. Medium cells were defined as cells possessing a VAP of between 25 μm/sec and 10 μm/sec. Slow cells defined as those with either VAP <10 μm/sec, a VSL <10 μm/sec, or both. Static cells showed no forward movement. Bars represent ± SEM. Same letter superscripts denote statistically significant difference (P ≤ 0.05) between groups.

**FIG. 5.** VCL distributions of uncapacitated sperm from Chrna7<sup>+/+</sup> mice and from mice lacking the CHRNA7 subunit (Chrna7<sup>−/−</sup>) as determined by CASA (plotted in 60-μm/sec increments; n = 4). Bars represent ± SEM. Statistically significant differences (P ≤ 0.05) between groups are denoted by different letter superscripts.

**FIG. 4.** Comparison of VAP, VSL, and VCL values of uncapacitated (Uncap) and capacitated (Cap) sperm from Chrna7<sup>+/+</sup> mice and from mice lacking the CHRNA7 subunit (Chrna7<sup>−/−</sup>) as determined by CASA. Bars represent ± SEM. Same letter superscripts denote statistically significant difference (P ≤ 0.05) between groups.

**FIG. 6.** Hyperactivated motility in Chrna7<sup>+/−</sup> mice and from mice lacking the CHRNA7 subunit (Chrna7<sup>−/−</sup>) as determined by CASA following a 2-h capacitation period. Hyperactivation was defined as VCL ≥180 μm/sec, ALH ≥9.5 μm, and LIN ≤38%. Bars represent ± SEM. Same letter superscripts denote statistically significant difference (P ≤ 0.05) between groups.

ACh may be generated by sperm themselves to act in a paracrine or autocrine manner. The epithelia of the female reproductive may produce ACh to influence sperm function and motility. To date, within the female reproductive tract, ACh secretion has been detected from cells of the vaginal mucosa, cultured granulosa cells, and the ovary in both the mouse and human [42, 50].

Fertility is a multifactorial phenomenon. Reduced litter sizes suggest the fecundity of the Chrna7<sup>−/−</sup> mouse is impaired (communication with representatives at the Jackson Laboratories, Bar Harbor, ME). A recent study has suggested that this impairment can be attributed solely to asynchronous estrous cycles in female Chrna7<sup>−/−</sup> [51]. However, this study did not examine sperm motility or the contribution of female reproductive tract in active sperm transport. A residual population of Chrna7<sup>−/−</sup> sperm retaining relatively normal motility may be sufficient to achieve near normal levels of pregnancy when Chrna7<sup>−/−</sup> males are mated with Chrna7<sup>+/−</sup> females.

Sperm motility is a complex trait. The loss of nicotinic ACh receptors containing the CHRNA7 subunit leads to an
intermediate phenotype; sperm from \textit{Chrna7}^−/− mice initially appear to swim normally but fail to maintain motility, with only a small population retaining rapid swimming and displaying hyperactivated motility following in vitro cultivation. A low level of residual α-BTX binding sites on sperm from \textit{Chrna7}^−/− mice suggests at least one additional nicotinic ACh receptor subunit, possibly a CHRNA9 subunit, may be expressed on mouse sperm. A number of different nicotinic ACh receptor subunits are expressed on human sperm, localized around the postacrosomal, neck, and midpiece regions [18]. If the same is true of mouse sperm, the functional redundancy arising from the expression of a range of different nicotinic ACh receptor subunits and their ability to promiscuously associate to form functional receptors may compensate to a degree for the absence of a particular subunit. The present study provides the first genetic evidence that sperm nicotinic ACh receptors are important for the maintenance normal sperm motility.

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