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Engineering neuronal nicotinic acetylcholine receptors with functional sensitivity to α -bungarotoxin: A novel $\alpha 3$ -knock-in mouse

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Abstract

We report here the construction of a novel knock-in mouse expressing chimeric $\alpha 3$ nAChR subunits with pharmacological sensitivity to α -bungarotoxin (α BTX). Sensitivity was generated by substituting five amino acids in the loop C ($\beta 9$ – $\beta 10$) region of the mouse $\alpha 3$ subunit with the corresponding residues from the $\alpha 1$ subunit of the muscle type receptor from *Torpedo californica*. To demonstrate the utility of the underlying concept, expressed $\alpha 3[5]$ subunits were characterized in the superior cervical ganglia (SCG) of homozygous knock-in mice, where the synaptic architecture of postsynaptic $\alpha 3$ -containing nAChR clusters could now, for the first time, be directly visualized and interrogated by live-staining with rhodamine-conjugated α BTX. Consistent with the postsynaptic localization of ganglionic nAChRs, the α BTX-labeled puncta colocalized with a marker for synaptic varicosities. Following *in vivo* deafferentation, these puncta persisted but with significant changes in intensity and distribution that varied with the length of the recovery period. Compound action potentials and excitatory postsynaptic potentials recorded from SCG of mice homozygous for $\alpha 3[5]$ were abolished by 100 nM α BTX, even in an $\alpha 7$ null background, demonstrating that synaptic throughput in the SCG is completely dependent on the $\alpha 3$ -subunit. In addition, we observed that the genetic background of various inbred and outbred mouse lines greatly affects the functional expression of $\alpha 3[5]$ -nAChRs, suggesting a powerful new approach for exploring the molecular mechanisms underlying receptor assembly and trafficking. As α BTX-sensitive sequences can be readily introduced into other nicotinic receptor subunits normally insensitive to α BTX, the findings described here should be applicable to many other receptors.

Keywords

acetylcholine; superior cervical ganglion; trafficking; autonomic

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Introduction

Multiple nicotinic acetylcholine receptor (nAChR) subtypes populate the peripheral and central nervous systems of vertebrates. At present, 5 neuromuscular nicotinic subunits ($\alpha 1$, $\beta 1$, γ , δ , and ϵ) and 12 neuronal nicotinic subunits ($\alpha 2$ – $\alpha 10$ and $\beta 2$ – $\beta 4$) have been identified and cloned (Karlin, 2002; Colquhoun et al., 2003; Millar and Gotti, 2009). Unlike muscle-type nAChRs, which are constrained to a single pentameric arrangement in adult tissue, neuronal nAChRs can assemble with varied subunit compositions and stoichiometries, resulting in an undetermined number of receptor isoforms (McGehee and Role, 1995; Role and Berg, 1996; Hogg et al., 2003). Alterations in nicotinic receptor function are thought to contribute to the pathological mechanisms underlying a wide range of neurological and psychiatric disorders, including Alzheimer's disease, Parkinson's disease, schizophrenia, epilepsy, and drug addiction. Despite their recognized importance, the relationships between the subunit composition of most neuronal nAChR isoforms and their physiological roles *in vivo* remain unclear. New tools and approaches would help address these critical questions (Gotti et al., 2006; Gotti et al., 2007; Hogg and Bertrand, 2007).

Neuronal nAChRs containing the $\alpha 3$ subunit mediate fast excitatory neurotransmission in both sympathetic and parasympathetic autonomic ganglia in mammals (Mandelzys et al., 1995; Perry et al., 2002; Rassadi et al., 2005). Of all the neuronal nAChR genes, $\alpha 3$ is the only single subunit that produces a lethal phenotype when deleted in inbred mice (Xu et al., 1999). There is also mounting evidence that $\alpha 3$ -containing receptors are expressed in select regions of the central nervous system, including the medial habenula, pineal gland, interpeduncular nucleus, dorsal horn of the spinal cord, hypothalamus, cerebellum, locus coeruleus, inferior colliculus, and hippocampus (Sudweeks and Yakel, 2000; Yeh et al., 2001; Perry et al., 2002; Whiteaker et al., 2002; Liu et al., 2003; Hernandez et al., 2004; Turner and Kellar, 2005; Grady et al., 2009).

Previously, using heterologous expression studies in *Xenopus* oocytes, we demonstrated that the $\alpha 3$ neuronal nAChR subunit can be mutated into a α BTX-sensitive form by substituting from one to five amino acids in the loop C region of the $\alpha 3$ subunit with the corresponding residues from the $\alpha 1$ subunit of *Torpedo californica* (Levandoski et al., 1999). In the oocyte expression system, these chimeric $\alpha 3$ -containing nAChRs exhibited EC₅₀ values for ACh ranging from 50–500 μ M and IC₅₀ values for block by α BTX ranging from 20–200 nM, depending on (1) the specific amino acid substituted, (2) the total number of residues mutated, and (3) whether $\beta 2$ or $\beta 4$ was co-expressed. These studies led us to hypothesize that minimally mutated α BTX-sensitive $\alpha 3$ nAChR subunits could be utilized as part of a knock-in strategy for the study of $\alpha 3$ -containing neuronal nAChRs *in vivo*. Toward this goal, a gene-targeting vector was designed to introduce five mutations in the mouse $\alpha 3$ nAChR subunit: Y184W, E187W, I188V, K189Y, and N191T (*Torpedo* numbering) (Levandoski et al., 1999). Here we report that these mutations have been introduced into the genome of mouse ES cells using gene replacement technology and that functional, α BTX-sensitive $\alpha 3[5]$ -containing nAChRs are expressed in SCG neurons, opening the door to new possibilities of probing the function and anatomical distribution of $\alpha 3$ -containing nAChRs in the central nervous system. Furthermore, this novel knock-in mouse has increased our understanding of the well-characterized SCG system by enabling an imaging analysis of the dynamics of neuronal nAChR cluster size and distribution following preganglionic denervation at a level of analysis previously unattainable.

Materials and Methods

Preparation of the $\alpha 3[5]$ targeting construct

A gene-targeting vector containing genomic, mouse $\alpha 3$ nAChR DNA and a neomycin-resistance cassette in pBluescript SK (–) (Stratagene, La Jolla, CA) was the generous gift of

Dr. Changhai Cui, Salk Institute. The genomic DNA sequence in this construct included exon V of the *CHRNA3* gene along with ~2,000 to 3,000 of flanking, homologous nucleotides. PCR-based mutagenesis (QuikChange XL, Stratagene) was used to generate two alterations in the targeting vector. First, seven nucleotides were altered to produce the $\alpha 3[5]$ substitutions in the $\alpha 3$ gene product (YKHEIKYN to WKHWVYYT), along with a *Bst*Z17I restriction enzyme cleavage site. Mutagenic PCR primers were designed according to the guidelines in the QuikChange technical manual: forward (5'-GCCATCATTAAGCCCCGGGCTGGAAACATTGGGTAT ACTACACCTGCTGT GAGGAGATCTACC-3') and reverse (5'-GGTAGATCTCCTCACAGCAGGTGTAGT ATACCAATGTTTCCAGCC CGGGGCTTTAATGATGGC-3'). Positive clones were identified by a restriction analysis using *Bst*Z17I.

Second, a 57 nucleotide region containing a Cre-recombinase recognition sequence (*loxP*) positioned just 5' to exon V in the original targeting vector was removed. The sequences of the mutagenic primers used for the *loxP* removal were as follows: forward (5'-GGTGATAAGTGTGGCAAATTATGTGCC AGCAGAGGCGGG GGTGGTGG TGGTGAATAACCAATGTGGG-3') and reverse (5'-CCCACATTGGTTATTC ACCACCACCACCCCGCCTCTGCTGGCACAT AATTTGCCACACTTATCACC-3'). Positive clones were identified by standard PCR methods. Plasmid DNA was isolated using Mini or Maxi Plasmid Kits (Qiagen Venlo, NL).

Gene targeting in embryonic stem (ES) cells

AB2.2 ES cells derived from a 129S7 male embryo were grown on mitotically inactive SNL76/7 cells and used for targeting. Ten million ES cells were electroporated with 25 μ g of $\alpha 3[5]$ targeting vector linearized with *Sa*I, and G418 selection was initiated after 24 h. One-hundred and ninety-two neomycin resistant clones were selected for further analysis. Correctly targeted ES cell clones were identified as described (Ramirez-Solis et al., 1995).

Generation of mice

All breeding and procedures were carried out according to approved institutional procedures at Brown University Animal Facility and in agreement with the NIH Guide for the Use and Care of Laboratory Animals. The targeted ES cells were grown to 90% confluence and trypsinized before injection. E3.5 blastocysts were derived from C57BL/6-*Tyr^{c-Brd}* female mice and injected with 12–20 ES cells. The injected blastocysts were implanted into the uteri of day 2.5 pseudo-pregnant females for generation of chimeras. About 8–10 injected embryos were implanted per uterine horn.

The resulting male chimeras were mated with C57BL/6-*Tyr^{c-Brd}* females to obtain F1 progeny. Thus, the strain carrying the germ line transmitted $\alpha 3[5]$ allele (designated *Chrna3^{tm1.0Hwrt}*) (Fig. 1A) was obtained and initially maintained on a mixed C57BL/6-129S7 background. To obtain mutant mice carrying $\alpha 3[5]$ with Neo cassette deleted (allele designated *Chrna3^{tm1.1Hwrt}*), heterozygous *Chrna3^{tm1.0Hwrt/+}* mutants were mated with 129S1-*Hprt1^{tm1(cre)Mnn/J}* mice (The Jackson Laboratory, Bar Harbor, ME) and the offspring were screened for the presence of the $\alpha 3[5]$ mutations and for the deletion of Neo (*Chrna3^{tm1.1Hwrt/+}* mice). Congenic mice were generated by 10 consecutive backcrosses of *Chrna3^{tm1.1Hwrt/+}* to wild-type C57BL/6 mice. To obtain *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* *Chrna7^{tm1Bay/tm1Bay}* double homozygous animals ($\alpha 3[5]$ -homozygous $\alpha 7(-/-)$), *Chrna3^{tm1.1Hwrt/+}* animals were mated with *Chrna7^{tm1Bay/tm1Bay}* mice (B6.129S7-*Chrna7^{tm1Bay/J}*; the Jackson Laboratory, Bar Harbor, ME) and double heterozygous mice were selected. In the next step *Chrna3^{tm1.1Hwrt/+}* *Chrna7^{tm1Bay/+}* animals were intercrossed and screened for *Chrna3^{tm1.1Hwrt/+}* *Chrna7^{tm1Bay/tm1Bay}*. In the final step, *Chrna3^{tm1.1Hwrt/+}*

Chrna7^{tm1Bay/tm1Bay} and *Chrna3^{tm1.1Hwrt/+}* *Chrna7^{tm1Bay/+}* animals were intercrossed and offspring selected for *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* *Chrna7^{tm1Bay/tm1Bay}*.

Southern blot analysis

ES cell clones and transgenic animals were screened for the knock-in allele using Southern blot analysis. The Southern strategy utilized a *KpnI* restriction cleavage site in the targeting construct. Digestion with *KpnI* was expected to produce 17.4 kb fragment from the wild-type allele, and 8.9 kb and 10.5 kb fragment in the case of the mutated allele. Two 0.6 kb nucleotide probes were designed to anneal outside of the targeted region of *Chrna3*. The Southern probes were generated by PCR, purified using QIAquick PCR Purification Kit (Qiagen). The probes were labeled with ³²P-dCTP by the random primer labeling method according to the manufacturer's protocol in the Megaprime DNA Labeling Systems (Amersham, GE Healthcare UK). *KpnI* digested genomic DNA (10–15 µg /sample) from ES cell clones or transgenic animals was separated by agarose gel electrophoresis (1% gel; run at 50V for 16 h) and transferred to a nylon membrane, Hybond-XL (Amersham, GE Healthcare UK), by the capillary blotting method. Southern hybridizations were performed using standard protocols.

Animals

Mice were housed in Brown University Animal Care Facility and McGill University Animal Resource Center mouse rooms. Routine cage maintenance, including feeding and watering, was performed by the animal facility staff. Mice were maintained on a 12 hour light/dark cycle. Mating cages were typically set up with one male and one female. Pups were weaned at 21 days. Tail biopsies were used as tissue sources for DNA extraction and genotyping. All survival surgeries were performed using sterile procedures within the animal care facility mouse rooms. In cases in which euthanasia was necessary, mice were asphyxiated with CO₂ according to Brown University's policy on the "Use of CO₂ as a Euthanasia Agent for Small Laboratory Animals" and guidelines established by the Canadian Council on Animal Care. All animal use procedures described in this study were reviewed and approved by the designated institutional animal use committee.

Genotyping

Pups were screened using two PCR-based strategies. The first method screened directly for the wild-type or recombinant allele using the following forward primers: GCTGGAAACATTGGGTATACTACACC specific for the knock-in allele and GGCTACAAACATGAAATCAAGTACAACCTGC specific for the wild-type allele. The same reverse primer positioned 400 bp downstream of the targeted region CCGTAGAAGTTCCTCGTCTTTGGG was used in both reactions. "Triple-Master PCR System" (Eppendorf, Hamburg, DE) was used for all PCR-based screens.

The second PCR-based method for genotyping the α3[5]-containing allele utilized a *BstZ17I* restriction site in the mutated region. The digestion-based PCR screen amplified a ~650 bp product with an internal restriction site. A forward primer GTGACCTACTTCCCGTTTGACTACC was designed to anneal ~250 bp upstream of the mutated region. The reverse primer CCGTAGAAGTTCCTCGTCTTTGGG was placed ~400 bp downstream of the targeted region. The PCR products were subjected to restriction digestion using *BstZ17I* (NE Biolabs, Ipswich, MA) and agarose gel electrophoresis (2% gels). Reaction products from wild-type alleles were resistant to digestion, whereas PCR products from heterozygous or homozygous mutants were partially or completely digested with *BstZ17I*, respectively.

Primers GGATCTCCTGTCATCTCACCTTGCT and ATCCTGATCGACAAGACCGGCTTC were used to identify mice positive for the Neo

cassette. Primers CTTGTCCATCGTCATCACAGTCT TTGTG and GAGTCTAATTTTCTAACCTCTGCCCTATGC were used to identify mice negative for Neo.

Primers TGCTGTTTCACTGGTT GTGCGGCG and TGCCTTCTCTACACCTGCGGTGCT were used to identify mice positive for the Cre-recombinase expression cassette. Primers CCTGATTTTATTTCTATAGGACTGAAAGAC and TAAGTAATTATACTTACACAGTAGCTC TTC were used to identify mice negative for Cre.

The *Chrna7^{tm1Bay}* allele in $\alpha 7$ knockout mice (B6.129S7-*Chrna7^{tm1Bay}*/J) was detected by PCR as recommended by the Jackson Laboratory (Bar Harbor, ME) using primers IMR1002, IMR1003 and IMR1004.

Reverse-transcriptase PCR

SCG tissue from wild-type and knock-in mice was dissected and placed immediately into ice-cold RNA-later solution (Ambion, Austin, TX) and stored at 4° overnight. RNA isolation was performed using RNeasy-later (Ambion) according to the manufacturer's protocol. In brief, SCG tissue was disrupted in 500 μ l of lysis/binding buffer using a 1 ml Potter-Elvehjem tissue grinder (Kontes, Vineland, NJ). DNase I treatment was performed by adding 7 μ l of 10X DNase buffer to 1 μ l of DNase. To precipitate the RNA, 2.5 volumes of 100% EtOH were added, and the mixture was stored overnight at -20°C. The pellet was resuspended in 12 μ l of RNase-free H₂O. Total RNA concentration was measured by UV absorbance using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcriptase (RT) reactions were performed using the RETROscript Kit (Ambion, Austin, TX) according to manufacturer's protocol for 2-step RT-PCR without heat denaturation of RNA. 0.4–1 μ g of total RNA was used per RT reaction. Random primers were used for all RT reactions. PCR primer pairs were designed to generate ~480 bp products from wild-type and mutant cDNA templates. If genomic DNA was present and amplified, ~8500 bp PCR products would be expected. A wild-type forward primer (CATCCAGTTTGAGGTGTCTATGTCTCAG) was designed to anneal within exon II of mouse *Chrna3*. Mutant and wild-type allele-specific reverse primers were designed to anneal within the targeted region of exon V (wild-type, GCAGTTGTACTTGATTTCATGTTTGTAGC and $\alpha 3[5]$, CAGGTGTAGTATACCCAATGTTTCCAGC). TripleMaster PCR System (Eppendorf, Hamburg, DE) was used for all amplification reactions, which were assembled using 600 ng of total cDNA per reaction. Positive control primers designed to amplify the *rig/S15* "housekeeping" gene sequence were supplied by Ambion and negative controls included reagent-only and RNA-only reactions.

Live-staining of SCG

To label recombinant $\alpha 3[5]$ -containing receptors, freshly dissected ganglia from $\alpha 3[5]$ -homozygous mice (>2 months of age) asphyxiated with CO₂ were placed in L15 Air solution containing 100 nM rhodamine conjugated α BTX (rhoBTX, Sigma Chemical) for 1 h. Next, ganglia were placed in 0.5% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for 1 h, transferred into a solution of 3% Triton-X in phosphate buffered saline (PBS) for 3 h, and then incubated overnight with primary antibodies (polyclonal goat anti-VACHT (1:750, Chemicon, Temecula, CA) and monoclonal mouse anti-neurofilament (NFM; 1/10000, Sternberger) or polyclonal goat anti-post synaptic density 93 (PSD93; 1/100, Synaptic Systems) dissolved in PBS containing 10% normal donkey serum at 4°C. The next day ganglia were rinsed twice for 15 min in PBS and placed in secondary antibodies Alexa 488 donkey anti-goat antibodies (1/1000, Molecular Probes) or Alexa 402 donkey anti-mouse antibodies (1/1000, Molecular

Probes) in PBS containing 10% normal donkey serum for 2.5 h at room temperature. The ganglia were then rinsed twice for 15 min with PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Nonspecific staining was assessed by processing the sections without primary antibody.

Imaging

A series of z stacks (0.3 $\mu\text{m}/\text{plane}$) was obtained with a confocal microscope (LSM 510; Zeiss) and a 63x, 1.4 numerical aperture Plan Neofluor oil-immersion objective. In experiments with denervated ganglia, we set values for detector gain, detector offset, and amplifier offset by imaging control ganglia from the same mouse stained with rhoBTX and anti-VAcHT and found values that captured the distribution of intensities within an image depth of 8 bits. We then used these acquisition parameters to image denervated ganglia. For colocalization experiments where the intensities of the signals were less important, we adjusted the acquisition settings to optimize the staining for synaptic proteins.

Images were quantified off-line with MetaMorph (Universal Imaging Corporation, Downingtown, PA, USA). The representative images in figures of rhoBTX stained and VAcHT-stained SCG were created by averaging three consecutive image planes from a z stack, cropping the averaged image, splitting the color components of the cropped image, thresholding each color channel individually to 2x the value of background intensity, and adding the two component images back together. Representative images of NFM/VAcHT/rhoBTX triple stained ganglia were processed in a similar way. Occasionally, we used the low pass filter tool with a 2x2 convolution kernel to remove single pixel noise from our images.

Extracellular and intracellular recordings

Ganglia from mice greater than 2 months of age were pinned down in a small recording chamber (1.5 ml volume) with minutia pins, perfused continuously at 3–4 ml/min with oxygenated modified Tyrode's solution supplemented with glucose (5.6 mM) and choline (0.01 mM) (pH = 7.3–7.4) at 36–37°C, and viewed through a dissecting microscope (SMZ-10; Nikon, Tokyo, Japan). The preganglionic nerve in the cervical sympathetic trunk was connected to a stimulator (Warner instruments) with a suction electrode, and the postganglionic trunk was connected to an alternating current differential amplifier (DP-301; Warner Instruments, Hamden, CT) with a suction electrode. The postganglionic compound action potentials were amplified (1000x), filtered at 10 Hz (low-pass cutoff) and 3 kHz (high-pass cutoff), digitized, displayed, and stored on a Pentium II-based personal computer with Patchkit (Alembic Software, Montreal, Quebec, Canada). The data were analyzed off-line with Igor (WaveMetrics, Lake Oswego, OR). All drugs were added directly to the oxygenated Ringer's solution.

Glass microelectrodes with a resistance of 40–80 M Ω (G150F-4; Warner Instruments) made with a DMZ universal puller (Zeitz Instruments, Munich, Germany) and filled with 1 M potassium acetate (KAc) were used for intracellular recordings from ganglion cells. Stable intracellular recordings were achieved with a high inertial precision microdrive (Inchworm 8200; EXFO, Vanier, Quebec, Canada) attached to a micromanipulator (SM11; Narishige, Tokyo, Japan) to drive the electrode through the ganglion. The recording electrode was connected by a thin silver chlorided wire to the head stage of an Axoclamp 2A amplifier (Axon Instruments, Union City, CA) used in current-clamp mode. Depolarizing or hyperpolarizing constant current pulses were applied through the recording electrode, and membrane potentials were filtered at 10 kHz, sampled, displayed, and stored on a Pentium II-based personal computer. Stimulation and acquisition was done with Patchkit software (Alembic Software), and the data were analyzed with IGOR. The preganglionic nerve was stimulated with brief (0.1–0.2 ms) voltage pulses applied to the cervical sympathetic trunk through the suction electrode. αBTX (100 nM) and methyllycaconitine (MLA; 50 nM) were dissolved in

oxygenated Tyrode's solution modified as above. Only neurons with membrane potentials more negative than -40 mV were included in this study.

Acute dissociation and culturing of SCG neurons

SCG ganglia were removed under sterile conditions from neonatal mice (P12–P14) following asphyxiation with CO_2 . The ganglia were incubated at 37°C in a trypsin solution (1 mg/ml of trypsin from Worthington Biochemical Corp. Lakewood, NJ, USA, 3X crystallized TRL3) in Hank's Balanced Salt Solution (HBSS) without Ca^{+2} or Mg^{+2} . After 1 hour the ganglia were triturated gently with a fire polished glass pipette. The resultant solution was centrifuged for 5 minutes and rinsed with growth media (L-15 media supplemented with vitamins, cofactors, penicillin-streptomycin, 5% rat serum and NGF (25–50 ng/ml). The cells were then plated on laminin-coated coverslips and incubated at 37°C (5% CO_2) until needed. The neurons were transferred to 30°C for 24 h prior to recording.

Whole-cell electrophysiology

Whole-cell patch-clamp recordings were made with an Axopatch 200B integrated patch clamp. Patch electrodes had a resistance of between 2–5 M Ω . Data were recorded with Axon Clampex 9.0 and visualized on Axon Clampfit 9.0. Axon Patch electrodes were filled with 65 mM KF, 55 mM KAc, 5 mM NaCl, 0.2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM EGTA, and 10 mM HEPES, pH 7.4. The cells were continuously perfused with extracellular recording media (140 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO_4 , 0.44 mM KH_2PO_4 , 2.8 mM CaCl_2 , 0.18 mM MgCl_2 , 10 mM HEPES, 5.6 mM glucose, 2 mM glutamine, 5 $\mu\text{g/ml}$ phenol red and 1–2 μM atropine, pH 7.4). The agonist ACh was dissolved in extracellular recording media and delivered through an Eppendorf Femtojet pressure perfusion system at 50 kPa for 1 sec through a glass capillary drawn to a diameter of 5–10 μm . Alternatively, in some experiments a 2 sec pulse of ACh was applied to cells through a gravity-fed multibarrel micropipette system.

Denervation

For denervation experiments, we modified procedures described by McFarlane and Cooper (1992) for neonatal rats. Briefly, mice older than 2 months of age were anesthetized with Ketamine-Xylazine by intraperitoneal injection. The preganglionic cervical sympathetic trunk on one side was exposed and transected approximately halfway between the SCG and the first rib. After the surgery, the mice were returned to their cages for different times up to 2 wk. We observed that all denervated animals exhibited pronounced ptosis of the eye on the operated side. In most animals, we confirmed that the denervation was complete by electrophysiologically recording from the postganglionic nerve with extracellular electrodes while stimulating the preganglionic stump. All denervation procedures were carried out at McGill University in accordance with the guidelines established by the Canadian Council on Animal Care.

Statistical methods

The statistical test applied to the analysis of genotype distribution was the one-way Chi-square test. The statistical test applied to analysis of electrophysiological measurements was the unpaired t-test using standard tables and online software provided through The Free Statistics Calculator Website (<http://www.danielsoper.com/statcalc>).

Results

Generation and characterization of $\alpha 3[5]$ knock-in mice

Mice with αBTX -sensitive $\alpha 3$ -containing nAChRs (*Chrna3^{tm1.0Hwrt}*) were generated by introducing mutations to the $\alpha 3$ gene (*Chrna3*) located on chromosome 9. Following

electroporation of the targeting DNA into mouse ES cells, a total of 192 G418-resistant candidates were collected and screened. Six recombinant ES cell clones were successfully identified using Southern blot analysis (Fig. 1B). The 3' probe detected a single band of ~17.4 kb in the wild-type sample, and a ~17.4 kb band along with a band of ~10.5 kb in the correctly targeted clones. With the 5' probe, a ~17.5 kb band was detected from the wild-type allele and an ~8.9 kb band from the targeted allele.

Genomic DNAs from the correctly targeted ES cell clones were further analyzed by the *KpnI* restriction site-based PCR detection assay and allele-specific PCR to confirm the presence of the $\alpha 3[5]$ mutation (data not shown). Although the neomycin resistance cassette was detected by Southern blot and PCR in all six of the homologous recombinants, the $\alpha 3[5]$ mutations could only be identified in three of six ES cell clones (A6, D4, and D12). These clones were used to generate chimeras and F1 and F2 *Chrna3^{tm1.0Hwrt/+}* animals.

Next, we removed the neomycin cassette by mating *Chrna3^{tm1.0Hwrt/+}* males and 129S1-*Hprt1^{tm1(cre)Mnn/J}* females. The 129S1-*Hprt1^{tm1(cre)Mnn/J}* strain carries an X-linked Cre-recombinase expression cassette that mediates the excision of DNA segments flanked by *loxP* sites (Tang et al., 2002). The Neo-deleted offspring (*Chrna3^{tm1.1Hwrt/+}*) were intercrossed to generate homozygous *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* pups, which were identified by Southern blot analysis (Fig. 1C). Initial breeding attempts indicated that ~87% of *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* animals died within two weeks of birth (data not shown) and exhibited a phenotype resembling that previously reported for $\alpha 3$ -null ($-/-$) mutants (Xu et al., 1999), with the exception that the ptosis in $\alpha 3[5]$ homozygotes appeared less severe.

To ensure that $\alpha 3[5]$ mRNA was being produced in knock-in animals, we performed RT-PCR using RNA isolated from the SCG of *Chrna3^{tm1.1Hwrt/+}*, *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}*, and wild-type mice. With the wild-type allele-specific primers, an expected ~480 bp product was detected from *Chrna3^{tm1.1Hwrt/+}* ($\alpha 3[5]/+$) and wild-type cDNAs, but not from *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* ($\alpha 3[5]/\alpha 3[5]$) cDNA. The $\alpha 3[5]$ allele-specific primers amplified a 480 bp product from both mutant samples, but not from the wild-type template (Fig. 1D). These results indicated that the mutated allele was actively transcribed and mRNA produced in the SCG of *Chrna3^{tm1.1Hwrt/+}* and *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice.

Crosses with inbred and outbred strains demonstrate the influence of genetic background on the phenotype of $\alpha 3[5]$ homozygotes

Our $\alpha 3[5]$ knock-in mice were maintained initially on a mixed S129S7/C57BL/6 background. After removing the neomycin cassette, we backcrossed them >10 times into C57BL/6 strain to generate congenic *Chrna3^{tm1.1Hwrt/+}* animals. The transmissibility of the mutant allele was then characterized in these congenic mice (Table I). Matings of *Chrna3^{tm1.1Hwrt/+}* animals with wild-type mice produced the expected Mendelian distribution of genotypes (1:1). As *Chrna3^{tm1.1Hwrt/+}* animals were fertile and had normal appearance and behavior for at least the first 12 months of life, no obvious dominant effects resulted from the presence of the mutant allele. Intercrosses of *Chrna3^{tm1.1Hwrt/+}* males and females, however, failed to produce the expected proportion of *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* pups, indicating that about 50% of homozygous mutants died before genotyping was conducted (at 1–2 weeks of age). The surviving *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* animals in the C57BL/6 background typically died within three months of birth. Pathologic examination performed on a deceased *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* animal at the age of three months identified mild hydronephrosis of the kidney. However, other major organs were determined to be within normal limits, and the cause of premature death remains unknown. All together, our mating analyses indicate that the five amino acid substitution in the $\alpha 3$ subunit of nAChR constitutes a recessive, hypomorphic mutation that reduces lifespan in the C57BL/6 inbred background.

To increase viability and lifespan of mutant mice, it is sometimes advantageous to transfer the generated mutation on an outbred background. Outbred mice maintain maximum heterozygosity and can offer numerous advantages over inbred strains, such as longer life spans, disease resistance, and more robust breeding (Silver, 1995). To test whether outbred genetic backgrounds would influence the observed phenotypes, we outcrossed *Chrna3^{tm1.1Hwrt/+}* mice with two outbred strains: ICR (Taconic, Hudson, NY) and CD-1 (Charles River Canada, St. Constante, Qc). Intercrosses between ICR outbred *Chrna3^{tm1.1Hwrt/+}* animals are summarized in Table II. Although in the sample matings tested the proportion of homozygous *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice was somewhat lower than expected, the differences were not statistically significant ($P > 0.05$). Moreover, lifespan was increased significantly to an average of five months following outcrosses with the ICR strain. Similar results were obtained with mice outcrossed to the CD-1 strain. For these reasons, outbred-derived $\alpha 3[5]$ homozygotes were chosen for our initial characterizations of synaptic function in intact SCG.

α BTX-binding sites colocalize with synaptic markers in sympathetic ganglia from $\alpha 3[5]$ -homozygous mice

In murine sympathetic ganglia, $\alpha 3$ -containing nAChRs are essential for fast synaptic transmission (Rassadi et al., 2005; Krishnaswamy and Cooper, 2009). Therefore, to determine whether $\alpha 3[5]$ subunits were surface-expressed at synapses, we stained living SCG with rhodamine-conjugated α BTX (rhoBTX). The ganglia were then post-fixed and stained with anti-neurofilament (NFM-H) to locate the preganglionic axons, and with anti-vesicle ACh transporter (VACHT) to locate the presynaptic terminals. Sympathetic neurons in SCG obtained from mature (2–3 month) *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice yielded bright α BTX-labeled clusters that colocalized with presynaptic varicosities on the soma and dendrites of sympathetic neurons (Fig. 2A,C,D,E). Moreover, these α BTX-labeled clusters colocalized with PSD93 (Fig. 2B,D,E), indicating that $\alpha 3[5]$ -containing receptors are clustered at synapses. At many of the PSD-93 and VACHT sites, the signal from the clusters of $\alpha 3[5]$ -containing receptors was too low to be resolved from background, leading to less than complete correlation (Fig. 2E). In all cases, fluorescent labeling by α BTX was completely abolished by preincubation with 100 nM unlabeled α BTX (Fig. 2C).

Rodent sympathetic neurons express $\alpha 7$ mRNA (Mandelzys et al., 1995; De Koninck and Cooper, 1995) and have 125 I- α BTX-binding sites (Smolen 1983; De Koninck and Cooper, 1995; Del Signore et al., 2004). This raised the possibility that the α BTX labeling in $\alpha 3[5]$ -containing ganglia from *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice could be due to $\alpha 7$ nAChRs rather than $\alpha 3[5]$ -containing receptors. To rule out this possibility, we crossed $\alpha 3[5]$ mice with $\alpha 7$ null mice and generated *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}Chrna7^{tm1Bay/tm1Bay}* double mutants (i.e., $\alpha 3[5]$ -homozygous and $\alpha 7(-/-)$). Punctate α BTX labeling on sympathetic neurons from these $\alpha 3[5]$ -homozygous $\alpha 7(-/-)$ ganglia was similar in appearance and density to that on neurons from $\alpha 3[5]$ -homozygous $\alpha 7(+/+)$ ganglia (Fig. 2F), indicating that $\alpha 7$ nAChRs are not contributing to the observed fluorescent signal (Fig. 2C). Moreover, preincubation with 50 nM MLA, a specific $\alpha 7$ antagonist, had no effect on rhoBTX labeling in $\alpha 3[5]$ -homozygous ganglia from mice in the $\alpha 7(+/+)$ background (Fig. 2C). Mice with wild-type $\alpha 3$ and $\alpha 7$ failed to give rise to any detectable rhoBTX signal (Fig. 2F).

Synaptic transmission in SCG of $\alpha 3[5]$ -homozygous mice is sensitive to α BTX block

The results above indicate that $\alpha 3[5]$ -containing postsynaptic receptors are located at synapses in the SCG of *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice. Next, we asked whether α BTX blocked synaptic activity in the SCG of these mice. To measure synaptic transmission, we stimulated the preganglionic nerve and recorded the nerve-evoked compound action potentials (CAP) extracellularly from the postganglionic trunk. In wild-type ganglia, stimulating the

preganglionic nerve produced a 1.5–3 mV CAP (n=12). In line with previous studies, the CAP in wild-type ganglia was unaffected by α BTX (100 nM for 20–30 min; n=6) (Fig. 3A). The nerve-evoked CAPs recorded from *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice were smaller (0.2–0.3 mV; n=15) than those from wild-type ganglia. However, unlike CAPs from wild-type ganglia, the CAPs from α 3[5]-homozygous ganglia were completely blocked by α BTX (Fig. 3A). Similar levels of block were observed in all preparations tested (n=12).

To further investigate synaptic transmission of intact SCG, we recorded excitatory postsynaptic potentials (EPSPs) intracellularly from these sympathetic neurons. With SCG from wild-type mice, the nerve-evoked EPSPs on sympathetic neurons were large (20–30 mV; n=20) and suprathreshold (Fig. 3B). As with the CAPs, none of the EPSPs recorded from wild-type ganglia were blocked by α BTX (100 nM for 20–30 min; n=6). In contrast, the majority (>80%) of nerve-evoked EPSPs on sympathetic neurons in *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* SCG were subthreshold (n=25) with peak amplitudes of only 3.6 ± 0.8 mV (n=25). Most relevantly, however, all EPSPs (10/10) on α 3[5]-homozygous neurons were blocked by α BTX (Fig. 3B).

To ensure that the nerve-evoked EPSPs on α 3[5]-homozygous neurons were not due to resident α 7-containing nAChRs (Cuevas et al., 2000; Severance et al., 2004), we examined nerve-evoked EPSPs in ganglia from *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}Chrna7^{tm1Bay/tm1Bay}* (α 3[5]-homozygous and α 7(-/-)) mice. All nerve-evoked EPSPs on sympathetic neurons in the SCG of these homozygous double mutants were similarly blocked by α BTX (n=12), indicating that the α BTX-sensitive EPSPs were mediated by α 3[5]-containing receptors (Fig. 3B).

Although we observed reproducible labeling of α 3[5] subunits at synapses, we wondered why the nerve-evoked EPSPs on α 3[5]-homozygous sympathetic neurons were smaller than those on wild-type neurons. Conceivably, replacing 5 amino acids in the α 3 subunit with corresponding residues from α 1 of *Torpedo californica* may have interfered with the function or surface expression of α 3[5]-containing receptors. To examine the possibility that the α 3[5] mutation significantly altered the EC₅₀ for ACh, we carried out a dose response study on cultured α 3[5]-homozygous SCG neurons (Fig. 4). We obtained an EC₅₀ of 48 ± 2 μ M using cultured SCG neurons isolated from *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice, and SCG neurons from wild-type mice yielded an EC₅₀ of 64 ± 3 μ M, which is in line with published values (Kristufek et al., 1999). Thus, the smaller evoked responses observed in intact SCG are not due to a decrease in affinity or efficacy of the α 3[5]-containing nAChRs. In order to define the association and dissociation characteristics of the observed α BTX block in SCG, we carried out a study with cultured dissociated SCG neurons to follow the time course of onset of block, and the time course of recovery of function following a washout of the α BTX (Fig. 5). Full block was achieved within 3–5 minutes upon application of 100 nM α BTX, and full recovery was obtained 15 minutes after the α BTX was removed. The dissociation of α BTX is relatively rapid, certainly with respect to α BTX binding to the neuromuscular junction, but complex formation is empirically stable enough to allow fluorescence labeling under most conditions using live cells (e.g., Fig. 2).

To examine the possibility that the α 3[5] mutation adversely affects surface expression, we carried out additional electrophysiological studies measuring ACh-evoked currents from cultured SCG neurons isolated from neonatal *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice that were fully congenic (N10) on the C57BL/6 inbred background. No ACh-evoked currents could be detected from α 3[5]-homozygous neurons under standard conditions of culture at 37°C (Fig. 6B). However, ACh-evoked currents were detectable following a shift in culture incubation temperature from 37°C to 30°C for 24 h (Fig. 6B), consistent with studies showing that reduced temperature increases the surface expression of some nAChR subtypes (Ross et al., 1991; Cooper et al., 1999; Wanamaker & Green, 2007). The ACh-evoked current densities recorded from cultured *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* neurons at 30°C were approximately 6% of

those from control neurons and were blocked by α BTX (100 nM) (Fig. 6B). Our analysis of SCG neurons from double homozygous mutant mice (α 3[5]-homozygous α 7(-/-)) yielded similar results (Fig. 6B), further indicating that the observed ACh-evoked currents were not mediated by α 7-containing nAChRs. In comparison, a 24 hr incubation at 30°C of dissociated SCG neurons obtained from wild-type mice did not result in any significant change in current densities nor in the expected insensitivity to α BTX block. These results suggest that the amino acid substitutions in α 3[5] likely have a negative impact on some critical aspect of receptor subunit assembly and/or surface expression, which can be relieved in part by a decrease in incubation temperature.

Next, considering the unexpected differences in surface expression of α 3[5]-containing nAChRs in C57BL/6 (Fig. 6B) versus outbred ICR/CD-1 backgrounds (Fig. 2), we sought to more fully explore the influence of genetic background on surface expression of functional α 3[5]-containing nAChRs. To do this, we crossed fully congenic C57BL/6 *Chrna3^{tm1.1Hwrt/+}* mice with wild-type α 3 mice from the following strains: C3H, DBA, BALB/cJ, and ICR. Heterozygous F1 offspring from each of the above-mentioned strains were obtained, from which F2 *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice were generated through intercrosses. We then prepared primary cultures of SCG neurons from neonatal *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice in these various backgrounds and measured ACh responses following culture incubation at 30°C. The ACh-evoked current densities of SCG neurons from *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice derived from the outbred ICR background were dramatically increased compared to neurons from congenic C57BL/6 mice (Fig. 6C). Of several inbred strains tested in this fashion, the BALB/cJ background appears to support a significant increase in ACh response amplitude. In addition, we observe that lifespan is greatly extended in *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice on the BALB/cJ background. Whereas <2 % of *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice on the C57BL/6 or S129 genetic background survive beyond 70 days of age, more than 60% of the homozygous mice on the BALB/cJ background survive to that point. In addition, none of the *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice on the BALB/cJ background are born runted, whereas all such homozygotes are born runted on the C57BL/6 background. In all outward appearances, the *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice on the BALB/cJ background are indistinguishable from mice with wild-type α 3. Furthermore, in preliminary studies, cultured SCG neurons from α 3[5] homozygotes produced from early backcrosses onto the C57BL/6-BALB/cJ background and incubated from 24 to 48 hours at 30°C have produced ACh responses approaching near wild-type levels (data not shown). Moreover, in contrast to the situation with C57BL/6, we have observed ACh-evoked whole-cell currents in SCG neurons from older BALB/cJ- and ICR-derived α 3[5]-homozygous mice without the necessity of a 30°C incubation period.

Preganglionic denervation confirms postsynaptic localization and reveals an unexpected short-term increase in cluster size and density

The colocalization of α 3[5]-containing receptor clusters with presynaptic terminals at ganglionic synapses, as demonstrated in Fig. 2, prompted us to ask whether the presynaptic nerve has a direct role in clustering receptors, similar to the role of presynaptic terminals at the neuromuscular junction (Sanes and Lichtman, 1999; Gingras et al., 2002). Therefore, we cut the preganglionic nerve to the SCG in *Chrna3^{tm1.1Hwrt/tm1.1Hwrt}* mice and examined denervated neurons at different times up to 2 weeks. Within 2 days of denervation, we observed a significant increase in the size of the α BTX-labeled clusters as well as an increase in the density of clusters compared to control (Fig. 7A,B,E). These increases likely reflect a response similar to denervation supersensitivity, a process observed in many other synaptic systems (Sharpless, 1975; Cangiano, 1985). Interestingly, these changes in clustering were transient, and, by one week after denervation, the receptor clusters were not significantly different from those on neurons in the contralateral control ganglia (Fig. 7C,E). Over the following week, the clusters continued to decrease in size and density (Fig. 7D,E). We did not determine whether this

decrease in cluster-labeling, as summarized in Fig. 7E, reflects a dispersal of receptors similar to what is observed on autonomic neurons in PSD93-null mice after denervation (Parker et al., 2004), or whether it reflects a decrease in the total number of receptors at the surface. This analysis confirmed the postsynaptic localization of the rhoBTX-labeled clusters and revealed new insights into the architecture and dynamics of nAChR clustering following disruption of normal preganglionic input.

Discussion

Efforts to understand fully the physiological roles of specific heteromeric nAChR subtypes in the central nervous system are limited by lack of reliable subtype-selective ligands. Antibodies against neuronal nAChR subunits are often problematic for isolating receptors from intact tissue or neurons, as most endogenous antigenic determinants are localized to the cytoplasmic portion of the subunits. Moreover, studies with knock-out mice have raised serious questions concerning the specificity of several types of neuronal nAChR antibodies (Herber et al., 2004; Moser et al., 2007). Although some α -conotoxins have demonstrated remarkable selectivity among particular nAChR subunit interfaces, additional strategies are still needed to address the critical questions concerning subunit identification in the physiological setting (Millard et al., 2009).

The $\alpha 1$ subunit from the muscle-type nAChR contains the major determinants for binding the classic nicotinic antagonist, α BTX, while most neuronal nAChRs, apart from the $\alpha 7$ subgroup, are completely insensitive to α BTX. Given the considerable homologies shared by the genes encoding the muscle and neuronal nAChR subunits, we hypothesized that functional sensitivity to α BTX could be conferred with minimally mutated neuronal nAChR subunits. This approach has been validated in principle with the neuronal rat $\alpha 3$ subunit as studied in *Xenopus* oocytes (Levandoski et al., 1999). For decades, α BTX has been used extensively for the study of nicotinic receptors. Fluorescent-labeling with α BTX has many practical advantages over staining with antibodies: α BTX is a relatively small, cell-impermeant protein with numerous commercially available conjugates. Antibodies, on the other hand, are relatively large, divalent molecules which may cap or cluster surface antigens and compromise surface trafficking (Sekine-Aizawa and Haganir, 2004). Efforts in our lab have been focused on extending the experimental advantages of α BTX to the study of α BTX-insensitive neuronal nAChRs. Recently, we introduced an 11 amino acid $\alpha 1$ -derived, α BTX-binding sequence into the loop C region of the $\beta 4$ neuronal nAChR subunit. Co-injection of cRNAs from wild-type $\alpha 3$ together with the chimeric $\beta 4/\alpha 1[11]$ resulted in functional channels that were allosterically inhibited by α BTX (Sanders & Hawrot, 2004). Similar results have been achieved using an analogous chimeric $\beta 2$ subunit (Sanders & Hawrot, manuscript in preparation) as well as with an analogous chimeric $\alpha 5$ subunit (Chew, Sanders & Hawrot, manuscript in preparation). In such a context, we refer to these ectopic α BTX-binding sequences as “pharmatopes,” as the pharmacological block is localized to subunit-interfaces not normally involved in agonist binding (Sanders & Hawrot, 2004).

High-affinity α BTX-binding sequences have been used in a variety of trafficking studies involving non-nicotinic receptor proteins. Sekine-Aizawa and Haganir (2004) inserted a 13-mer peptide sequence known as HAP-1 (Kasher et al., 2001, Fuchs et al., 2003) at the N-terminus of the GluR2, AMPA receptor subunit. This strategy facilitated the monitoring of surface expression and trafficking dynamics of the α BTX-tagged AMPA receptor subunits expressed in HEK cells using radioactive, fluorescent, and biotinylated α BTX-conjugates. Bogdanov et al. (2006) utilized a comparable approach for investigating the membrane-trafficking of GABA_A receptors in hippocampal neurons. Guo et al. (2006) engineered a 13 amino acid α BTX-binding sequence into the N-terminus of a metabotropic glutamate receptor, mGluR8a, and visualized surface-expressed receptors in rat sympathetic neurons using

fluorescein-conjugated α BTX. More recently, Wilkins et al. (2008) monitored the cell-surface mobility of α BTX-tagged GABA_B subunits (R1a) in real time in transfected hippocampal neurons.

Our present study expands on the simple tagging approach by introducing pharmacological sensitivity to is α BTX *in vivo*. We provide the first example of a gain-of- α BTX-sensitivity knock-in mouse in which the primary determinants for α BTX-binding are incorporated into the homologous region of a native nAChR subunit that is normally insensitive to α BTX. Our studies show that α BTX-sensitive $\alpha 3[5]$ subunits are incorporated into functional nicotinic receptors in the SCG neurons of homozygous knock-in mice when the appropriate genetic background is used (see discussion below). Live surface-staining with rhodamine-conjugated α BTX revealed high-density receptor clusters in ganglia from $\alpha 3[5]$ -homozygous animals (Fig. 2). All α BTX-labeled clusters colocalized with VAcHT (Fig. 2D), a marker for presynaptic varicosities and with PSD-93 (Fig. 2D), a marker for the postsynaptic membrane shown previously to be associated with nAChR clusters in the mouse SCG (Parker et al., 2004).

Our novel knock-in mouse allowed us to take advantage of the α BTX binding ability of the $\alpha 3[5]$ -containing nAChRs to carry out a quantitative analysis of nAChR clusters in the postsynaptic membrane (Fig. 2D,E,F). In addition, we were able to determine the dynamics of nAChR cluster distribution and intensity following preganglionic denervation at a resolution and level of analysis not previously possible in the SCG (Fig. 7). Our denervation experiments (Fig. 7) demonstrate that presynaptic terminals play a role in clustering $\alpha 3[5]$ -containing receptors to synapses in SCG neurons, similar to their role at the neuromuscular junction (Sanes and Lichtman, 1999; Gingras et al., 2002). We found that cutting the presynaptic nerve leads to an initial increase in α BTX-labeled clusters within 2 days, followed by cluster dispersal over the next 1–2 weeks. Previous studies on rat SCG indicated that following preganglionic nerve crush there was no significant effect on the number of nAChRs in intact SCG as measured by total radioactive epibatidine binding and by radioactive α BTX binding (Del Signore et al., 2004). This rat study measured binding on intact SCG and would not have detected changes at the level of postsynaptic nAChR cluster size or density. In a denervation study using mouse submandibular ganglia (Parker et al., 2004), antibody-labeled nAChR clusters appeared to be disassembling two days after denervation and cluster dispersal continued to the end of the study, five days later. At that point, only ~23% of the postganglionic neurons contained nAChR clusters. Our findings reported here are more similar to the mouse submandibular ganglia study than the rat SCG study, but with important differences. In SCG examined two days post denervation, we see a significant increase in cluster size and in puncta density, together with a ~21% decrease in mean puncta intensity. These results would suggest that postganglionic nAChR clusters are initially spreading in size along the lines of what has been reported for muscle type nAChRs following motor nerve denervation (Sharpless, 1975; Cangiano, 1985). Beyond two days, nAChR cluster size returns to the control level, and puncta density decreases over the next two weeks to arrive at a level ~40% of the control (Fig. 7E). Denervation of autonomic neurons in amphibians and chick has been reported to lead to a similar dispersal of receptor clusters (Jacob and Berg, 1987; Sargent and Pang, 1988; McEachern et al., 1989; Levey and Jacob, 1996). Importantly, the unique features of our knock-in mouse have permitted a detailed examination of nAChR cluster architecture and dynamics not previously possible with existing approaches.

Consistent with our prediction that $\alpha 3[5]$ -containing nAChRs in SCG neurons would be sensitive to blockade by α BTX, we found that the amplitudes of both the CAPs and fast EPSPs in SCG neurons containing exclusively $\alpha 3[5]$ subunit, although decreased to approximately 10–20% of wild-type control levels, were completely blocked by α BTX and with a rapid rate of block onset (Fig. 4). We were able to rule out the possibility that the decrease in amplitude of the nerve-evoked EPSPs might be due to a decrease in the EC₅₀ of the $\alpha 3[5]$ -containing

nAChRs in SCG neurons (Fig. 3) by showing that the EC_{50} for ACh of $\alpha 3[5]$ -containing nAChRs in cultured SCG neurons was very similar to the EC_{50} of WT controls. Based on studies with SCG neurons in culture (Fig. 5), we now believe that the number of functional, surface-expressed $\alpha 3[5]$ -containing nAChRs is greatly decreased, in most genetic backgrounds (see below), as compared to nAChRs in wild-type ganglia. A decreased level of surface expression could help explain our observations that not all VAcHT or PSD93-labeled puncta in SCG were labeled with RhoBTX (Fig. 2E), although another possible explanation for this observation would be incomplete labeling of all $\alpha 3(5)$ -containing receptors due to the relatively rapid off-rate of α BTX binding (Figure 5). It is possible that the $\alpha 3[5]$ mutations may have affected the integrity of pentamer assembly or membrane trafficking, or both, possibly leading to retention in the endoplasmic reticulum (Millar & Harkness, 2008). Previous studies have suggested that the N-terminal extracellular region can regulate the association of nAChR subunits (Sumikawa, 1992). It also is possible that the loop C mutations could affect the surface stability of fully assembled receptors, leading to enhanced receptor turnover once receptors reach the surface. Additional investigations will be required to determine which of these possible mechanisms explains the observed decrease in functional responsiveness of $\alpha 3[5]$ -containing nAChRs in SCG.

The replacement of $\alpha 3$ with $\alpha 3[5]$ within a pure C57BL/6 genetic background resulted in SCG neurons that did not respond to ACh application when cultured at 37°C (Fig. 6B). Following a shift to 30°C, however, we observed a significant increase in the number of functional receptors, with ACh-responses reaching a level of about 6% of that seen with wild-type neurons cultured under similar conditions. These results are in line with previous studies demonstrating that reduced temperature can increase the surface-expression of neuronal nAChRs in certain situations (Cooper et al., 1999; Nelson et al., 2003). Interestingly, we discovered that the observed effects of lower incubation temperature appear to vary greatly among different strains of mice. For example, whole-cell recordings of 30°C-treated SCG neurons prepared from outbred ICR-derived mice show considerable increases in their ACh-evoked current densities compared to neurons from mice in the congenic C57BL/6 background (Fig. 6C), but with great individual to individual variability as might be expected in an outbred line that is bred for maximal levels of heterozygosity in its strain-delimited gene pool. The results of our most recent observations with early generation backcrosses into the BALB/cJ strain show some ACh responses approaching near wild-type levels in 30°C-treated SCG neurons prepared from several older backcrossed mice (>P20). With these early generation backcrossed mice, we have occasionally observed significant ACh responses in SCG neurons maintained in culture at 37°C with no shift to lower temperature (data not shown). The prospect that the expression of functional $\alpha 3[5]$ -containing nAChRs varies among different inbred genetic backgrounds, as observed between the C57BL/6 and BALB/cJ strains, is exciting, as this would provide a powerful new strategy for future genetic studies aimed at dissecting the molecular mechanisms underlying the assembly and trafficking of neuronal nAChRs.

The present study has demonstrated that it is possible to transform an α BTX-insensitive neuronal nAChR subunit into an α BTX-sensitive variant through a knock-in strategy. This was accomplished by replacing five amino acids in the Loop C region of the murine $\alpha 3$ subunit with the corresponding residues from the $\alpha 1$ subunit of *Torpedo californica*. The work described here establishes a new mammalian model for the study of $\alpha 3$ nAChRs in the adult nervous system as few $\alpha 3$ knock-out mice survive past weaning in standard inbred lines (Xu et al., 1999). Our studies also demonstrate the great potential of the “pharmatope” approach as a general strategy for investigating neuronal nAChRs in an *in vivo* setting.

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Abbreviations

α BTX	α -bungarotoxin
nAChR	nicotinic acetylcholine receptor
<i>Chrna3</i>	$\alpha 3$ nAChR gene
ES cell	embryonic stem cell
SCG	superior cervical ganglia
VACHT	vesicular acetylcholine transporter
NFM	neurofilament
CAP	compound action potential
EPSP	excitatory postsynaptic potential
rhoBTX	rhodamine-conjugated α BTX

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Figure 1. Overview of the $\alpha 3[5]$ knock-in targeting strategy and supporting genetic analysis
(A) A genetic map depicts significant landmarks in the WT *Chrna3* allele, $\alpha 3[5]$ targeting DNA, and recombinant allele before (*Chrna3^{tm1.0Hwrt}*) and after (*Chrna3^{tm1.1Hwrt}*) deletion of the neomycin cassette, along with anticipated regions of homologous recombination and restriction cleavage sites. WT exons are represented as solid rectangles. The mutated ExonV is shown as a hatched rectangle. **(B)** Southern analysis of targeted ES cell clone. A single band at ~17.5 kb is detected by the 3' probe in the WT control (left, lane 1). In targeted ES cell clone A6, the ~17.5 kb WT band is detected along with a second band at ~10.5 kb, the anticipated size of the 3' KpnI fragment in the recombinant allele (left, lane 2). A single band is detected at ~17.5 kb in the WT sample with the 5' probe (right, lane 1). In targeted ES cell clone A6,

the ~17.5 kb band appeared along with a second band at ~8.9 kb, the anticipated size of the 5' KpnI fragment in the mutant allele (right, lane 2). **(C)** Southern analysis of $\alpha 3[5]$ knock-in mice. Following deletion of the neomycin cassette, the mutant allele is reduced by ~2 kb from 8.9 kb in an $\alpha 3[5]$ heterozygote (+neo, lane 1) to 6.9 kb in an $\alpha 3[5]$ homozygote (-neo, lane 2). **(D)** RT-PCR analysis of SCG tissue extract. cDNAs were prepared from WT (+/+), $\alpha 3[5]$ -heterozygous mice (+/tm), and $\alpha 3[5]$ -homozygous mice (tm/tm). The WT-specific primer amplified 480 bp PCR products from the $\alpha 3[5]$ (+/tm) and WT samples, but not with cDNAs from the $\alpha 3[5]$ (tm/tm). The $\alpha 3[5]$ -specific primer amplified 480 bp PCR-products with cDNAs prepared from heterozygote and homozygote mice, and no bands are observable with WT cDNA. *Chrna3^{tm1.1Hwrt}* mice (i.e., neo-deleted) on a mixed C57BL/6-129S background at age <P20 were used in the RT-PCR experiments.

Figure 2. Labeling of nicotinic $\alpha 3[5]$ -containing receptors using rhodamine-conjugated α BTX in $\alpha 3[5]$ -homozygous mice

(A) Rhodamine-labeled α BTX (rhoBTX, red) clusters colocalize with a majority of the staining for vesicular acetylcholine transporter (VACHT, green; see panel D below) in SCG from $\alpha 3[5]$ -homozygous mice (left, $\alpha 3^{tm/tm}$) but not in SCG from WT mice (right). Immunostaining for neurofilament (NFM, blue) shows the preganglionic axons. These results were seen in 5/5 mice and indicate that $\alpha 3[5]$ -containing receptors are targeted to synapses. Inset shows regions of NFM-labeled axons that have VACHT-positive varicosities that colocalize with receptor clusters. (B) RhoBTX-labeled clusters (red) colocalize with postsynaptic density 93 (PSD93, green; see panel D below). All rhoBTX-clusters colocalize with PSD93, but many PSD93 puncta do not colocalize with detectable rhoBTX clusters. (C) RhoBTX-labeling is abolished by pre-incubating SCG from $\alpha 3[5]$ -homozygous mice with unlabeled α BTX (100 nM, left), but is normal when SCG are pre-incubated with MLA (50 nM, middle). RhoBTX-labeled clusters present on SCG neurons from double homozygous ($\alpha 3[5]$ -homozygous and $\alpha 7^{-/-}$) mice (right). *Chrna3^{tm1.1Hwrt}* mice on a mixed C57BL/6-CD-1 background aged 2–3 months were used in these experiments. Scale bar is 2 μ m for A, B, and C, and 0.5 μ m for the insets in A. (D) Quantitation of puncta from images obtained in the studies described in panels A, B and C indicate that all rhoBTX labeled clusters are colocalized with VACHT and PSD93 labeled puncta. (E) Quantitative analysis of puncta from images obtained in the studies described in panels B and C indicate that ~60% of VACHT stained puncta have α BTX clusters associated with them and ~30% of the PSD93 stained puncta have α BTX clusters associated with them. (F) Quantitative analysis of α BTX labeled puncta obtained in the studies described in panels

A, B, and C indicate no detectable puncta in WT $\alpha 3$ mice, but comparable densities of α BTX labeled puncta in $\alpha 3[5]$ -homozygous mice in either the null $\alpha 7$ ($-/-$) genetic background or with wild-type $\alpha 7$ background.

Figure 3. Fast synaptic transmission in SCG from $\alpha 3[5]$ -homozygous mice is blocked by α BTX
(A) Extracellular compound action potentials (CAP) recorded from the sympathetic trunk in 2-month old WT (top row) and a 2-month old $\alpha 3[5]$ -homozygous mouse (bottom row); the traces on the right show CAPs recorded from the same ganglia in the presence of 100 nM α BTX. CAPs recorded from $\alpha 3[5]$ -homozygous SCG are approximately 10–20% the amplitude of control and are completely blocked by α BTX. Similar results were seen in 12 WT mice and in 15 C57BL/6/CD-1 $Chrna3^{tm1.1Hwrt}$ homozygous mice greater than 2 months of age. **(B)** Nerve-evoked EPSPs recorded intracellularly from sympathetic neurons in 2-month old WT (top row), 2-month old C57BL/6/CD-1 $Chrna3^{tm1.1Hwrt}$ homozygous (middle row) and 5-month old homozygous double mutant ($\alpha 3[5]$ -homozygous $\alpha 7(-/-)$) SCG (bottom row).

Suprathreshold stimulation of the preganglionic nerve evokes small ~3mV EPSPs on sympathetic neurons from 5-month old $\alpha 3[5]$ -homozygous SCG that are completely blocked by α BTX. Similar results were seen for 20 EPSPs from WT neurons and 30 EPSPs from $\alpha 3[5]$ -homozygous SCG neurons dissected from mice greater than 2 months of age.

ACh dose-response in SCG neurons

Figure 4. The dose-response characteristics of $\alpha 3/\alpha 1[5]$ -containing receptors compared to WT $\alpha 3$ nicotinic receptors measured in cultured SCG neurons

Chrna3^{tm1.1Hwrt} mice congenic in the C57BL/6 strain were crossed with BALB/cJ mice and resulting heterozygotes were mated in an F2 cross to prepare *Chrna3^{tm1.1Hwrt}* homozygotes in the mixed C57BL/6/BALB/cJ background. WT $\alpha 3$ F2 littermates were used as the source of WT $\alpha 3$ for comparison with $\alpha 3[5]$ -containing receptors. Dissociated SCG primary cultures were prepared and maintained at 37°C. Two-three days before the ACh dose-response study, the cultures were shifted to 30°C. The fitted curves yielded an EC_{50} of $48 \pm 2 \mu\text{M}$ for $\alpha 3[5]$ -containing receptors and a value of $64 \pm 3 \mu\text{M}$ for WT $\alpha 3$ receptors. Data points shown are the mean $\pm$ SEM ($n=3-4$ per data point).

Figure 5. Time course of α BTX block and washout in cultured SCG neurons from *Chrna3^{tm1.1Hwrt}* homozygous mice

Chrna3^{tm1.1Hwrt} mice congenic in the C57BL/6 strain were crossed with BALB/cJ mice and resulting heterozygotes were mated in an F2 cross to prepare *Chrna3^{tm1.1Hwrt}* homozygotes in the mixed C57BL/6/BALB/cJ background. WT $\alpha 3$ F2 littermates were used as the source of WT $\alpha 3$ for comparison with $\alpha 3[5]$ -containing receptors. Dissociated SCG primary cultures were prepared and maintained at 37°C. Two-three days before recording, the cultures were shifted to 30°C. The data points show normalized peak currents evoked by brief (2 sec) application of 200 μ M ACh. The current density of the ACh evoked responses in the SCG neurons prior to α BTX application was 11.8 ± 2.1 pA/pF ($n=3$). Immediately after the first control data point was obtained, the cells were perfused with external solution containing 100 nM α BTX for 5 minutes. Current responses were recorded during this 5 minute time interval with a co-application of 200 μ M ACh and 100 nM α BTX. At time point 0, the washout of the α BTX was begun, and test pulses of 200 μ M ACh were applied to monitor recovery from α BTX block. Error bars indicate SEM, $n=3$.

Figure 6. ACh-evoked whole-cell currents in cultured SCG neurons from neonatal $\alpha 3[5]$ -homozygous C57BL/6 mice are sensitive to α BTX block and their detection is facilitated by short term culture at 30°C

(A) Bar graph showing whole-cell current density measurements recorded from cultured SCG neurons from WT C57BL/6 mice (<P20). Reducing the incubation temperature to 30°C for 1 day has no significant effect on the mean current density in WT SCG neurons (middle, $P=0.10$). Under these conditions, the mean current density is unaffected following a 1 h incubation with 100 nM α BTX (right, $P=0.74$). Values represent the mean ($n=15$) and SEM from 3 mice (5 cells/mouse). (B) SCG neurons from $\alpha 3[5]$ -homozygous mice congenic on C57BL/6 (<P20) are unresponsive to ACh application when cultured at 37°C (left). Following 1 day at 30°C,

SCG neurons from $\alpha 3[5]$ -homozygous mice generate ACh-evoked currents (note change in scale of y-axis) which are unaffected by the presence or absence of the $\alpha 7$ subunit (middle, P values >0.70). Under these conditions, the ACh responses are greatly decreased following 1 h incubation with 100 nM α BTX (right). No significant differences in the levels of α BTX block were observed among the three $\alpha 7$ genotypes (P values >0.1). Values represent the mean and SEM ($n=5$) from 1 mouse. (C) Data points representing ACh-evoked current densities in cultured SCG neurons either from $\alpha 3[5]$ -homozygous mice ($<P20$) congenic on C57BL/6, from F2 mixed-background inbred (C3H, DBA, and BALB/cJ), or from outbred (ICR) strains. P values were calculated using an unpaired t test.

Figure 7. Time course of α BTX-labeled nAChR cluster size, average cluster intensity, and surface density in the SCG following preganglionic denervation

(A) Shows the size distribution of rhoBTX labeled receptor clusters in innervated $\alpha 3[5]$ -homozygous SCG (n=5 ganglia). (B) Size distribution of rhoBTX labeled receptor clusters in $\alpha 3[5]$ -homozygous SCG two days after denervation (n=5 ganglia). There is an increase in the number and the size of puncta. (C) Size distribution of rhoBTX labeled receptor puncta in $\alpha 3[5]$ -homozygous SCG one week after denervation (n=5 ganglia). The number and the size of clusters one week following denervation are decreased slightly compared with control. (D) Size distribution of rhoBTX labeled receptor cluster in $\alpha 3[5]$ -homozygous SCG two weeks after denervation (n=5 ganglia). Cluster number and size are markedly decreased two weeks

following denervation. For A–D, The red lines are gaussian fits of the data, and the insets show representative images following live staining with rhoBTX and post-fix immunostaining for VACHT. Scale bars are 1 μ m. (E) Mean cluster density and intensity (a.u.) at different times post denervation. (number of clusters counted for each time point is >1000). *Chrna3^{tm1.1Hwrt}* mice on a mixed C57BL/6-CD-1 background were used in these experiments.

Table I

Distribution of genotypes in offspring from backcrosses and intercrosses of congenic $\alpha 3[5]$ carriers ($n \geq 9$).

male x female	heterozygous	wild-type	homozygous	<i>P</i>
<i>Chrna^{tm1.1Hwrt/+}</i> x <i>Chrna^{tm1.1Hwrt/+}</i>	177 (58%)	86 (28%)	41 (14%)	<i>P</i> <0.005
<i>Chrna^{tm1.1Hwrt/+}</i> x wild-type (C57BL/6)	117 (50%)	118 (50%)	-	<i>P</i> >0.95

Table II

Distribution of genotypes in offspring obtained from ICR-outcrossed parents.

male x female	heterozygous	wild-type	homozygous	<i>P</i>
<i>Chrna^{tm1.1Hwrt/+}</i> x <i>Chrna^{tm1.1Hwrt/+}</i>	99 (58%)	42 (25%)	30 (18%)	0.06> <i>P</i> >0.05

* *P* values were calculated using the one way Chi-square method