Journal of Neuroscience Research 86:972–981 (2008)

Nicotinic Acetylcholine Receptor-Subunit mRNAs in the Mouse Superior Cervical Ganglion Are Regulated by Development but Not by Deletion of Distinct Subunit Genes

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Mice with deletions of nicotinic ACh receptor (nAChR) subunit genes are valuable models for studying nAChR functions. We could previously show in the mouse superior cervical ganglion (SCG) that the absence of distinct subunits affects the functional properties of receptors. Here, we have addressed the question of whether deletions of the subunits α5, α7, or β2 are compensated at the mRNA level, monitored by reverse transcription and quantitative real-time polymerase chain reaction. Relative to our reference gene, α3, which is expressed in all SCG nAChRs, mRNA levels of β4 showed little change from birth until adult ages in intact ganglia of wild-type mice. In contrast, α4 declined sharply after birth and was barely detectable in adult animals. α5, α7, and β2 subunit message levels also declined, though more slowly and less completely than α4. The subunits α6 and β3 were detected by conventional polymerase chain reaction at very low levels, if at all, whereas α2 was never seen in any of our samples. The developmental profile of nAChR mRNA levels in the three knockout strains did not differ markedly from that of wild-type mice. Likewise, message levels of nAChR subunits were similar in cultures prepared from either wild-type or knockout animals. Our observations indicate a developmental regulation of nAChR subunit mRNAs in the SCG of mice after birth that was not affected by the three knockouts under investigation.

Key words: sympathetic nervous system; knockout mouse; gene regulation

Neuronal nicotinic ACh receptors (nAChRs) assemble as pentamers from nine homologous subunits (α2–α7 and β2–β4) in the mammalian nervous system (Cordero-Erausquin et al., 2000). To examine the role of receptors in general and of individual subunits in particular, mouse models with deletions of distinct nAChR genes have been generated. Considering the widespread distribution of receptors, deletions of individual subunits are, with the exception of α3, surprisingly well tolerated (Cordero-Erausquin et al., 2000; Picciotto et al., 2001; Wang et al., 2002b).

Several mechanisms may explain this paradox. Thus, nAChRs may play just a subtle modulating role on prevalent neurotransmitter systems (Champtiaux and Changeux, 2004); cholinergic neurotransmission may act in parallel with other neurotransmitters (Nilsson et al., 1988); a missing subunit might be filled in by normally transcribed subunits (Champtiaux et al., 2002); or deletion of a nAChR subunit gene might be compensated by regulation of other nAChR subunit mRNAs.

mRNA levels of nAChR subunits studied in the central nervous system by in situ hybridization seem unaffected in mice lacking the subunits α4 (Marubio et al., 1999), α5 (Salas et al., 2003), α6 (Champtiaux et al., 2002), β2 (Picciotto et al., 1995), and β3 (Cui et al., 2003). Observations in these studies were, however, restricted to the central nervous system; similar studies on α7 KO mice are missing, and potential developmentally dependent

Contract grant sponsor: Austrian Ministry for Education, Science and Culture; Contract grant number: GZ 70.072/2-PR/4/2000 (to S.H.).

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Received 20 April 2007; Revised 24 May 2007; Accepted 24 August 2007

Published online 1 November 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21559

effects in the knockouts were not considered. Interestingly, levels of α3 were found to be reduced by quantitative real-time polymerase chain reaction (qRT-PCR) in brains of adult mice lacking the nAChR subunits α5 or β4 (Kedmi et al., 2004).

Here, by using qRT-PCR, we have compared nAChR subunit mRNA in the superior cervical ganglion (SCG) of wild-type (WT) mice and of mice with deletions of the α5, α7, or β2 subunit genes. SCGs of mice and rats contain two types of nAChRs: homopentamers made up by the α7 subunit and heteropentameric receptors consisting of the subunits α3, α4, α5, β2, and β4 (this study; Mandelzys et al., 1994; Rust et al., 1994; Klimaschewski et al., 1994; Zoli et al., 1995; Del Signore et al., 2002; Skok, 2002; Severance et al., 2004; Mao et al., 2006).

The subunit α3 is indispensable for nAChR function in the SCG, and mice lacking this subunit die soon after birth from vegetative failure (Xu et al., 1999a; Rassadi et al., 2005). The subunit β4, which combines with α3 to form the backbone of nAChRs in the adult rat SCG (McGehee and Role, 1995; Del Signore et al., 2004; Mao et al., 2006), seems to be replaced by β2 in a β4 mouse KO model (Xu et al., 1999b). Mechanisms compensating for the absence of α5, α7, or β2 have not been investigated in the SCG.

In this study, we analyzed both intact ganglia and mouse SCG neurons kept in culture for 5 days. Levels of specific subunit mRNAs were calculated relative to α3, because recent studies demonstrate that, with the exception of α7 receptors, nAChRs in mouse or rat SCG always include the α3 subunit (Del Signore et al., 2002, 2004; Mao et al., 2006). Our analysis covered developmental periods from newborn to adulthood and thus crucial stages of high plasticity, when intraganglionic synapses are formed and synaptic contacts are made between the sympathetic axon terminals and their target organs (De Champlain et al., 1970; Smolen and Raisman, 1980). We reason that compensation at the level of mRNA for a missing subunit may be more reliably detected by covering a broader range of SCG development. Our experiments revealed a developmental regulation of the nAChR subunit mRNAs α4, α5, α7, and β2 relative to α3. Deletions of distinct nAChR subunit genes had no significant effect on mRNA levels of other nAChR subunits that occur in the SCG of KO animals.

MATERIALS AND METHODS

Acute Preparation of Ganglia

Experiments were performed on WT C57Bl/6 mice and mice with deletions of one of the nAChR subunit genes α5 (Wang et al., 2002a; Salas et al., 2003), α7 (Orr-Urtreger et al., 1997), or β2 (Picciotto et al., 1995), bred from homozygous parent animals. Mice used in this study were backcrossed onto a C57Bl/6 background for 7 (α5, α7) or 12 generations (β2). All animals were kept in thermostable rooms (21°C) on a light-dark schedule of 10:14 hr in group cages, with food and water freely accessible. Postnatal day 1, 5, and 9 (P1, P5, P9) and adult (8 weeks old) mice were sacrificed by decapitation. The adult animals were deeply anesthetized with CO_2 beforehand. SCGs were collected in ice-cold phosphate-buffered saline (PBS; mM concentrations: NaCl, 137; KCl, 2.7; Na_2HPO_4, 10.1; KH_2PO_4, 1.76; pH adjusted to 7.4 with HCl). After removal of the PBS, ganglia were flash frozen with liquid nitrogen and stored at −80°C for later use.

Cell Cultures of SCG Neurons

SCGs were dissected from 5-day-old mouse pups. Ganglia were enzyme-treated, dispersed by trituration in culture medium to a single-cell suspension, and plated on laminin-coated glass coverslips as described previously (Fischer et al., 2005). For the analysis of 6-hr cultures, 40,000–60,000 cells were seeded into 19-mm glass rings. Cultures kept for 3 days and 5 days in vitro (DIV) had 10,000 cells per 8-mm glass ring, with two glass rings per glass coverslip. The culture medium was Neurobasal A medium, supplemented with 2% B-27 additive (Gibco-Invitrogen, Grand Island, NY), 1.5 mM L-glutamine (Sigma, St. Louis, MO), 25 U/ml penicillin, 25 μg/ml streptomycin (Gibco-Invitrogen), and 20 ng/ml nerve growth factor (NGF; R&D systems, Minneapolis, MN). Cultures were maintained at 36.5°C, 5% CO_2, and 95% humidity and harvested for RNA extraction with RLT buffer (Qiagen, Hilden, Germany) containing β-mercaptoethanol.

Reverse Transcription

Total RNA was extracted from ganglia or cultured cells with RNeasy Mini- or Micro-Kit, respectively (Qiagen). Typically, 600 ng RNA was reverse transcribed using random hexamer primers and ThermoScript RT-PCR reagents (Invitrogen). According to the protocol provided by the manufacturer, RNA samples were denatured by heating to 65°C for 3 min, chilled on ice, and incubated with reverse transcriptase at 25°C for 20 min, followed by a temperature increase of 1°C per minute up to 55°C. After an additional 30 min of incubation at 55°C, the reaction was terminated by heating samples to 94°C for 5 min. cDNA was diluted with sterile water and stored at 4°C for later use, if required.

PCR

cDNA (corresponding to 10 ng reverse-transcribed RNA) was amplified in a PCR with the following reagents at indicated final concentrations: 2 mM $MgCl_2$, 800 μM dNTPs, 1 unit recombinant Taq-polymerase (Fermentas, Ontario, Canada), and 250 nM of each 5′ and 3′ primer: malpha2_500UP 5′-AGACGGGGAGTTTGCGGTGA-3′ and malpha2_892LP 5′-AGGCAGGAGATGAGCAGGCA-3′ (α2-subunit); malpha6_334UP 5′-GGCTGCGTCACATCTGGAAG-3′ and malpha6_834LP 5′-GGCAGGGGATGATGAGGTTG-3′ (α6-subunit); mbeta3_35UP 5′-TACGGGTCTTCTTGGCTCTC-3′ and mbeta3_672LP 5′-CGCTGGATTTCACAATGGCC-3′ (β3-subunit). The primer combinations used for the subunits α3 and α4 are provided below (see qRT-PCR). PCR conditions were as follows: preincubation at 95°C for 3 min; 30 sec denaturing at 95°C; 25 sec annealing at 58°C; and 30 sec elongation at 72°C. The PCR was terminated by 2 min at 72°C.

Amplified products were analyzed by using 2% agarose gel electrophoresis, run at 10 V/cm. DNA was visualized by including ethidium bromide (0.2 μg/ml) in the agarose. Brains (for the α2-subunit) and dorsal root ganglia (for the subunits α6 and β3) of P5 animals served as positive controls. PCR products were confirmed by sequencing (VBC Genomics, Vienna, Austria).

nAChR Constructs

With template cDNA prepared from SCGs of 1-day-old mice, we cloned the cDNA for the mouse nAChR subunits α3, α4, α5, α7, β2, and β4. The PCR products were subcloned into the expression vector pCI (Promega, Madison, WI) and sequenced (VBC Genomics). The following primers and restriction enzymes were used for the cloning of the individual subunits: α3-subunit: mnAChRa3_12UP (EcoRI): 5′-GAT GAATTCCTTGCCTGCCTGGGGTTCGT-3′ and mnA ChRa3_1711LP (SalI) 5′-AGTGTCGACGCATCAGCACAG-GTGAGCGA (amplifying 1,700 bp containing the entire coding region of α3, plus 158 bp 5′UTR and 42 bp 3′UTR); α4-subunit: mnAChRa4_21UP (EcoRI) 5′-CGAGAATTC GCGACACGGGGCATGAAGT-3′ and mnAChRa4_2121LP (SalI) 5′-TAGGTCGACGGTCAACCAGGGCAGCATC-3′ (2,101 bp containing the entire coding region of α4, plus 95 bp 5′UTR and 116 bp 3′UTR); α5-subunit: mnAChRa5_ 517UP (EcoRI) 5′-CGTGAATTCGGACGCAGCCAGCAA ACTAC-3′ and mnAChRa5_1417LP (XbaI) 5′-CTATCT AGAGCGGACAGTGTTGGTACTCA-3′ (1,098 bp containing 924 bp of the α5 C-terminal coding region plus 174 bp 3′UTR). α7-subunit: mnAChRa7_19UP (EcoRI) 5′-CCG GAATTCGGACGGCGGGACACGG-3′ and mnAChRa7_ 1780LP (SalI) 5′-TGAGTCGACGGTGCCCCTGCCGAG T-3′ (1,761 bp containing the entire coding region of α7, plus 32 bp 5′UTR and 221 bp 3′UTR); β2-subunit: mnAChRb2_ 21UP (EcoRI) 5′-ACA GAATTCGAAGAGCAGCCGGGA-CC-3′ and mnAChRb2 1747LP (SalI) AACGTC GACGGT-CACGGGATGA GTAGC-3′ (1,726 bp containing the entire coding region of β2, plus 187 bp 5′UTR and 33 bp 3′UTR); β4-subunit: mnAChRb4_1UP (XhoI) 5′-TCTC TCGAGC-ATTGTGGGGTGACCGGCAG-3′ and mnACh Rb4_1557LP (XbaI) 5′-TATTCTAGAACTACAACCCAG GGCCCCAT-3′ (1,563 bp fragment, containing the entire coding region of β4, plus 34 bp 5′UTR and 41 bp 3′UTR).

The plasmid DNAs used as templates for RNA generation were isolated from bacterial cultures with a JetStar Midi-Prep Kit (Genomed, Löhne, Germany). DNA at low concentrations was determined by means of the PicoGreen dsDNA Quantitation Kit (Invitrogen).

Assessment of RT Efficiency

For an assessment of the efficiency of RT reactions, mRNA standards were prepared by in vitro synthesis (mMessage Machine T7 Kit, Ambion, Austin, TX) from subunit-specific nAChR constructs cloned into pCI-Vector (see above). RNA was capped with a G nucleotide at the 5′ end and polyadenylated at its 3′ end. The purity and integrity of RNA were verified by denaturing RNA agarose gel electrophoresis. RNA concentrations (i.e., the number template molecules) were assessed with the RiboGreen RNA Quantization Kit (Invitrogen) and UV spectrophotometry.

The RT reactions of mRNA standards were performed in the presence of 600 ng carrier RNA using the protocols described above. The carrier RNA isolated from liver of 5-day-old mice was devoid of nAChR subunits (data not shown). cDNA levels of the RT reactions were measured by qRT-PCR, and results were compared with the number of template molecules of the mRNA standards. The resultant RT efficiencies were gene specific, ranging from 15% to 20% for the nAChR subunits α3, α4, β2, and β4, whereas mRNA of subunits α5 and α7 were reverse transcribed less well (5–7%, data not shown).

Quantitative Real-Time PCR

cDNA levels of the genes of interest were quantified in triplicate (fivefold measurements for cell culture materials) by SYBR Green I-based quantitative real-time PCR (qRT-PCR) using an iCycler (Bio-Rad Laboratories, Hercules, CA). cDNA samples (typically corresponding to 20 ng reverse-transcribed RNA) were adjusted to reaction volumes of 20 μl each. As a rule, all cDNA samples used to load one 96-well microtiter plate were derived from one RT reaction run with the same master mix of reverse transcriptase.

The master mix for the qRT-PCR contained 5′ and 3′ primers at a final concentration of 125 nM and two different products of commercially available blends of buffer, dNTP, and enzyme: Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and FailSafeReal-Time PCR System (Epicentre, Madison, WI). Protocols for the Brilliant SYBR Green QPCR Master Mix require an initial enzyme denaturing for 10 min at 94°C. qRT-PCR conditions were: 30 sec denaturing at 94°C; 25 sec annealing at 58°C; and 30 sec elongation at 72°C. The PCR was terminated after 50 cycles, and a melting curve was produced. Fluorescence signals were processed and transformed into gene transcript concentrations by the iCycler software.

SYBR Green I-based qRT-PCR cannot discriminate between nonspecific and desired PCR products. Hence, to optimize PCR conditions, we varied annealing temperatures and primer concentrations and tested several commercially available qRT-PCR kits, such as Brilliant SYBR Green QPCR Master Mix (Stratagene), qPCR MM (Eurogentec), and FailSafe Real-Time PCR System (Epicentre). Best results were obtained with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) for analysis of the nAChR subunit gene transcripts α4, α5, α7, β2, β4; and FailSafe Real-Time PCR System (Epicentre) for the gene transcripts of the nAChR subunit α3. At optimized conditions, PCR products showed at a low cycle threshold (named C_t value) and were devoid of nonspecific products and primer dimers (melting curves not shown; see agarose gel electrophoresis, Fig. 1).

To calibrate the assays, we prepared stocks (10^9 molecules per microliter) by PCR from the nAChR subunit plasmids mentioned above. Frozen stocks were thawed and diluted to standard concentrations ranging from 250 to 250,000 molecules per microliter. Appropriate standards were included in every qRT-PCR assay. PCR amplification effi-

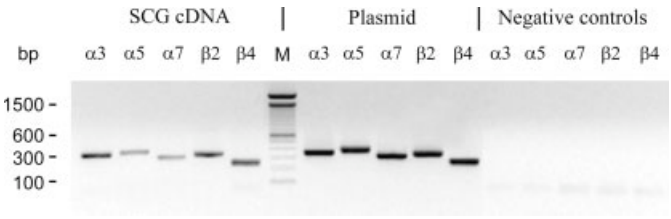

Fig. 1. PCR analysis by DNA agarose gel electrophoresis. PCR products (36 cycles) were generated at optimized PCR conditions (see Materials and Methods). SCG and plasmid DNA served as templates for the nAChR subunits α3, α5, α7, β2, and β4. Probes for the PCR negative controls contained water instead of template DNA. M, molecular weight marker (100-bp DNA ladder; Invitrogen).

ciencies thus determined were consistently found to be between 95% and 100% of the theoretical ideal situation (i.e., doubling of template in each cycle).

Subunit-specific, exon-spanning synthetic oligonucleotides used in this study were: malpha3_229UP 5′-CGCCTG TTCCAGTACCTGTT-3′ and malpha3_588LP 5′-CTTAAA GATGGCCGGAGGGA-3′ (α3-subunit); malpha4_183UP 5′-TCAGATGTGGTCCTTGTCCG-3′ and malpha4_436LP 5′-AGGTGGGCTTTGGTTAGGTG-3′ (α4-subunit); malpha5_570UP 5′-GAGCAAGGGGAACCGGAC-3′ and malpha5 958 LP 5′-TCTTACGAACCCACGGCG-3′ (α5-subunit); malpha7_409UP 5′-TGCCACATTCCACACCAACG-3′ and malpha7_726LP 5′-CTACGGCGCATGGTTACTGT-3′ (α7-subunit); mbeta2_211UP 5′-CTCCTTTGGCTGTGTTCAGG-3′ and mbeta2_556LP 5′-AGGAGACTTCGTACATGCCG-3′ (β2-subunit); mbeta4_149UP 5′-TACAACAACCTGATCCGC CC-3′ and mbeta4_407LP 5′-CATAGGTCCCATCGGCAT-TG-3′ (β4-subunit). All PCR products were confirmed by sequencing (VBC Genomics).

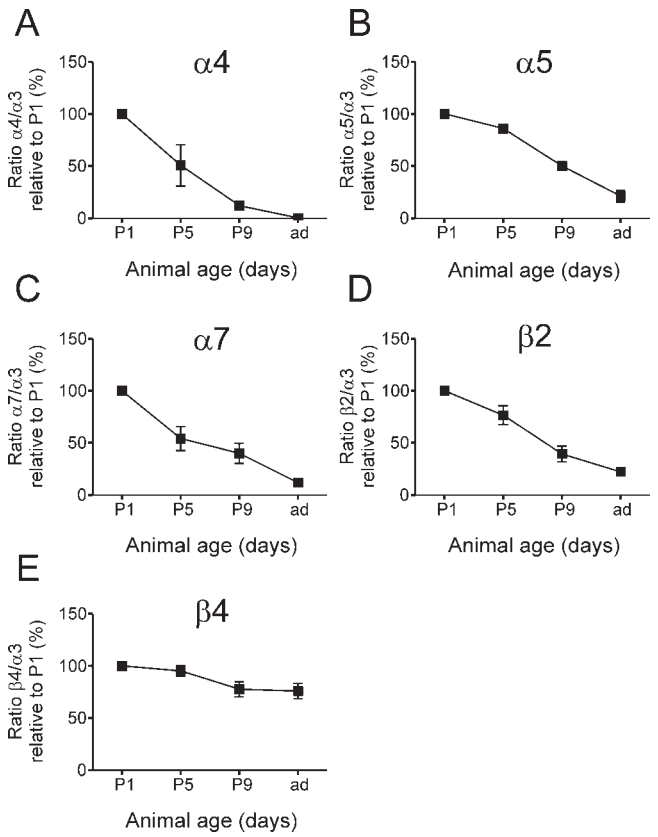

Fig. 2. Developmental profile of nAChR subunit mRNAs in the SCG of WT mice. α4 (**A**), α5 (**B**), α7 (**C**), β2 (**D**), and β4 (**E**) mRNA levels were calculated as ratios of α3. Ratios at postnatal day 1 (P1) were defined as 100%. Ratios for P5, P9, and adult were calculated relative to this reference point. The data points (means ± SEM) are averaged ratios from three to five different qRT-PCR assays.

Data Analysis

RNA samples obtained by extraction from the SCGs of one litter (this RNA being termed one "biological sample") were typically adjusted to 600 ng for the RT reaction. To reduce sampling errors introduced by the processing of the RNA, we took two precautions. First, RNA of WT animals and one KO strain (α5, α7, or β2) at the different developmental stages were reverse-transcribed in one experiment with the same master mix of buffer, primers, and enzyme. Second, levels of a gene of interest (e.g., α4) were measured in parallel with our reference gene (the nAChR subunit α3) in one comprehensive qRT-PCR assay. We then calculated ratios relative to the standard (e.g., α4/α3) in one and the same cDNA sample. To combine several such experiments, the ratio of a gene of interest (e.g., α4) to our reference gene (α3) as determined for WT animals at P1 was set to 100% (see Fig. 2). This protocol not only reveals the developmental time course of levels of a gene relative to α3 but also allows a comparison between WT and KO animals (see Fig. 3). Recent evidence suggests that nAChRs in SCG of both mice and rats always include the α3 subunit (Del Signore et al., 2002, 2004; Mao et al., 2006).

For a statistical comparison, the ratios of an experiment were assessed as follows. Message quotients were determined for one subunit from KO materials (e.g., α4/α3, equal to value A-KO) and WT (α4/α3 equal to value B-WT), and a ratio (A/B) was calculated for each developmental stage (P1, P5, P9, and adult). As shown in Figure 4, the hypothesis that ratios (values A by values B) differed from 1 was then tested by the signed-rank test (Wilcoxon).

RESULTS

We assessed mRNA levels of the nAChR subunits α3, α4, α5, α7, β2, and β4 in acute preparations of postnatal mouse SCGs by qRT-PCR. At our reference point, P1 (i.e., the day after birth), we found $2.7 \times 10^6 \pm 0.8 \times 10^6$ copies of the nAChR subunit α3 in 600 ng SCG RNA (means ± SEM, n = 9 biological samples) in WT animals. SCG ganglia at P1 had an average 329 ± 17 ng RNA (n = 9 biological samples), so this adds up to about 1.5×10^6 copies of α3 mRNA per ganglion. At P1, 600 ng SCG RNA furthermore contained $1.1 \times 10^5 \pm 0.3 \times 10^5$ copies of the α4 subunit (means ± SEM,

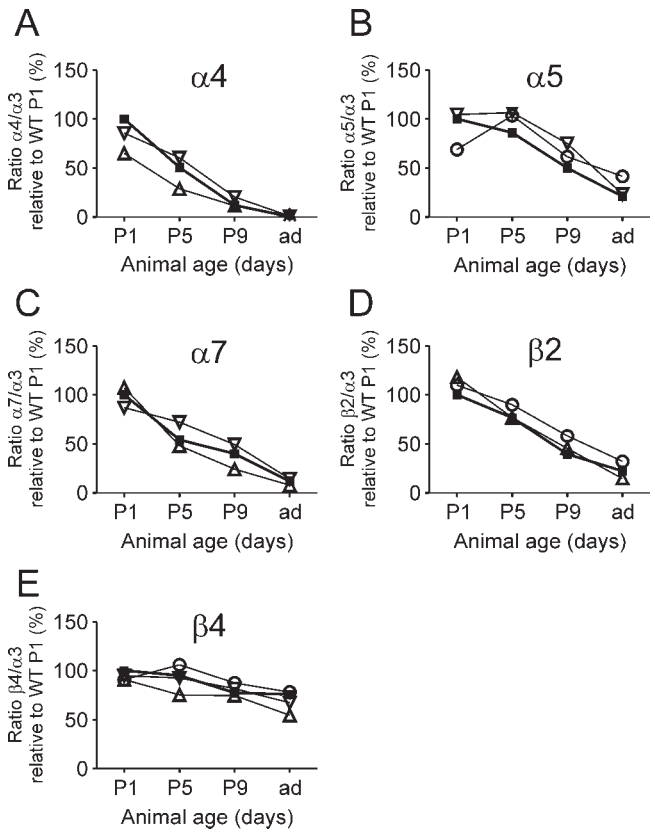

Fig. 3. **A–E:** Absence of effects by deletions of distinct nAChR subunit genes during development. Data points for WT animals (squares) were taken from Figure 2. The quotients of genes relative to α3 WT at postnatal day 1 (P1) were defined as 100%. Ratios for the KO animals (α5 KO: upward triangle; β2 KO: downward triangle; α7 KO: circle) at indicated developmental ages were set in relation to this reference. The KO data were derived from one (α5 KO in A; α7 KO in B,D,E) or two (averaged) qRT-PCR assays (α5 KO in C–E; β2 KO in A–C,E).

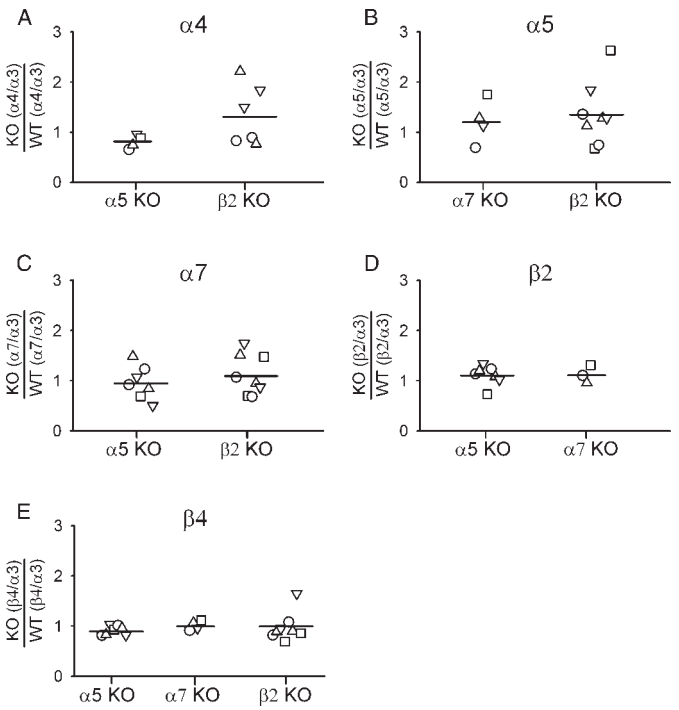

Fig. 4. **A–E:** Statistical comparison of subunit mRNA levels (relative to α3) for indicated genes in WT and KO animals. Data points were generated by assessing ratios of gene transcripts relative to the α3 subunit in WT animals as well as in indicated KOs of the same age. The two ratios were then divided and plotted. If, for example, the α4/α3 ratio at P1 was 0.019 in the α5 KO and 0.029 in WT, division of the two values (0.65) yielded one data point in A, "α5 KO." The three other data points for this calculation were derived by dividing α4/α3 ratios (from α5 KO as well as WT) at P5, P9, and adult, respectively. Bars indicate the mean. Taken together, the ratios of the data set "α5 KO" in A did not differ significantly from 1 (at $P < 0.05$, signed-rank test, Wilcoxon). Data sets containing more than four points (e.g., the set designated "β2 KO" in A) indicate that a second experiment (that may or may not have covered all four developmental stages) was processed in the same way. None of the data sets in A–E differed significantly from 1 (at $P < 0.05$, signed-rank test, Wilcoxon). Circles show ratios at P1; upward triangles ratios at P5; downward triangles ratios at P9; squares ratios at adult ages. The results in this figure are based on experiments shown in Figure 3.

n = 10 biological samples); $7.7 \times 10^5 \pm 4.4 \times 10^5$ copies of the α5 subunit (n = 5); $8.3 \times 10^5 \pm 1.7 \times 10^5$ copies of the α7 subunit (n = 11); $5.3 \times 10^5 \pm 0.6 \times 10^5$ copies of the β2 subunit (n = 9); and $1.2 \times 10^6 \pm 0.2 \times 10^6$ copies of the β4 subunit (n = 13).

Postnatal Regulation of nAChR Subunits in the SCG of WT Mice

The absolute number of copies of the nAChR subunit α3 increased to $7.9 \times 10^6 \pm 4.1 \times 10^6$ per 600 ng SCG RNA at adult ages (i.e., by factor of 2.9 compared with P1; means ± SEM, four biological samples). However, absolute numbers varied considerably, and we thus routinely processed our data by comparing levels of a gene of interest (e.g., α4) with our standard α3 in one and the same cDNA preparation (see above under Data analysis). α3 as a reference gene is well-suited for studying the part of distinct subunits in fully assembled nAChRs, because available evidence suggests that all heteropentameric receptors in the SCG contain α3 (Xu et al., 1999a; Del Signore et al., 2002; Mao et al., 2006).

α4/α3 ratios were already low at P1 (0.07, means of three qRT-PCR experiments), fell by 88% at P9, and approached zero in the adult (Fig. 2A). A significant developmental regulation was also seen for the nAChR subunits α5, α7, and β2. The ratios of these genes relative to α3 at P1 were 0.67 (α5/α3, means of three qRT-PCR experiments), 0.33 (α7/α3, means of four qRT-PCR experiments), and 0.09 (β2/α3, means of three qRT-PCR experiments). These ratios fell by 79% (α5/α3), 88% (α7/α3), and 78% (β2/α3), respectively, between P1 and adulthood (Fig. 2). β4 mRNA started off with a β4/α3 ratio of 0.44 at P1 (means of five

qRT-PCR experiments) and remained quite constant throughout development (Fig. 2E).

mRNA Levels of nAChR Subunits in the SCG of Mice Lacking the α5, α7, or β2 nAChR Subunit Gene

Deletion of the α5, α7, or β2 nAChR subunit genes had moderate effects, if any, on message levels (in relation to α3) of the nAChR subunits α4, α5, α7, β2, or β4 (Fig. 3). Although the graph (e.g., Fig. 3A, α5 KO) seems to indicate some deviation from values calculated for WT animals, such differences probably are due to the inherent variability of the method (Stahlberg et al., 2004). In fact, comparisons of KO with WT data when pooled across the four developmental stages (Fig. 4) did not show significant differences for any of the nAChR genes (signed-rank test, Wilcoxon).

mRNA Profile of nAChR Subunits in Cell Cultures of the SCG

Our previous functional analysis of SCG neurons suggested that the subunit compositions of nAChRs change with the age of cultures as well as deletion of the α5 (Fischer et al., 2005) and the β2 subunit gene (Huck, unpublished observations; see Discussion). We have thus determined mRNA levels of the nAChR subunits α4, α5, α7, β2, and β4 relative to α3 by qRT-PCR in cell cultures prepared from SCGs of 5-day-old WT mice and of mice with a null mutation of the α5 and the β2 nAChR subunit gene.

In relation to α3, the levels of α5 and β4 remained stable throughout the period of our observation (6 hr after plating until 5 DIV; Fig. 5). In contrast, levels of α4 declined rapidly, as did levels of the nAChR subunit α7. A similar time-dependent decline of α7 has previously been observed in rat SCG neurons kept in culture (De Koninck and Cooper, 1995). No significant difference was seen between cultures prepared from WT or any one of the KO mice (Fig. 5).

Absence of Subunits α2, α6, and β3 in the Mouse SCG

We checked for the presence of additional nAChR subunits in extracts prepared from intact ganglia of WT, α5, α7, and β2 knockout mice by conventional PCR and did not detect α2 with 35 cycles of the PCR (three independent experiments; data not shown). The subunits α6 and β3 showed up weakly in some preparations at 35 or 36 cycles, although the presence of PCR products was neither associated with a specific genotype nor dependent on the age of the animals (Fig. 6; two additional independent experiments, not shown). α6 And β3 amplicons were not seen with 30 cycles of the PCR, when faint bands of the low-copy gene α4 could reliably be demonstrated (Fig. 6).

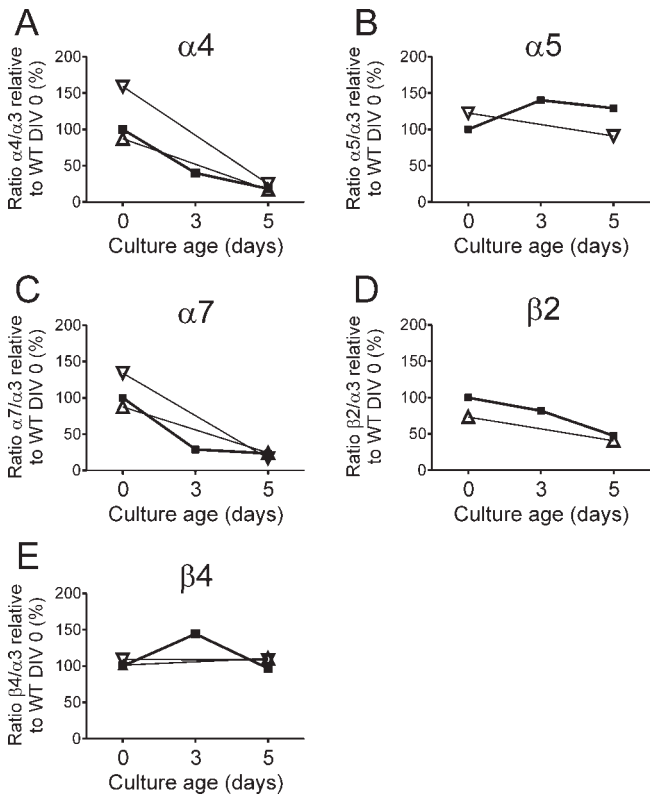

Fig. 5. **A–E:** Transcript profiles of indicated nAChR subunits in SCG cell cultures of WT (squares), α5 KO (upward triangles), and β2 KO mice (downward triangles). Neurons were kept for 6 hr (culture age 0 days) and 3 and 5 DIV. mRNA levels were calculated as ratios to α3. Ratios relative to α3 WT at DIV 0 were defined as 100%. Ratios for DIV 3 and DIV 5 were calculated relative to this reference point. Data points are one RT experiment followed by qRT-PCR measurements (B,C) or means of two (D) or three (A,E) such experiments.

DISCUSSION

We have chosen the mouse SCG to study a question of principal significance for nAChR function: How does the organism compensate for a missing subunit that is a regular constituent of the receptor? The SCG contains two types of nAChRs: homopentamers made up by the α7 subunit and heteropentameric receptors consisting of the subunits α3, α4, α5, β2, and β4. [^{3}H]epibatidine binding, in conjunction with immunoprecipitation experiments, revealed the presence of three subtypes of heterooligomeric receptors in the adult rat SCG: α3β4, α3β4β2, and α3β4α5, with incidences of 55–60%, 10–15%, and 25–30%, respectively (Mao et al., 2006). Similar to the case in rat, about 83% and 25% of [^{3}H]epibatidine-labeled receptors were immunoprecipitated in membrane extracts prepared from adult mouse SCG by anti-α3 and anti-β2 antibodies, respectively (Del Signore et al., 2002). The lower frequency of β4-containing receptors (only 53% of [^{3}H]epibatidine-labeled receptors were immunoprecipitated by anti-β4

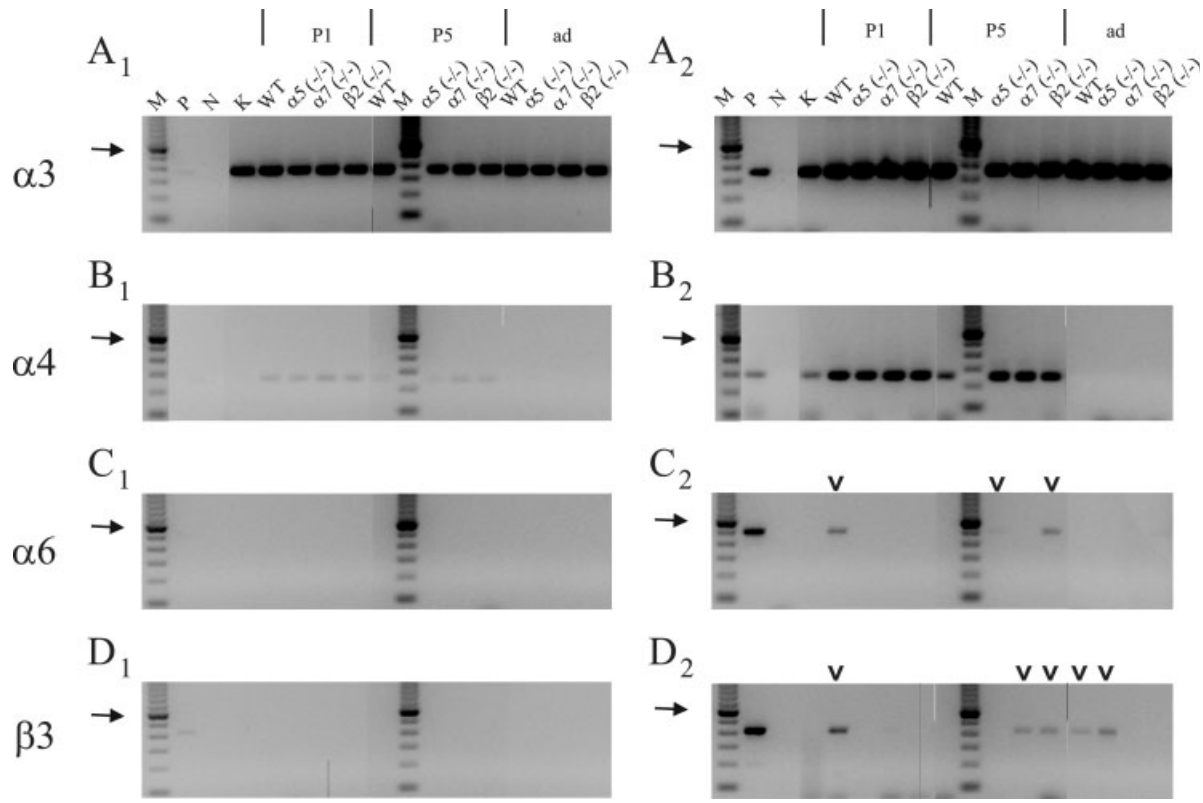

Fig. 6. DNA agarose gel electrophoresis of PCR products of nAChR subunits α3, α4, α6, and β3. PCR was performed with primers as described in Materials and Methods in parallel for 30 (**A1,B1,C1,D1**) or 36 (**A2,B2,C2,D2**) cycles. PCR products were analyzed for confirmation of the expected sequences (α3: 359 bp; α4: 254 bp; α6: 500 bp; β3: 420 bp). M, molecular weight marker (GeneRuler 100-bp DNA ladder; Fermentas; arrows indicate 500 bp). P, positive control: cDNA corresponding to 6 ng total RNA obtained from dorsal root ganglia of 5-day-old mice. N, negative control: PCR in the absence of template. K: cDNA from SCG cell cultures, 5 DIV. A qRT-PCR analysis run in parallel revealed that an identical cell culture sample contained 18,600 and 136 cDNA template molecules of α3 and α4, respectively. cDNA corresponding to 5 ng total RNA obtained from SCG of 1 day (P1), 5 day (P5), and adult (ad) animals were from WT, α5 KO, α7 KO, and β2 KO, respectively. α3 cDNA was clearly detected at 30 cycles, whereas α4 showed faint bands at 30 cycles and robust signals at 36 cycles. α6 And β3 were not detected in the SCG at 30 cycles and showed occasional weak bands at 36 cycles (arrowheads).

antibodies) reported in the latter study may reflect a species difference, though an independent confirmation of this finding due to recent concerns about the affinity and subunit specificity of anti-nAChR antibodies (Gotti et al., 2006; Moser et al., 2007) is to be awaited.

mRNA Levels of nAChR Subunits in α7 KO Mice

α7 Null mice have rather subtle vegetative symptoms in showing a decreased baroreflex (Franceschini et al., 2000). The absence of gross symptoms in these animals indicates that either α7 is not a crucial determinant for transganglionic transmission or its absence is balanced by some unknown mechanism. Our results suggest that compensation does not take place at the level of mRNA of subunits that constitute heteropentameric receptors, in that we did not observe significant changes in the ratios of α4, α5, β2, or β4 relative to α3 mRNA in the α7 KO. We cannot, however, exclude that these subunits are up- or down-regulated along with α3 in the KO animals.

Deletion of the β2 or the α5 nAChR Subunit Gene Does Not Affect mRNA Levels of Accompanying nAChR Subunits

Mice that lack the α5 or β2 nAChR subunit gene do well and show no gross impairment of autonomic nervous system function (Picciotto et al., 1995; Xu et al., 1999b; Wang et al., 2002a,b). Our results indicate that deletion of either the α5 or the β2 subunit had no effect on mRNA levels of the subunits present in the SCG of these mice (α4, α5, α7, and β4) in relation to α3.

We applied conventional RT-PCR to check for the presence of mRNA of the subunits α2, α6, and β3 in WT animals and in our three KO models. Our results

indicate that, irrespective of the genotype and the age of animals, mRNA copies of α6 and β3 may be present in very low numbers, if at all. The few copies could stem either from single SGC neurons or from a contamination by other cell types that express nAChRs, such as lymphocytes (Kawashima and Fujii, 2000; Skok et al., 2003), isolated along with the ganglia. We did not, however, find α2 mRNA in any of our samples. No mRNA message coding for the subunits α2, α6, or β3 has been detected by either RNAse protection assay or in situ hybridization in the rat SCG (Mandelzys et al., 1994; Rust et al., 1994).

Transient Expression of α4 mRNA After Birth

Our experiments provide clear evidence for the presence of α4 mRNA at early postnatal stages. However, mRNA levels of α4 declined sharply from P1 to P9 compared with our standard α3 and were hardly detectable in adult animals. In contrast to our observations in mice, the presence of the α4 subunit in the rat SCG is an unsettled issue (Mandelzys et al., 1994; Rust et al., 1994; Klimaschewski et al., 1994; Zoli et al., 1995).

The two studies reporting the presence of α4 were performed in adult rats (Rust et al., 1994; Klimaschewski et al., 1994), whereas analyses covering the range between E15 and P4 (Zoli et al., 1995) and between P1 and P14 (Mandelzys et al., 1994) yielded negative results. Three of these studies employed in situ hybridization for the analysis (Rust et al., 1994; Klimaschewski et al., 1994; Zoli et al., 1995), whereas mRNA contents were assessed with an RNAse protection assay by Mandelzys et al. (1994). If verified, these observations would indicate a reverse developmental regulation of α4 in the two species, rat and mouse. α4 (but not α2) mRNA has also been found in sympathetic ganglia of 10- and 17-day-old chick embryos (Listerud et al., 1991). However, the treatment of embryonic chick sympathetic neurons with α4 antisense oligonucleotides had no effect on ACh-induced currents, which indicates a minor contribution of the α4 subunit to nAChR function in these cells (Listerud et al., 1991).

The transient presence of α4 during the first postnatal days offers an attractive explanation for the short-term survival of mice with a deletion of the α3 subunit (Xu et al., 1999a; Rassadi et al., 2005). Since neither α2 nor α6 subunits are present in significant amounts in the mouse SCG, α4 is the only subunit that can possibly substitute for a missing α3 in forming functional receptors. Studies on the SCG of P7 to P10 animals have shown that disrupting the α3 gene eliminates fast excitatory postsynaptic potentials and the response evoked by acetylcholine in cultured SCG neurons (Rassadi et al., 2005). Likewise, SCG neurons freshly dissociated from 5–6-day-old α3 null mice show reduced channel openings in response to acetylcholine (Xu et al., 1999a). It will thus be interesting to see whether significant numbers of α4-containing nAChRs are expressed within the first week after birth. Such receptors might transiently compensate for the missing α3 subunit in this KO model.

Developmental Regulation of β2, α5, and α7 mRNA After Birth

We saw less dramatic changes in mRNA levels of the other nAChR subunits. Hence, β4/α3 ratios remained fairly constant, whereas mRNA coding for α5, α7, and β2 declined (relative to α3) more slowly and less completely than α4 between P1 and adult ages. A decrease of α5 and β2 during development leaves less mRNA of these subunits for translation into protein and may thus result in fewer nAChRs containing these subunits in adult animals.

mRNA levels have previously been analyzed in the SCG of rats between P1 and P14 by RNAse protection assays (Mandelzys et al., 1994). At P1, ratios of β2 (0.5) and β4 (1.5) relative to α3 seem higher, whereas the α5/α3 ratio (0.2) seems lower in rats compared with our mouse data, although these variations could result from inherent differences of the two techniques employed. Mandelzys et al. (1994) furthermore reported a 3.6-fold rise of α3 mRNA over this 2-week period not paralleled by the other subunits. Hence, similar to our observation in mice, levels of α5, α7, and β2 mRNA decline in relation to α3 in the rat SCG during development.

nAChR mRNA in Cultured SCG Neurons

nAChRs on cultured SCG neurons are more potently activated by cytisine than by 1,1-dimethyl-4-phenylpiperazinium (DMPP) 1 day after plating, whereas the two nicotinic agonists become almost equipotent after 5 DIV (Fischer et al., 2005). In contrast, SCG neurons in culture were more potently activated at any time by DMPP than by cytisine when taken from α5 KO animals (Fischer et al., 2005), whereas cytisine was consistently more potent than DMPP in SCG neurons from β2 KO mice cultured for up to 9 days (Huck, unpublished observation). Pharmacological and biophysical properties of nAChRs are determined primarily by their subunit composition (Luetje and Patrick, 1991; Covernton et al., 1994; McGehee and Role, 1995; Fischer et al., 2005), so these observations indicate rearrangements of the subunit composition of nAChRs not only in the KO but in a time-dependent manner also in WT SCG neurons kept in culture.

Cytisine is a partial agonist on nAChRs containing the β2 subunit (Luetje and Patrick, 1991; Papke and Heinemann, 1994), and a higher potency of DMPP compared with cytisine as seen in the α5 KO might thus be caused not only by the absence of receptors that lack α5 but also by more β2-containing nAChRs (if β2 takes the place of the missing α5 subunit). This consideration in a reciprocal manner also applies to the β2 KO model. The absence of prominent differences at the mRNA level suggests, however, that the effects of the KOs on functional properties of nAChRs in cultured

SCG neurons might be caused by posttranscriptional mechanisms.

CONCLUSIONS

Taking advantage of the mouse KO models α5, α7, and β2, we have tested whether compensation for a missing subunit is achieved by up-regulation of mRNA of nAChR subunits in the SCG. Our observations based on qRT-PCR indicate no significant effects on the levels (relative to the subunit α3) of α4, α5, α7, or β4 (for the β2 KO); α4, α7, β2, or β4 (for the α5 KO), or α4, α5, β2, or β4 (for the α7 KO) analyzed at developmental stages ranging from newborn to adult. Likewise, mRNAs coding for α2, α6, or β3 were not differentially expressed in the KO compared with WT animals.

We conclude that nAChR subunits translated at normal rates in the SCG are substituting for α5 or β2 at the protein level in the assembly of heteropentameric receptors. This hypothesis, along with the role of the α4 subunit and development-dependent changes in the subunit composition of nAChRs will be verified by [^{3}H]epibatidine binding, combined with immunoprecipitation/immunodepletion techniques. When developing the antibodies required for these experiments, we will take into consideration recent concerns about subunit specificity of antibodies (Gotti et al., 2006; Moser et al., 2007) and employ tissue from KO animals for a rigorous testing.

ACKNOWLEDGMENTS

Expert technical assistance was provided by Gabriele Koth and Karin Schwarz.

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