α4β2 Nicotinic Acetylcholine Receptors in the Early Postnatal Mouse Superior Cervical Ganglion

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ABSTRACT: **Heteropentameric nicotinic acetylcholine receptors (nAChR) mediate fast synaptic transmission in ganglia of the autonomic nervous system. It is undisputed that α3 and β4 are the predominant subunits in the superior cervical ganglion (SCG); however, reports on the presence of receptors that contain α4 have been controversial. Here, we have searched for the presence of α4-containing nAChRs in the postnatal rat and mouse SCG. We now show by immunoprecipitation combined with radioligand binding that α4-containing receptors constitute about 20% of hetero-oligomeric nAChRs in postnatal Day 3 (P3) mice. However, already by P9, the level of α4 approaches zero. In contrast, the number of α4-containing receptors is close to zero in the rat SCG at all times investigated. Deletion of the β2 subunit by using α5β2-double knockout (KO) mice removes all α4-containing receptors, suggesting that in the postnatal mouse SCG, α4 co-assembles only with β2 but not with β4. α4β2 receptors are, on the other hand, up-regulated in the SCG of P3 α5β4-double KO mice, where they make up about 50% of receptors that bind [^{3}H]-epibatidine. Nonetheless, receptors on the surface of SCG neurons from α5β4-double KO mice maintained for one to two days in culture comprise <10% of α4β2 and >90% of α3β2, as determined by patch clamp recordings with α4β2- and α3β2-specific ligands. We propose that in the P3 SCG of wild type mice, α3β4 (±α5) represent about 62% of receptors, whereas 17% are α3β2β4, and 21% are α4β2 (±α5) receptors.** © 2011 Wiley Periodicals, Inc. Develop Neurobiol 71: 390–399, 2011

Keywords: **acetylcholine receptor [AChR]; subunit composition; immunoprecipitation; knockout; patch clamp**

INTRODUCTION

Neuronal nicotinic acetylcholine receptors nAChR are pentameric ion channels consisting of five identical (homopentamers) or different (heteropentamers) subunits. They are widely distributed in the central and peripheral nervous system, but are also found in non-neuronal tissue as diverse as the immune system, intestinal epithelium, the lung, or the skin (Gahring and Rogers, 2005). nAChRs in the central nervous system are involved both in physiological functions (including cognition, reward, motor activity, and

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analgesia) and pathological conditions such as Alzheimer's disease, Parkinson's disease and schizophrenia (Gotti et al., 2006).

The predominant hetero-oligomeric nAChR in the CNS contain the subunits $\alpha4\beta2$, whereas $\alpha3\beta4$ prevail in the PNS. However, the expression of these subunits varies not only by region but also during development. For example, $\alpha3$ is initially (E12-13) expressed in the rat cerebral cortex but down-regulated and replaced by $\alpha4$ thereafter (Zoli et al., 1995). $\alpha3$ mRNA levels keep falling after birth not only in the cortex, but also in the hippocampus, striatum, brain stem, and the cerebellum (Zhang et al., 1998). According to this report, $\alpha4$ mRNA levels are stable from birth to adult in the cortex but steeply drop in the cerebellum, whereas $\beta2$ is stable during postnatal development both in the cerebral cortex and the cerebellum (Zhang et al., 1998). nAChRs particularly abundant in layer VI of the prefrontal cortex of postnatal rodents have been implied in the establishment of corticothalamic connectivity (Tribollet et al., 2004; Kassam et al., 2008). In the human cortex, levels of $\alpha4$, $\alpha5$, $\alpha7$, and $\beta2$ (but not of $\alpha3$) are significant lower at old (71 ± 4 years) ages compared to the prenatal (9.5–11 weeks of gestation) cortex (Hellström-Lindahl et al., 1998).

The developmental profile of neuronal nAChRs in the peripheral nervous system has been studied, in particular, in (chick and rodent) sympathetic and in chick ciliary ganglia. In the chick ciliary ganglion, the number of transcripts per neuron coding for $\alpha3$, $\alpha5$, $\alpha7$, $\beta2$, and $\beta4$ increases significantly between embryonic Days 8 and 18, as does the number of mAb 35- and α-bungarotoxin-nAChRs (Corriveau and Berg, 1993). Likewise, nAChRs containing the subunits $\alpha3$, $\alpha5$, $\alpha7$, and $\beta4$ are up-regulated in the developing chick sympathetic nervous system, with expression levels that depend, in addition, on retrograde signals from innervated target organs (Devay et al., 1999).

Rat sympathetic neurons express constant levels of $\alpha3$, $\beta2$, and $\beta4$ mRNA between embryonic day 15 to postnatal Day 4 (Zoli et al., 1995). Between postnatal Days 1 and 14, the mRNA coding for $\alpha5$, $\beta2$, and $\beta4$ increases moderately, whereas steep increases of $\alpha3$ and $\alpha7$ have been observed at this crucial period of target innervation (Mandelzys et al., 1994). $\alpha4$ mRNA was not detected in either of these studies, and no $\alpha4$-containing receptors were found in the adult rat and mouse SCG (Mao et al., 2006; David et al., 2010). In contrast, $\alpha4$ mRNA and $\alpha4$ at the protein level was found in the adult rat SCG (Rust et al., 1994; Klimaschewski et al., 1994) and in cultured rat SCG neurons (Skok et al., 1999), respectively, leaving some ambiguity regarding the presence of $\alpha4$ in the SCG.

We have recently reported that $\alpha4$ mRNA present in mice P1 superior cervical ganglia (SCG) is rapidly down-regulated by P9 and not detectable any more in 8 week old (adult) animals (Putz et al., 2008). Since nAChR subunit expression does not necessarily parallel mRNA levels (Moretti et al., 2004) we assessed the number of $\alpha4$-containing receptors in the SCG of postnatal rats and WT mice with [^{3}H]-epibatidine binding and immunoprecipitation. Using immunoprecipitation we now show $\alpha4$-containing receptors in the SCG of P3 mice capable of binding [^{3}H]-epibatidine. $\alpha4$-containing receptors in the SCG require the presence of the $\beta2$ subunit and disappear soon after birth.

METHODS

Animals and Acute Preparation of Ganglia

Experiments were performed on wild type C57Bl/6J (WT) mice, and on mice with deletions of distinct nAChR subunit genes. $\alpha5\beta4$-double KO mice (Kedmi et al., 2004) were provided by Avi Orr-Urtreger. Double KO mice lacking both $\alpha5$ and $\beta2$ were generated by crossing the two single KO lines of $\alpha5$ (Wang et al., 2002) and $\beta2$ (Picciotto et al., 1995) provided by Avi Orr-Urtreger and J.-P. Changeux (Pasteur Institute, Paris), respectively. $\alpha5\alpha7\beta4$-triple KO mice were obtained by crossing $\alpha5\beta4$-double with $\alpha7$-single KO (Orr-Urtreger et al., 1997) animals purchased from Jackson Laboratory. Mice used in this study were backcrossed onto C57Bl/6J background for 6 ($\alpha5\beta4$), 7 ($\alpha5$, $\alpha7$), or 12 ($\beta2$) generations after germ line transmission.

Sprague-Dawley rats (*Oncins France strain* A, OFA) were obtained from the Institute of Biomedical Research, Medical University of Vienna (Himberg, Austria) and bred in-house. All animals were kept in thermo stable rooms (21°C) on a light-dark schedule of 10:14 h in group cages with food and water freely accessible. Mice up to the age of five days (P2, P3, and P5) and two-day-old (P2) rat pups were killed by decapitation, 9 (P9), and 18 days old (P18, range 17–19 days) animals were deeply anesthetized with CO_2 prior to decapitation. Superior cervical ganglia (SCG) were collected in Ca^{2+}-free Tyrode's solution: 150 m*M* NaCl, 4 m*M* KCl, 2.0 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES, pH 7.4. After removal of the Tyrode's solution, ganglia were flash-frozen with liquid nitrogen and stored at −80°C for later use.

Cell Culture of SCG Neurons

SCGs were dissected from three- or six-day-old (P3, P6) mouse pups killed by decapitation. The use of enzymes, the trituration protocol, and the culture conditions were slightly modified to published procedures (Fischer et al., 2005): Ganglia were freed from adhering connective tissue and

blood vessels and incubated in a combination of collagenase IA (0.25 mg/mL, Sigma) and dispase (0.5 mg/mL, Roche Applied Science) for 10 min at room temperature. Subsequently, the ganglia were rinsed with Ca^{2+}-free Tyrode solution once and trypsinized (0.25% trypsin in Tyrode solution; Worthington) for 10 min at room temperature. After a 10-min incubation in culture medium (see Fischer et al., 2005) supplemented with 10% fetal calf serum, cells were dispersed by trituration. Dispersed neurons were plated in culture medium (without fetal calf serum) either onto tissue culture dishes (Nunc) for patch-clamp recordings, or on glass coverslips for immunocytochemistry. Glass coverslips were treated by submersion in concentrated nitric acid for two days and thoroughly rinsed thereafter with distilled water. Tissue culture dishes as well as glass coverslips were coated with poly-DL-lysine (25 μg/mL, Sigma) followed by laminin (Becton Dickinson, dissolved to 0.01 mg/mL in Neurobasal A medium).

We seed 10.000 cells into 8 mm glass rings in order to confine the cells to the center of 35 mm tissue culture dishes (Nunc). For patch clamp recordings, cells were cultured at 5% CO_2 and 36.5°C for 1-2 days before use.

Patch Clamp Recordings

We used standard techniques for either conventional whole cell or perforated patch clamp recordings as previously described (Fischer et al., 2005). The internal (pipette) solution consisted of 75 m*M* K_2SO_4, 55 m*M* KCl, 8.0 m*M* $MgCl_2$, and 10 m*M* HEPES, adjusted to pH = 7.3 with KOH. For the perforated patch clamp technique, access to cells was achieved by including 200 μg/mL amphotericin B (Rae et al., 1991). Cultured neurons (one to two days *in vitro*) were voltage-clamped at −70 mV. Substances were dissolved in external (bathing) solution consisting of: 120 m*M* NaCl, 3.0 m*M* KCl, 2.0 m*M* $CaCl_2$, 2.0 m*M* $MgCl_2$, 20 m*M* glucose, 0.1 mg/mL bovine serum albumin, 10 m*M* HEPES, and 0.5 μ*M* tetrodotoxin (TTX, Latoxan), adjusted to pH = 7.3 with NaOH. The substances were applied by means of a DAD-12 solenoid-controlled superfusion system (ALA Scientific Instruments) with a tip diameter of 100 μm and reservoirs set to a pressure of 250 mmHg. For recording and signal processing we used an Axopatch 200B patch clamp amplifier, a Digidata 1320A data acquisition system, and the pCLAMP 10 software (all from Molecular Devices).

Membrane Preparation

We homogenize SCG tissue in ice-cold homogenization buffer (10 m*M* HEPES, 1 m*M* EDTA, 300 m*M* sucrose, pH = 7.5, supplemented with Aprotinin (1 μg/mL), Leupeptin (0.5 μg/ml), Pepstatin (0.5 μg/mL) and 1 m*M* PMSF). Exactly three pulses of 5 s duration with the power level set to 30% were delivered by an ultrasonic homogenizer (Bandelin Sonopuls UW2200). We took great care to avoid excessive foam formation by precise positioning of the MS73 sonotrode tip. Following centrifugation of the homogenate for 30 min at 4°C and 50,000*g*, the pellet was resuspended in homogenization buffer without sucrose, incubated on ice for 30 min, and centrifuged again for 30 min at 50,000*g*. Membrane preparations were always used the same day.

Immunoprecipitation of [^{3}H]-Epibatidine Labeled Receptors

Receptors were solubilized by resuspending membrane preparations (described above) in 2% Triton X-100 lysis buffer: 50 m*M* Tris-HCl pH = 7.5, 150 m*M* NaCl, 2% Triton X-100, supplemented with one complete mini protease inhibitor cocktail tablet (Roche) per 10 mL buffer. Following two ultrasound pulses of 5 s duration at 30% energy level, samples were left for 2 h at 4°C and thereafter centrifuged at 16,000*g* for 15 min at 4°C. Totally, 130 μL clear supernatant containing the membranes of 3 SCG (WT, $\alpha5\beta2$ KO), 5 SCG (rat) or 10 SCG ($\alpha5\beta4$ KO), respectively, were incubated with 20 μL 10 n*M* [^{3}H]-epibatidine and 7 μg antibody in 15–20 μL phosphate-buffered saline (PBS: 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, 2.7 m*M* KCl, 140 m*M* NaCl, pH = 7.4) on a shaking platform at 4°C over night. Unspecific binding was determined by adding 300 μ*M* nicotine to half of the samples.

Heat-killed, formalin-fixed *Staphylococcus aureus* cells carrying protein A (Standardized Pansorbin-cells, Calbiochem) were centrifuged at 2300*g* for 5 min at 4°C. The pellets were washed twice with IP-High (50 m*M* Tris-HCl pH = 8.3, 600 m*M* NaCl, 1 m*M* EDTA, 0.5% Triton X-100), once in IP-Low (50 m*M* Tris-HCl pH = 8.0, 150 m*M* NaCl, 1 m*M* EDTA, 0.2% Triton X-100), and resuspended with IP-Low. 20 μl of this suspension of Pansorbin cells were added to the above mentioned cocktail containing the antibody, solubilized receptors, and [^{3}H]-epibatidine for 2 h at 4°C on a shaking platform. Samples were centrifuged at 2300*g* for 5 min at 4°C and washed twice with IP-High and once with IP-Low at 2300*g* for 1 min at 4°C. Pellets were resuspended in 200 μL 1*N* NaOH and subjected to liquid scintillation counting.

Quantification of Protein Contents in Membrane Preparations and Lysates

All protein quantifications were performed using the Micro BCA Protein Assay Reagent Kit (Pierce) following the manufacturer's instructions.

Immunofluorescent Staining

Cultured SCG neurons were fixed with 4% (w/v) paraformaldehyde in PBS for 20 min, incubated with 100 m*M* glycine in PBS for another 10 min and afterwards blocked and permeabilized using 3% (w/v) normal donkey serum and 0.2% (v/v) Triton X-100 in PBS for 30 min. Antibody staining was performed by incubation for 1 h with primary antibodies washed three times and incubated 1 h with secondary antibody, all antibodies diluted in blocking buffer.

The following antibodies were used: rabbit anti-α3 and anti-α4 nAChR antibodies (David et al., 2010) both diluted to 0.4 μg/mL, and mouse anti-actin (Millipore MAB1501, diluted 1:1000). Secondary antibodies were Alexa goat anti-rabbit-488 and goat anti-mouse-568 (Invitrogen, diluted 1:1000). After the antibody incubation, cells were washed 3× with PBS, 1× with H_2O and mounted with Prolong Gold Anti-Fade (Invitrogen). All pictures are single confocal sections averaged four times to reduce noise. Fluorescent images of neurons were acquired using the Leica TCS SP5 confocal microscope and the Leica application Suite (LAS) software.

Reagents

General chemical reagents were from Merck-VWR-Jencons. Substances not particularly mentioned were from Sigma-Aldrich. RJR 2403 oxalate (#1053) was from Tocris.

Data Analysis

All data are presented as means ± SEM. Statistical analyses was done with GraphPad Prism version 4.0 (GraphPad Software). Student's *t*-test or one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test, were performed when appropriate.

RESULTS

α4-Containing nAChRs in the Postnatal Rat and Mouse SCG

We assessed the number of α4-containing receptors in the SCG of postnatal rats and WT mice with [^{3}H]-epibatidine binding and immunoprecipitation. The experiments were performed with our anti-α4 nAChR antibodies which specifically and efficiently precipitate α4-containing receptors (for a full characterization of antibodies see David et al.., 2010; the efficacy in precipitating α4-containing nAChRs of the rat is documented in the supplementary material). Binding to SCG membrane homogenates of postnatal P18 WT mice is maximal at 1 n*M* epibatidine (David et al., 2010), and was therefore the concentration used for all immunoprecipitation experiments in this study. Epibatidine binds with high affinity in the picomolar range to hetero-oligomeric nAChRs (Houghtling et al., 1995), and with much lower affinity (greatly in excess to 1 n*M*) to α7 homo-oligomeric nAChRs (Sharples et al., 2000). In the mouse SCG, [^{3}H]-epibatidine binding by α4-containing receptors decreased from 163.2 ± 78.1 fmol/mg protein at two days after birth (P2) to levels close to zero at P9 and P18 (see Fig. 1). In contrast, the number of α4-containing receptors in the rat SCG is at the limits of detection at all times investigated (see Fig. 1).

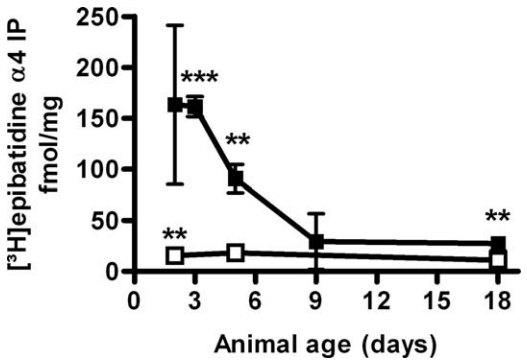

Figure 1 α4-containing nAChRs in the mouse SCG decline soon after birth and are barely detectable in the rat nAChRs from SCG membranes of 2, 3, 5, 9, or 18 day old WT mice and 2-, 5-, or 18-day-old rats were solubilized, labeled with 1 n*M* [^{3}H]-epibatidine, and immunoprecipitated with our anti-α4 antibodies. Nonspecific binding was measured in the presence of 300 μ*M* nicotine and subtracted from overall to obtain the specific binding shown. Data are the mean ± SEM of three to six independent experiments, each performed with duplicate (P3, P9) or triplicate (P2, P5, P18) measurements. *, **, ***: significantly different from zero ($p < 0.05$, 0.01, and 0.001, respectively; one sample Students *t*-test). The specificity and efficacy of our anti-α4 antibodies is documented in David et al., 2010 (for mouse nAChRs) and in the supplementary material (for rat nAChRs).

We see α4 also in SCG cell cultures prepared from P3 (see Fig. 2). The signal obtained by indirect immunofluorescence is most intense in cultures maintained for one to two days (Fig. 2 B1) but fades by six days *in vitro* (not shown). In keeping with our observation that α4-containing receptors in the SCG decline with increasing age of the animals, cultures maintained for six days *in vitro* that were prepared from P6 mice show a very weak fluorescent signal only (Fig. 2 B2). It is worth noting that radioligand binding requires receptors with intact binding sites, whereas immunofluorescence unveils just the presence of a particular protein that may or may not be functional (see our patch clamp experiments below).

nAChRs in P3 WT Mice

Since hetero-oligomeric nAChRs contain the β2 or the β4 subunit (Champtiaux and Changeux, 2004), a combined use of anti β2- plus anti-β4 antibodies will precipitate 100% of receptors (David et al., 2010). We found that 19% of the receptors contain the subunit α4, whereas 82% of receptors have the subunit α3

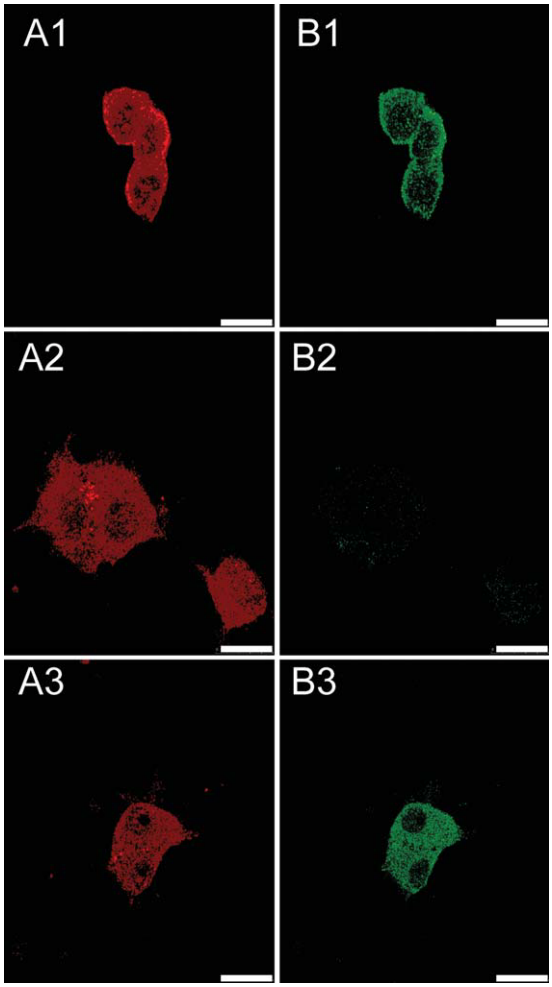

Figure 2 The nAChR subunit α4 is present in short-term SCG cell cultures from P3 mice Indirect immunofluorescence of actin (A1-A3) and the α4 (B1-B2) and α3 (B3) nAChR subunits as detected by mouse anti-actin (1:1000), rabbit anti-α4, and rabbit anti-α3 (each 0.4 μg/ml). Since the experiments were designed to parallel the patch clamp recordings shown in Fig. 5, cultures were prepared from α5α7β4-triple KO mice. A1 and B1: Cell culture one day *in vitro* prepared from P3 α5α7β4-triple KO mice. A2, A3, B2, and B3: Cell cultures six days *in vitro* prepared from P6 α5α7β4-triple KO mice. Note that the intensity of the fluorescent signal due to α4 is greatly attenuated in cells taken from P6 animals and cultured for six days (B2). Panel B3 shows the fluorescent signal due to α3 for comparison. Calibration: 25 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(see Fig. 3), adding up to a total of 101%. From this observation it is tempting to conclude that two populations of nAChRs with either the α3 or the α4 subunit are present in the P3 mouse SCG. Furthermore, α4-containing receptors seem to assemble with just β2, since the combined use of anti-α4 and anti-β2 antibodies does not precipitate more receptors than the sole use of anti-β2 antibodies (see Fig. 3). The somewhat larger number of receptors precipitated with anti-β2 relative to anti-α4 antibodies is in keeping with our previous observation (David et al., 2010) that the β2 subunit coassembles with α3 not only in the SCG of P19 but also of P3 mice.

nAChRs in P3 KO Mice

We probed the above mentioned hypothesis that the α4 subunit only co-assembles with β2 by using α5β2-double KO mice. In accordance with our assumption, all [^{3}H]-epibatidine binding by α4-containing receptors was lost in this genotype, whereas the expression of α3-containing receptors was not significantly affected (see Fig. 4). Quite

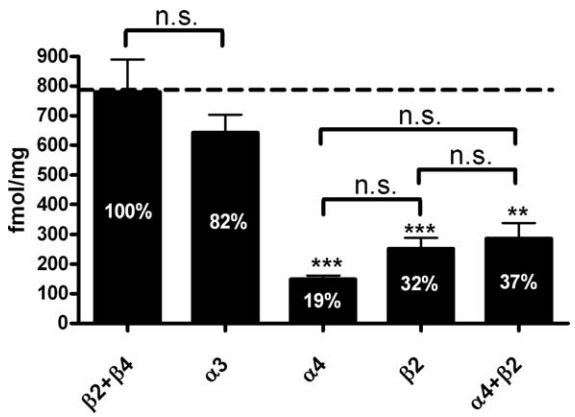

Figure 3 Subunit composition of P3 wild-type mice nAChRs. nAChRs from SCG membranes of three-day-old wild-type mice were solubilized, labeled with 1 n*M* [^{3}H]-epibatidine and immunoprecipitated with each of the subunit-specific antibodies indicated at the abscissa. Nonspecific binding was measured in the presence of 300 μM nicotine and subtracted from overall to obtain the specific binding shown in the figure. Data are the mean ± SEM of three to five independent experiments, each performed with duplicate measurements. The dashed line indicates the total number of receptors which are precipitated by the combined use of anti-β2 and anti-β4 antibodies. Although comparisons do not reach levels of significance, the results suggest that separate α3- (82%) and α4-containing receptors (19%) make up for 100% of the receptors. Likewise, α4-containing receptors seem to be less frequent than β2-containing receptors, whereas the combined use of anti- α4 and anti-β2 antibodies does not precipitate more receptors than the single use of anti-β2 antibodies. Statistical significance between data points was calculated by one-way ANOVA, followed by Bonferroni's *post-hoc* multiple comparison test (n.s.: $p > 0.05$; **, ***: significantly different from β2 + β4 with $p < 0.01$ and $p < 0.001$, respectively).

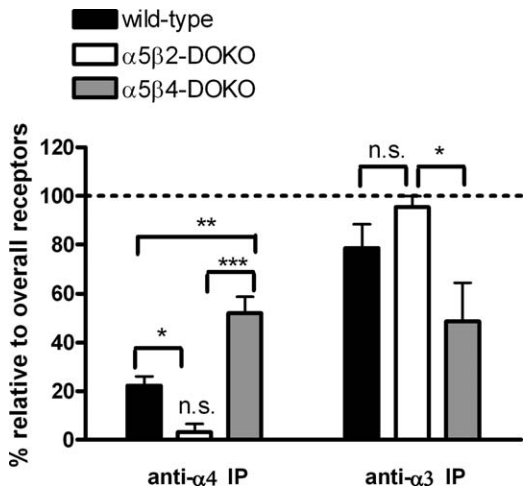

Figure 4 β subunits markedly affect the expression of both α3- and α4-containing receptors. nAChRs from SCG membranes of WT (black bars), α5β2 (white bars), and α5β4 (gray bars) KO mice were solubilized, labeled with 1 n*M* [^{3}H]-epibatidine, and immunoprecipitated with either anti-α3 or anti-α4 antibody. Nonspecific binding was measured in the presence of 300 μM nicotine and subtracted from overall to obtain the specific binding shown in the figure. Data are the mean specific binding relative to immunoprecipitation with anti-β2 + anti-β4 (for WT), anti-β2 (for α5β4 KO), and anti-β4 (for α5β2 KO) antibodies, respectively (dashed line, 100%) ± SEM of three to six independent experiments, each performed with duplicate measurements. Note that α4 is lost (n.s.: not significantly different from zero, $p > 0.05$, one sample Students *t*-test) in the SCG of α5β2 KO and significantly upregulated in α5β4 KO mice. α3-containing receptors are, on the other hand, significantly reduced in α5β4 KO mice. Statistical significance between data points was calculated by one-way ANOVA, followed by Bonferroni's *post-hoc* multiple comparison test (n.s.: $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

the opposite, deletions of α5 and β4 increased the relative frequency of α4-containing receptors but decreased receptors made by the α3 subunit (see Fig. 4). Unlike in WT mice, where α3-containing receptors are much more common than α4-containing receptors, α3β2 and α4β2 receptors occur with about equal frequency in the α5β4 KO. Please note, however, that similar to P19 animals (David et al., 2010), the deletion of the β4 subunit (in the α5β4-double KO) also causes a large reduction of the overall number of receptors in the P3 mice (WT: 760.5 fmol/mg protein; α5β4-double KO: 137.3 fmol/mg protein, Fig. 6). In contrast, and in keeping with observations on P19 animals, deletion of the β2 subunit (in the α5β2-double KO) does not affect the overall number of receptors (α5β2-double KO: 764.9 nmol/mg protein, Fig. 6).

Few α4-Containing nAChR Reach the Cell Surface

To assess the number of functional surface receptors, we used patch clamp measurements of currents in response to the α4β2 specific agonist RJR 2403 (Papke et al., 2000) as well as to nicotine in the presence and absence of α-conotoxin MII, an α3β2 (and α6β2) specific antagonist (Cartier et al., 1996; Champtiaux et al., 2003; David et al., 2010). The experiments were performed on β4-deficient SCG neurons taken from P3 animals and cultured for one to two days. Neurons in such cultures show clear immunofluorescence upon labeling with anti-α4 antibodies (see Fig. 2).

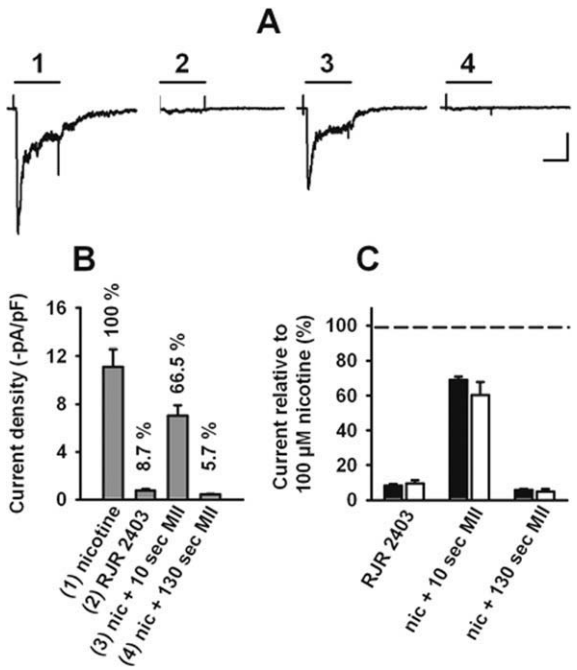

Figure 5 Few α4-containing nAChR reach the cell surface. A: Patch clamp recordings (perforated patch) from cultured postnatal Day 3 SCG neurons (α5β4α7-triple KO), kept for two days in culture. Agonists and α-conotoxin MII were always applied in the indicated order: (1) 100 μM nicotine, the 2-s application indicated by bar. (2) 300 μM RJR 2403, the 2 s application indicated by bar. (3) 100 μM nicotine after 10 s of application of 100 n*M* α-conotoxin MII. (4) 100 μM nicotine after 130 s of application of 100 n*M* α-conotoxin MII. Calibration bars: 100 pA; 1 s. B. Pooled observations from 38 cells. Data are mean peak current densities ± SEM. Figures on top of the bars indicate current densities relative to the currents induced by nicotine (in the absence of α-conotoxin MII). C. Comparison of effects obtained in α5β4α7-triple KO (filled bars; $n = 32$) and α5β4-double KO mice (open bars; $n = 6$). Peak amplitudes by indicated protocols relative to 100 μM nicotine did not differ between the two genotypes ($p > 0.05$, Student's *t*-test).

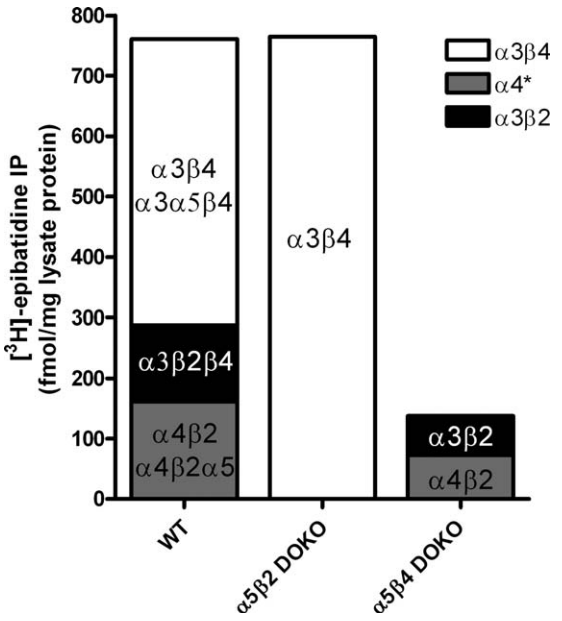

Figure 6 Proposed nAChR subtypes occurring in the SCG of P3 WT, $\alpha5\beta2$-, and $\alpha5\beta4$ KO mice. $\alpha5\beta2$-double KO (white bars) mice also lack the $\alpha4$ subunit (see Fig. 4), which leaves only $\alpha3\beta4$ hetero-oligomeric receptors in this genotype. Compared with WT mice, the overall number of receptors remains unaffected by the KO. *$\alpha5\beta4$*-double KO (gray bars) mice contain equal numbers of $\alpha3$- and $\alpha4$-containing receptors (see Fig. 4). In the absence of $\beta4$, the two subunits share the $\beta2$ subunit at about identical rates of recurrence (see Fig. 4). Compared with WT mice, the overall number of receptors is significantly reduced in the KO. WT (black bars): About 80% of receptors contain the subunit $\alpha3$, versus 20% of receptors with $\alpha4$ (see Fig. 3). $\beta2$ subunits (which occur in about 40% of the receptors, Fig. 3) that do not coassemble with $\alpha4$ (about 50% according to experiments shown in Fig. 2) assemble with $\alpha3$. The model infers from our observations in P19 WT animals (David et al., 2010) that $\alpha3\beta2$ receptors always contain the $\beta4$ subunit, whereas $\beta2$ and $\alpha5$ never occur in the same receptor. It also infers that $\alpha4$ only coassembles with $\beta2$ but not with $\beta4$ (see Fig. 4). We did not probe $\alpha5$-containing receptors which may concur with $\alpha3$ and $\beta4$ as in P19 animals (David et al., 2010). The $\alpha5$ subunit could, in principle, also coassemble with $\alpha4\beta2$ receptors (Gotti et al., 2006). According to this model, 62% of receptors in P3 WT animals consist of $\alpha3\beta4$ with and without $\alpha5$, 17% are of the $\alpha3\beta2\beta4$ type, and 21% are $\alpha4\beta2$ receptors with our without $\alpha5$.

We recorded from 32 SCG neurons taken from $\alpha5\beta4\alpha7$-triple KO, and 6 SCG neurons from $\alpha5\beta4$-double KO mice. The $\alpha5\beta4\alpha7$-triple KO mice were preferred because currents by $\alpha7$ receptor activation can be excluded. However, the results did not significantly differ between the two genotypes [Fig. 5(C)], and the data were therefore pooled [Fig. 5(B)]. We have previously reported that in the absence of the positive allosteric modulator PNU-120596, currents due to $\alpha7$ nAChR activation are missing in cultured SCG neurons from P5-6 animals (David et al., 2010).

SCG neurons from WT animals do have $\alpha4$-containing receptors (see Fig. 3), but their potential contribution to overall currents will be hard to determine because of the sheer size of currents due to $\alpha3\beta4$ receptor activation and because of the lack of potent $\alpha3\beta4$-specific antagonists to unveil $\alpha4\beta2$ activation. We do not expect $\alpha4$-containing ($\alpha4\beta4$) receptors in SCG neurons obtained from $\alpha5\beta2$ KO mice, since our IP with anti-$\alpha4$ antibodies did not show receptors in this genotype that bind [^{3}H]-epibatidine (see Fig. 4).

In the $\alpha5\beta4\alpha7$-triple- and the $\alpha5\beta4$-double KO, more than 90% of the current induced by 100 μM nicotine is blocked after a brief (130 s) application of 100 nM α-conotoxin MII [Fig. 5(A–C)]. α-conotoxin MII specifically blocks $\alpha3\beta2$ as well as $\alpha6\beta2$ receptors (Cartier et al., 1996; Champtiaux et al., 2003; David et al., 2010), but since the $\alpha6$ subunit is missing in the P1 mouse SCG (Putz et al., 2008) our data suggest that $\alpha4\beta2$ only contribute a small percentage to receptors expressed on the cell surface. In keeping with this observation, 300 μM of the $\alpha4\beta2$-selective agonist RJR 2403 induced currents that reached about 8.7% of the peak amplitude of currents induced by nicotine [Fig. 5(A–C)]. This is less than predicted by our IP experiments, which indicate about equal numbers of $\alpha3\beta2$ and $\alpha4\beta2$ receptors in the $\alpha5\beta4$-double KO mice (see Fig. 4). However, IP experiments were prepared from intact ganglia, and the number of $\alpha4\beta2$ receptors might have dropped by keeping the cells in culture (Putz et al., 2008). Alternatively, relatively more $\alpha4\beta2$ receptors compared with $\alpha3\beta2$ receptors might stay in the intracellular pool without reaching the cell surface.

DISCUSSION

Our observations on the early postnatal day mouse SCG show the presence of $\alpha4$-containing nAChR that specifically bind [^{3}H]-epibatidine. The $\alpha4$ subunit appears to assemble exclusively with $\beta2$, since $\alpha4$-containing nAChR are lost entirely in the $\alpha5\beta2$ KO. By combining this result with our previous observations in P19 animals (David et al., 2010) we propose that in the WT mouse SCG, (about) 62% of receptors consist of $\alpha3\beta4$; 17% are of the $\alpha3\beta2\beta4$ type, and 21% are $\alpha4\beta2$ receptors (see Fig. 6). We did not include anti-$\alpha5$ antibodies in our current IP experiments and thus have no information on the frequency of $\alpha3\beta4$ and $\alpha4\beta2$ receptors that contain the $\alpha5$ subu-

nit. About 24% of $\alpha3\beta4$ receptors in the SCG of P19 WT mice have the $\alpha5$ subunit (David et al., 2010).

By postnatal Day 9, and in parallel to the decline of mRNA (Putz et al., 2008), the number of $\alpha4$-containing receptors drops to a level not significantly different from 0. We do not know whether this regulation serves a biological purpose or whether it is merely the remains of a mechanism not needed any more later in development. However, the marked decay leads us to speculate that $\alpha4$-containing receptors may be even more numerous earlier in development, where they might contribute to the shaping of synaptic contacts in the ganglion. The rat SCG receives its preganglionic input as early as E12/13, with the first synaptic contacts being largely axosomatic. Thereafter, and upon the ensuing elaboration of ganglionic cell dendrites, synaptogenesis becomes focused upon these processes (Rubin, 1985).

Nicotinic signaling by $\alpha7$ nAChR in the mouse hippocampus and by both $\alpha7$ and non-$\alpha7$ nAChRs in the (chick) spinal cord and ciliary ganglion has been shown to transform GABAergic excitation into inhibition, which in turn affects neuronal morphology and innervation (Liu et al., 2006). Likewise, nicotinic signaling by $\beta2$-containing nAChRs is crucial not only for the generation of retinal waves in early postnatal mice but also for the normal development of the visual system (Bansal et al., 2000). Since deletion of the $\alpha3$ subunit slightly changes the spatiotemporal characteristics of the spontaneous retinal activity only (without abolishing it), and since the $\alpha3\beta2$- and $\alpha6\beta2$-specific antagonist α-conotoxin MII potently inhibits retinal waves (Bansal et al., 2000), nAChRs containing these subunits may equally contribute to the normal segregation process of the visual system (Champtiaux and Changeux, 2004). We have additional striking examples where nAChR signaling plays a key role in the tuning of neuronal circuits during development (Hanson and Landmesser, 2004; Myers et al., 2005; Poorthuis et al., 2009). It is thus conceivable that cholinergic nicotinic transmission by $\alpha4$-containing receptors play a role in the maturation of synaptic contacts in the SCG early in development. $\alpha3\beta4$ receptors are likely also present in the embryonic SCG (Zoli et al., 1995), but ACh is about four times more potent in activating $\alpha4\beta2$ compared to $\alpha3\beta4$ (Chavez-Noriega et al., 1997; Fenster et al., 1997), meaning that much lower concentrations of the transmitter are required for $\alpha4\beta2$ activation.

Because of the robust transganglionic transmission in the sympathetic nervous system (McLachlan, 2009), rather subtle changes that may be caused by the absence of $\alpha4$ may, however, pass without readily noticeably affecting its function. Hence, even in the $\alpha5\beta4$ mouse KO model where more than 80% of receptors are lost, compound action potentials recorded from postganglionic nerves of adult mice have normal amplitudes (David et al., 2010).

$\alpha4$-containing receptors may, on the other hand, play a role in mice with deletions of the $\alpha3$ subunit gene. These mice have morphologically normal synapses, but when recorded at postnatal Day 7, lack transganglionic transmission in the SCG due to the complete absence of hetero-oligomeric nAChRs (Rassadi et al., 2005; Krishnaswamy and Cooper, 2009). Though almost all $\alpha4$-containing receptors will be lost by this time, these receptors may retain some residual synaptic activity that keeps the majority of inbred $\alpha3$ KO mice alive for a few days (Xu et al., 1999; Rassadi et al., 2005).

In conclusion, our work has shown the presence of $\alpha4$-containing receptors in the mouse SCG until early after birth. In WT mice, about 20% of nAChRs have the $\alpha4$ subunit, versus 80% of receptors that contain $\alpha3$. The subunits $\alpha3$ and $\alpha4$ do not occur in one and the same receptor, and $\alpha4$ coassembles with just $\beta2$ but not with $\beta4$. $\alpha4\beta2$ receptors are absent in adult animals and thus play no role in normal trans-synaptic transmission of the SCG.

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