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$\alpha 4\beta 2$ 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 $\alpha 3$ and $\beta 4$ are the predominant subunits in the superior cervical ganglion (SCG); however, reports on the presence of receptors that contain $\alpha 4$ have been controversial. Here, we have searched for the presence of $\alpha 4$ -containing nAChRs in the postnatal rat and mouse SCG. We now show by immunoprecipitation combined with radioligand binding that $\alpha 4$ -containing receptors constitute about 20 % of hetero-oligomeric nAChRs in postnatal day 3 (P3) mice. However, already by P9, the level of $\alpha 4$ approaches zero. In contrast, the number of $\alpha 4$ -containing receptors is close to zero in the rat SCG at all times investigated.

Deletion of the $\beta 2$ subunit by using $\alpha 5\beta 2$ -double knockout (KO) mice removes all $\alpha 4$ -containing receptors, suggesting that in the postnatal mouse SCG, $\alpha 4$ co-assembles only with $\beta 2$ but not with $\beta 4$. $\alpha 4\beta 2$ receptors are, on the other hand, up-regulated in the SCG of P3 $\alpha 5\beta 4$ -double KO mice, where they make up about 50 % of receptors that bind [³H]-epibatidine. Nonetheless, receptors on the surface of SCG neurons from $\alpha 5\beta 4$ -double KO mice maintained for 1-2 days in culture comprise < 10 % of $\alpha 4\beta 2$ and > 90 % of $\alpha 3\beta 2$, as determined by patch clamp recordings with $\alpha 4\beta 2$ - and $\alpha 3\beta 2$ -specific ligands. We propose that in the P3 SCG of wild type mice, $\alpha 3\beta 4$ ($\pm \alpha 5$) represent about 62 % of receptors, whereas 17 % are $\alpha 3\beta 2\beta 4$, and 21 % are $\alpha 4\beta 2$ ($\pm \alpha 5$) receptors.

Keywords

Acetylcholine receptor [AChR]; subunit composition; immunoprecipitation; knockout; patch clamp

INTRODUCTION

Neuronal nicotinic acetylcholine receptors nAChR are pentameric ion channels consisting of 5 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

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and Rogers, 2005). nAChRs in the central nervous system are involved both in physiological functions (including cognition, reward, motor activity and 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 $\alpha 4\beta 2$, whereas $\alpha 3\beta 4$ prevail in the PNS. However, the expression of these subunits varies not only by region but also during development. For example, $\alpha 3$ is initially (E12-13) expressed in the rat cerebral cortex but down-regulated and replaced by $\alpha 4$ thereafter (Zoli *et al.*, 1995). $\alpha 3$ 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, $\alpha 4$ mRNA levels are stable from birth to adult in the cortex but steeply drop in the cerebellum, whereas $\beta 2$ 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 $\alpha 4$, $\alpha 5$, $\alpha 7$, and $\beta 2$ (but not of $\alpha 3$) 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 $\alpha 3$, $\alpha 5$, $\alpha 7$, $\beta 2$, and $\beta 4$ increases significantly between embryonic days 8 and 18, as does the number of mAChR 35- and α -bungarotoxin-nAChRs (Corriveau and Berg, 1993). Likewise, nAChRs containing the subunits $\alpha 3$, $\alpha 5$, $\alpha 7$, and $\beta 4$ 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 $\alpha 3$, $\beta 2$, and $\beta 4$ mRNA between embryonic day 15 to postnatal day 4 (Zoli *et al.*, 1995). Between postnatal day 1 and 14, the mRNA coding for $\alpha 5$, $\beta 2$, and $\beta 4$ increases moderately, whereas steep increases of $\alpha 3$ and $\alpha 7$ have been observed at this crucial period of target innervation (Mandelzys *et al.*, 1994). $\alpha 4$ mRNA was not detected in either of these studies, and no $\alpha 4$ -containing receptors were found in the adult rat and mouse SCG (Mao *et al.*, 2006; David *et al.*, 2010). In contrast, $\alpha 4$ mRNA and $\alpha 4$ 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 $\alpha 4$ in the SCG.

We have recently reported that $\alpha 4$ 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 $\alpha 4$ -containing receptors in the SCG of postnatal rats and WT mice with [^{3} H]-epibatidine binding and immunoprecipitation. Using immunoprecipitation we now show $\alpha 4$ -containing receptors in the SCG of P3 mice capable of binding [^{3} H]-epibatidine. $\alpha 4$ -containing receptors in the SCG require the presence of the $\beta 2$ 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. $\alpha 5\beta 4$ -double KO mice (Kedmi *et al.*, 2004) were provided by Avi-Orr-Urtreger. Double KO mice lacking both $\alpha 5$ and $\beta 2$ were generated by crossing

the two single KO lines of $\alpha 5$ (Wang *et al.*, 2002) and $\beta 2$ (Picciotto *et al.*, 1995) provided by Avi-Orr-Urtreger and J.-P. Changeux (Pasteur Institute, Paris), respectively. $\alpha 5\alpha 7\beta 4$ -triple KO mice were obtained by crossing $\alpha 5\beta 4$ -double with $\alpha 7$ -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 ($\alpha 5\beta 4$), 7 ($\alpha 5$, $\alpha 7$) or 12 ($\beta 2$) 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 hr in group cages with food and water freely accessible. Mice up to the age of 5 days (P2, P3 and P5) and 2 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 CO₂ prior to decapitation. Superior cervical ganglia (SCG) were collected in Ca²⁺-free Tyrode's solution: 150 mM NaCl, 4 mM KCl, 2.0 mM MgCl₂, 10 mM glucose, and 10 mM 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 3 or 6 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 Ca²⁺-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 2 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 % CO₂ 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 mM K₂SO₄, 55 mM KCl, 8.0 mM MgCl₂ and 10 mM 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 (1-2 days *in vitro*) were voltage-clamped at -70 mV. Substances were dissolved in external (bathing) solution consisting of: 120 mM NaCl, 3.0 mM KCl, 2.0 mM CaCl₂, 2.0 mM MgCl₂, 20 mM glucose, 0.1 mg/ml bovine serum albumin, 10 mM HEPES, and 0.5 µM 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 mm Hg. 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 mM HEPES, 1 mM EDTA, 300 mM sucrose, pH = 7.5, supplemented with Aprotinin (1 µg/ml), Leupeptin (0.5 µg/ml), Pepstatin (0.5 µg/ml) and 1 mM PMSF). Exactly three pulses of 5 seconds 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 re-suspended in homogenization buffer without sucrose, incubated on ice for 30 minutes, and centrifuged again for 30 min at 50 000 g. Membrane preparations were always used the same day.

Immunoprecipitation of [³H]-epibatidine labeled receptors

Receptors were solubilized by re-suspending membrane preparations (described above) in 2 % Triton X-100 lysis buffer: 50 mM Tris-HCl pH = 7.5, 150 mM 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 seconds duration at 30 % energy level, samples were left for 2 hours at 4° C and thereafter centrifuged at 16000 g for 15 min at 4° C. 130 µl clear supernatant containing the membranes of 3 SCG (WT, α5β2 KO), 5 SCG (rat) or 10 SCG (α5β4 KO), respectively, were incubated with 20 µl 1 nM [³H]-epibatidine and 7 µg antibody in 15-20 µl phosphate-buffered saline (PBS: 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 140 mM NaCl, pH = 7.4) on a shaking platform at 4° C over night. Unspecific binding was determined by adding 300 µM 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 mM Tris-HCl pH = 8.3, 600 mM NaCl, 1 mM EDTA, 0.5 % Triton X-100), once in IP-Low (50 mM Tris-HCl pH = 8.0, 150 mM NaCl, 1 mM EDTA, 0.2 % Triton X-100), and re-suspended with IP-Low. 20 µl of this suspension of Pansorbin cells were added to the above mentioned cocktail containing the antibody, solubilized receptors, and [³H]-epibatidine for 2 hours 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 re-suspended 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 mM 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 (see 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 3x with PBS, 1x with H₂O and mounted with Prolong Gold Anti-Fade (Invitrogen). All pictures are single confocal sections averaged 4 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 $\pm$ 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

α 4-containing nAChRs in the postnatal rat and mouse SCG

We assessed the number of α 4-containing receptors in the SCG of postnatal rats and WT mice with [³H]-epibatidine binding and immunoprecipitation. The experiments were performed with our anti- α 4 nAChR antibodies which specifically and efficiently precipitate α 4-containing receptors (for a full characterization of antibodies see David *et al.*, 2010; the efficacy in precipitating α 4-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 nM 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 nM) to α 7 homo-oligomeric nAChRs (Sharples *et al.*, 2000). In the mouse SCG, [³H]-epibatidine binding by α 4-containing receptors decreased from 163.2 ± 78.1 fmol/mg protein at 2 days after birth (P2) to levels close to zero at P9 and P18 (Fig. 1). In contrast, the number of α 4-containing receptors in the rat SCG is at the limits of detection at all times investigated (Fig. 1).

We see α 4 also in SCG cell cultures prepared from P3 (Fig. 2). The signal obtained by indirect immunofluorescence is most intense in cultures maintained for 1-2 days (Fig. 2 B1) but fades by 6 days *in vitro* (not shown). In keeping with our observation that α 4-containing receptors in the SCG decline with increasing age of the animals, cultures maintained for 6 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 β 2 or the β 4 subunit (Champtiaux and Changeux, 2004), a combined use of anti β 2- plus anti- β 4 antibodies will precipitate 100 % of receptors (David *et al.*, 2010). We found that 19 % of the receptors contain the subunit α 4, whereas 82 % of receptors have the subunit α 3 (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 α 3 or the α 4 subunit are present in the P3 mouse SCG. Furthermore, α 4-containing receptors seem to assemble with just β 2, since the combined use of anti- α 4 and anti- β 2 antibodies does not precipitate more receptors than the sole use of anti- β 2 antibodies (Fig.

3). The somewhat larger number of receptors precipitated with anti- $\beta 2$ relative to anti- $\alpha 4$ antibodies is in keeping with our previous observation (David *et al.*, 2010) that the $\beta 2$ subunit co-assembles with $\alpha 3$ 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 $\alpha 4$ subunit only co-assembles with $\beta 2$ by using $\alpha 5\beta 2$ -double KO mice. In accordance with our assumption, all [^3H]-epibatidine binding by $\alpha 4$ -containing receptors was lost in this genotype, whereas the expression of $\alpha 3$ -containing receptors was not significantly affected (Fig. 4). Quite the opposite, deletions of $\alpha 5$ and $\beta 4$ increased the relative frequency of $\alpha 4$ -containing receptors but decreased receptors made by the $\alpha 3$ subunit (Fig. 4). Unlike in WT mice, where $\alpha 3$ -containing receptors are much more common than $\alpha 4$ -containing receptors, $\alpha 3\beta 2$ and $\alpha 4\beta 2$ receptors occur with about equal frequency in the $\alpha 5\beta 4$ KO. Please note, however, that similar to P19 animals (David *et al.*, 2010), the deletion of the $\beta 4$ subunit (in the $\alpha 5\beta 4$ -double KO) also causes a large reduction of the overall number of receptors in the P3 mice (WT: 760.5 fmol/mg protein; $\alpha 5\beta 4$ -double KO: 137.3 fmol/mg protein, Fig. 6). In contrast, and in keeping with observations on P19 animals, deletion of the $\beta 2$ subunit (in the $\alpha 5\beta 2$ -double KO) does not affect the overall number of receptors ($\alpha 5\beta 2$ -double KO: 764.9 nmol/mg protein, Fig. 6).

Few $\alpha 4$ -containing nAChR reach the cell surface

In order to assess the number of functional surface receptors, we used patch clamp measurements of currents in response to the $\alpha 4\beta 2$ specific agonist RJR 2403 (Papke *et al.*, 2000) as well as to nicotine in the presence and absence of α -conotoxin MII, an $\alpha 3\beta 2$ (and $\alpha 6\beta 2$) specific antagonist (Cartier *et al.*, 1996; Champiaux *et al.*, 2003; David *et al.*, 2010). The experiments were performed on $\beta 4$ -deficient SCG neurons taken from P3 animals and cultured for 1 to 2 days. Neurons in such cultures show clear immunofluorescence upon labelling with anti- $\alpha 4$ antibodies (Fig. 2).

We recorded from 32 SCG neurons taken from $\alpha 5\beta 4\alpha 7$ -triple KO, and 6 SCG neurons from $\alpha 5\beta 4$ -double KO mice. The $\alpha 5\beta 4\alpha 7$ -triple KO mice were preferred because currents by $\alpha 7$ receptor activation can be excluded. However, the results did not significantly differ between the two genotypes (Fig. 5C), and the data were therefore pooled (Fig. 5B). We have previously reported that in the absence of the positive allosteric modulator PNU-120596, currents due to $\alpha 7$ nAChR activation are missing in cultured SCG neurons from P5-6 animals (David *et al.*, 2010).

SCG neurons from WT animals do have $\alpha 4$ -containing receptors (Fig. 3), but their potential contribution to overall currents will be hard to determine because of the sheer size of currents due to $\alpha 3\beta 4$ receptor activation and because of the lack of potent $\alpha 3\beta 4$ -specific antagonists to unveil $\alpha 4\beta 2$ activation. We do not expect $\alpha 4$ -containing ($\alpha 4\beta 4$) receptors in SCG neurons obtained from $\alpha 5\beta 2$ KO mice, since our IP with anti- $\alpha 4$ antibodies did not show receptors in this genotype that bind [^3H]-epibatidine (Fig. 4).

In the $\alpha 5\beta 4\alpha 7$ -triple- and the $\alpha 5\beta 4$ -double KO, more than 90 % of the current induced by 100 μM nicotine is blocked after a brief (130 sec) application of 100 nM α -conotoxin MII (Fig. 5A-C). α -conotoxin MII specifically blocks $\alpha 3\beta 2$ as well as $\alpha 6\beta 2$ receptors (Cartier *et al.*, 1996; Champiaux *et al.*, 2003; David *et al.*, 2010), but since the $\alpha 6$ subunit is missing in the P1 mouse SCG (Putz *et al.*, 2008) our data suggest that $\alpha 4\beta 2$ only contribute a small percentage to receptors expressed on the cell surface. In keeping with this observation, 300 μM of the $\alpha 4\beta 2$ -selective agonist RJR 2403 induced currents that reached about 8.7 % of the peak amplitude of currents induced by nicotine (Fig. 5A-C). This is less than predicted by our IP experiments, which indicate about equal numbers of $\alpha 3\beta 2$ and $\alpha 4\beta 2$ receptors in the

$\alpha 5\beta 4$ -double KO mice (Fig. 4). However, IP experiments were prepared from intact ganglia, and the number of $\alpha 4\beta 2$ receptors might have dropped by keeping the cells in culture (Putz *et al.*, 2008). Alternatively, relatively more $\alpha 4\beta 2$ receptors compared with $\alpha 3\beta 2$ 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 $\alpha 4$ -containing nAChR that specifically bind [^3H]-epibatidine. The $\alpha 4$ subunit appears to assemble exclusively with $\beta 2$, since $\alpha 4$ -containing nAChR are lost entirely in the $\alpha 5\beta 2$ 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 $\alpha 3\beta 4$; 17 % are of the $\alpha 3\beta 2\beta 4$ type, and 21 % are $\alpha 4\beta 2$ receptors (Fig. 6). We did not include anti- $\alpha 5$ antibodies in our current IP experiments and thus have no information on the frequency of $\alpha 3\beta 4$ and $\alpha 4\beta 2$ receptors that contain the $\alpha 5$ subunit. About 24 % of $\alpha 3\beta 4$ receptors in the SCG of P19 WT mice have the $\alpha 5$ subunit (David *et al.*, 2010).

By postnatal day 9, and in parallel to the decline of mRNA (Putz *et al.*, 2008), the number of $\alpha 4$ -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 $\alpha 4$ -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 focussed upon these processes (Rubin, 1985).

Nicotinic signalling by $\alpha 7$ nAChR in the mouse hippocampus and by both $\alpha 7$ and non- $\alpha 7$ 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 signalling by $\beta 2$ -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 $\alpha 3$ subunit slightly changes the spatiotemporal characteristics of the spontaneous retinal activity only (without abolishing it), and since the $\alpha 3\beta 2$ - and $\alpha 6\beta 2$ -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 signalling plays a key role in the tuning of neuronal circuits during development (Penn *et al.*, 1998; Hanson and Landmesser, 2004; Myers *et al.*, 2005; Poorthuis *et al.*, 2009). It is thus conceivable that cholinergic nicotinic transmission by $\alpha 4$ -containing receptors play a role in the maturation of synaptic contacts in the SCG early in development. $\alpha 3\beta 4$ receptors are likely also present in the embryonic SCG (Zoli *et al.*, 1995), but ACh is about four times more potent in activating $\alpha 4\beta 2$ compared to $\alpha 3\beta 4$ (Chavez-Noriega *et al.*, 1997; Fenster *et al.*, 1997), meaning that much lower concentrations of the transmitter are required for $\alpha 4\beta 2$ activation.

Due to the robust transganglionic transmission in the sympathetic nervous system (McLachlan, 2009), rather subtle changes that may be caused by the absence of $\alpha 4$ may, however, pass without readily noticeably affecting its function. Hence, even in the $\alpha 5\beta 4$ 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).

$\alpha 4$ -containing receptors may, on the other hand, play a role in mice with deletions of the $\alpha 3$ 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 $\alpha 4$ -containing receptors will be lost by this time, these receptors may retain some residual synaptic activity that keeps the majority of inbred $\alpha 3$ KO mice alive for a few days (Xu *et al.*, 1999; Rassadi *et al.*, 2005).

In conclusion, our work has shown the presence of $\alpha 4$ -containing receptors in the mouse SCG until early after birth. In WT mice, about 20 % of nAChRs have the $\alpha 4$ subunit, versus 80 % of receptors that contain $\alpha 3$. The subunits $\alpha 3$ and $\alpha 4$ do not occur in one and the same receptor, and $\alpha 4$ co-assembles with just $\beta 2$ but not with $\beta 4$. $\alpha 4\beta 2$ receptors are absent in adult animals and thus play no role in normal trans-synaptic transmission of the SCG.

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Figure 1. $\alpha 4$ -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 nM [^{3} H]-epibatidine, and immunoprecipitated with our anti- $\alpha 4$ 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 $\pm$ SEM of 3-6 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- $\alpha 4$ antibodies is documented in David et al., 2010 (for mouse nAChRs) and in the supplementary material (for rat nAChRs).

Figure 2. The nAChR subunit $\alpha 4$ is present in short-term SCG cell cultures from P3 mice
 Indirect immunofluorescence of actin (A1-A3) and the $\alpha 4$ (B1-B2) and $\alpha 3$ (B3) nAChR subunits as detected by mouse anti-actin (1:1000), rabbit anti- $\alpha 4$, and rabbit anti- $\alpha 3$ (each 0.4 $\mu\text{g}/\text{ml}$).

Since the experiments were designed to parallel the patch clamp recordings shown in Fig. 5, cultures were prepared from $\alpha 5\alpha 7\beta 4$ -triple KO mice.

A1 and B1: Cell culture 1 day *in vitro* prepared from P3 $\alpha 5\alpha 7\beta 4$ -triple KO mice.

A2, A3, B2, and B3: Cell cultures 6 days *in vitro* prepared from P6 $\alpha 5\alpha 7\beta 4$ -triple KO mice.

Note that the intensity of the fluorescent signal due to $\alpha 4$ is greatly attenuated in cells taken

from P6 animals and cultured for 6 days (B2). Panel B3 shows the fluorescent signal due to $\alpha 3$ for comparison. Calibration: 25 μm .

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 nM [^{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 μ M nicotine and subtracted from overall to obtain the specific binding shown in the figure. Data are the mean $\pm$ SEM of 3-5 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- β 2 and anti- β 4 antibodies. Though comparisons don't reach levels of significance, the results suggest that separate α 3- (82 %) and α 4-containing receptors (19 %) make up for 100 % of the receptors. Likewise, α 4-containing receptors seem to be less frequent than β 2-containing receptors, whereas the combined use of anti- α 4 and anti- β 2 antibodies does not precipitate more receptors than the single use of anti- β 2 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 β 2 + β 4 with $P < 0.01$ and $P < 0.001$, respectively).

Figure 4. β subunits markedly affect the expression of both $\alpha 3$ - and $\alpha 4$ -containing receptors nAChRs from SCG membranes of WT (black bars), $\alpha 5\beta 2$ (white bars), and $\alpha 5\beta 4$ (grey bars) KO mice were solubilized, labeled with 1 nM [^3H]-epibatidine, and immunoprecipitated with either anti- $\alpha 3$ or anti- $\alpha 4$ 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- $\beta 2$ + anti- $\beta 4$ (for WT), anti- $\beta 2$ (for $\alpha 5\beta 4$ KO), and anti- $\beta 4$ (for $\alpha 5\beta 2$ KO) antibodies, respectively (dashed line, 100 %) $\pm$ SEM of 3-6 independent experiments, each performed with duplicate measurements.

Note that $\alpha 4$ is lost (n.s.: not significantly different from zero, $P > 0.05$, one sample Students t -test) in the SCG of $\alpha 5\beta 2$ KO and significantly upregulated in $\alpha 5\beta 4$ KO mice. $\alpha 3$ -containing receptors are, on the other hand, significantly reduced in $\alpha 5\beta 4$ KO mice. Statistical significance between data points was calculated by one-way ANOVA, followed by Bonferroni's *post-hoc* multiple comparison test (n.s.: $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Fig. 5. Few $\alpha 4$ -containing nAChR reach the cell surface

A. Patch clamp recordings (perforated patch) from cultured postnatal day 3 SCG neurons ($\alpha 5 \beta 4 \alpha 7$ -triple KO), kept for 2 days in culture. Agonists and α -conotoxin MII were always applied in the indicated order: (1) 100 μ M nicotine, the 2 sec application indicated by bar. (2) 300 μ M RJR 2403, the 2 sec application indicated by bar. (3) 100 μ M nicotine after 10 sec of application of 100 nM α -conotoxin MII. (4) 100 μ M nicotine after 130 sec of application of 100 nM α -conotoxin MII. Calibration bars: 100 pA; 1 sec.

B. Pooled observations from 38 cells. Data are mean peak currents $\pm$ 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 $\alpha 5 \beta 4 \alpha 7$ -triple KO (filled bars; $n = 32$) and $\alpha 5 \beta 4$ -double KO mice (open bars; $n = 6$). Peak amplitudes by indicated protocols relative to 100 μ M nicotine did not differ between the two genotypes ($P > 0.05$, Student's t -test).

Fig. 6. Proposed nAChR subtypes occurring in the SCG of P3 WT, $\alpha 5\beta 2$ -, and $\alpha 5\beta 4$ KO mice
 $\alpha 5\beta 2$ -double KO (white bars) mice also lack the $\alpha 4$ subunit (see Fig. 4), which leaves only $\alpha 3\beta 4$ hetero-oligomeric receptors in this genotype. Compared to WT mice, the overall number of receptors remains unaffected by the KO.
 $\alpha 5\beta 4$ -double KO (grey bars) mice contain equal numbers of $\alpha 3$ - and $\alpha 4$ -containing receptors (see Fig. 4). In the absence of $\beta 4$, the two subunits share the $\beta 2$ subunit at about identical rates of recurrence (Fig. 4). Compared to WT mice, the overall number of receptors is significantly reduced in the KO.
WT (black bars): About 80 % of receptors contain the subunit $\alpha 3$, *versus* 20 % of receptors with $\alpha 4$ (Fig. 3). $\beta 2$ subunits (which occur in about 40 % of the receptors, Fig. 3) that do not co-assemble with $\alpha 4$ (about 50 % according to experiments shown in Fig. 2) assemble with $\alpha 3$. The model infers from our observations in P19 WT animals (David *et al.*, 2010) that $\alpha 3\beta 2$ receptors always contain the $\beta 4$ subunit, whereas $\beta 2$ and $\alpha 5$ never occur in the same receptor. It also infers that $\alpha 4$ only co-assembles with $\beta 2$ but not with $\beta 4$ (Fig. 4). We did not probe $\alpha 5$ -containing receptors which may concur with $\alpha 3$ and $\beta 4$ as in P19 animals (David *et al.*, 2010). The $\alpha 5$ subunit could, in principle, also co-assemble with $\alpha 4\beta 2$ receptors (Gotti *et al.*, 2006). According to this model, 62 % of receptors in P3 WT animals consist of $\alpha 3\beta 4$ with and without $\alpha 5$, 17 % are of the $\alpha 3\beta 2\beta 4$ type, and 21 % are $\alpha 4\beta 2$ receptors with or without $\alpha 5$.