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Developmental changes in the expression of nicotinic acetylcholine receptor α-subunits in the rat heart

Abstract Neuronal nicotinic acetylcholine receptors
(nAChR) are ligand-gated ion channels that consist of
various subunits. During ontogeny, muscular and neuronal
nAChR undergo changes in the distribution and subunit
composition in skeletal muscle and brain, respectively.
Here, we have investigated the occurrence of the ligand-
binding α-subunits of neuronal nAChR by means of
reverse transcription/polymerase chain reaction and im-
munohistochemistry in the rat heart during prenatal and
postnatal development and after capsaicin-induced sensory
denervation. mRNAs coding for the α4, α5, α7 and α10
subunits were detected throughout all developmental
stages. Messenger coding for the α2 subunit was first
detectable at developmental stage E20; α3 subunit mRNA
was expressed throughout all prenatal developmental
stages, whereas it was restricted postnatally to the atria.
mRNA for α6 was observed at E14-P8 but was absent
thereafter. At no developmental stage could an unequi-
vocal signal for α9 nAChR subunit mRNA be obtained.
The expression pattern was unchanged by capsaicin
treatment. Immunohistochemistry demonstrated α7 sub-
units on cardiac neurons, fibroblasts and cardiomyocytes
and α2/4 subunits on cardiomyocytes with a postnatal
redistribution to intercalated discs, as shown by cryo-
immunoelectron microscopy. Our results indicate an
additional non-neuronal expression of nAChR subunits
in the rat heart that, as in skeletal muscle, precedes
functional innervation and then undergoes changes in its
distribution on the surface of cells.

Keywords Cardiomyocyte · Development · Heart ·
Nicotinic acetylcholine receptor · Rat (Wistar)

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Introduction

Nicotinic acetylcholine receptors (nAChR) are ligand-
gated ion channels with a pentameric structure and a
central pore with a cation gate. They consist of various
subunits. To date, 17 nAChR subunit genes that encode
both neuronal and skeletal muscle nAChRs have been
cloned from vertebrates (Sargent 1993; Lukas et al. 1999;
Elgohyen et al. 2001). In skeletal muscle, this receptor is
classified into two types, a junctional type consisting of
four different subunits, i.e. α1, β1, δ and ε, in adult
muscle, and an extrajunctional type in embryonic and
adult denervated muscle, in which an γ subunit replaces
the ε subunit (Mishina et al. 1986; Brehm and Henderson
1988; Karlin 1993; Duclert and Changeux 1995). By
contrast, the neuronal types of nAChR consist of different
arrangements of α2–α10 and β2–β4 subunits (Lukas et al.
1999; Elgohyen et al. 2001), among which the α8 subunit
has been described only in chick (Schoepfer at al. 1990).
These receptors have been detected in neurons of the
central and peripheral nervous system and, additionally, in
several non-neuronal cell types including skeletal muscle
fibres (Chini et al. 1992; Sargent 1993; Corriveau et al.
1995; Grando et al. 1995; Elgohyen et al. 2001;
Brüggmann et al. 2003). During ontogeny, nAChRs as a
population undergo changes in their distribution, subunit
composition, and function in the nervous system (Mar-
giotta and Gurantz 1989; Zoli et al. 1995; Winzer-Serhan
and Leslie 1997; Charpantier et al. 1999) and in skeletal
muscle (Corriveau et al. 1995; Brehm and Henderson
1988; Fischer et al. 1999).

To date, limited data are available concerning the
expression of these genes in the various cell types of rat

heart. Cultured rat intracardiac neurons express $\alpha2$–$\alpha9$ and $\beta2$–$\beta4$ subunits (Poth et al. 1997) and electrophysiological and pharmacological data indicate that functional $\alpha7$ nAChRs also operate in these neurons in situ (Cuevas and Berg 1998; Ji et al. 2002). In the present study, we have investigated the expression of nAChR α-subunits in rat heart during prenatal and postnatal development by reverse transcription/polymerase chain reaction (RT-PCR) and immunohistochemistry. Our results indicate an additional non-neuronal expression of nAChR subunits, with particular expression of subunits $\alpha2$, $\alpha4$ and $\alpha7$ in ventricles; immunohistochemistry has revealed their occurrence on cardiomyocytes.

The distribution of nAChRs in skeletal muscle development undergoes changes that are linked with innervation (Frank and Fischbach 1979) and that have been suggested to be at least partially governed by agrin, an extracellular matrix protein, and calcitonin gene-related peptide (CGRP; Fontaine et al. 1987; Nitkin et al. 1987; Daniels 1997). Moreover, CGRP-receptors are present on cardiomyocytes (Dvorakova et al. 2003). We have therefore asked whether similar mechanisms may also operate in the heart. To this end, we have treated animals with capsaicin to deplete hearts of sensory nerve fibres that are the only known source of agrin and CGRP in the rat heart (Stone and Nikolics 1995; Wharton et al. 1986).

Materials and methods

Animals

All animals used in this study were cared for in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. Timed pregnant Wistar rats, with the day of mating defined as embryonic day 0 (E0), were killed by chloroform inhalation and the embryos removed at E14, E16, E18 and E20. The hearts from the embryos were frozen in liquid nitrogen (for RNA isolation) and in isopentane (for immunofluorescence). Rats at postnatal day (P) 1, P3, P8 and P30 and adult rats (body weight higher than 250 g) were killed by chloroform inhalation and their hearts were excised. Atria and ventricles were processed separately and frozen in liquid nitrogen. Seven to nine animals were used for each developmental stage. For cryo-immunoelectron microscopy, three adult rats were killed by inhalation of halothane and transcardially perfused with rinsing solution (Forssmann et al. 1977) followed by 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M phosphated buffer, pH 7.4. Segments of heart ventricle were dissected, incubated for 4 h in fixative, washed in 0.1 M sodium phosphate buffer and cryoprotected in 1.9 M sucrose in 0.1 M phosphate buffer for 16 h and in 2.3 M sucrose for 24–48 h. Specimens were frozen in liquid nitrogen.

Additionally, rats were treated with capsaicin (Sigma, Deisenhofen, Germany) in two doses at P1 and P2 (total dose: 100 mg/kg) and controls were injected with the vehicle (10% ethanol and 10% Tween 80 diluted in physiological saline solution (0.9% NaCl; all reagents Sigma) alone (Nagy et al. 1983). Rats at P3, P8 and P10 and adult rats (body weight >250 g) were killed by decapitation and their hearts were quickly removed. Atria and ventricles were separated and the ventricles were treated as described above. Eight animals were investigated at each time point.

RT-PCR procedure

Total RNA was isolated from four to five hearts per developmental stage by using RNazol (WAK-Chemie, Bad-Homburg, Germany) following the protocol of the

Table 1 Characteristics of primer pairs used for RT-PCR detection of nAChR α-subunits (*for* forward, *rev* reverse). A primer pair specific for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as an efficiency control for RNA isolation

Amplicon	Range		Primer sequence	Product length	Genebank accession number
$\alpha2$ nAChR	1,710–1,864 bp	for	cttcggtgaaggaagactgg	155 bp	L 10077
		rev	ggagccaacatgaaggacat		
$\alpha3$ nAChR	241–396 bp	for	cgcctgttccagtacctgtt	156 bp	X 03440
		rev	cttcagccacacaggttggttt		
$\alpha4$ nAChR	409–606 bp	for	gactttgcagtcacccacct	198 bp	L 31620
		rev	cggctatgcatgctcactaa		
$\alpha5$ nAChR	1,686–1,832 bp	for	accctaccaatttgcaacca	147 bp	NM 017078
		rev	gacccaaagacccattctga		
$\alpha6$ nAChR	1,276–1,418 bp	for	tggtgttaaggaccccaaaa	143 bp	L 08227
		rev	gctgctggcttaacctcttg		
$\alpha7$ nAChR	443–641 bp	for	atctgggcattgccagtatc	199 bp	S 53987
		rev	tcccatgagatcccatcctc		
$\alpha9$ nAChR	365–506 bp	for	cgtgggatcgagaccagtat	142 bp	U 12336
		rev	tcatatcgcagcaccacatt		
$\alpha10$ nAChR	1,561–1,728 bp	for	tctgacctcacaacccacaa	168 bp	AF 196344
		rev	tcctgtctcagcctccatgt		
GAPDH	165–464	for	cgtcttcaccaccatggaga	300 bp	XM 213991.2
		rev	cggccatcacgccacagctt		

manufacturer. Total RNA was then treated with DNase I (Life Technologies, Karlsruhe, Germany) in the presence of 20 mM TRIS–HCl (pH 8.4), 2 mM $MgCl_2$ and 50 mM KCl for 15 min at 25°C. Single-stranded cDNA was synthesised from 1 μg total RNA in 20 μl superscript synthesis buffer containing 3 mM $MgCl_2$, 75 mM KCl, 50 mM TRIS–HCl (pH 8.3), 10 mM dithiothreitol, 2.5 mM each of dNTP (Life Technologies), 0.5 μg oligo(dT) primer (MWG Biotech, Ebersberg, Germany) and 200 U of Superscript RNase H⁻ reverse transcriptase (Life Technologies) for 50 min at 42°C. PCR amplification was conducted in 25 μl PCR buffer II containing 1 μl or 2 μl cDNA, 2 μl $MgCl_2$, 0.625 μl dNTPs (6.25 mM each), 0.25 μl AmpliTaq Gold polymerase (all reagents from Applied Biosystems, Überlingen, Germany) and 0.625 μl each primer (12.5 pM). The primers were designed to amplify the sequence corresponding to the published rat cDNA sequence for the α2–α7, α9 and α10 nAChR subunits (Table 1). The efficiency of RNA isolation and PCR was tested with primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; (Table 1). RNA isolated from rat brain was used as a positive control. Reactions without template were included as negative controls. The PCR amplification products were separated by 2.0% agarose gel electrophoresis.

Immunofluorescence

Three to four hearts per developmental stage were cut on a cryostat (Jung Frigocut 1900E, Leica, Bensheim, Germany). Sections (8 μm thick) were placed onto gelatinised slides, fixed for 10 min in acetone and air-dried. Sections were covered for 1 h with phosphate-buffered saline (PBS) containing 10% normal porcine serum, 0.1% bovine serum albumin and 0.5% Tween 20 to saturate unspecific binding sites. Immunofluorescence was performed with a goat antiserum raised against the α2/4 nAChR subunit (1:20, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and a mouse monoclonal antibody against the α7 nAChR subunit (1:400, clone mAb 306, Sigma-Aldrich, Taufkirchen, Germany). Primary antibodies were applied overnight at 4°C, followed by washing steps (2×10 min in PBS) and a subsequent 1-h incubation with fluorescein isothiocyanate (FITC)-labelled donkey anti-mouse-IgG (1:80, Dianova, Hamburg, Germany) or mouse anti-goat-IgG conjugated to FITC (1:80, Sigma). Thereafter, slides were washed in PBS and coverslipped in carbonate-buffered glycerol at pH 8.6. To establish the specificity of α2/4 nAChR immunolabelling, the antibody was pre-absorbed overnight with its corresponding antigen (20 μg/ml diluted serum; antigen from Santa Cruz Biotechnology) prior to immunolabelling. Application of non-related isotype (IgG_1)-matched mouse immunoglobulin served as a specificity control for the mouse monoclonal α7 subunit antibody. Immunolabelling was evaluated on an epifluorescence microscope (BX 60F, Olympus, Hamburg, Germany) equipped with appropriate filter combinations.

Cryo-electron microscopy

Ultrathin cryosections were cut at 100 nm (Reichert Ultracut S equipped with a Reichert FC R cryo chamber; Leica, Mannheim, Germany) and transferred to 100-mesh nickel grids (Plano, Wetzlar, Germany). Sections were washed (4×5 min) in 0.05 M PBS and then incubated for 20 min with 100% normal porcine serum and for 90 min with goat-anti-α2/4 antibody (1:20, Santa Cruz Biotechnology). After being washed in PBS, sections were incubated with colloidal gold-conjugated (5 nm, Sigma) rabbit anti-goat-Ig in 10% normal porcine serum and washed again in PBS. The sections were fixed in 2.5% glutaraldehyde in 0.1 M PBS for 5 min, counterstained, embedded with 0.3% uranyl acetate in 2% methylcellulose (10 min at 4°C) and viewed with a EM 902 transmission electron microscope (Zeiss, Jena, Germany). The specificity of the secondary antiserum was validated by incubation without the primary antiserum.

Results

RT-PCR procedure

Nicotinic AChR α-subunit expression was studied in the rat heart during prenatal and postnatal development. Additionally, postnatal stages from rats neonatally treated with capsaicin to cause sensory denervation were investigated. The developmental expression profile of neuronal α-subunits of nAChRs in the rat heart is presented in Fig. 1. Messenger RNA coding for the α2 nAChR subunit was first detectable at developmental stage E20; α3 nAChR subunit mRNA was present in the hearts of all prenatal developmental stages investigated, whereas postnatally it was demonstrated solely in the atria. The mRNAs coding for α4, α5, α7 and α10 nAChR subunits were detected throughout all developmental stages. The mRNA for α6 nAChR was observed at developmental stages E14-P3. At P8, α6 nAChR subunit mRNA was still detectable in atria but not in ventricles and was absent in atria and ventricles of developmental stage P30 and older. At no developmental stages could an unequivocal signal for α9 nAChR subunit mRNA be obtained. No obvious difference in α-subunit nAChR composition was found between hearts from vehicle-treated animals and those treated with capsaicin to induce sensory denervation (Fig. 2).

Immunohistochemistry

In view of the prominent expression of subunits α2, α4 and α7 in ventricles that lacked neuronal cell bodies, antisera recognising these subunits were employed for immunohistochemistry to unravel their tissue distribution.
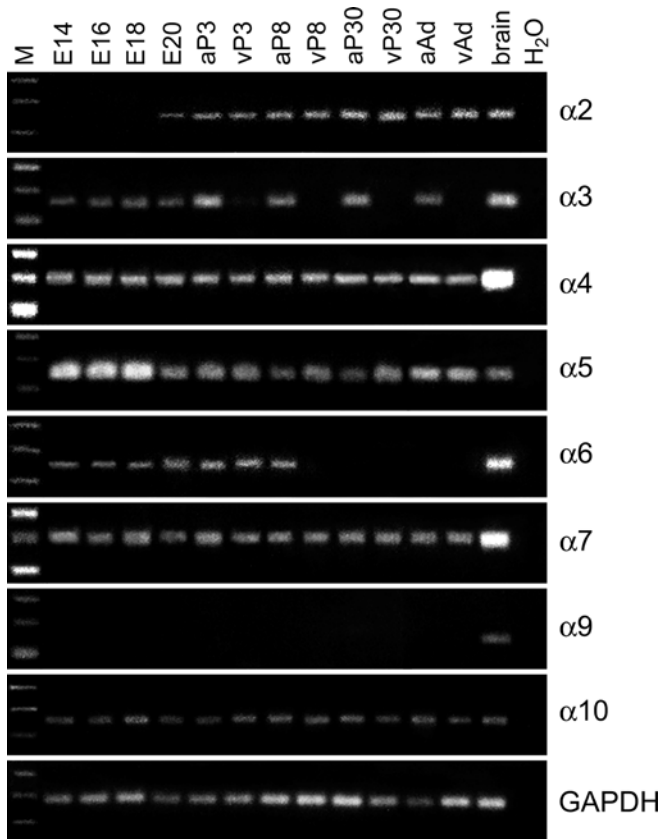
Fig. 1 RT-PCR products on an ethidium-bromide-stained gel; developmental expression of nAChR subunit mRNAs. mRNA for the α2 nAChR subunit is detectable from embryonic (E) day 20, whereas mRNA for the α3 nAChR subunit is detected in RNA isolated from entire hearts at all prenatal stages and, postnatally, in samples from the atria (*a*) but not from the ventricles (*v*). mRNAs for α4, α5, α7 and α10 are expressed throughout all the developmental stages. The α6 subunit is present from E14 up to postnatal day (P) 8 but is absent thereafter. At no developmental stages was a signal for α9 nAChR subunit mRNA obtained. RNA isolated from rat brain served as a positive control. H_2O was used as a negative control in which the template was replaced by water. *M* Marker (100, 200, 300 bp for nAChR subunits; 200, 300, 400 bp for GAPDH)

Fig. 2 RT-PCR products on an ethidium-bromide-stained gel; postnatal expression of nAChR subunit mRNAs in ventricles of capsaicin-treated versus vehicle-treated animals. Neither vehicle nor capsaicin treatment altered the normal postnatal expression pattern (cf. Fig. 1). RNA isolated from rat brain served as a positive control. H_2O was used as a negative control, template being replaced by water. *M* Marker (100, 200, 300 bp for nAChR subunits; 200, 300, 400 bp for GAPDH)

Immunoreactivity for the α2/4 nAChR subunit

Cardiomyocytes displayed immunoreactivity at all developmental stages with no obvious differences between the individual chambers of the heart (Fig. 3). In the prenatal and early postnatal stages (E14-P8), α2/4-nAChR-immunoreactive spots formed clusters arranged in cross-striations (Fig. 3e). These clusters were less prominent in later postnatal stages (P30, adult rat) in which immunolabelling was redistributed to the intercalated discs (Fig. 3f, g). This localisation was confirmed at the electron-microscopical level (Fig. 4). In addition to labelling of cardiomyocytes, α2/4 nAChR staining of smooth muscle cells of the coronary arteries was observed (Fig. 3f, g), whereas, consistent with a previous report (Brüggmann et al. 2003), smooth muscle cells of large elastic arteries (aorta, truncus pulmonalis) remained unlabelled (data not
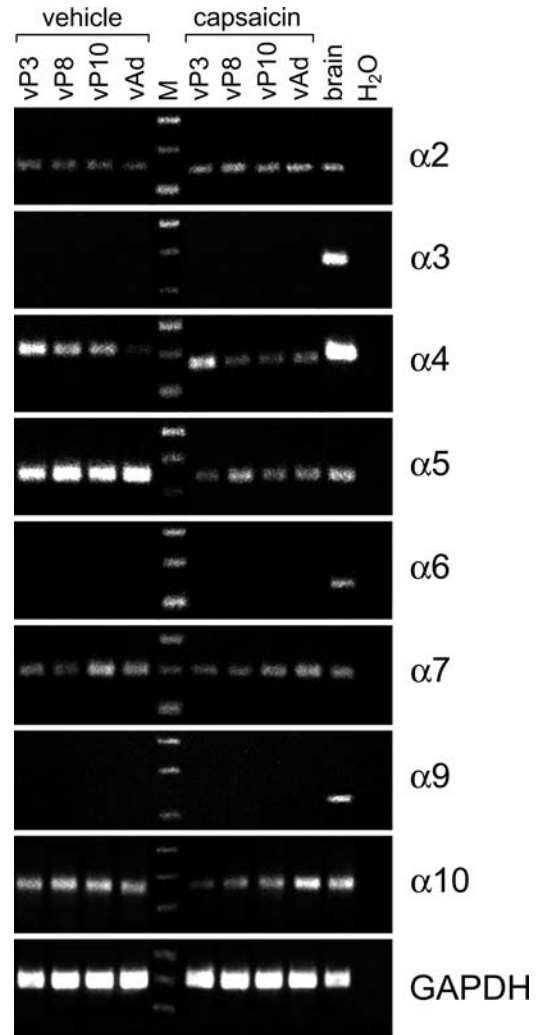
shown). No specific labelling of cardiac ganglionic neurons and nerve fibre bundles was observed (Fig. 5a).

Differences in the tissue distribution of immunoreactivity for the α2/4 nAChR subunit after capsaicin treatment were not noticed (data not shown).

Immunoreactivity for the α7 nAChR subunit

Cardiomyocytes exhibited immunoreactivity for subunit α7 at all developmental stages, although an age-related decline in the intensity of the immunostaining was noted postnatally (Fig. 6). Smooth muscle cells of coronary arteries also showed immunolabelling (Fig. 6f) as did cardiac ganglionic neurons (Fig. 5b). Throughout the

Fig. 3 Immunohistochemistry for the detection of the α2/4 nAChR subunit in developing rat heart. Strong immunoreactivity of cardiomyocytes at E14 (**a**); this is abolished by preabsorption with the corresponding peptide (**b**). The receptor clusters on the cardiomyocyte surface at E14 (**c**) and E18 (**d**) are arranged in cross-striations at P3 (**e**) and are observed at intercalated discs (*arrows*) at P30 (**f**) and in adult rat (**g**). Smooth muscle cells (*arrowheads*) of coronary arteries are labelled at P30 and thereafter (**f**, **g**). *Bars* 50 μm (**a**, **b**), 20 μm (**d–g**)
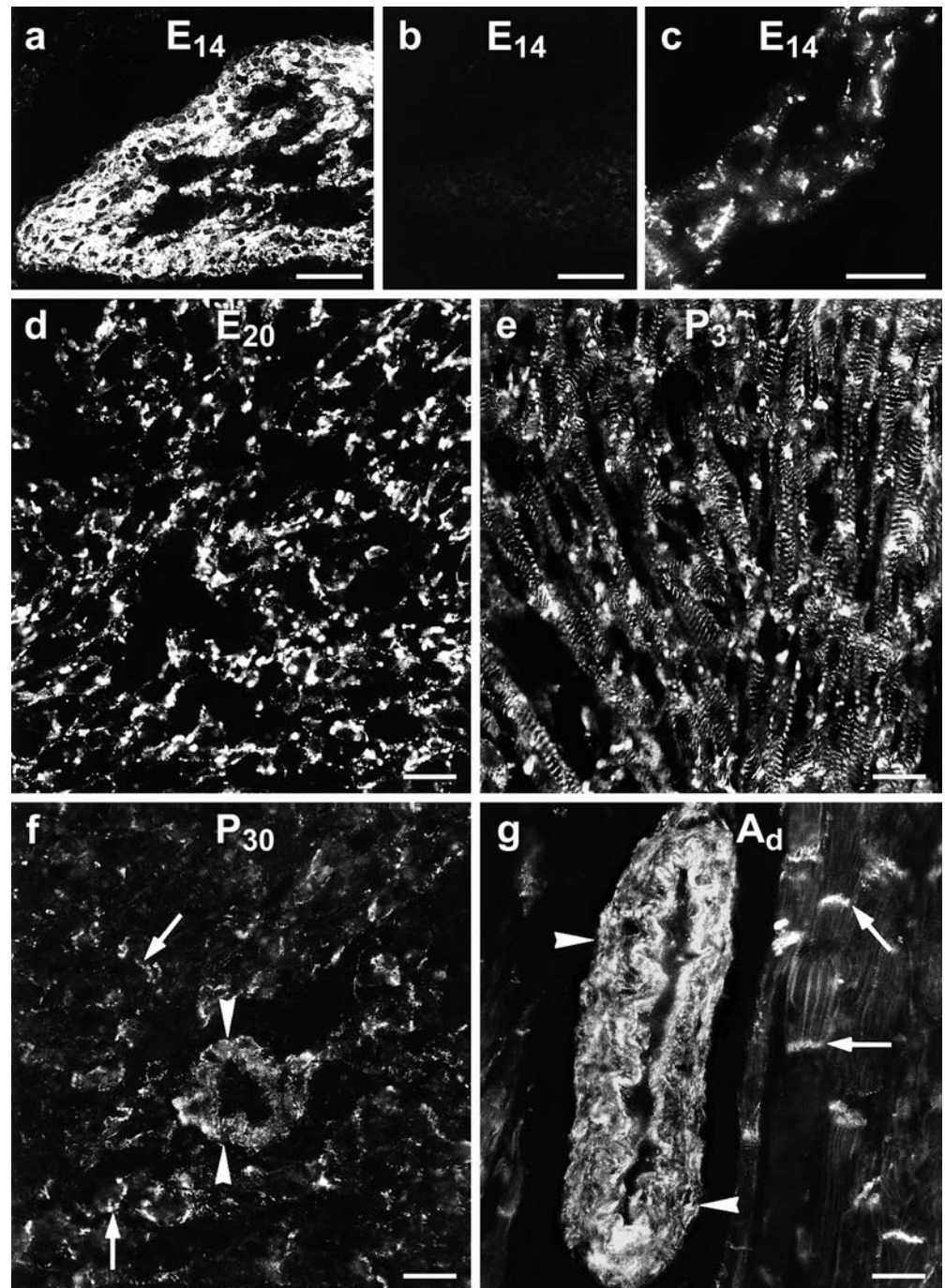
heart, additional cells were labelled that probably represented fibroblasts (Fig. 6c).

Differences in the tissue distribution of immunoreactivity for the α7 nAChR subunit after capsaicin treatment were not noticed (data not shown).

Discussion

The present study establishes the expression of multiple nAChR subunits in various tissue components of the developing rat heart. Previous studies have focused on the parasympathetic cardiac ganglia in which fast synaptic transmission is mediated via acetylcholine and nAChRs, demonstrating the α-subunits 2, 3, 4, 5 and 7 by means of RT-PCR with respect to neurons cultured from these ganglia of 2- to 7-day-old rats (Poth et al. 1997) and immunoreactivity to α-subunit 3 and 7 in canine cardiac neurons (Bibevski et al. 2000). These data and electrophysiological findings have revealed the heterogeneous expression pattern of nAChR subunits among individual neurons (Poth et al. 1997; Bibevski et al. 2000). Similarly, we have not observed α2/4 immunoreactivity in nerve cell bodies, whereas each neuron is immunolabelled by the α7

Fig. 4 Immunohistochemical detection of $\alpha2/4$ nAChR subunit protein at the intercalated discs of cardiomyocytes by means of cryo-electron microscopy (*arrows* undulating course of the cardiomyocyte membrane at unlabelled stretches). **a** Colloidal gold particles indicate sites of immunoreactivity. **b** Specificity of the detection system is demonstrated by the absence of colloidal gold labelling in the absence of primary antibody (*M* mitochondrion). *Bar* 100 nm
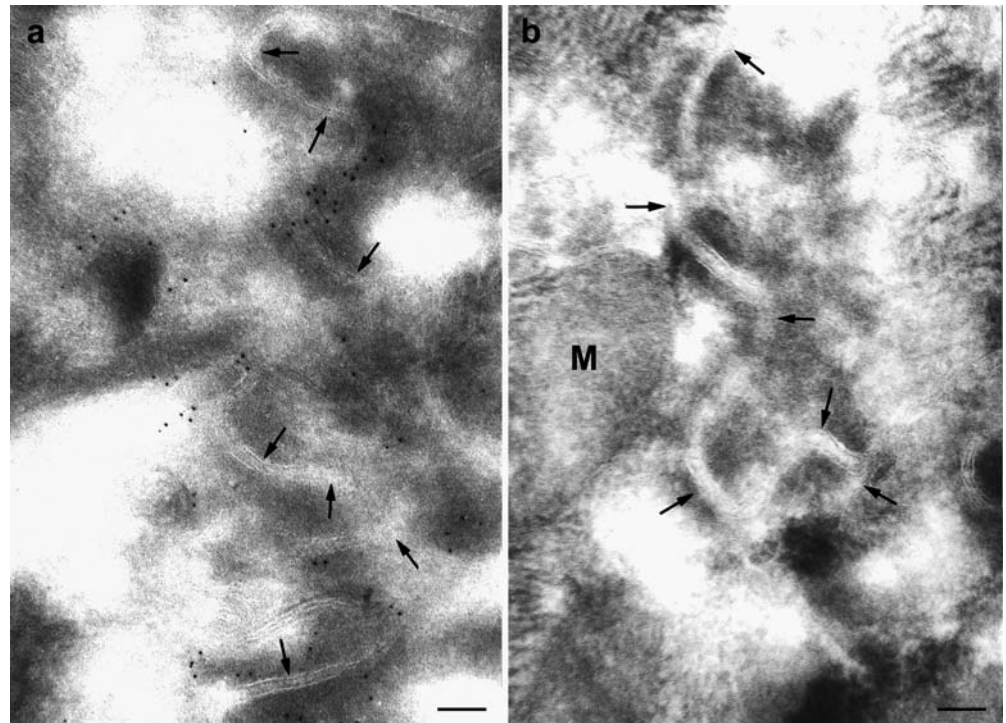
antibody. The main new aspect of the present study is that, in addition to these ganglionic receptors, multiple nAChR α-subunits also occur on non-neuronal cells of the heart. At all postnatal stages, the atria and ventricles have been separately processed for RT-PCR and, from this time point on, subunit $\alpha3$ has been detected in atria only, while subunits $\alpha2$, $\alpha4$, $\alpha5$ and $\alpha7$ appear to be expressed both in atria and in ventricles. This suggests an exclusive expression of $\alpha3$ in cardiac neurons, which are overwhelmingly located in the atria of the rat heart (Pardini et al. 1987; Richardson et al. 2003), and both expression studies and electrophysiological data have pointed to a predominant role of $\alpha3$-containing nAChRs in ganglionic transmission (Poth et al. 1997; Bibevski et al. 2000). Moreover, $\alpha6$ mRNA has been detected only in atria at P8. This subunit forms heteropentamers together with $\alpha3$-subunits and $\beta2$-subunits (Gerzanich et al. 1997; Fucile et al. 1998; Kuryatov et al. 2000) and, thus, may be an assembly partner of $\alpha3$ in prenatal and early postnatal ganglia. Since

$\alpha3\alpha6\beta2$ heteropentamers exhibit a reduced potency to acetylcholine compared with $\alpha3\beta2$ heteropentamers (Fucile et al. 1998; Kuryatov et al. 2000), the cessation of $\alpha6$ expression after the first postnatal week may be the cause of the facilitation of ganglionic cholinergic transmission at this time point (Slavikova and Tucek 1982). On the other hand, the sustained ventricular presence of $\alpha2$, $\alpha4$, $\alpha5$, $\alpha7$ and $\alpha10$ indicates a non-neuronal expression of these subunits; this could be verified by immunohistochemistry, in that immunoreactivity for both the $\alpha2/4$-subunit and the $\alpha7$-subunit has been detected on vascular smooth muscle cells and cardiomyocytes. Cardiac muscle labelling is most prominent, especially at early developmental stages, and hence the following discussion will focus on nAChRs on cardiomyocytes.

Developing cardiac muscle cells express multiple nAChR α-subunits before a functional innervation is established. As early as E14, mRNAs coding for the α-subunits 3, 4, 5, 6, 7 and 10 have been detected by RT-

Fig. 5 Immunohistochemistry for $\alpha2/4$ (**a**) and $\alpha7$ (**b**) nAChR subunits in cardiac ganglia. Nerve fibres and all neuronal cell bodies are stained with the $\alpha7$ nAChR subunit antibody (**b**), whereas they are not labelled with the $\alpha2/4$ antiserum (*arrows* $\alpha2/4$-immunoreactive cardiomyocytes, *stars* unstained perikarya **a**). *Bar* 20 μm
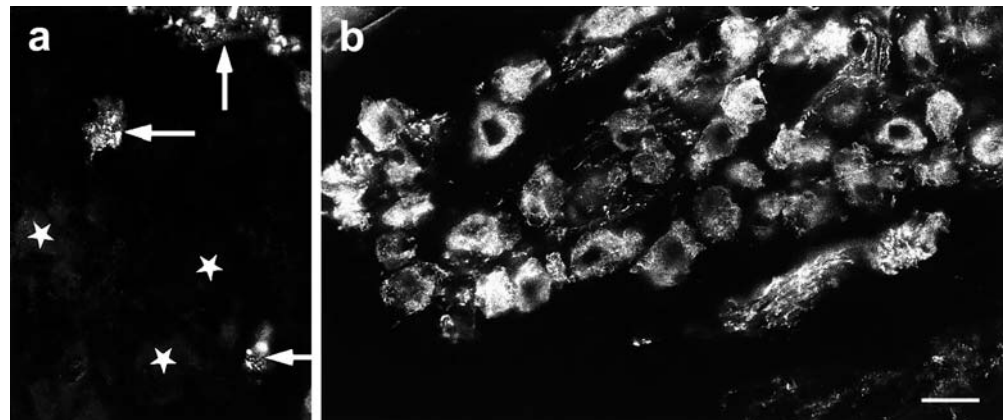
Fig. 6 Immunohistochemistry for the α7 nAChR subunit in developing rat heart. Immuno-reactive spots for α7 form clusters on the cardiomyocyte surface at E14 (**a**), E18 (**b**), P1 (**c**), P3 (**d**) and P30 (**e**). In addition to cardiomyocytes (*CM*), fibroblasts (*F*) of the fibrous ring of the heart also exhibit α7 nAChR labelling (**c**). In the adult rat (**f**), immunore-activity of cardiomyocytes is weak, whereas smooth muscle cells of a coronary artery show staining. *Bars* 20 μm
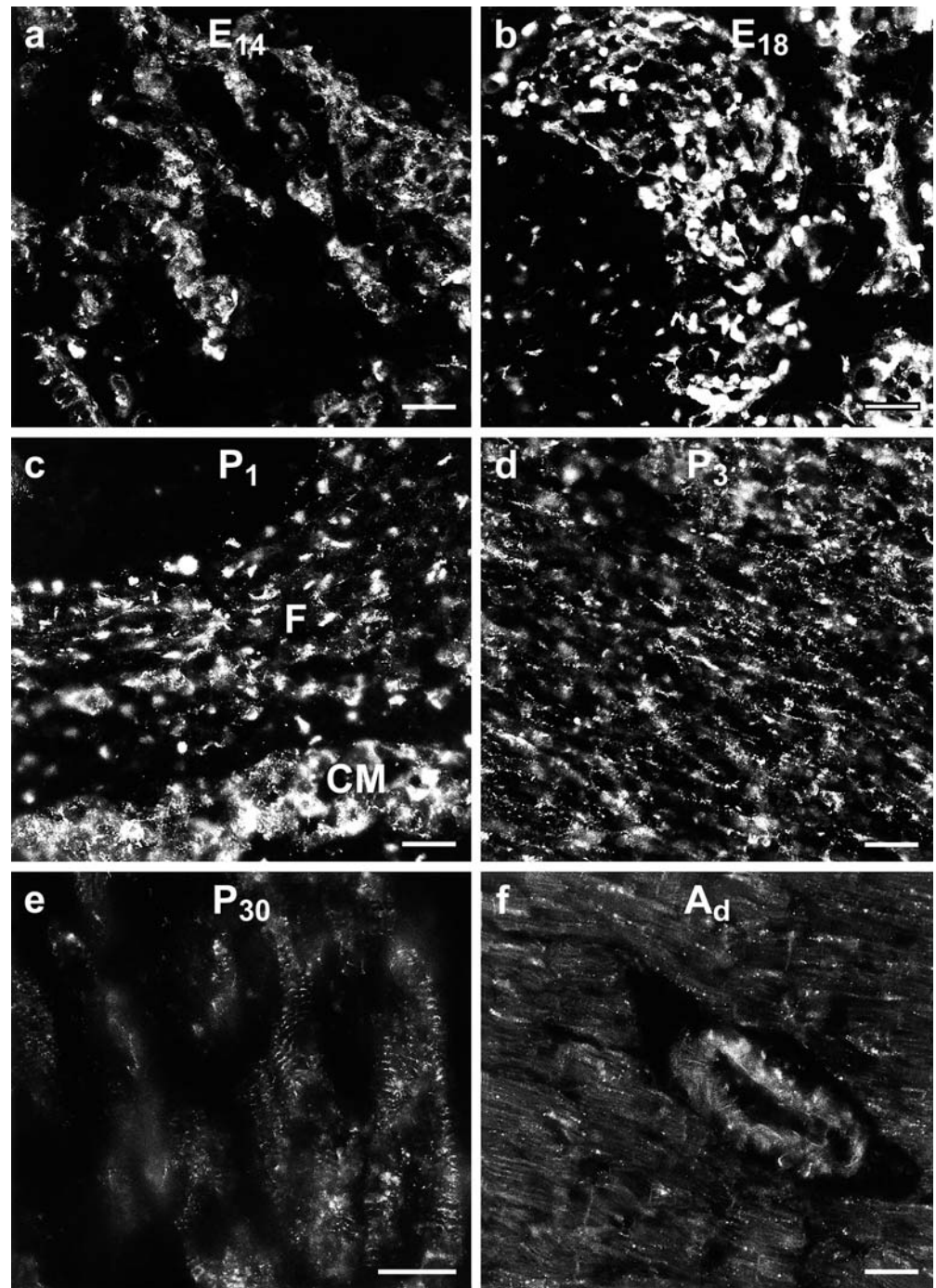
PCR and cardiomyocytes are immunoreactive for subunits α2/4 and α7. This developmental stage is the youngest age at which the first ganglia appear in the right atrial wall, whereas the first nerve endings near myocardial cells are not detected earlier than E15 by light microscopy (Gomez 1958). Immunohistochemically, cholinergic parasympa-thetic and sensory CGRP-immunoreactive nerve terminals are first detected in the myocardium at E15 and E19, respectively (Franco et al. 1997; Shoba and Tay 2000) and the first electrophysiological evidence for a functional cholinergic innervation can be found at P3 (Quigley et al. 1996). Hence, the onset of nAChR gene expression in the developing rat heart is neither triggered by cholinergic innervation nor by neurally released CGRP. In this aspect, the heart parallels skeletal muscle in which myotubes start to express nAChR subunit genes before the first neurites arrive (for a review, see Sanes and Lichtman 2001). Subsequently, however, functional innervation resulting in electrical activity and the release of agrin and CGRP increases the synthesis of nAChR subunits in skeletal muscle cells and leads to a clustering of α1-subunit containing nAChRs in the postjunctional membrane (Sanes and Lichtman 2001). At first sight, a striking resemblance can be seen to the situation found in the heart,

since we have observed a redistribution of immunoreactive α2/4-subunits at the surface of cardiomyocytes after the first postnatal week, i.e. the period in which maturation of the cholinergic innervation occurs (Quigley et al. 1996). Some notable differences are however present in the redistribution mechanisms between skeletal and cardiac muscle. (1) In skeletal muscle, a direct contact of the motor nerve terminal with the basal lamina of the myotube has to be formed to induce these changes (Sanes and Lichtman 2001), whereas this is certainly not the case in the rat heart, at least not in the ventricles, which are sparsely innervated by cholinergic fibres (Schäfer et al. 1998). (2) Induced clustering of nAChRs is strictly localised to the postjunctional membrane in skeletal muscle fibres (Sanes and Lichtman 2001), whereas cardiac α2/4-subunits are redistributed from spots and striations at the surface of the cardiomyocytes to the intercalated discs.

Furthermore, we could not find any evidence for a significant involvement of the neuropeptide CGRP in the regulation of cardiac nAChRs, in that chemical destruction of CGRP-containing nerve fibres by capsaicin application altered neither the α-subunit composition determined by RT-PCR nor the postnatal redistribution of α2/4-subunits to intercalated discs. Moreover, by destroying CGRP-containing sensory neurons, capsaicin also depletes the only known source of the heparan sulphate proteoglycan agrin in the heart (Stone and Nikolics 1995); this is essential for the maintenance of postjunctional nAChR aggregation in skeletal muscle (Gautam et al. 1996; Lin et al. 2001).

In conclusion, the non-neuronal expression of nAChR in the rat heart, like that in skeletal muscle, precedes functional innervation and then undergoes changes in its distribution on the surface of cells.

References

Bibevski S, Zhou Y, McIntosh JM, Zigmond RE, Dunlap ME (2000) Functional nicotinic acetylcholine receptors that mediate ganglionic transmission in cardiac parasympathetic neurons. J Neurosci 20:5076–5082

Brehm P, Henderson LP (1988) Regulation of acetylcholine receptor channel function during development of skeletal muscle. Dev Biol 129:1–11

Brüggmann D, Lips KS, Pfeil U, Haberberger RV, Kummer W (2003) Rat arteries contain multiple nicotinic acetylcholine receptor α-subunits. Life Sci 72:2095–2099

Charpantier E, Besnard F, Graham D, Sgard F (1999) Diminution of nicotinic receptor alpha 3 subunit mRNA expression in aged rat brain. Brain Res Dev Brain Res 118:153–158

Chini B, Clementi F, Hukovic N, Sher E (1992) Neuronal-type alpha-bungarotoxin receptors and the alpha 5-nicotinic receptor subunit gene are expressed in neuronal and nonneuronal human cell lines. Proc Natl Acad Sci USA 89:1572–1576

Corriveau RA, Romano SJ, Conroy WG, Oliva L, Berg DK (1995) Expression of neuronal acetylcholine receptor genes in vertebrate skeletal muscle during development. J Neurosci 15:1372–1383

Cuevas J, Berg D (1998) Mammalian nicotinic receptors with α7 subunits that slowly desensitize and rapidly recover from a-bungarotoxin blockade. J Neurosci 18:10335–10344

Daniels MP (1997) Intracellular communication that mediates formation of the neuromuscular junction. Mol Neurobiol 14:143–170

Duclert A, Changeux JP (1995) Acetylcholine receptor gene expression at the developing neuromuscular junction. Physiol Rev 75:339–368

Dvorakova M, Haberberger RV, Hagner S, McGregor GP, Slavikova J, Kummer W (2003) Expression and distribution of the calcitonin receptor-like receptor in the developing rat heart. Anat Embryol 207:307–315

Elgohyen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF, Boulter J (2001) Alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. Proc Natl Acad Sci USA 98:3501–3506

Fischer U, Reinhardt S, Albuquerque EX, Maelicke A (1999) Expression of functional alpha7 nicotinic acetylcholine receptor during mammalian muscle development and denervation.Eur J Neurosci 11:2856-2864

Fontaine B, Klarsfeld A, Changeux JP (1987) Calcitonin generelated peptide and muscle activity regulate acetylcholine receptor alpha-subunit mRNA levels by distinct intracellular pathways. J Cell Biol 105:1337–1342

Forssmann WG, Ito S, Weihe E, Aoki A, Dym M, Fawcett DW (1977) An improved perfusion fixation method for the testis. Anat Rec 188:307–314

Franco D, Moorman AF, Lamers WH (1997) Expression of the cholinergic signal-transduction pathway components during embryonic rat heart development. Anat Rec 248:110–120

Frank E, Fischbach GD (1979) Early events in neuromuscular junction formation in vitro: induction of acetylcholine receptor clusters in the postsynaptic membrane and morphology of newly formed synapses. J Cell Biol 83:143–158

Fucile S, Matter JM, Erkman L, Ragozzino D, Barabino B, Grassi F, Alema S, Ballivet M, Eusebi F (1998) The neuronal alpha6 subunit forms functional heteromeric acetylcholine receptors in human transfected cells. Eur J Neurosci 10:172–178

Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell 85:525–535

Gerzanich V, Kuryatov A, Anand R, Lindstrom J (1997) "Orphan" alpha6 nicotinic AChR subunit can form a functional heteromeric acetylcholine receptor. Mol Pharmacol 51:320–327

Gomez H (1958) The development of the innervation of the heart in the rat embryo. Anat Rec 130:53–71

Grando S, Horton R, Pereira E, Diethelm B, Okita P, George PM, Albuquerque EX, Conti-Fine B (1995) A nicotinic acetylcholine receptor regulating cell adhesion and motility is expressed in human keratocytes. J Invest Dermatol 105:774–781

Ji S, Tosaka T, Whitfield BH, Katchman AN, Kandil A, Knollmann BC, Ebert SN (2002) Differential rate responses to nicotine in rat heart: evidence for two classes of nicotinic receptors. J Pharmacol Exp Ther 301:893–899

Karlin A (1993) Structure of the nicotinic acetylcholine receptors. Curr Opin Neurobiol 3:299–309

Kuryatov A, Olale F, Cooper J, Choi C, Lindstrom J (2000) Human α6 AchR subtypes: subunit composition, assembly, and pharmacological responses. Neuropharmacology 39:2570–2590

Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee KF (2001) Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature 410:1057–1064

Lukas RJ, Changeux JP, Le Novere N, Albuquerque EX, Balfour DJK, Berg DK, Bertrand D, Chiappinelli VA, Clarke PBS, Collins AC, Dani JA, Grady SR, Kellar KJ, Lindstrom JM, Marks MJ, Quik M, Taylor PW, Wonnacott S (1999) International Union of Pharmacology. XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. Pharmacol Rev 51:397–401

Margiotta JF, Gurantz D (1989) Changes in the number, function, and regulation of nicotinic acetylcholine receptors during neuronal development. Dev Biol 135:326–339

Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321:406–411

Nagy JI, Iversen LL, Goedert M, Chapman D, Hunt SP (1983) Dose-dependent effects of capsaicin on primary sensory neurons in the neonatal rat. J Neurosci 3:399–406

Nitkin RM, Smith MA, Magill C, Fallon JR, Yao YM, Wallace BG, McMahan UJ (1987) Identification of agrin, a synaptic organizing protein from *Torpedo* electric organ. J Cell Biol 105:2471–2478

Pardini BJ, Patel KP, Schmid PG, Lund DD (1987) Location, distribution and projections of intracardiac ganglion cells in the rat. J Auton Nerv Syst 20:91–101

Poth K, Nutter TJ, Cuveas J, Parker MJ, Adams DJ, Luetje CW (1997) Heterogeneity of nicotinic class and subunit mRNA expression among individual parasympathetic neurons from rat intracardiac ganglia. J Neurosci 17:586–596

Richardson RJ, Grkovic I, Anderson CR (2003) Immunohistochemical analysis of intracardiac ganglia of the rat heart. Cell Tissue Res 314:337–350

Quigley KS, Shair HN, Myers MM (1996) Parasympathetic control of heart period during early postnatal development in the rat. J Auton Nerv Syst 56:75–82

Sanes JR, Lichtman JW (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2:791–805

Sargent PB (1993) The diversity of neuronal nicotinic acetylcholine receptors. Annu Rev Neurosci 16:403–443

Schäfer MK, Eiden LE, Weihe E (1998) Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter. II. The peripheral nervous system. Neuroscience 84:361–376

Schoepfer R, Conroy WG, Whiting P, Gore M, Lindstrom J (1990) Brain alpha-bungarotoxin binding protein cDNAs and Mabs reveal subtypes of this branch of the ligand-gated ion channel gene superfamily. Neuron 5:35–48

Shoba T, Tay SS (2000) Nitrergic and peptidergic innervation in the developing rat heart. Anat Embryol 201:491–500

Slavikova J, Tucek S (1982) Postnatal changes of the tonic influence of the vagus nerves on the heart rate, and of the activity of choline acetyltransferase in the heart atria of rats. Physiol Bohemoslov 31:113–120

Stone DM, Nikolics K (1995) Tissue- and age-specific expression patterns of alternatively spliced agrin mRNA transcripts in embryonic rat suggest novel developmental roles. J Neurosci 15:6767–6778

Wharton J, Gulbenkian S, Mulderry PK, Ghatei MA, McGregor GP, Bloom SR, Polak JM (1986) Capsaicin induces a depletion of calcitonin gene-related protein (CGRP)-immunoreactive nerves in the cardiovascular system of the guinea pig and rat. J Auton Nerv Syst 16:289–309

Winzer-Serhan UH, Leslie FM (1997) Codistribution of nicotinic acetylcholine receptor subunit alpha 3 and beta 4 mRNAs during rat brain development. J Comp Neurol 386:540–554

Zoli M, LeNovere N, Hill JA Jr, Changeux JP (1995) Developmental regulation of nicotinic ACh receptor subunit mRNAs in the rat central and peripheral nervous systems. J Neurosci 15:1912–1939