REPRODUCTION
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The cholinergic system in rat testis is of non-neuronal origin

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Abstract

The cholinergic system consists of acetylcholine (ACh), its synthesising enzyme, choline acetyltransferase (CHAT), transporters such as the high-affinity choline transporter (SLC5A7; also known as ChT1), vesicular ACh transporter (SLC18A3; also known as VAChT), organic cation transporters (SLC22s; also known as OCTs), the nicotinic ACh receptors (CHRN; also known as nAChR) and muscarinic ACh receptors. The cholinergic system is not restricted to neurons but plays an important role in the structure and function of non-neuronal tissues such as epithelia and the immune system. Using molecular and immunohistochemical techniques, we show in this study that non-neuronal cells in the parenchyma of rat testis express mRNAs for *Chat*, *Slc18a3*, *Slc5a7* and *Slc22a2* as well as for the CHRN subunits in locations completely lacking any form of innervation, as demonstrated by the absence of protein gene product 9.5 labelling. We found differentially expressed mRNAs for eight α and three β subunits of CHRN in testis. Expression of the α7-subunit of CHRN was widespread in spermatogonia, spermatocytes within seminiferous tubules as well as within Sertoli cells. Spermatogonia and spermatocytes also expressed the α4-subunit of CHRN. The presence of ACh in testicular parenchyma (TP), capsule and isolated germ cells could be demonstrated by HPLC. Taken together, our results reveal the presence of a non-neuronal cholinergic system in rat TP suggesting a potentially important role for non-neuronal ACh and its receptors in germ cell differentiation.

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Introduction

The cholinergic system consists of acetylcholine (ACh), its synthesising enzymes, transporters and receptors. ACh is synthesised by choline acetyltransferase (CHAT). Ongoing ACh synthesis requires the uptake of choline into cholinergic cells via a high-affinity choline transporter (SLC5A7; also known as ChT1; Okuda *et al.* 2000). In neurons, ACh is transported into synaptic vesicles via the vesicular ACh transporter (SLC18A3; also known as VAChT; Erickson *et al.* 1996, Parsons 2000) and released via exocytosis upon stimulation. In addition, ACh release can occur through bidirectional transport of ACh, utilising organic cation transporters (SLC22s; also known as OCTs) with SLC22A2 being the most likely candidate for non-neuronal transport (Wessler *et al.* 2001, Lips *et al.* 2005). ACh can stimulate five different G-protein-coupled muscarinic receptor subtypes (CHRMs, also known as mAChR; Nathanson 2008) and an unknown variety of ionotropic nicotinic ACh receptors (CHRN; also known as nAChR) with subtype-specific arrangements of nine α- and four β-subunits in mammals (Albuquerque *et al.* 2009). It is now known that the cholinergic system, traditionally associated with neurotransmission, is not restricted to neurons but plays an important role in the structure and function of non-neuronal tissues such as epithelia and the immune system (Fujii *et al.* 2008, Kummer *et al.* 2008, Wessler & Kirkpatrick 2008). For example, in epithelia, CHRNs are involved both in maintaining the integrity of the epithelial layer and in the development of neoplastic changes (Grando *et al.* 2003, Grozio *et al.* 2007, Paleari *et al.* 2009).

Several observations indicate the presence of a cholinergic system within the mammalian testis: functional ACh receptors are found on male germ cells and Sertoli cells; mice lacking CHRN subtypes or with reduced ACh levels reveal reduced sperm motility (Borges *et al.* 2001, Bray *et al.* 2005); and high nicotine levels in the blood lead to reduced sperm production and fertility (Dwivedi & Long 1989, Yamamoto *et al.* 1998). Published reports suggest that there is little, if any,

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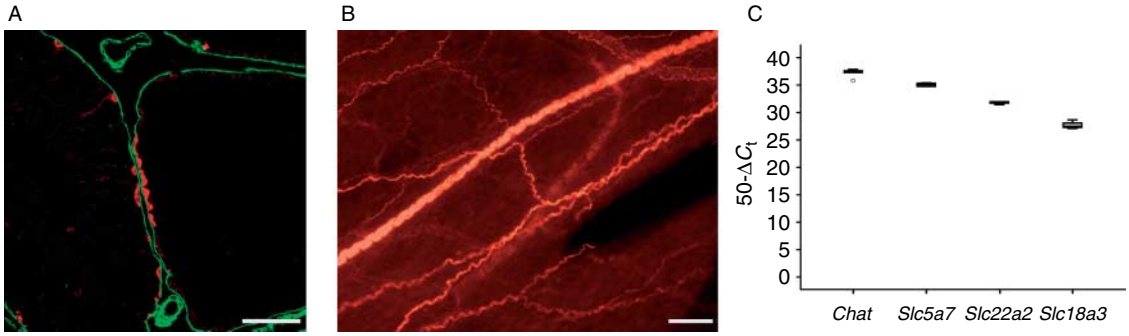

Figure 1 (A) Innervation within the testicular parenchyma (TP) was examined with an antibody against protein gene product 9.5 (PGP 9.5, red). α-smooth muscle actin antibody labels peritubular myoid cells and interstitial blood vessels (green). Nerve fibres were absent in rat TP, but PGP 9.5 appears to stain a spermatogonial subpopulation. Bar, 75 μm. (B) The mesostructures showed a dense innervation with PGP 9.5 positive nerve fibres and nerve fibre bundles. Bar, 100 μm. (C) Box plot showing the relative mRNA expression levels of choline acetyltransferase (*Chat*), high-affinity choline transporter (*Slc5a7*), vesicular ACh transporter (*Slc18a3*) and organic cation transporter 2 (*Slc22a2*) in TP. The ΔC_T values were subtracted from 50 showing higher values with higher expression.

cholinergic innervation of most testicular tissue suggesting the presence of a non-neuronal cholinergic system. However, neither the sites of testicular ACh synthesis nor the cells targeted by locally synthesised ACh are known. Therefore, we have used molecular and immunohistochemical techniques to determine the levels of ACh, and the expression patterns of CHAT, SLC5A7, SLC18A3, SLC22A2 and CHRN subunits in rat testicular tissue.

Results

The synthesising enzyme and the transporters of the cholinergic system are expressed in testicular parenchyma

No protein gene product (PGP) 9.5 positive nerve fibres could be detected in the parenchyma of rat testes, whereas non-neuronal basal cells of seminiferous tubules showed PGP 9.5 immunoreactivity (IR; Fig. 1). In contrast to the parenchyma, the mesostructures that connect testis with epididymis and ductus deferens were densely supplied with PGP 9.5 positive nerve fibres (Fig. 1). However, PCR revealed significant expression of mRNA for *Chat*, *Slc5a7*, *Slc18a3* and *Slc22a2* in testicular parenchyma (TP). After removal of the testicular capsule (TC), the relative mRNA expression profiles in the parenchyma had a rank order of *Chat*> *Slc5a7*> *Slc22a2*> *Slc18a3* (Fig. 1 and Table 1).

We further determined the expression of mRNAs for cholinergic elements in isolated populations of testicular cells. *Chat* mRNA was localised in cells of the seminiferous tubules (Fig. 2). High or moderate expression levels of mRNA for *Chat*, *Slc5a7*, *Slc18a3* and *Slc22a2* occurred in pachytene spermatocytes, round spermatids and, except for *Slc18a3*, in residual bodies (Fig. 2). In contrast, no product corresponding to *Chat*, *Slc5a7*, *Slc18a3* or *Slc22a2* mRNA could be detected in Sertoli cells, peritubular cells and spermatogonia. Products corresponding to *Chat* and *Slc5a7* were present in total RNA from isolated testicular macrophages (TM). A PCR product corresponding to *Chat* mRNA could be detected in Leydig cells (Fig. 2).

CHAT and SLC5A7 proteins are present in seminiferous tubules

IR for CHAT, SLC5A7, SLC22A2 and SLC18A3 was detected mainly in the seminiferous epithelium. CHAT-IR was detected also in endothelial cells of small interstitial arteries. The labelling was absent after pre-absorption with the corresponding antigen. Strong IR for CHAT and SLC5A7 was detected in spermatids and in some basal cells of the seminiferous tubules, presumably spermatogonia (Fig. 3). Similar cells were also positive for SLC18A3 (Fig. 3), while only barely detectable SLC18A3 labelling was present in

Table 1 Relative expression levels for CHRN subunits, and *Chat*, *Slc5a7*, *Slc18a3* and *Slc22a2* in testicular parenchyma.

CHRN subunit	Samples (*n*)	Testicular parenchyma (mean±S.D.)
α1	3	30.1±0.17
α2	5	29.2±0.54
α3	5	28.9±0.92
α4	5	39.8±0.19
α5	3	33.5±0.42
α6	5	8.7±11.94*
α7	3	34.6±0.55
α9	5	32.9±0.39
α10	3	29.0±0.94
β1	3	34.8±0.40
β2	3	35.8±0.68
β3	3	33.9±0.19
β4	5	0.0
Slc22a2	3	31.8±0.28
Chat	3	37.2±0.81
Slc5a7	7	35.0±0.30
Slc18a3	3	27.7±0.81

The asterisk indicates the absence of mRNA in three out of five replicates, while the other two replicates revealed very weak expression.

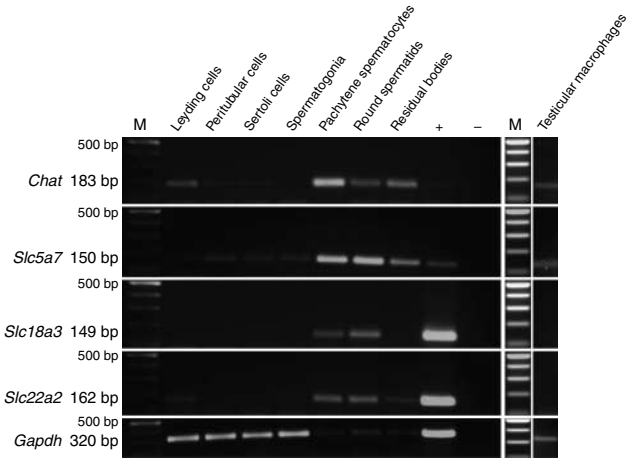

Figure 2 Ethidium bromide-stained agarose gels showing the PCR products for choline acetyltransferase (*Chat*, product length 183 bp), high-affinity choline transporter (*Slc5a7*, product length 150 bp), vesicular ACh transporter (*Slc18a3*, product length 149 bp) and organic cation transporter 2 (*Slc22a2*, product length 162 bp) in isolated cell populations as examined by RT-PCR. *Gapdh* (product length 320 bp) served as the reference gene. +, rat brain (positive control); −, water control (negative control) and M, marker.

other cell types of the seminiferous epithelium (Fig. 3). SLC22A2 IR was visible in Leydig cell clustered in the interstitium, but not within the seminiferous tubules (Fig. 3).

Multiple CHRN subunits are expressed in different cell types of the TP

To determine potential targets of ACh in the testis, we first analysed the relative mRNA expression patterns of CHRN subunits in TP. In the parenchyma, mRNA for 11 out of 13 CHRN subunits, including the supposedly 'muscle specific' α1- and β1-subunits, was detected (Fig. 4 and Table 1). The mRNAs for CHRN α6- and β4-subunits could not be detected in the TP but were present in the TC (Fig. 4). The highest levels of expression were observed for mRNA for α4-subunit followed by α7-, α5- and α9-subunits and β-subunits 1–3 (Fig. 4 and Table 1).

The mRNAs for the α7- and α4-subunits of CHRN are expressed in the seminiferous tubule

As mRNAs for α4- and α7-subunits of CHRN were strongly expressed in the testis and α7-subunit of CHRN has been reported to be associated with sperm motility (Bray *et al*. 2005), we investigated the mRNA expression patterns of both subunits in isolated cell preparations. PCR products corresponding to mRNAs of both subunits were present in peritubular and Leydig cells, whereas Sertoli cells contained mRNA for the α7-, but not the α4-subunit of CHRN. Spermatogonia as well as pachytene spermatocytes expressed mRNAs for α4- and α7-subunits; however, mRNA for α7-subunit could not be detected in later stage spermatocytes. The PCR product corresponding to mRNA for the α7-subunit was found in residual bodies, but not in round spermatids (Fig. 5). TM expressed mRNAs for both α4- and α7-subunits (Fig. 5). Since subtype-specific CHRN antisera were not available (Herber *et al*. 2004, Moser *et al*. 2007), it was not possible to further analyse the distribution of receptor subunit proteins. However, *in situ* hybridisation localised mRNA for the α7-subunit of CHRN in pachytene spermatocytes (Fig. 6).

ACh is present in testis

ACh was measured in rat testis. Comparison of the ACh content in 16-day-old immature testes as well as in adult testes showed that ACh was present in all

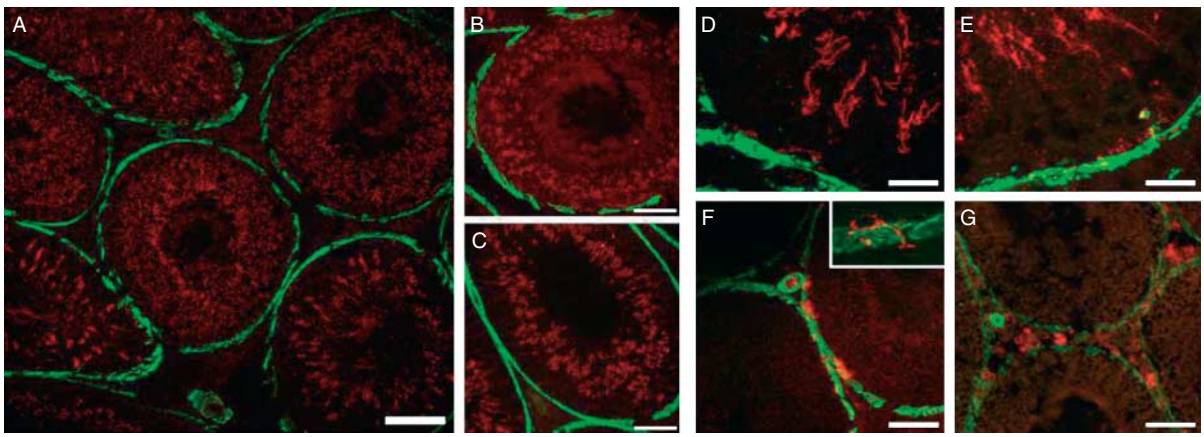

Figure 3 Multiple labelling immunohistochemistry. (A, B and D) Choline acetyltransferase (CHAT, in red), (C and E) high-affinity choline transporter (SLC5A7, in red), (F) vesicular acetylcholine transporter (SLC18A3, in red), (G) organic cation transporter 2 (SLC22A2). CHAT and SLC5A7 are present in spermatids (A–C), with CHAT-antiserum additionally staining round spermatids and some meiotic cells (D), while SLC18A3 is present in spermatogonia (F, inset). SLC22A2 is present in interstitial cells (G). α-Smooth muscle actin was visualised with FITC-coupled antibody (green in A–G). Bars A, 100 μm; B, C, F and G, 75 μm; D and E, 20 μm.

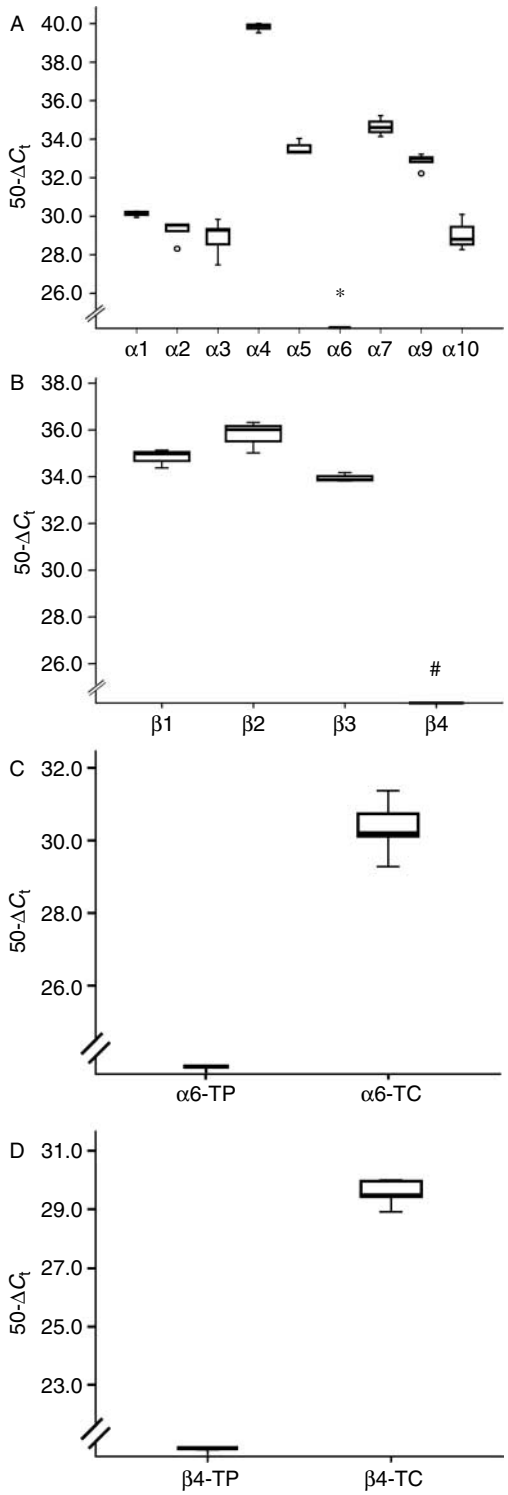

Figure 4 The mRNA expression level of the nicotinic ACh receptor (CHRN) subunits, α (A) and β (B), in testicular parenchyma (TP) was determined by RT-qPCR. The asterisk indicates the absence of mRNA in three out of five replicates, while the other two replicates revealed very weak expression. #, no mRNA detectable. (C and D) Comparison of the α6- and β4-subunits of CHRN expression in testicular capsule (TC) and testicular parenchyma (TP). The ΔC_t values were subtracted from 50 showing higher values with higher expression.

samples. It was found at lower levels in TC and parenchyma of 16-day-old animals, whereas adult rats showed extremely high ACh levels in the parenchyma (3068.3 ± 764.94 vs 3.8 ± 1.62 pmol/mg protein, Table 2 and Fig. 7) and the ACh levels were similar in adults and 16-day-old rats in the capsule. Freshly isolated germ cells of adult rats also contained ACh; however, significantly higher levels were present in the supernatant (11.24 ± 3.65 vs 47.74 ± 23.79 pmol/mg protein, Table 2 and Fig. 7).

Discussion

ACh synthesising enzyme and transporters are present in TP

This study demonstrates that non-neuronal cells in the parenchyma of rat testis have all the components necessary for cholinergic signalling. We detected mRNAs for the ACh synthesising enzyme, transporters and ACh receptors in locations completely lacking any form of innervation as was demonstrated by the absence of PGP 9.5 labelling (see also Zhu *et al.* (1995)).

Cells of the germinal epithelium can synthesise ACh

Previous studies have demonstrated *Chat* mRNA and activity in human and rat spermatozoa (Sastry *et al.* 1981, Ibanez *et al.* 1991), while the ACh degrading enzyme ACh esterase, AChE, is expressed in rat spermatozoa with higher enzymatic activity in testis than in epididymis (Egbunike 1980). We now have found CHAT and SLC5A7 mRNAs and proteins in isolated germ cells *in vitro* as well as *in situ*. In addition, mRNAs for two cholinergic transporters, *Slc18a3* and *Slc22a2*, were present in isolated spermatocytes and spermatids. SLC5A7 is the rate-limiting factor in ACh synthesis (Ribeiro *et al.* 2006), whereas SLC18A3 and SLC22A2 are involved in vesicular and non-vesicular ACh release respectively (Erickson *et al.* 1996, Ribeiro *et al.* 2006). Taken together, these observations strongly support the possibility that the germinal epithelium has the capability to synthesise and release ACh. However, it cannot be excluded that the testicular ACh synthesising machinery is not functional. Lonnerberg & Ibanez (1999) described truncated non-functional forms of CHAT in rat testis. Our expression analysis demonstrated the presence of mRNA and protein for ACh synthesis, and data are supported by the detection of ACh in rat testis and in isolated germ cells. ACh was present in isolated cells at about 11 pmol/mg protein and at higher levels of about 48 pmol/mg protein in the supernatant. The levels are comparable to the ACh content determined in non-neuronal cells. ACh concentrations in mononuclear cells were 0.35 pmol/1 million cells (Fujii & Kawashima 2001), 1.21 pmol/1 million cells (Innis 2011) and 0.83 pmol/1 million cells (Hecker *et al.*

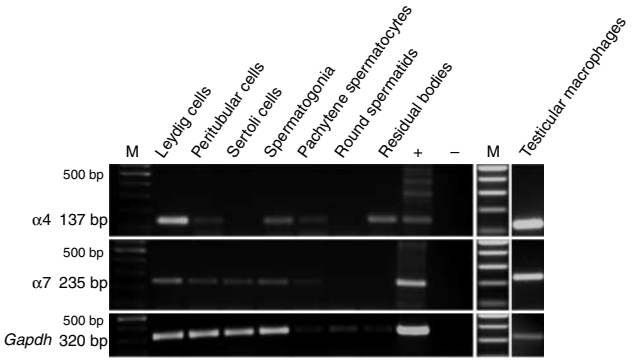

Figure 5 The presence of nicotinic ACh receptor (CHRN) subunits α4 (product length 137 bp) and α7 (product length 235 bp) was determined in isolated testicular cell populations using RT-PCR. *Gapdh* (product length 320 bp) served as the reference gene. +, positive control rat brain; −, negative water control; M, marker.

2009). In the absence of nerve fibres, our results clearly indicate a local non-neuronal source of ACh in rat testis. The two interesting observations in this study are the high ACh levels in the supernatant of germ cells and the strong increase in ACh levels in adult rat testis. The high level in the supernatant of freshly isolated germ cells may be related to constant ACh synthesis and release from the germ cells itself. This is supported by the fact that the molecule responsible for storage of ACh, SLC18A3, could not be detected in the germ cell population. This is similar to human and murine leukocytes and crypt cells in the distal rat colon, which also do not express SLC18A3, but contains or synthesises ACh (Kawashima & Fujii 2000, Yajima *et al*. 2010). In addition, vascular endothelial cells, TM and Sertoli cells could also be a potential source of endogenous ACh. Endothelial cells have been shown to contain the machinery responsible for ACh and release ACh (Haberberger *et al*. 2000, Kirkpatrick *et al*. 2003), and macrophages and Sertoli cells express *Chat* and *Slc5a7* mRNA. These cells could synthesise and release ACh in the absence of a functional ACh synthesis in germ cells.

Testicular cells can be targets for non-neuronal ACh

The TP contains a large variety of potential targets for non-neuronally released ACh. We found the mRNA for eight α and three β-subunits of CHRN in testicular cells, including mRNAs for the supposedly 'muscle specific' α1- and β1-subunits. These subunits are also expressed in human skin, where their function remains elusive (Spies *et al*. 2006). Expression of the α7-subunit of CHRN was widespread in spermatogonia, spermatocytes within seminiferous tubules as well as within Sertoli cells. Spermatogonia and spermatocytes also expressed the α4-subunit of CHRN, potentially as part of the heteropentameric α4β2 CHRN, which is one of the major CHRN subtypes in neuronal tissue (Albuquerque *et al*. 2009).

The adverse effects of smoking on reproductive function such as preterm delivery and abortion are well established. In the male, nicotine, a major component of cigarette smoke, induced impairment of spermatogenesis and steroidogenesis, the latter probably by affecting steroidogenic acute regulatory protein (StAR), the rate-limiting factor in sex steroid synthesis (Gocze & Freeman 2000, Bose *et al*. 2007). However, the identification of the mRNAs for at least the α4- and α7-subunits of CHRN in Leydig cells in this study proposes an additional mechanism of suppression of androgen production. Effects on spermatogenesis and sperm function parameters can be explained by the presence of α3-, α5-, α7-, α9- and β4-subunits of CHRN in ejaculated human spermatozoa, which are indicative of an earlier production during germ cell development in the testis, a finding that is partially supported by our mRNA expression data. Functionally, mouse sperm deficient of the α7-subunit of CHRN shows impaired motility (Bray *et al*. 2005) and the ACh-triggered acrosome

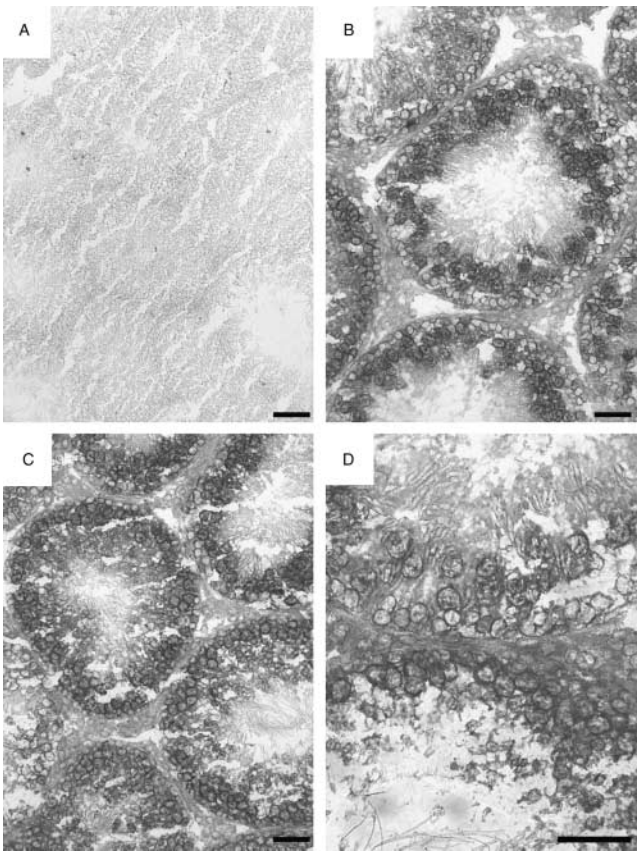

Figure 6 Localisation of α7-subunit of CHRN mRNA expression was examined in rat testis using *in situ* hybridisation. (A) Sense and (B–D) antisense probes. Staining for the α7-subunit of CHRN mRNA was mainly visible in primary spermatocytes, with only few round spermatids weakly labelled. Interstitial cells were negative. No staining was obtained using the sense probe (A). Bars, 20 μm.

Table 2 Acetylcholine content in germ cell preparations and testicular capsule (TC) and testicular parenchyma (TP).

Source	Samples (*n*)	Ratio (%) ACh/choline	Protein (μg/ml)	ACh (pmol/mg protein)
Germ cells	5	67	425.00	11.24±3.65
Germ cells supernatant	6	200	841.67	47.74±23.79
TC d16	6	36	666.67	6.33±2.41
TP d16	6	08	1662.50	3.61±1.32
TC adult	2	18	5850.00	3.8±1.62
TP adult	2	45	212.50	3068.3±764.94

reaction was suppressed by antagonists of tyrosine phosphorylation (Kumar & Meizel 2005). Other CHRN subunits seem to have no direct impact on fertility since mice with deficiency in the subunits α4, β2 (Marubio *et al*. 1999), α5 (Wang *et al*. 2002) and β4 (Wang *et al*. 2003) were reported with no abnormalities with respect to litter size and fertility. As spermatozoa are exclusively transported in the luminal compartment of the male and female reproductive tract separated by epithelial cells from nerve endings that could release ACh, the studies of Bray *et al*. (2005) and Kumar & Meizel (2005) are a clear indication for the necessity of a non-neuronal cholinergic system in the male gonad and during fertilisation.

Albeit information from toxicological studies and transgenic mouse models have provided some insight in the pathophysiological functions of the cholinergic system, its role in the normal testis is still unclear. Observations from other systems suggest that CHRN can mediate effects on cell division, metabolism and motility also in testicular tissue. The proximity of cellular ACh synthesising and reception sites in the testis favours an autocrine or paracrine mode of regulating testicular function by the non-neuronal cholinergic system, comparable to what is observed in the lymphatic system and the skin (Kawashima & Fujii 2000, Kurzen *et al*. 2007). Taken together, our results are a clear indication for a functional non-neuronal cholinergic system in the testis and add important data to further understand the pathophysiological consequences of smoking on male reproductive function.

Materials and Methods

Tissue preparation

Specimens for RT-PCR were obtained from 8- to 10-week-old male Wistar-Firth rats. The animals were killed by a lethal dose of isoflurane, and the testis was removed and decapsulated. Approximately 30 mg TP and the complete capsule were snap-frozen separately in liquid nitrogen and stored at −80 °C until required. For *in situ* hybridisation, the testis was snap frozen in liquid isopentane and fixed as required after sectioning. For immunohistochemistry, TP and capsule were immersion fixed in Zamboni's fixative (2% formaldehyde, 15% picric acid in 0.2 M phosphate buffer, pH 7), washed overnight in PBS, cryoprotected in 18% sucrose and frozen in optimal cutting temperature compound. All animal procedures were performed in accordance with approval from the Flinders University Animal Welfare Committee.

Isolation of cells from the rat testis

Leydig cells, Sertoli cells, spermatogonia, pachytene spermatocytes, round spermatids and residual bodies were prepared from rat testes as described previously (Guillaume *et al*. 2001). TM were isolated from testes without any enzymatic treatment. The testes were decapsulated into 10 ml pre-warmed endotoxin-free DMEM–F12 medium (PAA Laboratories, Morningside, QLD, Australia). The seminiferous tubules were gently separated, and the volume was adjusted to 50 ml. After gentle stirring, the tubule fragments were allowed to settle for 5 min before the supernatant was centrifuged at 300 ***g*** for 10 min. The pellet containing interstitial cells and TM was resuspended in 5 ml DMEM–F12 and incubated at 32 °C and 5% CO_2 for 30 min. TM and the remaining interstitial cells were separated by the more rapid adherence of TM to surfaces.

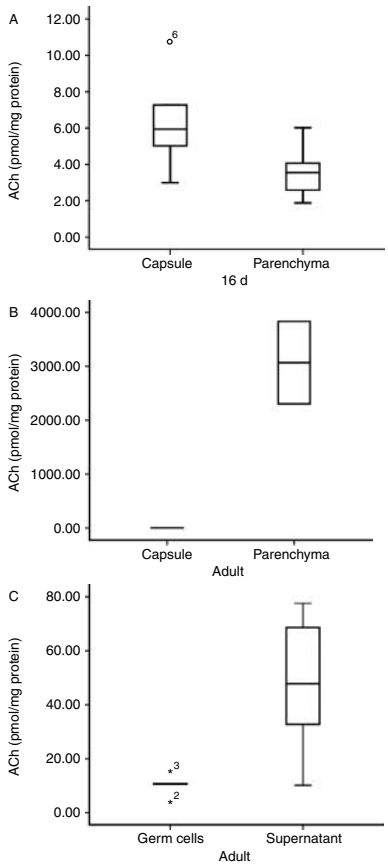

Figure 7 Detection of ACh in rat germ cells, testicular capsule and testicular parenchyma (A–C). Comparison of the ACh content in 16-day-old immature and adult testes showed that ACh was present at low levels in testicular capsule and testicular parenchyma of 16-day-old animals (A), while adult rats showed very high ACh levels in the parenchyma and low levels in the capsule (B). The box plots in C show the presence of ACh in freshly isolated germ cells and in the supernatant.

After 30 min, non-adherent cells were removed by washing with fresh medium. Subsequently, TM were washed a second time, by pipetting directly on the surface. Purity of TM (80%) was determined by immunolabelling using monoclonal antibodies ED1 and ED2 (AbD Serotec, Oxford, UK) directed against monocytes/macrophages.

RT-PCR

RT-PCR was used to detect mRNAs in isolated populations of testicular cells (PTC 200; Peqlab, Erlangen, Germany). Quantitative real-time PCR (RT-qPCR) was used to quantify relative expression levels of mRNAs using cDNA from TP and capsule (Corbett Roto-cycler, Sydney, NSW, Australia). Capsule and parenchyma were lysed in RLT lysis buffer (Qiagen) using a tissue lyser (Qiagen). RNA was extracted (RNeasy Mini Kit, Qiagen), and quantity and quality of the RNA were determined. Isolated testicular cells were lysed in RLT buffer, homogenised with a 21G syringe and subsequently used for RNA extraction according to the manufacturer's instructions (RNeasy Mini Kit, Qiagen). Total RNA from brain served as positive control. In negative controls, the reverse transcriptase was replaced by water (negative water control). The extraction was followed by DNase digestion and RT (iScript, Bio-Rad). The cDNA was used for subsequent PCR and RT-qPCR. RT-PCR for isolated testicular cells was performed as for standard PCR in a 25 µl reaction volume with 1 µl cDNA, 0.2 mM dNTP mix, 1 mM $MgCl_2$, 0.2 pmol each primer (Table 3), buffer and GoTaq Polymerase according to the manufacturer's protocol (Promega). *Gapdh* was used as the reference gene. All RT-qPCR were performed in duplicate or triplicate from three to six animals using a ready-to-use kit according to the

Table 3 Primer pairs used for RT-PCR.

Primer	Sequence (5′ → 3′) F – forward and R – reverse		Product length (bp)	Accession no.
Chat	F	TGAACGCCTGCCTCCATTCGGCCTGCTGA	272	XM_224626
	R	GTGCCATCTCGGCCCACCACGAACTGCA		
Chat	F	CAACCATCTTCTGGCACTGA	183	XM_224626
	R	TAGCAGGCTCAATAGCCATT		
Slc18a3	F	GCCACATCGTTCACTCTCTTG	149	X80395
	R	CGGTTCATCAAGCAACACATC		
Slc22a2	F	GCCTCCTGATCCTGGCTG	226	X98334
	R	GGTGTCAGGTTCTGAAGAGAG		
Slc5a7	F	ATGGCTCTACCAGCCATTTG	189	AB030947.1
	R	GGACATGACAGCAGCAGAAA		
Slc5a7	F	CAAGACCAAGGAGGAAGCAC	150	AB030947.1
	R	GCAAACATGGAACTTGTCGA		
Rpl19	F	CATGGAGCACATCCACAAAC	216	NM_031103.1
	R	CCATAGCCTGGCCACTATGT		
18S rRNA	F	CCGCAGCTAGGAATAATGGA	245	M11188
	R	AGTCGGCATCGTTTATGGTC		
Gapdh	F	CATTGTTGCCATCAACGACC	320	NM_017008
	R	TCACACCCATCACAAACATG		
α1	F	AACTTCATGGAGAGCGGAGA	285	NM_024485.1
	R	CAGCTCCACAATGACGAGAA		
α2	F	GGAGCAGATGGAGAGGACAG	216	NM_133420.1
	R	AGCACAGTGAGGCAGGAGAT		
α3	F	GCCAACCTCACAAGAAGCTC	208	NM_052805.2
	R	CCAGGATGAAAACCCAGAGA		
α4	F	GGACCCTGGTGACTACGAGA	137	NM_024354
	R	CATAGAACAGGTGGGCCTTG		
α5	F	CACGTCGTGAAAGAGAACGA	112	NM_017078.2
	R	TCCCAATGATTGACACCAGA		
α6	F	ACAGCTCTTCCACACGCTCT	286	NM_057184.1
	R	GAAGTCACCGACGGCATTAT		
α7	F	GGCTCTGCTGGTATTCTTGC	286	NM_012832.3
	R	AAACCATGCACACCAGTTCA		
α7	F	ACATTGACGTTCGCTGGTTC	235	L31619
	R	CTACGGCGCATGGTTACTGT		
α9	F	CGTGGGATCGAGACCAGTAT	242	NM_022930.1
	R	AAAGGTCAGGTTGCACTGCT		
α10	F	CTGCTGACTCTGGGGAGAAG	317	NM_022639.1
	R	GGCTGACTCTAGTGGCTTGG		
β1	F	CATCGAGTCTCTCCGTGTCA	206	NM_012528.1
	R	TGCAATTCTGCCAGTCAAAG		
β2	F	AAGCCTGAGGACTTCGACAA	142	NM_019297.1
	R	TGCCATCATAGGAGACCACA		
β3	F	CACTCTGCGCTTGAAAGGAA	196	NM_133597.1
	R	GCGGACCCATTTCTGGTAAC		
β4	F	CTCCTGAACAAAACCCGGTA	371	NM_052806.2
	R	ACCTCAATCTTGCAGGCACT		

Table 4 Primer used for generation of *in situ* hybridisation probes. T7 binding site TAATACGACTCACTATAGGG; SP6 binding site ATTTAGGTGACACTATAGAA.

Primer	Sequence (5′ → 3′) F – forward, R – reverse	Product length (bp)
Chat_F	TAATACGACTCACTATAGGGTGAACGCCTGCCTCCATTCGGCCTGCTGA	312
Chat_R	ATTTAGGTGACACTATAGAAGTGCCATCTCGGCCCACCACGAACTGCA	
α7_F	TAATACGACTCACTATAGGGGGCTCTGCTGGTATTCTTGC	326
α7_R	ATTTAGGTGACACTATAGAAAAACCATGCACACCAGTTCA	

manufacturer's protocol (iQ SYBR green Supermix, Bio-Rad). Primers specific for mRNA sequences were designed using Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 3). All primers spanned introns except the primers for the α2-subunit and *Slc18a3*. All PCR products were sequenced (Flinders University Sequencing Facility) and showed 100% homology with the predicted target sequence. Primers specific for the rat reference genes *Rpl19* (Adams *et al*. 2007) and *18S* rRNA were used for normalisation. The RT-qPCR data were normalised by subtracting the threshold cycle (C_T) levels between the genes of interest and the mean of *Rpl19* and *18S* rRNA (Livak & Schmittgen 2001). The ΔC_T values were subtracted from 50 so that higher values reflect higher expression levels.

In situ *hybridisation*

In situ hybridisation probes were prepared using digoxigenin (DIG) labelling (Roche). The primers contained binding sites for either the T7 or SP6 RNA polymerase (Table 4). Cryosections (12 µm) were fixed in 4% paraformaldehyde (PFA), permeabilised with proteinase K (2 µg/ml) for 7 min, fixed again in PFA and acetylated (0.1 M triethanolamine containing 0.25% (v/v) acetic anhydride). After prehybridisation, hybridisation was performed overnight at 65 °C. Subsequently, the sections were washed in decreasing concentrations of SSC. The DIG-labelled probes were detected using alkaline phosphate-conjugated anti-DIG antibody and subsequent visualisation using NBT/BCIP as substrate (Roche). Colour development was allowed to proceed in the darkness for 4–16 h. The reaction was terminated by immersion in tap water.

Immunohistochemistry

Rat testes were serially cryosectioned at a thickness of 12 µm, fixed with methanol and subsequently preincubated for 1 h with PBS (145.4 mM NaCl (8.5 g/l), 7.54 mM Na_2HPO_4 (1.07 g/l), 2.5 mM $NaH_2PO_4H_2O$ (0.39 g/l), pH 7.1) containing 10% normal donkey serum, 0.1% BSA and 0.5% Tween 20. Indirect immunofluorescence was performed by overnight incubation with antisera directed against either PGP 9.5, CHAT, SLC5A7 or SLC18A3 in combination with an FITC-conjugated anti-smooth muscle actin antibody (anti-SMA-FITC; Table 5) diluted in PBS with doubled concentration NaCl at room temperature followed by washing in PBS and subsequent incubation with appropriate combinations of secondary reagents (Table 5) for 1 h at room temperature. After incubation with the secondary reagents, the slides were washed in PBS and coverslipped in carbonate-buffered glycerol at pH 8.6 and evaluated by fluorescence microscopy or sequential scanning using a confocal laser scanning microscope (TCS SP5, Leica, Bensheim, Germany). Specificity of the CHAT, SLC18A3 and SLC22A2 antisera was tested by pre-absorption with the corresponding antigen. In addition, the specificity of the SLC5A7 antibody has been shown in murine cochlea, brain and spinal cord (SLC5A7, Brandon *et al*. 2004, Bergeron *et al*. 2005).

ACh measurement

Freshly prepared tissue from testes was homogenised in ten volumes of a mixture of ice-cold acetone (85%) and 1 M formic acid (15%) as described previously (Klein *et al*. 1993). Cultured cells and cell media (supernatant) were taken in 96% ethanol. Homogenates were centrifuged at 10 000 ***g***. Aliquots of the supernatant were taken to dryness in a vacuum centrifuge, taken in HPLC buffer and injected into a Eicom HTEC-500 microbore system coupled to a Shimadzu SIL-20AC auto-sampler. The buffer composition was 5 g $KHCO_3$, 400 mg sodium decanesulfonate, and 50 mg EDTA in 1 l Aqua Dest. (pH 8.3). At a flow rate of 0.15 ml/7 min, retention times were 7.8 min for choline and 13.2 min for ACh. The limit of detection was 1–2 fmol analyte per 5 µl injection volume. ACh chloride and choline chloride (purity >99% each) were purchased from Sigma–Aldrich.

Table 5 Primary and secondary antisera, nuclear staining.

Antibody	Host	Conjugate	Supplier	Dilution
Primary				
Anti-rat-SLC5A7	Polyclonal, rabbit		Chemicon	1:100
Anti-rat-CHAT	Polyclonal, sheep		Chemicon	1:2000
Anti-rat-SLC18A3	Polyclonal, goat		Chemicon	1:800
Anti-SMA	Monoclonal, mouse	FITC	Sigma	1:800
Anti-rat-PGP 9.5	Polyclonal, rabbit		Neuromics	1:500
Secondary				
Anti-rabbit-Ig	Donkey	Cy3	Jackson	1:100
Anti-sheep/goat-Ig	Donkey	Cy3	Jackson	1:100
Hoechst 333258			Mol. Probes	1:2000

Chemicon, Boronia, Australia; Neuromics, Medina, MN, USA; Jackson Immuno Research, West Grove, PA, USA; Molecular Probes (Invitrogen), Mulgrave, VIC, Australia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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