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Murine embryonic stem cell line CGR8 expresses all subtypes of muscarinic receptors and multiple nicotinic receptor subunits: Down-regulation of α4- and β4-subunits during early differentiation

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ABSTRACT

Non-neuronal acetylcholine mediates its cellular effects via stimulation of the G-protein-coupled muscarinic receptors and the ligand-gated ion channel nicotinic receptors. The murine embryonic stem cell line CGR8 synthesizes and releases non-neuronal acetylcholine. In the present study a systematic investigation of the expression of nicotinic receptor subunits and muscarinic receptors was performed, when the stem cells were grown in the presence or absence of LIF, as the latter condition induces early differentiation. CGR8 cells expressed multiple nicotinic receptor subtypes (α3, α4, α7, α9, α10, β1, β2, β3, β4, γ, δ, ε) and muscarinic receptors (M1, M3, M4, M5); M2 was detected only in 2 out of 8 cultures. LIF removal caused a down-regulation only of the α4- and β4-subunit. In conclusion, more or less the whole repertoire of cholinergic receptors is expressed on the murine embryonic stem cell line CGR8 for mediating cellular signaling of non-neuronal acetylcholine which acts via auto- and paracrine pathways. During early differentiation of the murine CGR8 stem cell signaling via nicotinic receptors containing α4- or β4 subunits is reduced. Thus, the so-called neuronal α4 nicotine receptor composed of these subunits may be involved in the regulation of pluripotency in this murine stem cell line.

1. Introduction

Acetylcholine and essential components of the non-neuronal cholinergic system (choline uptake, choline acetyltransferase, nicotinic and muscarinic receptors, esterases) have been demonstrated in epithelial, endothelial, mesothelial and immune cells as well as smooth muscle fibers [1–5]. Non-neuronal acetylcholine acts as a local cell molecule signaling autocrine and paracrine mechanisms to control basic cell functions such as proliferation, differentiation, maturation, migration, secretion, organization of the cytoskeleton and cell–cell contact [1–5].

Synthesis of acetylcholine as well as m- and n-AChRs have also been demonstrated in murine embryonic stem cells [6–8]. There is increasing evidence that non-neuronal acetylcholine has a functional role within embryonic stem cells. Applied acetylcholine increased the viability but reduced the proliferation rate of CGR8 cells [7]. Nicotine stimulates the expression of the transcription factors Oct-4 and Rex-1 [9]. In murine induced pluripotent stem cells nicotine promotes DNA synthesis and cell proliferation via stimulation of α4- and α7 subtype receptors [10]. Experimental hypoxia limits DNA synthesis and increases apoptosis in murine embryonic stem cells, and the application of exogenous acetylcholine prevented these cell-damaging effects by an atropine-sensitive mechanism [11]. Finally, during early embryogenesis muscarinic receptors are involved in the regulation of primitive streak and mesoderm formation [12]. All these examples provide evidence for a regulatory role of nicotinic and muscarinic receptors (n- and m-AChRs) in stem cells and embryogenesis. It had previously been reported that the CGR8 stem cell line expresses all subtypes of muscarinic receptors and also the α3, α4, α7 and β2 subunits [6,7]. However, a systematic analysis of the subunits of all known nicotinic receptor subunits expressed on stem cells has not been performed so far. Moreover it is not known whether the expression of m- and n-AChRs changes during early differentiation which is induced by the removal of leukemia inhibitory factor [8]. At this vulnerable cell period multiple genes alter their activity state and it is of interest to investigate, whether cholinergic cell signaling can

Abbreviations: ACh, acetylcholine; BLAST, basic local alignment search tool; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6; Chmr1,2,3,4,5 cholinergic receptor, muscarinic 1,2,3,4,5, CNS [mus musculus]; Chrnα1,2,3,4,5,6,7,9,10 cholinergic receptor, nicotinic, alpha polypeptide 1,2,3,4,5,6,7, 9,10 (muscle) [mus musculus]; Chrnβ2,3,4 cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal), 3,4 [mus musculus]; Chrnγ, cholinergic receptor, nicotinic, gamma polypeptide [mus musculus]; Chrnδ, cholinergic receptor, nicotinic, delta polypeptide [mus musculus]; Chrnε, cholinergic receptor, nicotinic, epsilon polypeptide [mus musculus]; LIF, leukemia inhibitory factor; mAChRs, muscarinic receptors; nAChRs, nicotinic receptors; NCBI, National Center for Biotechnology Information; Oct-4, octamer binding transcription factor 4.

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in principle be involved by changing the expression levels of the m- and n-AChRs Therefore, in the present experiments the murine stem cell line CGR8 was cultured in the presence or absence of LIF, to investigate the expression of m- and nAChRs using subtype-specific primers.

2. Methods

2.1. Culture of CGR8 cells

The murine embryonic stem cell line CGR8 was a generous gift from Dr. J. Hescheler, University of Cologne, Germany. Undifferentiated cells were cultured under standard conditions in gelatinized flasks with Glasgow's buffered minimal essential medium (Gibco, Germany), supplemented with 100 U/ml leukemia inhibitory factor (LIF; Sigma-Aldrich, St. Louis, USA) to maintain pluripotency. When indicated, LIF was omitted during the last 5 culture days to investigate a possible change of the expression pattern. In addition, the medium contained 0.05 mM beta-mercaptoethanol, 2 mM L-glutamine, 100 units/ml penicillin, 0.01 mg/ml streptomycin and 10 vol% fetal calf serum (Sigma-Aldrich, Germany). Cells grown in 25 cm^2 flasks reached confluence after 4 days. Thereafter, CGR8 cells were passaged and seeded at a density of 1×10^6 cells on 75 cm^2 flasks. After an additional culture period (5–6 days) adherent cells were collected (0.05% trypsin/0.02% EDTA) for RNA extraction.

2.2. RNA isolation and quantification

The primers used for the different subtypes/subunits of muscarinic and nicotinic receptors are indicated in Table 1. Primers were designed at the nucleotide database of the NCBI website with the exception of one primer pair for GAPDH (short amplification product), which was taken from Wang et al. [13].

RNA was extracted from CGR8 cell pellets cultured in the presence or absence of LIF by the commercial GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Munich, Germany). 1 μg of extracted RNA was transcribed into cDNA according to a standard protocol using Omniscript RT PCR Kit (Qiagen, Hilden, Germany). The cDNA was amplified with gene-specific primers (see Table 1) using either (A) Power SYBR® Green PCR Master Mix (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany) or (B) Taq PCR Core Kit (Quiagen, Hilden, Germany). RT-PCR analysis (GeneAmp PCR System, Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany) was carried out using the following cycler program: 95 °C for 10 min, 95 °C for 30 s, 60° for 30 s , 72° for 30 s, 72° for 10 min, 40/45 cycles. 25 ng (A) or 10 ng (B) of cDNA was applied for each reaction together with 0.8 pmol/μl of one primer. The PCR products were separated by gel electrophoresis (2% agarose) and detected by ethidium bromide staining. GAPDH was used as endogenous control. In the present experiments primers were used to end up with short (<200 bp) or long (>200 bp) amplification products (for the receptor and housekeeping genes). Water controls were performed in parallel and were always negative. Primers were synthesized and purchased from Microsynth (Balgach, Switzerland). At least 3 to 5 different cell cultures were investigated for each condition, i.e. CGR8 cell culture performed in the presence or absence of LIF. For comparison brain, heart and striated muscle (innervated part of the diaphragm) of mice were homogenized in 500 μl TRIzol® for RNA extraction; a standard protocol was used for further preparation of RNA extraction by means of the commercial GenElute™ Mammalian Total RNA Miniprep Kit (see above). For quantification of the expression levels of the α4- and β4-subunits the ImageJ 1.4 program was used and the data were normalized to the GAPDH expression (see Fig. 3).

Table 1
Primers used for RT-PCR.

Muscarinic receptors	Sequence	Size (bp)
Chrm1 for	5′-TGCTCATCAGCTTTGACCGT-3′	196
Chrm1 rev	5′-AACTGGATGTAGCACTGCCC-3′	196
Chrm1 for	5′-AGAAGAGGCTGCCACGGTA-3′	198
Chrm1 rev	5′-CAGACCCCACCTGGACTTTA-3′	198
Chrm2 for	5′-CGGCTTTCTATCTGCCTG TC-3′	169
Chrm2 rev	5′-GGCATGTTGTTGTTGTTTGG-3′	169
Chrm3 for	5′-CACCGTGAACCCCGTGTGCT-3′	156
Chrm3 rev	5′-GCCTGCTCAGGCACTCGCTT-3′	156
Chrm4 for	5′-CCAACCCGGCGGTGACCTTC-3′	129
Chrm4 re	5′-GGCCCTCGGGTCGATGCTTG-3′	129
Chrm4 for	5′-GTCAAGAGTGTGTACCGGGG-3′	575
Chrm4 rev	5′-TTAGCATGGCCTCCCAGTTG-3′	575
Chrm5 for	5′-TCAGCCATCAAATGACCAAA-3′	180
Chrm5 rev	5′-AGTAACCCAAGTGCCACAGG-3′	180
Chrm5 for	5′-GAGAAAACCGGCTCACAGGA-3′	336
Chrm5 rev	5′-TTTTCAGTCCGAGGGCTCAC-3′	336
Nicotinic receptor subunits		
Chrnα1 for	5′-GTCTGAAACAGCAATGGGTCG-3′	168
Chrnα1 rev	5′-CCAGGAGCACCTTGGTGAAT-3′	168
Chrnα1 for	5′-TAACCCGGAAAGTGACCAGC-3′	266
Chrnα1 rev	5′-TGCAATGTACTTCACGCCCT-3′	266
Chrnα1 for	5′-CAATAACGCCGCTGAGGAATG-3′	303
Chrnα1 rev	5′-ATACAGCCGTGTGAGCAGAG-3′	303
Chrnα1 for	5′-GGAGGACCACCGTGAGATTG-3′	676
Chrnα1 rev	5′-CCAGGAGCACCTTGGTGAAT-3′	676
Chrnα2 for	5′-AGGCCTTACCCAGACTTCCT-3′	125
Chrnα2 rev	5′-TCATGTCTTGGGGTTTGACACA-3′	125
Chrnα2 for	5′-CCTGGACTCAAAGTCACGCT-3′	502
Chrnα2rev	5′-AACTCCCCGTCTGCATTGTT-3′	502
Chrnα3 for	5′-TGATCACCGAGACCATCCCT-3′	220
Chrnα3 rev	5′-GTGCTGGTTGGCCTAGTCAT-3′	220
Chrnα4 for	5′-ACACGGGGCATGAAGTTGG-3′	489
Chrnα4 rev	5′-CGTCCGCGTTGTTGTAGAG-3′	489
Chrnα5 for	5′-GCTGTCAGTCACAGTTGTCCT-3′	285
Chrnα5 rev	5′-GCTGGTTTTTCTCATCCACGTC-3′	285
Chrnα6 for	5′-ATACCATGCCCAAGTGGGTG-3′	366
Chrnα6 rev	5′-TCCAGTCGTCTTCTACCTCGT-3′	366
Chrnα7 for	5′-CGTGCCCTTGATAGCACAGTA-3′	171
Chrnα7 rev	5′-TTCATGCGCAGAAACCATGC-3′	171
Chrnα7 for	5′-CTCATGGGAATCCCTGGCAA-3′	660
Chrnα7 rev	5′-ATCATGTGTTGGGGAGCAGG-3′	660
Chrnα9 for	5′-TGCGCTATGATACCTCCCCA-3′	286
Chrnα9 rev	5′-TGCTGTCTCTACGGCTTTGA-3′	286
Chrnα9 for	5′-CGTCTCCAGTCATTCGGCTT-3′	648
Chrnα9 rev	5′-CGTCATCGGCCTTGTTGTAG-3′	648
Chrnα10 for	5′-CGCCATGAAGATTGGAAGCG-3′	125
Chrnα10 rev	5′-CCAGTTCCTGGCCCTTACAG-3′	125
Chrnα10 for	5′-AGTCATATGGAAAGGGACGGAA-3′	141
Chrnα10 rev	5′-TGGAAACCAGAGATTGCGGC-3′	141
Chrnβ1 for	5′-TTCACTGAGCGAAAGAGGCA-3′	117
Chrnβ1 rev	5′-ATCAGTTGGCCTTCGGCTTC-3′	117
Chrnβ1 for	5′-GCCGAAGGCCAACTGATTAAG-3′	669
Chrnβ1 rev	5′-GTAGAAGAGAGGCTTCCGCC-3′	669
Chrnβ2 for	5′-GTGTTCCCTAGAAGAGCAGCC-3′	271
Chrnβ2 rev	5′-CCAAAACCCCTGAACACAGC-3′	271
Chrnβ3 for	5′-CCTCCGACGAAGGGGAAAAA-3′	223
Chrnβ3 rev	5′-GCCATGGGATGGTACGTTGA-3′	223
Chrnβ4 for	5′-TCCTCGTCTCTCTGTTCGCT-3′	237
Chrnβ4 rev	5′-CAGTCCATTCCTGTTTCAGCC-3′	237
Chrnγ for	5′-AGAGACCTCAGCTCCTCTTGC-3′	241
Chrnγ rev	5′-TAGTCGCACCATTGCATCTCTAT-3′	241
Chrnδ for	5′-GGGGCCTGAATGAGGAACAA-3′	200
Chrnδ rev	5′-GCTGTCTACCCAGGCATGAT-3′	200
Chrnδ for	5′-GTGGATAGATCATGCCTGGGT-3′	240
Chrnδ rev	5′-TAGGTGACTGAGATGGGGCA-3′	240
Chrnε for	5′-CGGCTGCGCCAGATTTTATT-3′	196
Chrnε rev	5′-TCCAAGTTCCGTGCCGATG-3′	196
Housekeeping gene		
GAPDH for	5′-CCTCGTCCCGTAGACAAAATG-3′	124
GAPDH rev	5′-TGAAGGGGTCGTTGATGGC-3′	124
GAPDH for	5′-GGCTCATGACCACAGTCCAT-3′	547
GAPDH rev	5′-TGGGATAGGGCCTCTCTTGCT-3′	547