Regulation of gene expression by muscarinic acetylcholine receptors

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Abstract

In the brain, muscarinic acetylcholine receptors (mAChRs) are involved in higher cognitive functions including synaptic plasticity and memory. In Alzheimer’s disease (AD) patients the cholinergic nervous system is severely damaged. In order to reinforce the cholinergic system, clinical tests were started to use cholinomimetic drugs to treat AD patients. To identify the genes involved in mAChR signalling, we used a differential display approach and found 11 genes that were readily activated by mAChR with 1 hour of activation. These included the transcription factors Egr-1, Egr-2, Egr-3, c-Jun, Jun-D and Gos-3; the growth regulator hCyr61; the signalling factors NGFi-B (nerve growth factor induced gene-B) and Etr101; the unknown gene Gig-2 (for G-protein-coupled receptor induced gene 2); and the acetylcholinesterase gene (ACHE). Our data show that multiple immediate-early genes are under the control of mAChRs, and they suggest that these genes play important roles in coupling receptor stimulation to long-term neuronal responses. The results also suggest a feedback mechanism where up-regulated ACHE expression and accelerated breakdown of acetylcholine (ACh) at the cholinergic synapses limits increases in cholinergic transmission. Three hours after m1 mAChR activation a different pattern of gene expression was demonstrated. It included the novel genes Gig-3 and Gig-4, as well as the LIM-only protein LM04. Like ACHE, these genes are target genes which may be under the control of the above immediate-early genes. Together, our data show that muscarinic recep-

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tors induce a complex and sustained pattern of gene expression that may be involved in the regulation of cholinergic transmission as well as the control of cellular functions in post-synaptic cholinergic target cells. These results may contribute to a better understanding of the effects and side effects of cholinomimetic treatment in AD patients.

Introduction

Muscarinic acetylcholine receptors (mAChRs) are members of a superfamily of G-protein-coupled, cell-surface receptors [1,2]. Different subtypes of mAChRs include m1 and m3 mAChR, which are preferentially coupled to Gq/G11 proteins. They are present on somatodendritic plasma membranes of large pyramidal neurons throughout the cortex and the hippocampus, as well as on small cholinergic interneurons in the striatum. In contrast, m2 and m4 mAChRs are coupled to the Gi/G0 proteins, and are predominantly localized to presynaptic terminals and the axons of the large basal forebrain projection neurons that innervate cortical and hippocampal cholinergic target cells. Postsynaptic mAChRs trigger a variety of distinct short-term and intermediate signalling cascades. These include phospholipase D, adenylate cyclase, phospholipase A2, the generation of diacylglycerol, which activates protein kinase C and couples mAChRs to the ERK–MAP-kinase (extracellular signal-related protein kinase–mitogen-activated protein kinase) signalling cascade, activation of endoplasmic reticulum IP3 receptors and stimulation of ligand-operated cell-surface Ca2+ channels, as well as voltage-gated potassium channels [3–10]. mAChRs are also involved in the activity-dependent regulation of the processing of the β-amyloid precursor protein (APP) by α-secretase [11–13]. This is associated with reduced generation of β-amyloid (Aβ) peptides [14–16], the principal component of amyloid plaques in Alzheimer’s disease (AD) brains [17]. These experimental results together with the pathology of AD brains that show a progressive loss of cholinergic innervation from the basal forebrain to the cerebral cortex, hippocampus and amygdala [18–20], as well as the correlation of those changes to the cholinergic system with cognitive decline [21], led to the development of different therapeutic strategies that enhance cholinergic function [22,23]. One of these strategies is to use muscarinic agonists. Cellular responses of mAChRs include the activation of neurite outgrowth, the fine-tuning of membrane potentials and the regulation of mitogenic growth responses in cells that are not terminally differentiated [24]. In the brain, mAChRs are involved in long-term potentiation and synaptic plasticity [25]. Such plastic alterations in neuronal structure and function have been proposed to be associated with rapid and transient transcription of activity-dependent genes [26–29]. In order to identify genes that are regulated by mAChRs, we performed a differential mRNA display (DD) screen with HEK-293 cells stably transfected with m1 mAChR. We show here that several genes representing three functional groups of proteins are readily induced by mAChRs.
Materials and methods

Cell culture experiments, DD, Northern blots, Western blots, mobility shift assays, co-transfections and promoter analysis were carried out as described previously [30,31].

Semiquantitative reverse transcriptase-PCR

HEK-293 cells stably transfected with m1 mAChR were treated with carbachol and 1 hour after stimulation total RNA was prepared using TRIZOL® reagent (Life Technologies, Karlsruhe, Germany) according to the manufacturer’s instructions. Equal amounts of RNA (0.2 μg) were transcribed to cDNA in 20 μl reactions containing 20 μM dNTP, 10 μM DTT, 2 μl of Expand™ Reverse Transcriptase (Boehringer Mannheim, Mannheim, Germany), 1 μl of RNase OUT (Life Technologies, Karlsruhe, Germany) and 10 μM oligo-dT oligonucleotide. Reverse transcription was performed for 60 minutes at 42°C with a final denaturation step for 3 minutes at 95°C. Aliquots of 1 μl of the obtained cDNA were each subjected to PCR using primers corresponding to the hydrophilic form of human acetylcholinesterase {AChE; primer 1 = 1522(+), primer 2 = E6/2003(−) [32]} and corresponding to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; primer 1 = GTCATCAATGG AAATCCCATCACC, primer 2 = TGGCAGGTITTCTAGACGCGCAGG). PCRs were performed employing the corresponding primer (1 μM each), 2.5 mM MgCl₂, 100 μM dNTP (Amersham Pharmacia Biotech, Freiburg, Germany), and 0.5 units of Thermoprime Plus DNA polymerase (Advanced Biotechnologies, Hamburg, Germany) in a final volume of 25 μl. PCR cycle conditions were performed as follows: 94°C for 45 seconds, 62°C or 66°C (AChE or GAPDH respectively) for 1 minute and 72°C for 2 minutes. After each third cycle, starting with cycle number 24, one probe from each PCR was removed. DNA was separated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV illumination.

Results and discussion

mAChRs regulate expression of several immediate-early genes

In order to identify genes that are regulated by mAChRs, we developed an mRNA differential display (DD) approach that yielded highly consistent results [30]. A set of 64 distinct random primers was specifically designed to approach a statistically comprehensive analysis of all mRNA species in a defined cell population. One-base anchor primers in combination with several random primers in the modified DD protocol were used in reactions employing total RNA obtained from HEK-293 cells stably expressing m1 mAChRs. By using 81 of 192 possible primer combinations to analyse mRNAs generated in response to 1 hour of m1 mAChR stimulation, we obtained 38 differential PCR products. Twenty-five of these were analysed and revealed 10 distinct immediate-early genes: Egr-1, Egr-2, Egr-3, NGFi-B, Etr101, c-Jun, Jun-D, Gos-3, and hCyr61, as well as the previously unknown gene Gig-2 (Table 1).
All identified mAChR-inducible genes were immediate-early genes, consistent with the known mAChR-mediated induction of the transcription factor gene families Jun, Fos and Egr [33–41]. A more detailed study of the mAChR-coupled regulation of the Egr family showed that mAChRs can regulate Egr-1, Egr-2, Egr-3 and Egr-4 at the level of transcription, as well as functional protein synthesis [31]. Our data suggest that, among members of the Egr gene family, Egr-1 is the major target of the m1 receptor because competition experiments with Egr-1-specific antibodies almost completely blocked the binding of nuclear extracts to the Egr recognition sequence that is known to interact with all members of the Egr family. The ability of different mAChR subtypes to stimulate Egr-1 expression suggests that similar genes are controlled by ACh both in pre- and post-synaptic neuronal populations. Additional genes induced by m1 mAChR included NGFi-B, a transcription activator of the nuclear factor superfamily [42], and Etr101, an immediate-early gene with an unknown function [43]. Both genes were also found to be carbachol-inducible in independent studies [36,44,45].

**Table 1 Differentially expressed genes induced by m1 mAChR activation.**

Data represent numerical results from DD screens for m1 mAChR-inducible genes after 1 and 3 hours of receptor activation. DNA sequencing and database analysis identified genes and novel gene tags. Verification of regulation of identified genes was done by Northern blot analyses.

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Protein function</th>
<th>Number of representing DD bands</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>After 1 hour of m1 mAChR activation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egr-1</td>
<td>Transcription factor</td>
<td>4</td>
</tr>
<tr>
<td>Egr-2</td>
<td>Transcription factor</td>
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</tr>
<tr>
<td>Egr-3</td>
<td>Transcription factor</td>
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</tr>
<tr>
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<td>Transcription factor</td>
<td>3</td>
</tr>
<tr>
<td>Gos-3</td>
<td>Transcription factor</td>
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</tr>
<tr>
<td>Jun-D</td>
<td>Transcription factor</td>
<td>1</td>
</tr>
<tr>
<td>NGFi-B</td>
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</tr>
<tr>
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<tr>
<td>Gig-2</td>
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</tr>
<tr>
<td><strong>After 3 hours of m1 mAChR activation</strong></td>
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<td></td>
</tr>
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<td>Egr-1</td>
<td>Transcription factor</td>
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</tr>
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<tr>
<td>Gig-4</td>
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</tr>
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</table>
mACHRs regulate expression of hCyr61 and Gig-2

hCyr61, an immediate-early gene, is a member of the CCN gene family of CTGF (connective tissue growth factor), Cyr61 and Nov that encode secretory signalling factors [46]. Cyr61 binds to integrin $\alpha_v\beta_3$ at the cell surface [47], as well as to heparin-containing components of the extracellular matrix [48]. These interactions promote cell growth, adhesion, proliferation, chemotaxis and migration [49]. As a ligand of the integrin $\alpha_v\beta_3$, Cyr61 induces angiogenesis, neovascularization and tumour growth [50]. By activation of mACHRs, expression of hCyr61 was induced within 15 minutes with a maximum after 1 hour of stimulation [30].

Gig-2, a previously unknown gene, was found to be up-regulated by mACHR within 40 minutes of stimulation, and it attained a maximum within 60–100 minutes of stimulation. Receptor stimulation also triggered Gig-2 expression in presence of cycloheximide, indicating that Gig-2 is an immediate-early gene, and that receptor-coupled expression was independent of de-novo protein synthesis.

Both mACHR-overexpressing HEK-293 lines and primary cortical neurons responded to receptor stimulation with increased expression of both genes within 15 minutes, attained a maximum after 1 hour with sustained high expression for 4 hours. Increased expression of both hCyr61 and Gig-2 was coupled to mACHRs by protein kinase C, whereas cAMP failed to affect expression. In experiments in vitro, the muscarinic agonist pilocarpine strongly induced both hCyr61 and Gig-2 expression in neurons of several, but different, layers of the brain cortex, the hippocampal CA1 region and the putamen.

ACHE is an mACHR-regulated effector gene

Our studies show binding to, and activation of, Egr-promoter sequences followed by the synthesis of functional protein as a result of mACHR stimulation [31]. Egr-1 increases the promoter activity and gene expression of AChE, a serine hydrolase that catalyses the breakdown of ACh. Our data generated by using the AChE gene promoter fused to a luciferase reporter show that stimulation of m1 mACHR specifically increased AChE gene promoter activity [31]. Additional co-transfection experiments with the AChE gene promoter reporter construct together with a cytomegalovirus-driven expression plasmid for Egr-1 showed that overexpression of Egr-1 leads to an increase of transcription from the AChE gene promoter [31]. In order to analyse the transcription rate of AChE after mACHR activation, semi-quantitative reverse transcriptase-PCR experiments were performed with m1 HEK-293 cells stimulated by carbachol (Figure 1). By comparison, kinetic PCR experiments with primer combinations for GAPDH as a control revealed that m1 mACHR activation leads to a significant induction of AChE mRNA expression. Moreover, in vivo experiments using adult rats that were treated with the cholinergic immuno-toxin 192 IgG-saporin revealed a dramatic decrease of AChE activity in cortex, hippocampus, and in the cholinergic cell bodies in the medial septum [51]. If confirmed for the subcortical cholinergic projection system in the brain, EGR-dependent regulation of AChE transcription may be involved in a receptor-coupled feedback control of cholinergic transmission also in the post-
synaptic neurons. This would be an addition to the classical dogma that AChE is regulated in synaptic terminals of presynaptic neurons.

**mAChRs regulate genes for late-response proteins**

In order to identify additional mAChR-inducible target genes and late-response genes, we analysed, with DD, m1 HEK-293 cells 3 h after stimulation and identified 23 differential bands with 27 out of 192 possible primer combinations. Five of these revealed four distinct differentially expressed genes: Egr-1, LM04, and the two previously unknown genes Gig-3 and Gig-4 (Table 1). The differential band that corresponded to Egr-1 at this time was less intense than that after 1 hour of receptor activation. In contrast, Gig-4 expression was much stronger after 3 hours of receptor activation as compared to 1 hour (Figure 2). Similar results were revealed analysing time courses of gene expression of Gig-3 and LM04.

**mAChRs induce multiple genes**

Our studies show that the expression of many distinct genes is under the control of mAChRs. These results generate the hypothesis of mAChR regulation of gene expression (Figure 3). Activation of m1 AChRs induces the expression of two groups of immediate-early genes. These are: transcription factors including the Egr, Jun and Fos families, and early effector genes, including Cyr61 and Gig-2, that are not directly related to transcriptional regulation. The biological functions of early effector genes may be directly involved in such cellular responses as growth, adhesion and plasticity. Transcription fac-

![Figure 1](image-url)
tors induce a set of target genes that encode late effector proteins including AChE, LM04, Gig-3 and Gig-4. Both early and late effector proteins may act

Figure 2 m1 mAChRs increase mRNA expression of Gig-4. (a) Northern blot analysis of total RNA from unstimulated (control), carbachol-stimulated (hours of stimulation) m1 HEK-293 cells, and cells treated with atropine in parallel to carbachol. As compared to the GAPDH loading control, carbachol stimulation increased message levels of Gig-4 significantly after 3 hours of carbachol stimulation. Exposure time of the Gig-4 blot was 17 hours; exposure time of the GAPDH blot was 3 hours. (b) Quantification of Gig-4 mRNA level normalized with GAPDH mRNA level. Atr, Atropine; CCH, carbachol; Ctr., control.
together mediating such activity-dependent long-term cellular responses as neurite growth, synaptic plasticity, long-term potentiation, and memory and learning.

Cholinergic signalling in brains of Alzheimer’s disease patients is heavily impaired as a result of the early and massive degeneration of the long basal forebrain projection neurons to brain hippocampus and cortex. In as much as EGR-dependent genes in post-synaptic cholinergic target cells are regulated by mAChR activity, expression of such genes may be decreased in AD. Post-mortem studies are required to test this hypothesis. Drugs designed to activate mAChRs, including AChE inhibitors and m1 agonists currently tested in clinical trials for the treatment of AD, may be expected to stimulate transcription of
Egr genes along with EGR-dependent target genes. *In vivo* studies are required to test whether pharmacological treatments designed to stimulate brain mAChRs increase AChE gene expression, along with AChE enzyme activity, and accelerated breakdown of ACh.

**References**