In brain, muscarinic acetylcholine receptors (mAChRs) modulate neuronal functions including long term potentiation and synaptic plasticity in neuronal circuits that are involved in learning and memory formation. To identify mAChR-inducible genes, we used a differential display approach and found that mAChRs rapidly induced transcription of the immediate early gene CYR61 in HEK 293 cells with a maximum expression after 1 h of receptor stimulation. CYR61 is a member of the emerging CCN gene family that includes CYR61/CEF10, CTGF/FISP-12, and NOV; these encode secretory growth regulatory proteins with distinct functions in cell proliferation, migration, adhesion, and survival. We found that CYR61, CTGF, and NOV were expressed throughout the human central nervous system. Stimulation of mAChRs induced CYR61 expression in primary neurons and rat brain where CYR61 mRNA was detected in cortical layers V and VI and in thalamic nuclei. In contrast, CTGF and NOV expression was not altered by mAChRs neither in neuronal tissue culture nor rat brain. Receptor subtype analyses demonstrated that m1 and m3 mAChR subtypes strongly induced CYR61 expression, whereas m2 and m4 mAChRs had only subtle effects. Increased CYR61 expression was coupled to mAChRs by both protein kinase C and elevations of intracellular Ca\(^{2+}\). Our results establish that CYR61 expression in mammalian brain is under the control of cholinergic neurotransmission; it may thus be involved in cholinergic regulation of synaptic plasticity.

The cholinergic system in mammalian brain is involved in higher cognitive functions including attention, learning, and memory (1). A major source of the neurotransmitter acetylcholine are cholinergic neurons of the basal forebrain that innervate neurons throughout the cerebral cortex, the hippocampus, the amygdala, and some thalamic nuclei (2, 3). The degeneration of cholinergic neurons in the basal forebrain is associated with Alzheimer’s disease; lesions within this brain region, as well as mAChR1 antagonists, impair cognitive functions in mammals (4–7). Muscarinic AChRs are G protein-coupled cell surface receptors with seven transmembrane topologies (8). Five different subtypes are expressed in different brain regions on pre- and postsynaptic neuronal compartments (9). They have excitatory and inhibitory effects on cholinergic synapses by modulating the conductance of K\(^{+}\) and Ca\(^{2+}\) ion channels (10–12) and by coupling to several intracellular second messengers as follows: mAChR subtypes m1, m3, and m5 activate protein kinase C (PKC) (13, 14), increase intracellular Ca\(^{2+}\) and cAMP (13–15), stimulate the MAP kinase pathway (16, 17), and activate the phospholipases A2 (18) and D (19). In contrast, m2 and m4 mAChRs inhibit adenyl cyclase (13, 14, 16, 17, 20, 21).

The storage of long term memory is associated with the generation of long term potentiation and with changes in synaptic plasticity that are mediated by alterations in the expression of activity-dependent genes (22). These include immediate early transcription factors and activity-dependent genes with potential functions in modulating long term memory by structural modifications of preexisting synapses, in the generation of new neuronal connections, as well as in neurite extension and dendritic ramification (23–27). Cholinergic mechanisms are involved in learning and memory processes in that mAChRs promote the generation of long term potentiation (28) and activate the expression of the transcription factors c-fos, jun-B, Egr-1, Egr-2, Egr-3, and Egr-4 (29, 30).

To identify mAChR-inducible genes, we established a differential display screen of genes activated in response to mAChR stimulation with the acetylcholine analog carbachol. We identified the immediate early gene CYR61 that encodes an extracellular signaling molecule to be differentially regulated by mAChRs. CYR61 belongs to the emerging CCN gene family (CYR61/CEF10, CTGF/FISP-12, NOV, ELM-1, COP-1, and WISP-3) of secreted growth regulatory proteins that bind to components of the extracellular matrix and the cell surface (31, 32). CYR61 and CTGF (connective tissue growth factor) are both growth factor-inducible immediate early genes in fibroblasts (33, 34), whereas the protooncogene NOV (nephroblastoma overexpressed), involved in Wilms tumor, is expressed in...
quiescent cells and down-regulated after serum stimulation (35, 36). Consistent with their expression kinetics, secreted CYR61 and CTGF proteins exhibit growth-promoting cellular functions (37, 38), whereas the overexpression of NOV protein has growth-inhibitory effects and is associated with normal differentiation of the central nervous system and kidney in general (35, 39, 40). CTGF gene is a downstream target of TGF-β, mediating TGF-β-related actions in connective tissue cells during wound healing and skin disorders (42, 43, 44), and both proteins, CYR61 and CTGF, are regulators of angiogenesis, mediating cellular adhesion and migration of endothelial cells in an integrin-αβ-dependent manner (43, 44). Even though CYR61, CTGF, and NOV are expressed in the developing nervous system (40, 45), less is known about their function in adult brain.

We provide evidence that CYR61 and its family members CTGF and NOV are expressed throughout the mammalian brain and that transcription of CYR61, but not that of CTGF and NOV, is under the control of mAChR signaling in primary cortical neurons and in mammalian brain.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture and Drugs—**Wild-type HEK 293 cells and 293 cells stably transfected with muscarinic acetylcholine receptor subtypes m1, m2, m3, or m4 (13) were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 supplemented with 0.5% B27 (Invitrogen) and 1% Glutamax (Life Technologies, Inc.). Dissociated cells were suspended in 10% horse serum and a fire-polished Pasteur pipette in small volumes of Hanks’ balanced salt solution. Dissociated cells were cultured in parallel from the same passages, were used as controls. Effective receptor stimulation and transcriptional response were verified by using carbachol-stimulated (1 h) HEK 293 m1AChRs cells. The forward primer EMHP (5′ CCAAACCGGGGAAAGTTTCCAGGC3′) derived from the partial human sequence A33210 (EMBL accession number Z50168) that showed homology to the 5′ end of murine cyr61. The reverse primer EMHR (5′ TTTCCAGTTTACCTTCCCTCC3′) was homolog to the 3′ end of h-CYR61 identified by our differential display experiment. The PCR products were separated by agarose gel electrophoresis, purified, and cloned into the SWaI restriction site of pBluescript KS (Stratagene). The cloned PCR product was sequenced, and a putative human cDNA probe featuring the ZAP Express protocol (Stratagene).

**Human CYR61, CTGF, and NOV cDNA Fragments for Northern Blots and Library Screening—**We amplified a 1403-bp h-CYR61 cDNA fragment by using cDNA from carbachol-stimulated (1 h) HEK 293 m1AChRs cells. The forward primer EMHP (5′ CCAAACCGGGGAAAGTTTCCAGGC3′) derived from the partial human sequence A33210 (EMBL accession number Z50168) that showed homology to the 5′ end of murine cyr61. The reverse primer EMHR (5′ TTTCCAGTTTACCTTCCCTCC3′) was homolog to the 3′ end of h-CYR61 identified by our differential display experiment. The PCR products were separated by agarose gel electrophoresis, purified, and cloned into the SwaI restriction site of pBluescript KS (Stratagene). The cloned PCR product was sequenced, and a putative human cDNA probe featuring the ZAP Express protocol (Stratagene).

**RATIONALE**

**RNA Preparation and Reverse Transcription—**Total RNA was prepared by using either the RNAeasy Kit (Qiagen) or Trizol reagent (Life Technologies, Inc.) following the manufacturer’s instructions. RNA was stored at −80°C.

The RNA preparations from fetal rat lung (P1), adult rat thyroid, spleen, and brain were treated with DNase I (Roche Molecular Biochemicals) together with RNasin (Promega) for 30 min, followed by phenol protein extraction, and ethanol precipitation. 0.2 µg of RNA preparation was transcribed to cDNA by using the SuperScript™II Reverse Transcriptase (Life Technologies, Inc.) with equal amounts of one base anchor primers HT7,1 (5′ TGCCGAAGCAGCTTTTTTTTTTTTTTT3′), HT2,1 (5′ TGCCGAAGCAGCTTTTTTTTTTTTTTT3′), and HT7,2 (5′ TGCCGAAGCTTTTTTTTTTTTTTTTTTTT3′).

**Molecular Cloning**

**Northern Blotting—**5–10 µg of total RNA were separated in a formaldehyde-containing agarose gels, and the RNA was blotted onto nylon membranes (Hybond-N*, Amersham Pharmacia Biotech). Membrane-bound RNA was hybridized with labeled cDNA probes that were generated by using the Megaprime DNA labeling kit (Amersham Pharmacia Biotech). Membranes were washed under high stringent conditions, and X-ray films were exposed for 1–72 h. To control for equal loading of RNA, the identical membranes were probed with a 700-bp cDNA fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or with a β-actin cDNA fragment (CLONTECH).

**Construction of a m1 mAChr-stimulated ZAP Express cDNA Library—**HEK 293 m1AChRs cells were cultured with carbachol for 1 h, and poly(A+)- mRNA was prepared from total RNA by using the Poly(A)Tract IV kit (Promega). cDNA synthesis from poly(A+) mRNA was performed following a modified ZAP-cDNA synthesis protocol (Stratagene), and cDNA clones into zAP Express vector were done according to the manufacturer’s instructions (Stratagene). A library screen of 4 × 10⁶ clones was performed with a radiolabeled human CYR61 cDNA probe following the ZAP Express protocol (Stratagene).

**Human CYR61, CTGF, and NOV cDNA Fragments for Northern Blots and Library Screening—**We amplified a 1405-bp h-CYR61 cDNA fragment by using cDNA from carbachol-stimulated (1 h) HEK 293 m1AChRs cells. The forward primer EMHP (5′ CCAAACCGGGGAAAGTTTCCAGGC3′) derived from the partial human sequence A33210 (EMBL accession number Z50168) that showed homology to the 5′ end of murine cyr61. The reverse primer EMHR (5′ TTTCCAGTTTACCTTCCCTCC3′) was homolog to the 3′ end of h-CYR61 identified by our differential display experiment. The PCR products were separated by agarose gel electrophoresis, purified, and cloned into the SwaI restriction site of pBluescript KS (Stratagene). The cloned PCR product was sequenced, and a putative human cDNA probe featuring the ZAP Express protocol (Stratagene).

**In Vivo Experiments—**Adult male Harlan Sprague-Dawley rats were

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injected intraperitoneally with the mAChR agonist pilocarpine (25 mg/kg; Sigma) or with the vehicle PBS as an injection control. Animal behavior was monitored until the animals were decapitated 75 min after drug application. Brains were rapidly dissected and frozen on dry ice.

**In Situ Hybridization—**Rat CYR61 (nt 928–1532), r-CTGF (nt 644–1252), r-NOV (nt 294–1280) cDNA fragments were subcloned into pCR-BluntII-TOPO vector (Invitrogen). Plasmid DNA was digested by restriction enzymes BamHI or XhoI, separated by agarose gel electrophoresis, purified, and subjected to phenol protein extraction and ethanol precipitation. Linearized plasmid DNA was used as template to generate radiolabeled sense and antisense cRNA probes by using either T7 or SP6 RNA polymerase (Roche Molecular Biochemicals) together with [α-35S]UTP (NEN Life Science Products), GTP, ATP, and CTP, dithiothreitol, and RNase Out (Roche Molecular Biochemicals) for 2 h at 37 °C. Unincorporated nucleotides were removed by ProbeQuant G50 gel filtration (Amersham Pharmacia Biotech). Rat brains were cut on a Leica cryostat microtome in 16-μm slices, thawed on glass slides, and stored at −80 °C before use. The slices were fixed in 4% paraformaldehyde, acetylated, and prehybridized for 3 h at 55 °C with prehybridization buffer (50% formamide, 5× hybridization salts, 5× Denhardt’s, 0.2% SDS, 10 μg/ml dithiothreitol, 250 μg/ml salmon sperm DNA (Stratagene), 250 μg/ml yeast tRNA (Roche Molecular Biochemicals)) in a humidified chamber. Hybridization was performed for 18 h at 55 °C with radiolabeled cRNA probes (5000 cpn/μl) in prehybridization buffer containing 10% dextran sulfate. The slices were dehydrated, washed under non-stringent conditions in 4× SSC at room temperature, followed by RNase A treatment. Two stringent wash steps were performed in 2× SSC for 10 min at 55 °C. The slices were dehydrated, and an x-ray film (Kodak Biomax MR) was exposed for 3–14 days. Emulsion dips were prepared with NTB-2 photoemulsion (Kodak), exposed for 1–4 weeks, developed, and fixed. Sections were counterstained with Mayer’s Hemalaun (Merck) (51).

**RESULTS**

**Identification of Human CYR61 by Differential Display—**By using a differential display screen of mRNAs expressed in response to stimulation of m1 mAChR expressing HEK 293 cells, we identified two redundant bands of 450 and 540 bp in length, which were absent in parallel fractions of unstimulated cells (data not shown). Either band contained cDNA corresponding to human CYR61 (h-CYR61), a homolog of the murine growth factor-inducible immediate early gene cyr61 (33). To verify that mAChRs are coupled to the transcriptional activation of the h-CYR61 gene, we compared RNA from m1 mAChR-expressing cells that were stimulated with carbachol for different incubation times with unstimulated m1 mAChR-expressing cells. Northern blot analysis showed no detectable levels of h-CYR61 mRNA in unstimulated cells; h-CYR61 signal increased 15 min after stimulation, attained a maximum within 50–60 min, and decreased to lower but higher than control levels until 240 min after stimulation (Fig. 1A). An increase of h-CYR61 expression was also detectable in HEK 293 m1 cells that were treated with carbachol in the presence of the protein synthesis inhibitor cycloheximide (Fig. 1A).

**Expression of CYR61, CTGF, and NOV in Human Brain—**Our data demonstrate that carbachol stimulation rapidly increased h-CYR61 expression in a time-dependent manner. A Northern blot containing total RNA (5 μg per lane) from carbachol-treated (minutes of stimulation) and untreated m1 mAChR-expressing HEK 293 cells was probed with a radiolabeled 1403-bp h-CYR61 cDNA fragment. h-CYR61 stimulation increased h-CYR61 expression in the presence of cycloheximide. A Northern blot containing total RNA (5 μg per lane) from HEK 293 m1 mAChR cells that were treated for 1 h with carbachol alone or together with the protein synthesis inhibitor cycloheximide was probed with a radiolabeled 1403-bp h-CYR61 cDNA fragment. Cycloheximide was applied to the cells 15 min before carbachol treatment. To control for equal RNA loading, the blots were probed with a specific GAPDH cDNA. ctr, untreated HEK 293 m1 mAChR cells; CCh, carbachol; Chx, cycloheximide. X-ray film exposed to Northern blot: h-CYR61, 48 h; GAPDH, 1 h; kb, kilobase pairs.

**FIG. 1.** m1 mAChRs stimulation induced the expression of the immediate early gene h-CYR61, A, m1 mAChR stimulation rapidly increased h-CYR61 expression in a time-dependent manner. A Northern blot containing total RNA (5 μg per lane) from carbachol-treated (minutes of stimulation) and untreated m1 mAChR-expressing HEK 293 cells was probed with a radiolabeled 1403-bp h-CYR61 cDNA fragment. B, m1 mAChR stimulation increased h-CYR61 expression in the presence of cycloheximide. A Northern blot containing total RNA (5 μg per lane) from HEK 293 m1 mAChR cells that were treated for 1 h with carbachol alone or together with the protein synthesis inhibitor cycloheximide was probed with a radiolabeled 1403-bp h-CYR61 cDNA fragment. Cycloheximide was applied to the cells 15 min before carbachol treatment. To control for equal RNA loading, the blots were probed with a specific GAPDH cDNA. ctr, untreated HEK 293 m1 mAChR cells; CCh, carbachol; Chx, cycloheximide. X-ray film exposed to Northern blot: h-CYR61, 48 h; GAPDH, 1 h; kb, kilobase pairs.
Muscarinic AChRs Induce CYR61 Expression in Primary Neurons—To investigate r-CYR61, r-CTGF, and NOVR expression in neuronal cells that endogenously express mACHRs, we stimulated a mixed culture of primary cortical and hippocampal neurons from embryonic (E18) rat brains with carbachol for 1 or 3 h (Fig. 3A). We found all three genes to be constitutively expressed. The stimulation with carbachol for 1 h increased basal r-CYR61 mRNA levels 3.5-fold, and this expression was not influenced by mAChR stimulation, nor after 1 or 3 h of carbachol treatment (Fig. 3A and B). The inhibition of m2 mAChRs with the m2-antagonist gallamine together with carbachol failed to block the carbachol-induced r-CYR61 expression (data not shown).

mAChR-dependent Regulation of CYR61 in the Mammalian Brain—In the mammalian brain, neuronal cells in the cortex, the hippocampus, and some thalamic nuclei receive cholinergic input from the basal forebrain (2, 3). To investigate whether mACHR regulate CYR61 expression in vivo, the mACHR agonist pilocarpine was administered to adult rats and brains were prepared 75 min later. Brains of non-injected and PBS-injected animals were used as controls. In situ hybridization analyses of cryostat slices of forebrain tissue from control and pilocarpine-treated animals demonstrated that expression of r-CYR61, but not r-CTGF or NOVR readily increased in response to mAChR stimulation (Fig. 4A). In both non-injected and PBS-injected control animals, we detected a very low basal expression of r-CYR61 that increased after pilocarpine treatment in cortical layer VI as well as in some cells of cortical layer V and in thalamic nuclei (Fig. 4, A and B). Rat CTGF mRNA was predominantly expressed in cortical layer VI in both control and pilocarpine-treated rats (Fig. 4A). Comparable to r-CTGF, NOVR expression was not altered by mAChR stimulation. NOVR mRNA was detected in cortical layers II/III, V, and VI, the pyramidal cell CA1-CA3 hippocampal regions, and the amygdala (Fig. 4A).

Muscarinic AChR Subtypes m1–m4 Induced Transcription of Human CYR61—To determine which mAChR subtype can couple to h-CYR61 expression, we analyzed Northern blots of RNA obtained from carbachol-treated HEK 293 cells stably expressing m1, m2, m3, or m4 mACHRs as well as untransfected wild type cells. h-CYR61 expression was differentially affected by these receptor subtypes (Fig. 5A). m1 and m3 mACHRs increased cellular levels of h-CYR61 mRNA 5–10 times as compared with unstimulated control cells (Fig. 5B). In contrast, m2 and m4 mACHRs-expressing cells stimulated h-CYR61 expression only weakly. In carbachol-stimulated as well as in unstimulated wild type HEK 293 cells minimal h-CYR61 signals were detected on Northern blots.

Multiple Internal and External Signals Coupled CYR61 Expression to mACHRs in HEK 293 Cells—To determine the signal transduction pathways that couple CYR61 induction to mACHRs, we treated HEK 293 m1 mACHRs cells with activa-
tors or inhibitors of known signaling cascades. Alternatively, cells were treated with the mAChR agonist carbachol together with pharmacological inhibitors. Northern blots of RNA derived from these cells were hybridized with a h-CYR61 cDNA probe (Fig. 6A). The muscarinic antagonist atropine blocked the carbachol-induced increase in cellular levels of h-CYR61 mRNA in HEK 293 m1 cells. Stimulation of PKC with the phorbol ester phorbol myristate acetate increased h-CYR61 expression 3.0-fold (Fig. 6B), and this increase was blocked by the PKC inhibitor GF109203X along with carbachol stimulation, however, still led to transcriptional activation of h-CYR61 but to lower magnitudes as compared with carbachol alone. These results demonstrate that stimulation of PKC is sufficient but not necessary to induce h-CYR61 expression. In contrast, the activation of protein kinase A (PKA) by 8-bromo-cAMP failed to induce h-CYR61 transcription, and the PKA inhibitor H7 failed to block the carbachol-induced increase in CYR61 message. Moreover, activation of phospholipase A2 by mellitin increased h-CYR61 expression, and this effect was slightly decreased by the phospholipase A2 inhibitor DEDA, but DEDA failed to block the carbachol-induced increase. Both the increase of intracellular Ca2+ levels by ionomycin as well as serum induced h-CYR61 expression.

DISCUSSION

In brain, mAChR are associated with long term memory formation that depends on changes in synaptic plasticity, including modifications of preexisting synapses and the generation of new neuronal connections. These synaptic modifications are mediated by alterations in the expression of activity-dependent genes. Muscarinic AChR induce the expression of immediate early gene transcription factors c-fos, jun-B, Egr-1/ Krox20, and Egr-2/ Krox24 (53), and a variety of activity-dependent genes are known that proteins directly influence synaptic plasticity, like growth factors (β-activin, Ref. 54) and intracellular signaling molecules (Rheb, Ref. 55), as well as cytoskeletal-associated (Arc; Ref. 56), and secreted proteins that modify the extracellular matrix (tPA; Ref. 57) or promote neurite outgrowth (Neurtin; Ref. 58). The members of the CCN family CYR61, CTGF, and NOV are secreted extracellular signaling molecules with different functions in cellular growth, adhesion, migration, and survival in peripheral tissues (32), but their function in brain is still unknown. The results of this study show that CYR61, CTGF, and NOV were expressed throughout the human central nervous system. Among these three family members, the immediate early gene CYR61 was unique in that its expression was regulated by mAChR activity, both in primary cortical neurons and in adult rat brain.
Consistent with the fact that CYR61, CTGF, and NOV are expressed during development of the central nervous system (40, 45), we found basal levels of message for these three genes in primary cortical neurons prepared from E18 embryonic brains. Carbachol-induced stimulation of r-CYR61 of endogenously expressed mAChRs in primary cortical neurons was atropine-sensitive, indicating that muscarinic receptors mediated the activation of r-CYR61 expression. The failure of the m2-antagonist gallamine to block this response indicated that it was primarily mediated by m1, and possibly by m3 mAChRs. Receptor subtype analyses in HEK 293 cells underscored that finding in that m1 and m3 rather than m2, and m4 mAChR-mediated signaling induced the expression of CYR61. The G11-coupled m1 and m3 receptors strongly induced h-CYR61 expression. In contrast, the G/G coupling m2 and m4 mAChRs only lead to a very subtle induction of h-CYR61 that was still higher, however, than the absent response in untransfected control cells. This subtle increase may be related to the weak stimulation of PKC coupled to m2 and m4 mAChRs (14, 20). Consistent with our finding that mainly m1 and m3 mAChR subtypes induce CYR61 expression in receptor-transfected cells, we found two principal signaling mechanisms that may couple h-CYR61 expression to mAChR activation, PKC and Ca2+. These signal transduction mediators independently increased cellular levels of h-CYR61 mRNA, but our data do not exclude the possibility of cross-talk among them. Recent studies show that the expression of CYR61 is activated during the basic fibroblast growth factor-induced differentiation process in the embryonic hippocampal neuronal cell line H19–7 in a MAP kinase-dependent and -independent manner (61). In addition to PKC and Ca2+, muscarinic m1 receptors are known to activate MAP kinase signaling (62, 63) strongly suggesting that MAP kinase signaling may couple muscarinic receptor stimulation to CYR61 expression. To exclude that mAChR-dependent increases in cAMP or PKA regulate h-CYR61 expression, we treated cells with 8-bromo-cAMP, and we found that it failed to change basal levels of h-CYR61 mRNA. Likewise, inhibition of PKA with H7 failed to block the carbachol-induced stimulation of h-CYR61 expression. These data argue against a significant role of cAMP-PKA-CREB1 signaling in coupling h-CYR61 expression to mAChR in HEK 293 cells. Taken together our data are compatible with the concept that mAChRs induce h-CYR61 expression via PKC, MAP kinase, and Ca2+ signaling.

CYR61 and CTGF are both immediate early genes, but the expression of r-CTGF was unchanged in response to mAChR stimulation in primary neurons. The CTGF promoter contains consensus sequences that are characteristic for growth factor-inducible genes as well as a TGF-β response element, but different promoters have been postulated for CTGF in different tissues (34, 64). Our data indicate that mAChR-related signaling do not regulate r-CTGF expression. NOV is expressed in quiescent cells and known to be down-regulated after serum stimulation in chicken embryo fibroblasts (36). Even though this appears in a PKC-dependent manner, we could not detect any down-regulation of NOVR after either 1 or 3 h of mAChR stimulation.

To determine whether mAChRs stimulate the expression of r-CYR61, r-CTGF, and NOVR in vivo, we analyzed forebrain regions of pilocarpine- and vehicle-treated rats and found that our data from primary neurons were confirmed by the in vivo situation. Whereas r-CYR61 was rarely detectable in unstimulated rat brain, the stimulation of mAChRs strongly increased r-CYR61 mRNA levels in cortical layers V, VI, and in thalamic nuclei. This special pattern is consistent with the innervation of the cortex by cholinergic neurons from the basal forebrain.
and with the known expression pattern of m1 and m3 mAChRs throughout the cortex, predominantly in cortical layer VI (2, 3, 65, 66). Moreover, some thalamic nuclei are innervated by cholinergic neurons from the midbrain and nucleus basalis Meynert (2). In rat brain, mAChRs induce the expression of the immediate early genes c-fos, jun-B, and Egr-1/Krox24 in cortical layers IV, VI, and the hippocampus (53). Even though r-CYR61 revealed basal expression in hippocampus, the expression was not altered in response to mACHR stimulation in this brain region.

In contrast, r-CTGF message was predominantly present in the cortical layer VI, in both control and stimulated animals. Throughout the central nervous system, CTGF protein is found in astrocytes, as well as in neuronal cells of the cortex, where it supposedly mediates TGF-β-related functions in cell growth, development, and tissue remodeling following injury (67, 68).

**NOVr** was expressed in cortical layers II/III, V, and VI, in the CA1–CA3 hippocampus region and the amygdala, which are major parts of the basal forebrain cholinergic system, but its expression did not change in response to mAChR stimulation.

NOV is expressed during development of the central nervous system, and its protein is supposed to have important functions in the maintenance of differentiated neurons (40, 69). Interestingly, we found r-CYR61, r-CTGF, and NOVR to be expressed in cells of cortical layer VI near corpus callosum. Kondo et al. (67) detected CTGF protein in rat brain in cortical layers III and V, and Su et al. (40) localized NOV mRNA in cortical layers V and VI of 38-week human embryos. Efferents and afferents of neurons in cortical layer VI project to other cortical layers. Therefore, differences between mRNA and protein localization especially of CTGF may be related to neuronal protein transport rather than diffusion of the secreted proteins. In addition to that affecteds from pyramidal cells in cortical layer VI innervate the thalamus (70).

Our data suggest that CYR61 protein has a role in mediating mAChR-related alterations in synaptic plasticity. CYR61 encodes a secretory protein with functions in extracellular signaling; it binds to integrin αβ1 and αβ3, at the cell surface, as well as to heparin-containing components of the extracellular matrix (71–73). These interactions promote cell proliferation, adhesion, chemotaxis, and migration (37). If CYR61 has similar functions in brain cells, our studies suggest the possibility that it regulates the expression of extracellular matrix components. CYR61 may act, in concert with integrins, as a chemotactic factor that influences neurite outgrowth in response to a stimulating acetylcholine signal. This possibility is underscored by the known functions of brain integrins in neuronal differentiation, migration, neurite guidance, and long term potentiation (74–77). CTGF has CYR61-related functions; in addition to its regulation of extracellular matrix (71–73). These interactions promote cell proliferation, adhesion, chemotaxis, and migration (37).

Muscarinic signaling in cortex and hippocampus is impaired in Alzheimer’s disease, whereas binding of ligands to mAChRs appears to be largely intact (4, 5). This led to the development of cholinomimetic drugs and to the use of several acetylcholine esterase inhibitors for the therapy of Alzheimer’s disease (78).

Future studies are required to show whether h-CYR61 expression in brain is altered in response to the degeneration of subcortical projection neurons in Alzheimer’s disease or other neurodegenerative diseases, and whether treatments with cholinomimic-meets Our work reveals the potential of CYR61 for the therapy of Alzheimer's disease (78).
Muscarinic Receptors Induce CYR61 Expression

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J. Biol. Chem. 2000, 275:28929-28936.
doi: 10.1074/jbc.M003053200 originally published online June 13, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M003053200

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