

# Coincidence Signaling of Dopamine D<sub>1</sub>-Like and M<sub>1</sub> Muscarinic Receptors in the Regulation of Cyclic AMP Formation and CREB Phosphorylation in Mouse Prefrontal Cortex

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## Key Words

M<sub>1</sub> muscarinic receptors • Dopamine D<sub>1</sub>-like receptors • G $\beta\gamma$ -stimulated adenylyl cyclase isoforms • CREB phosphorylation • Mouse prefrontal cortex

## Abstract

In the prefrontal cortex, dopamine D<sub>1</sub>-like and M<sub>1</sub> muscarinic receptors are both involved in the regulation of attentional, cognitive and emotional processes but so far no information has been provided on their functional interaction. In the present study we show that in mouse medial prefrontal cortex, concomitant activation of M<sub>1</sub> muscarinic receptors potentiated D<sub>1</sub>-like receptor-induced cyclic AMP formation through a mechanism involving activation of G<sub>q/11</sub> and the release of G protein  $\beta\gamma$  subunits. Immunohistochemical studies indicated that the adenylyl cyclase isoforms AC2 and AC4 are expressed in mouse prefrontal cortex and that they colocalize with D<sub>1</sub>-like receptors with a greater association for AC4. In primary cultures of frontal cortex neurons, D<sub>1</sub>-like receptor-induced Ser133 phosphorylation of the transcription factor cyclic AMP-responsive element binding protein (CREB) was potentiated by concurrent stimulation of M<sub>1</sub> receptors. Suppression of AC4 expression with small interfering RNA transfection reduced D<sub>1</sub> stimulation of cyclic AMP formation and CREB phosphorylation and abolished the M<sub>1</sub> potentiation, whereas knockdown of AC2 had no significant

effects. These data indicate that in mouse prefrontal cortex G<sub>q/11</sub>-coupled M<sub>1</sub> receptor and G<sub>s</sub>-coupled D<sub>1</sub>-like receptor inputs converge on AC4 with a consequent enhancement of cyclic AMP formation and signaling to the nucleus.

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## Introduction

In the cerebral cortex both dopamine (DA) and acetylcholine (ACh) muscarinic receptors are involved in the control of cognitive functions and alterations of their expression and/or activity have been implicated in the pathogenesis of cognitive deficits associated with different neuropsychiatric diseases. In the prefrontal cortex, DA released from terminals of the mesocortical pathway acts on two receptor populations: the D<sub>1</sub>-like receptors, including D<sub>1</sub> and D<sub>5</sub> subtypes, and the D<sub>2</sub>-like receptors, comprising the D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> subtypes [1, 2]. Both receptor populations have been shown to be expressed on cortical pyramidal and nonpyramidal cells [3–5] and to be involved in DA regulation of recurrent excitation of prefrontal cortex neuronal circuits, which is thought to underlie the retention of information in working memory [2]. Particularly, an optimal DA D<sub>1</sub>-like receptor activity has been shown to be required for working memory function [6, 7]. At the cellular level, DA D<sub>1</sub>-like receptor ago-

nists regulate neuronal excitability and different forms of synaptic plasticity, such as long-term potentiation, a cellular correlate of long-term memory [8]. While DA D<sub>2</sub>-like receptors are coupled to inhibition of adenylyl cyclase (AC) activity [9], DA D<sub>1</sub>-like receptors transduce their signals via the stimulatory G protein G<sub>s</sub>, which activates AC leading to enhanced production of cyclic AMP and activation of protein kinase A (PKA) [10, 11]. The cyclic AMP/PKA pathway increases the phosphorylation state and activity of a variety of signaling molecules, including different ion channels, GABA<sub>A</sub> receptors and ionotropic glutamate receptors [10, 11]. Moreover, DA D<sub>1</sub>-like receptors trigger the phosphorylation on Ser133 and activation of the transcription factor cyclic AMP-responsive element binding protein (CREB), which is required for memory, mood regulation and addiction [12–14]. A hypofunctioning of the D<sub>1</sub> receptor/cyclic AMP/PKA pathway causes cognitive deficits and may underlie the cognitive dysfunction observed in aging, schizophrenia and hyperactivity/attention deficit disorder [15–17].

Among the different muscarinic receptor subtypes, the M<sub>1</sub> receptor is currently considered one of the major players in the cholinergic control of cognitive and attentional processes. Behavioral studies have shown that M<sub>1</sub> null mutant mice display selective cognitive dysfunctions, indicating that the M<sub>1</sub> receptor is specifically involved in memory processes requiring cortical and hippocampal interaction [18]. A blockade of prefrontal cortex muscarinic receptors by the local infusion of the M<sub>1</sub>/M<sub>4</sub>-preferring antagonist pirenzepine impaired spontaneous working memory in mice, whereas the M<sub>1</sub>-preferring agonist McN-A-343 had opposite effects [19]. Radioligand binding studies in postmortem brain and in vivo SPECT analysis have demonstrated that in schizophrenic patients there is a selective decrease of M<sub>1</sub> receptor density [20, 21]. Moreover, epidemiological studies have observed an association between the c.267C/C genotype at the c.267A/C polymorphism in the M<sub>1</sub> receptor gene with more severe cognitive deficits in schizophrenia, suggesting that a variation in the M<sub>1</sub> receptor sequence may contribute to the core symptom of this disorder [22]. Finally, clinical studies have shown that M<sub>1</sub> receptor agonists display beneficial effects in treating Alzheimer's dementia and exhibit promising efficacy against the cognitive deficits of schizophrenia [23–25]. At the molecular level, the M<sub>1</sub> receptor subtype is known to be preferentially coupled to the G proteins G<sub>q/11</sub> and to regulate phospholipase C $\beta$ , intracellular Ca<sup>2+</sup> mobilization and protein kinase C [26]. However, because of the limited availability of selective ligands, the signaling mechanisms mediating the procog-

nitve effects of the M<sub>1</sub> receptor are still largely unknown. Moreover, despite several pharmacological studies indicating an interaction between cortical DA and ACh [27–29], a functional interplay between DA D<sub>1</sub>-like and M<sub>1</sub> muscarinic receptors in controlling cyclic AMP signaling in the prefrontal cortex has not yet been described.

In the present study we report the occurrence in mouse prefrontal cortex of a coincidence signaling mechanism involving a specific form of AC, whereby concomitant activation of M<sub>1</sub> muscarinic receptors increases DA D<sub>1</sub>-like stimulation of cyclic AMP formation and CREB phosphorylation.

## Materials and Methods

### Materials

[ $\alpha$ -<sup>32</sup>P]ATP (30–40 Ci/mmol) and [2,8-<sup>3</sup>H]cyclic AMP (25 Ci/mmol) were obtained from Perkin Elmer (Boston, Mass., USA). SKF 81297 (6-chloro-2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine) hydrobromide was obtained from Tocris Bioscience (Bristol, UK), and SCH 23390 [R-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol] maleate from Schering Plough (Bloomfield, N.J., USA). Hydrophilic and detergent-free  $\beta\gamma$  subunits of transducin ( $\beta\gamma_t$ ) and the GDP-bound form of the  $\alpha$  subunit of transducin ( $\alpha_{tGDP}$ ) were purified from bovine retina and generously provided by Prof. Heidi E. Hamm. The purity of the transducin subunit preparations was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining as described in the online supplementary material (for all online supplementary material, see [www.karger.com/doi/10.1159/000335208](http://www.karger.com/doi/10.1159/000335208)). The compound YM 254890 was kindly provided by Dr. M. Yamasaki. The muscarinic toxin MT-7 and himbacine were generous gifts from Dr. E. Karlsson and Dr. W.C. Taylor, respectively. Carbachol (CCh), [( $\pm$ )-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine] hydrobromide [( $\pm$ )-APB], DA, oxotremorine-M (Oxo-M) and the other reagents were from Sigma Aldrich (St. Louis, Mo., USA).

### Tissue Dissection and Membrane Preparation

Male CD-1 mice (25–30 g) were obtained from Harlan Laboratories (S. Pietro al Natisone, Udine, Italy). Animals were housed in polypropylene cages in a temperature-controlled room (22  $\pm$  1°C) and maintained on a 12-hour light/dark cycle with food and water ad libitum. Experiments were performed according to the principles of laboratory animal care established by the European Communities Council Directive (86/609/EEC) and conformed to University of Cagliari guidelines on the ethical use of animals. Following light ether anesthesia, the mice were euthanized by cervical dislocation and the brains were rapidly excised from the cranial cavity and immersed in ice-cold phosphate-buffered saline (PBS). The brains were placed on the dorsal surface, the olfactory bulbs and the olfactory tracts were removed and, with the use of a tissue slicer, 300- $\mu$ m coronal slices were prepared starting from the frontal pole. Each tissue slice was immediately transferred into ice-cold PBS and kept in this medium until dissection. Six

coronal slices were obtained from each animal. Individual slices, starting from 2.60 mm anterior to the bregma according to the mouse brain atlas of Franklin and Paxinos [30], were transferred to a cold glass slide and, by using a dissecting microscope with a diascope illuminator base (Nikon type 102), the medial prefrontal cortex was dissected from each brain hemisphere by making a cut with small dissecting knives. Landmarks used for dissections were the rhinal fissure, the anterior commissure and nucleus accumbens, the lateral ventricle and the forceps minor of corpus callosum. The dissections included the infralimbic, prelimbic, dorsal and ventral anterior cingulate cortex and the medial cortical area.

Tissue fragments from individual slices were pooled and immediately homogenized in an ice-cold buffer containing 10 mM Hepes-NaOH, 1 mM EGTA, 1 mM MgCl<sub>2</sub> (pH 7.40) using a Teflon-glass tissue grinder. The homogenate was diluted with the same medium and centrifuged at 27,000 g for 20 min at 4°C. The pellet was resuspended in the same buffer and centrifuged as above. The final pellet was resuspended to a protein concentration of 0.5–0.7 mg/ml and the tissue preparation was used immediately for the AC assay.

Protein concentration was determined by Bradford's method [31] using bovine serum albumin (BSA) as a standard.

#### *Primary Cultures of Cells from Mouse Frontal Cortex*

Primary cultures of cells from mouse frontal cortex were prepared from 1-day-old CD-1 mice. The animals were anesthetized by hypothermia and sacrificed by decapitation. The brains were immediately immersed in an ice-cold complete Neurobasal medium containing B27 serum-free supplement, 0.5 mM L-glutamine, 50 μM β-mercaptoethanol, 50 U penicillin/ml, 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, Calif., USA). They were placed on the dorsal surface and the olfactory bulbs together with the olfactory tracts were removed. Following removal of the meninges, the frontal cortex was dissected from the brain by making a cut in the coronal plane at the level just anterior to the olfactory tubercles. The frontal lobes were transferred to a fresh medium and minced into small fragments under a dissecting microscope. The tissue fragments were collected by sedimentation and incubated in the presence of 0.2% trypsin (type IX from porcine pancreas; Sigma) dissolved in Neurobasal medium for 30 min at 30°C. Thereafter, soybean trypsin inhibitor (final concentration 1%; Sigma) and DNase type I (0.1 mg/ml; Sigma) were added and the incubation continued for an additional 5–10 min. Following gentle trituration with fire-polished Pasteur pipettes, the cell suspension was layered at the top of a 4% BSA solution in complete Neurobasal medium and centrifuged at 1,200 g for 10 min at room temperature. The cell pellet was again suspended in complete Neurobasal medium. Cells were plated on either glass coverslips (Bellco Brand, Electron Microscopy Sciences, Fort Washington, Pa., USA) or 6-well plates precoated with 0.01% L-poly-lysine (Sigma) at a density of  $1 \times 10^5$  cells and  $1 \times 10^6$ /well, respectively. The cells were placed in a humidified incubator and maintained at 37°C in 5% CO<sub>2</sub>. The medium was removed 6 h after plating. Thereafter the medium was changed every 4 days. Cells were used 8–10 days after plating.

#### *Transfection of Small Interfering RNA*

Primary cortical cells were transfected with control small interfering RNA (siRNA)-A (sc-37007), mouse AC2 siRNA (sc-

40318) or mouse AC4 siRNA (sc-29603) (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) using Lipofectamine RNAiMAX (Invitrogen) as a transfection reagent according to the manufacturer's instructions. Cyclase siRNAs were a pool of three target-specific siRNA duplexes. The sequences in 5'→3' orientation were: AC2 sense GGAUGAUCCAAGCAAUUGATT, antisense UCAAUUGCUUGGAUCAUCCTT, sense CGACUGCCAAU-GCAUCUAATT, antisense UUAGAUGCAUUGGCAGUCGTT, sense GAAGUUCAGUGGCGUUGAATT, antisense UUCAAC-GCCACUGAACUUCTT; AC4 sense CACGGCCAGAGAACA-CAAATT, antisense UUUGUGUUCUCUGGCCGUGTT, sense CUCCUGACGCUGUACUUCATT, antisense UGAAGUACAG-CGUCAGGAGTT, sense GCGAUCUACUUCUUAUCUTT, antisense AGAUGAAGAAGUAGAUCGCTT. Control siRNA consisted of a nontargeting sequence. Cells grown in either 6-well plates or glass coverslips placed in 24-well plates were incubated in an antibiotic-free medium for 24 h. The medium was replaced with Opti-MEM I reduced-serum medium (Invitrogen) and the cells were incubated with 20 nM siRNA duplexes for 4–5 h at 37°C. Thereafter, the medium was replaced by the growth medium and the cells were analyzed 48 h posttransfection. To determine transfection efficiency, parallel samples were transfected with fluorescein-conjugated control siRNA-A (sc-36869; Santa Cruz Biotechnology). An efficiency of 50–60% was obtained in 6 separate experiments.

#### *Preparation of Primary Cortical Cell Lysate*

For AC assays, cells were washed with ice-cold PBS (pH 7.4) and scraped in a hypotonic buffer containing 10 mM Hepes/NaOH (pH 7.4), 1 mM EGTA and 1 mM MgCl<sub>2</sub>. The cells were homogenized by using a hand-operated Dounce glass tissue grinder at ice-bath temperature and the homogenate was used immediately.

For Western blot analysis, cells were washed with PBS and scraped into a buffer containing PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 20 nM okadaic acid, 0.1% phosphatase inhibitor cocktail 1, 1% protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride (lysis buffer). The samples were sonicated for 5 s at ice bath temperature and stored at –80°C.

#### *Preparation of Tissue Extracts*

Tissue fragments of mouse medial prefrontal cortex dissected as described above were homogenized by sonication in an ice-cold lysis buffer supplemented with 1% (v/v) Triton X-100. The homogenate was centrifuged at 20,800 g for 10 min at 4°C and the supernatant was stored at –80°C for Western blot analysis.

#### *Western Blot Analysis*

Following protein content determination, aliquots of each sample containing equal amounts of protein (10–50 μg) were mixed with a sample buffer (300 mM Tris-HCl, 2% SDS, 40% glycerol, 5% β-mercaptoethanol and 0.16% bromophenol blue, pH 6.8) and subjected to SDS-PAGE. The proteins were then electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, Mass., USA). The efficiency of the transfer was controlled by staining of the gel with Coomassie brilliant blue and by following the transfer of prestained protein standards (Santa Cruz Biotechnology). Nonspecific binding sites



were blocked by incubating the membranes in a TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, and 0.05% Tween 20, pH 7.6) containing 2.5% low-fat dried milk (Blotting Grade Blocker, Bio-Rad Laboratories, Hercules, Calif., USA) and 50 mM NaF for 1 h at room temperature. After washing with the TBS-T buffer, the membranes were incubated overnight at 4°C with one of the following primary antibodies: rabbit monoclonal anti-phospho-Ser133-CREB (9198; 1:1,000; Cell Signaling Technology, Beverly, Mass., USA), rabbit polyclonal anti-AC2 (sc-587; 1:200), rabbit polyclonal anti-AC4 (sc-589; 1:600) and goat polyclonal anti-AC7 (sc-1966; 1:200; Santa Cruz Biotechnology) diluted in TBS-T containing 5% BSA. For the experiments reported in figure 4, anti-AC2 and anti-AC4 antibodies were preincubated with either vehicle or the cognate blocking peptide (sc-587P and sc-589P, respectively) at the ratio of 1:5 (w/w) overnight at 4°C before being used for immunoblotting. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:15,000; Santa Cruz Biotechnology) for 45 min at room temperature. Immunoreactive bands were detected by using an enhanced chemiluminescence system (ECL Plus) and ECL Hyperfilm (Amersham Biosciences, Piscataway, N.J., USA). For assurance that an equal amount of total protein was loaded in each lane, after the experiment the membranes were stripped of the antibodies by using Western-Rep probe reagent (Calbiochem, La Jolla, Calif., USA) and reprobed with either rabbit monoclonal anti-CREB (9197; 1:1,000) (Cell Signaling Technology) or, for AC immunoblots, rabbit polyclonal anti-actin antibody (1:500; Sigma). Band optical densities were determined by using the NIH Image software. The optical density of phospho-CREB and AC bands was normalized to the density of the corresponding CREB and actin band, respectively.

#### AC Assay

AC activity was assayed in a reaction mixture (final volume 100  $\mu$ l) containing 50 mM Hepes-NaOH (pH 7.4), 2.3 mM  $MgCl_2$ , 0.3 mM EGTA, 0.2 mM [ $\alpha$ - $^{32}P$ ]ATP (150 cpm/pmol), 0.5 mM [ $^3H$ ]cyclic AMP (80 cpm/nmol), 1 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 U/ml of creatine phosphokinase, 100  $\mu$ M GTP, 50  $\mu$ g of BSA, 10  $\mu$ g of bacitracin and 10 kIU of aprotinin. The reaction was started by the addition of the tissue preparation (15–20  $\mu$ g of protein) and was carried out at 30°C for 10 min. Cyclic AMP was isolated by sequential chromatography on Dowex and alumina columns. The recovery of [ $^{32}P$ ]cyclic AMP from each sample was calculated on the basis of the recovery of [ $^3H$ ]cyclic AMP. When the effects of  $\beta\gamma_t$  and  $\alpha_{tGDP}$  were investigated, 10  $\mu$ l of the tissue preparation were preincubated with an equal volume of a solution containing either the test compounds or their vehicle at ice bath temperature for 40–60 min. At the end of the incubation the receptor agonists were added, immediately followed by the addition of the reaction mixture to a final volume of 50  $\mu$ l. The concentrations of the transducin subunits reported in the figure legends refer to the final concentrations in the AC assay. For heat inactivation, the transducin subunits were boiled for 5 min. Assays were carried out in duplicate.

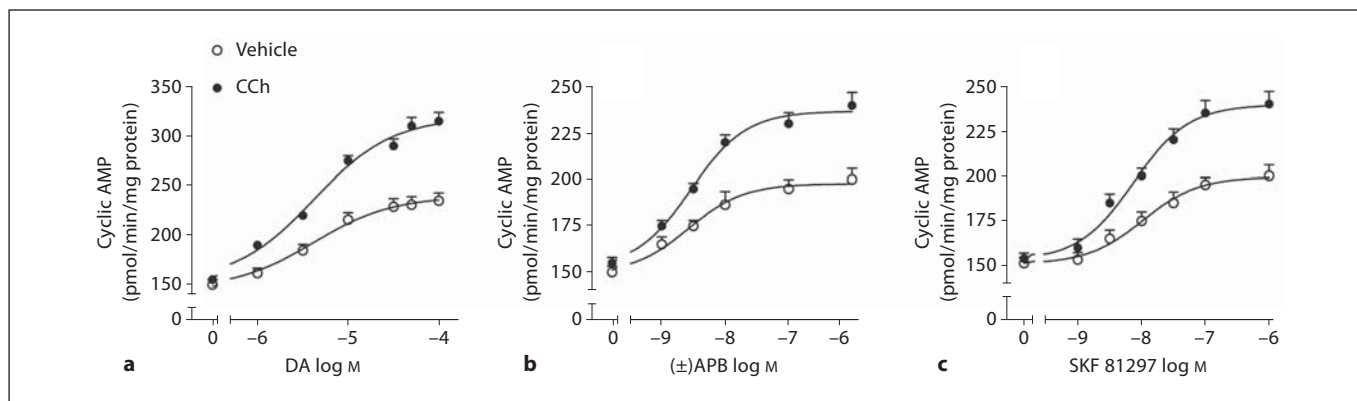
#### Immunofluorescence Analysis of CREB Phosphorylation

Cells from neonatal frontal cortex were cultured for 10–14 days. For phospho-CREB and neurofilament analysis, cultures were washed with complete Neurobasal A medium without B27 supplement and incubated in the same medium for 2 h in a CO<sub>2</sub>

incubator at 37°C. Thereafter, the incubation medium was replaced with DMEM-Ham's F12 (1:1; Invitrogen) and the cells were treated with the test compounds. Following a 30-min incubation, the medium was removed, the cells were washed once with ice-cold PBS and fixed with ice-cold 4% paraformaldehyde in PBS for 50 min at ice bath temperature. Following fixation, the cells were washed with PBS and incubated for 5 min in 0.2% Triton X-100 in PBS at room temperature. The cells were again washed with PBS and treated with 3% BSA and 1% normal goat serum for 1 h at room temperature. The cultures were then incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-phospho-Ser133-CREB (1:1,000; Upstate Biotechnology Inc., Lake Placid, N.Y., USA) and mouse monoclonal anti-neurofilament 160/200 (1:500; Sigma). Control samples were incubated in the absence of primary antibodies. After rinsing, the cells were incubated in the dark at room temperature with Alexa 594-conjugated goat anti-mouse IgG (1:4,000) and Alexa 488-conjugated goat anti-rabbit IgG (1:3,000; Invitrogen-Molecular Probes). After extensive washing, the cells were mounted in Gel Mount aqueous mounting medium (Sigma). Cells were analyzed with an Olympus BX61 microscope (Olympus Europe GmbH, Hamburg, Germany) and images were captured with an F-View II charge-coupled device camera. Images were analyzed using the program Cell P (Olympus Soft Imaging Solutions). For quantification of phospho-CREB immunoreactivity, digital images were acquired using a 40 $\times$  objective and constant camera settings within each experiment. At least 10 fields were analyzed for each sample and only cells showing an unobstructed nucleus and positive labeling for neurofilament were considered. In each selected cell, the average pixel intensity was measured within the region of the nucleus and in an adjacent area, which was used as a background value. This value was used to correct for differences in background intensity within and between images. In each experiment, nuclei were considered to be phospho-CREB-positive if the average pixel intensity was equal or above a threshold value corresponding to one standard deviation above the average pixel intensity of the nuclei of control samples. The percentage of positive nuclei was calculated as the number of positive nuclei/total number of neuronal nuclei  $\times$  100. No labeling was detected in samples treated without primary antibodies. Four separate culture preparations were analyzed by an investigator unaware of the treatment.

#### Colocalization of DA D<sub>1</sub>-Like Receptors with AC2 and AC4 in Mouse Prefrontal Cortex

The mice were anesthetized with chloral hydrate (400 mg/kg i.p.) and perfused with 4% paraformaldehyde. The brain was sunk in 20% sucrose at 4°C and embedded in OCT (Leica, Nusslock, Germany). Ten-micrometer-thick coronal sections corresponding to planes 11–16 of the mouse brain atlas of Franklin and Paxinos [30] were made by using a Leica CM3050S cryostat at –22°C. The sections were thaw-mounted, blocked in 10% normal goat serum and 0.3% Triton X-100 in PBS for 1 h at room temperature and then incubated with mouse monoclonal anti-DA D<sub>1</sub>-like receptor antibody (1:50; sc-33660; Santa Cruz), anti-AC2 antibody (1:50), anti-AC4 antibody (1:50) and a combination of the anti-D<sub>1</sub> receptor antibody with either anti-AC2 or anti-AC4 antibody for 24 h at 4°C. The antibodies were diluted in PBS containing 1% BSA, 1% normal goat serum and 0.3% Triton X-100. Following washing, the sections were incubated with



**Fig. 1.** Potentiation of DA  $D_1$ -like receptor-stimulated AC activity by CCh in membranes of mouse medial prefrontal cortex. The enzyme activity was assayed in the presence of the indicated concentrations of either DA (**a**), ( $\pm$ )APB (**b**) or SKF 81297 (**c**) in the absence and in the presence of 1 mM CCh. Values are the mean  $\pm$  SEM of 4 experiments conducted in 3 separate tissue preparations.

a mixture of Alexa 488-conjugated goat anti-rabbit IgG (1:3,000) and Alexa 594-conjugated goat anti-mouse IgG (1:1,000). After extensive washing, the cell nuclei were stained with 0.1  $\mu$ g/ml DAPI. Sections were analyzed with an Olympus BX 61 microscope and images were captured with an F-View II charge-coupled device camera in different regions of the medial prefrontal cortex by using a 40 $\times$  objective lens and mirror units for the detection of green fluorescence (U-MNIBA3; excitation 470–495, emission 510–550), red fluorescence (U-MNG2; excitation 530–550, emission 590) and blue fluorescence (U-MNUA2, excitation 360–370, emission 420–460). Images of sections stained with a single fluorophore were acquired with both green and red fluorescence filters to examine possible cross-talks. The specificity of the labeling was determined by either omitting the primary antibody for anti-DA  $D_1$ -like receptor antibody or preabsorption with the cognate blocking peptide for AC antibodies. Images of costained sections were analyzed using the colocalization module of the software Cell P. Colocalization was determined by loading the images with the green and red fluorescence into an image buffer and appointing a specific intensity threshold for each fluorescence. The program yielded colocalized and noncolocalized areas in false-color and a result sheet containing the following data: area (%) of each fluorescence, area (%) of colocalization and the ratio of area (%) of colocalization/area (%) of each fluorescence.

#### Statistical Analysis

Results are reported as mean  $\pm$  SEM, with  $n$  = number of experiments. Data from concentration-response curves were analyzed by the program GraphPad Prism (San Diego, Calif., USA), which yielded agonist concentration producing half-maximal ( $EC_{50}$  values) and maximal effects ( $E_{max}$  values). Statistical analysis of the difference between multiple groups was performed by ANOVA followed by Dunnett's post hoc test. Statistical analysis of the difference between the two groups was performed by the two-tailed unpaired Student  $t$  test.

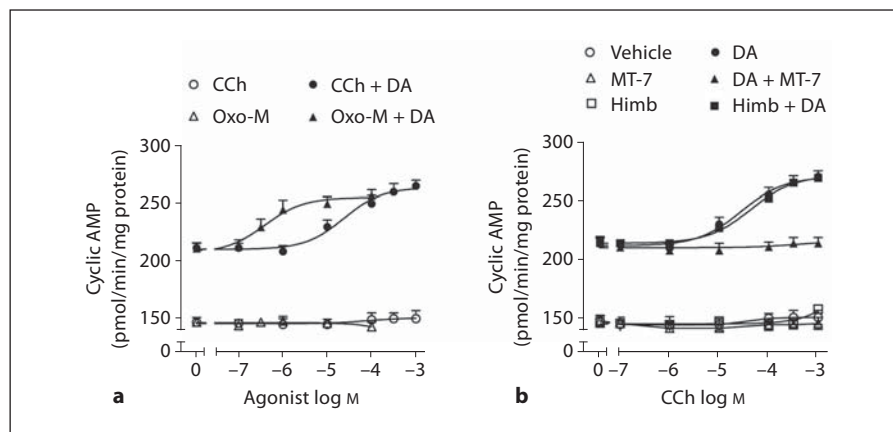
## Results

### Concomitant Activation of $M_1$ Muscarinic Receptor Enhances DA $D_1$ -Like Receptor Stimulation of Cyclic AMP Formation

In membranes of mouse medial prefrontal cortex, DA induced a concentration-dependent increase of cyclic AMP formation with an  $EC_{50}$  value of  $4.1 \pm 0.6 \mu$ M and an  $E_{max}$  value corresponding to  $64 \pm 5\%$  increase of basal activity ( $p < 0.001$ ,  $n = 20$ ) (fig. 1a). The DA stimulatory effect was mediated by activation of DA  $D_1$ -like receptors as the selective DA  $D_1$ -like receptor antagonist SCH23390 (1  $\mu$ M) completely blocked the increase of AC activity elicited by a maximal effective concentration (30  $\mu$ M) of DA (results not shown). The addition of the cholinergic agonist CCh (1 mM) failed to significantly affect basal cyclic AMP formation but markedly increased the stimulatory effect of DA (fig. 1a). CCh increased the DA  $E_{max}$  value by  $78 \pm 6\%$  ( $p < 0.001$ ,  $n = 4$ ) without changing the DA  $EC_{50}$  value ( $4.0 \pm 0.7 \mu$ M,  $p > 0.05$ ,  $n = 4$ ). CCh (1 mM) also potentiated the stimulatory effects elicited by the selective DA  $D_1$ -like receptor agonists ( $\pm$ )APB (fig. 1b) and SKF 81297 (fig. 1c) by  $74 \pm 5$  and  $79 \pm 6\%$  ( $p < 0.001$ ,  $n = 4$ ), respectively, without affecting their  $EC_{50}$  values [( $\pm$ )APB: vehicle  $2.6 \pm 0.7$  nM, CCh  $2.9 \pm 0.6$  nM; SKF 81297: vehicle  $10.5 \pm 2$  nM, CCh  $7.5 \pm 1.9$  nM,  $p > 0.05$ ,  $n = 4$ ].

Both CCh and Oxo-M, a selective muscarinic receptor agonist, enhanced DA-stimulated cyclic AMP formation in a concentration-dependent manner with  $EC_{50}$  values of  $23 \pm 3 \mu$ M and  $382 \pm 30$  nM, respectively (fig. 2a). At

**Fig. 2.** The cholinergic potentiation of DA  $D_1$ -like stimulation of cyclic AMP formation is concentration-dependent and blocked by the selective  $M_1$  receptor antagonist MT-7. **a** AC activity was assayed at the indicated concentrations of either CCh or Oxo-M in the absence and in the presence of 30  $\mu$ M DA. Values are the mean  $\pm$  SEM of 3 experiments. **b** Tissue membranes were incubated with either vehicle, 30 nM himbacine (Himb) or 10 nM MT-7 and AC activity was assayed at the indicated concentrations of CCh in the absence and in the presence of 30  $\mu$ M DA. Values are the mean  $\pm$  SEM of 3 experiments.



the concentrations tested, both cholinergic agonists failed to affect basal cyclic AMP production.

To define the receptor subtype mediating the muscarinic effect, we used the muscarinic toxin MT-7, which is the most potent and selective  $M_1$  antagonist so far identified [26, 32]. As shown in figure 2b, the CCh-induced potentiation of DA  $D_1$ -like receptor activity was completely suppressed by the addition of MT-7 (10 nM), whereas the addition of himbacine (30 nM), which preferentially blocks  $M_2$  and  $M_4$  receptor subtypes [26], was without effect.

#### *G<sub>q/11</sub> and G Protein $\beta\gamma$ Subunits Mediate $M_1$ Muscarinic Receptor Potentiation of DA $D_1$ -Like Receptor-Stimulated AC in Mouse Medial Prefrontal Cortex*

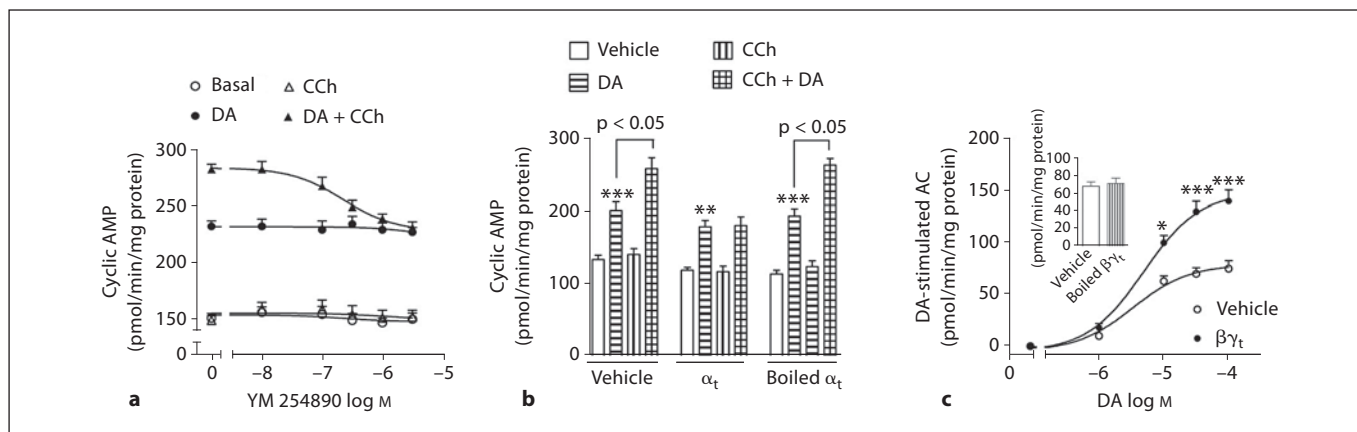
To investigate the molecular mechanisms mediating the muscarinic potentiation of DA  $D_1$ -like receptor signaling, we first examined the involvement of the G proteins  $G_{q/11}$ , which preferentially couple to the  $M_1$  receptor. As shown in figure 3a, YM-254890, a cyclic depsipeptide that blocks  $G_{q/11}$  activation by inhibiting the exchange of GDP with GTP in the  $\alpha$  subunit [33, 34], caused a concentration-dependent inhibition of CCh-induced enhancement of DA-stimulated cyclic AMP formation with an  $EC_{50}$  value of  $220 \pm 15$  nM. At the concentrations used, YM-254890 failed to affect either basal or DA-stimulated cyclic AMP formation. To assess the role of G protein  $\beta\gamma$  subunits ( $G\beta\gamma$ ), tissue membranes were treated with the  $G\beta\gamma$  scavenger  $\alpha_{tGDP}$  (500 nM) [35] and assayed for AC activity. The addition of  $\alpha_{tGDP}$  failed to significantly affect DA-stimulated cyclic AMP formation but markedly suppressed the potentiation elicited by CCh (fig. 3b). Heat-inactivated  $\alpha_{tGDP}$  did not antagonize the CCh effect. To determine whether the addition of exogenous

$G\beta\gamma$  could mimic the muscarinic potentiation, the effect of  $\beta\gamma_t$  on DA stimulation of cyclic AMP production was examined. As shown in figure 3c, like muscarinic receptor activation,  $\beta\gamma_t$  (400 nM) enhanced the  $E_{max}$  value of DA by  $82 \pm 5\%$  ( $n = 3$ ,  $p < 0.001$  by the two-tailed unpaired Student t test) without changing the agonist  $EC_{50}$  value (vehicle  $3.8 \pm 0.6$   $\mu$ M,  $\beta\gamma_t$   $4.8 \pm 0.7$   $\mu$ M,  $n = 3$ ,  $p > 0.05$  by the two-tailed unpaired Student t test). The addition of heat-inactivated  $\beta\gamma_t$  (400 nM) did not increase DA-stimulated cyclic AMP formation (fig. 3c, inset). One-dimensional SDS-PAGE analysis of the  $\alpha_{tGDP}$  and  $\beta\gamma_t$  preparations used in the AC assays indicated the presence in each preparation of a single protein band of 40 kDa for  $\alpha_{tGDP}$ , and 36 kDa for  $\beta\gamma_t$  (online suppl. fig. 1). These values correspond to the molecular mass of transducin  $\alpha$  and  $\beta$  subunits, respectively. The  $\gamma$  subunit (8 kDa) of the  $\beta\gamma_t$  dimer migrated at the dye front.

#### *DA $D_1$ -Like Receptors Colocalize with AC2 and AC4 in Mouse Medial Prefrontal Cortex*

Among the ten AC isoforms so far identified by molecular cloning, nine have been found to be expressed in the brain. The isoforms display a preferential regional distribution and are differentially regulated by  $Ca^{2+}$  and  $G\beta\gamma$ . For instance, AC1 is expressed in the hippocampus, cerebellum and cerebral cortex, AC3 is predominantly localized in the olfactory epithelium, AC2 and AC4 are known to be present in olfactory bulb layers and in the hippocampus and AC5 is concentrated in the basal ganglia [35, 36]. AC1, AC3 and AC8 are stimulated by  $Ca^{2+}$ /calmodulin, AC5 and AC6 are inhibited by  $Ca^{2+}$ , AC2, AC4 and AC7 are  $Ca^{2+}$ -insensitive, and AC9 is inhibited by calcineurin. On the other hand,  $G\beta\gamma$  stimulates AC2, AC4 and AC7 synergistically with activated  $G\alpha_s$ , inhibits



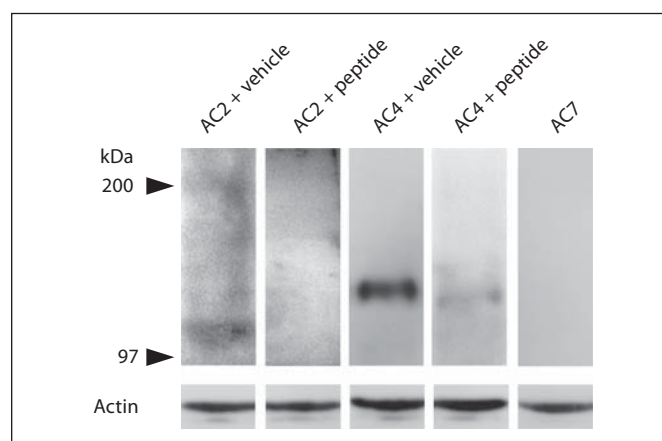


**Fig. 3.** Involvement of  $G_{q/11}$  and  $G\beta\gamma$  in  $M_1$  muscarinic potentiation of DA  $D_1$ -like receptor stimulation of cyclic AMP formation. **a** Tissue membranes were incubated with either vehicle or the indicated concentrations of the  $G_{q/11}$  antagonist YM 254890 and AC activity was assayed in the absence and in the presence of 30  $\mu$ M DA without and with 1 mM CCh. Values are the mean  $\pm$  SEM of 4 experiments. **b** Tissue membranes were pretreated for 40 min with either vehicle,  $\alpha_t$ GDP (500 nM) or boiled  $\alpha_t$ GDP (500 nM) and AC activity was assayed in the absence and in the presence of 30  $\mu$ M DA without and with 1 mM CCh. Values are the mean  $\pm$  SEM of 4 experiments for  $\alpha_t$ GDP and 3 experiments for boiled  $\alpha_t$ GDP.

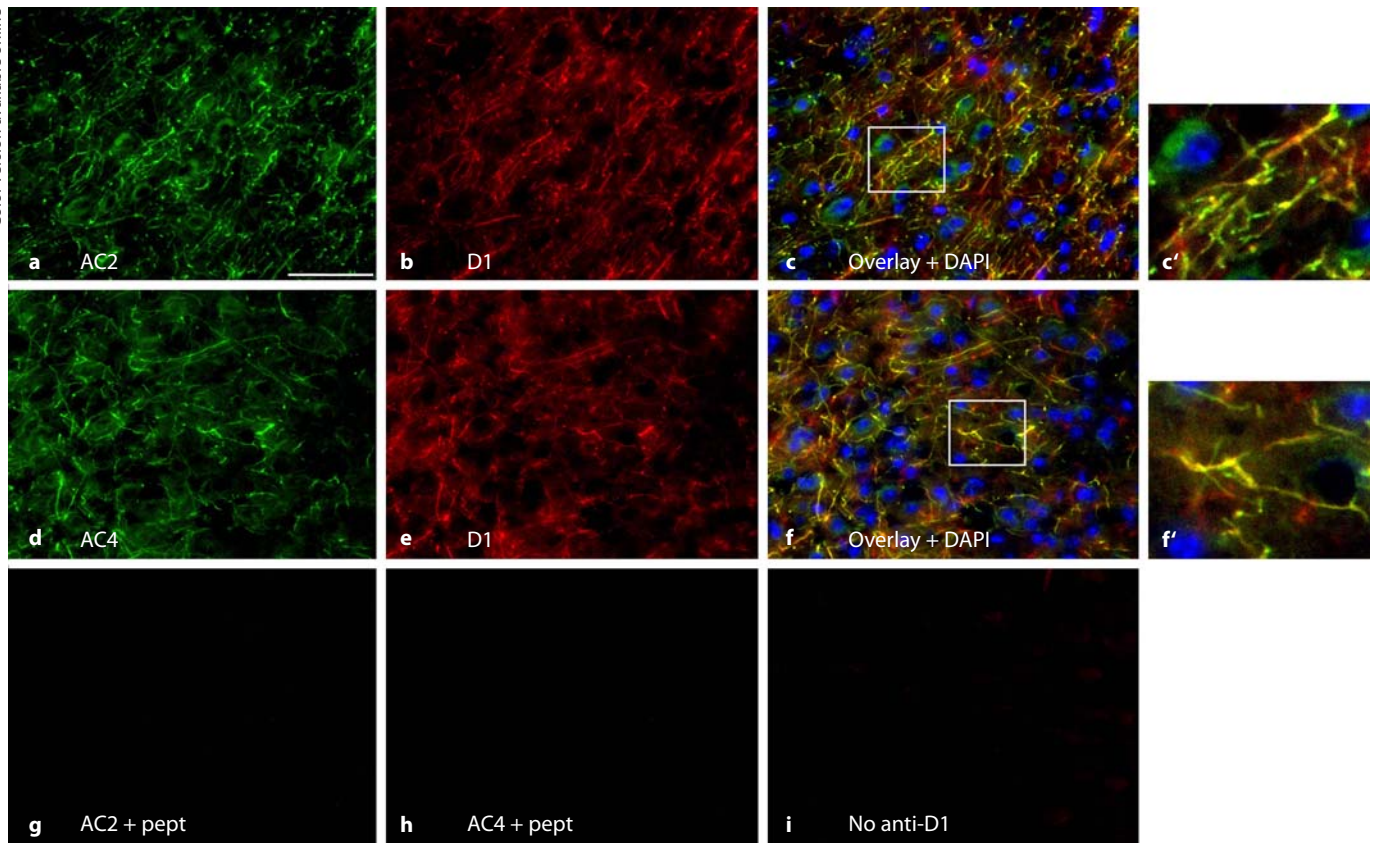
\*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  vs. the corresponding vehicle. **c** Tissue membranes were preincubated with either vehicle or 400 nM  $\beta\gamma_t$  for 60 min and AC activity was assayed in the presence of either vehicle or the indicated concentrations of DA. Values refer to the enzyme activity stimulated by DA in the absence and in the presence of  $\beta\gamma_t$  and are the mean  $\pm$  SEM of 3 experiments. \*  $p < 0.05$ , \*\*\*  $p < 0.001$  vs. DA + vehicle. **Inset** Tissue membranes were preincubated with either vehicle or boiled  $\beta\gamma_t$  (400 nM) for 60 min and AC activity was assayed in the presence of either vehicle or 30  $\mu$ M DA. Values are the mean  $\pm$  SEM of 3 experiments.

AC1, and may have indirect inhibitory effects on AC5 and AC6 [35, 36].

The finding that the addition of  $G\beta\gamma$  potentiated DA-stimulated cyclic AMP formation suggested the receptor coupling to  $G\beta\gamma$ -stimulated AC2, AC4 and AC7 isoforms. Western blot analysis of mouse medial prefrontal cortex tissue extracts detected the presence of AC2 and AC4 immunoreactive bands of approximately 110 and 160 kDa, respectively, but no AC7 immunoreactivity (fig. 4). The AC2 and AC4 labeling disappeared or was markedly suppressed when the blots were developed with primary antibodies preabsorbed with their cognate peptide (fig. 4). These results indicate that among the  $G\beta\gamma$ -stimulated AC isoforms, AC2 and AC4 are those predominantly expressed in mouse medial prefrontal cortex. Therefore, we investigated whether in this brain area DA  $D_1$ -like receptors colocalized with these AC isoforms. As shown in figure 5, DA  $D_1$ -like receptor, AC2 and AC4 immunoreactivities showed a diffuse distribution, labeling both the neuropil and neuronal cell bodies. AC2 immunoreactivity appeared to be localized in both neuronal cell bodies and processes, whereas the AC4 fluorescence was predominantly expressed in fiber-like structures, with relatively few neuronal cell bodies being visualized. Preabsorption with the



**Fig. 4.** Western blot analysis of AC2, AC4 and AC7 expression in mouse medial prefrontal cortex. Tissue extracts containing 50  $\mu$ g of protein were loaded onto 7.5% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes and incubated with anti-AC2 antibody (1:200) pretreated with either vehicle or the blocking peptide (AC2 + peptide), anti-AC4 antibody (1:600) pretreated with either vehicle or the blocking peptide (AC4 + peptide) and anti-AC7 antibody (1:200). The membranes were stripped of the antibodies and reprobed with antiactin antibody to control for equal protein loading. Molecular size markers are indicated on the left. Data are representative of 3 experiments performed on 3 separate tissue preparations.



**Fig. 5.** Double immunofluorescence histochemistry of AC2, AC4 and DA D<sub>1</sub> receptors in the medial prefrontal cortex. Mouse brain coronal sections (10  $\mu$ m) were labeled with the AC2 and AC4 antibodies each combined with the DA D<sub>1</sub> receptor antibody. **a** AC2 labeling. **b** DA D<sub>1</sub> receptor labeling in the same image shown in **a**. **c** Overlay of the images shown in **a** and **b** plus the nuclear staining with DAPI. AC2 immunoreactivity appears to be localized in neuronal soma and fiber-like structures, whereas DA D<sub>1</sub> receptor immunoreactivity is predominantly distributed throughout the neuropil. **c'** Higher magnification of merged image of the boxed area in **c** showing colocalization of AC2 and DA D<sub>1</sub> receptor immunoreactivities in fiber-like structures. **d** AC4 labeling. **e** DA D<sub>1</sub>

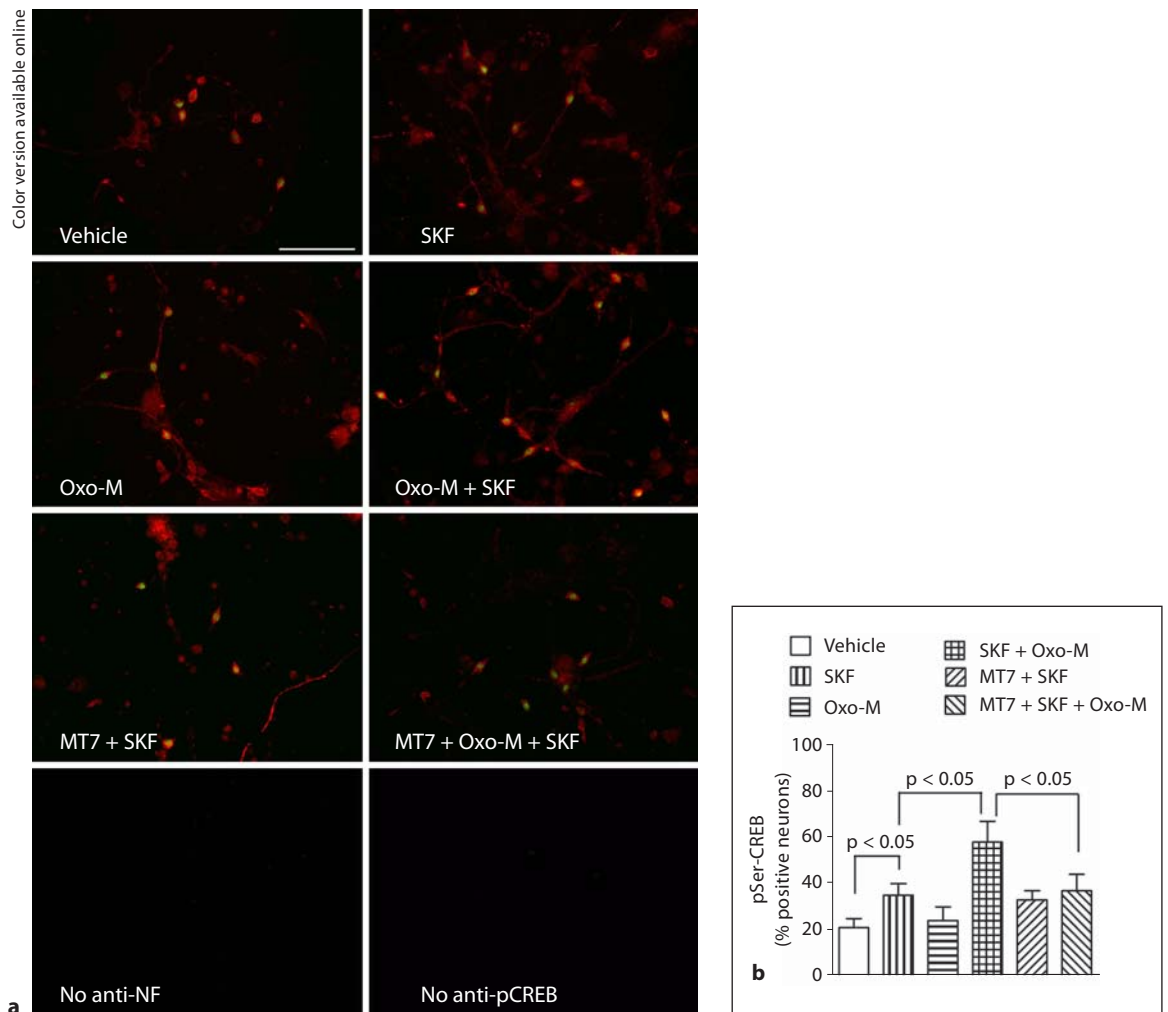
receptor labeling in the same image shown in **d**. **f** Overlay of the images shown in **d** and **e** plus the nuclear staining with DAPI. AC4 immunoreactivity appears to be mostly distributed in fiber-like structures. **f'** Higher magnification of merged image of the boxed area in **f** showing colocalization of AC4 and DA D<sub>1</sub> receptor immunoreactivities in neuronal processes. **g**, **h** Preabsorption with the cognate blocking peptide (pept) suppresses AC2 and AC4 labeling, respectively. **i** The tissue section was processed for DA D<sub>1</sub> receptor immunolabeling without the addition of a primary antibody. Data are representative of 3 tissue preparations. Bar = 50  $\mu$ m.

corresponding immune peptide suppressed both AC2 and AC4 immunolabeling (fig. 5g, h). The immunostaining for DA D<sub>1</sub>-like receptors was mainly diffuse in the neuropil and few neuronal somata were labeled in the different fields. This labeling was not observed in samples processed without a primary antibody (fig. 5i). Several fiber-like structures positive for DA D<sub>1</sub>-like receptors displayed a marked AC2 (fig. 5c, c') and AC4 (fig. 5f, f') immunoreactivity. Quantitative analysis indicated that  $64.9 \pm 6$  and  $37.0 \pm 10\%$  of DA D<sub>1</sub>-like receptor signal colocalized with AC4 and AC2 signals, respectively ( $p < 0.05$ ,  $n = 25$ ).

#### *Activation of M<sub>1</sub> Muscarinic Receptor Enhances DA D<sub>1</sub>-Like Receptor-Induced Stimulation of CREB Phosphorylation*

Exposure of primary cortical cells to 1  $\mu$ M SKF 81297 induced a significant increase of the neuronal cells positive for phospho-CREB (fig. 6). The addition of Oxo-M (3  $\mu$ M), which per se had little effect on phospho-CREB labeling, markedly enhanced the stimulatory effect of SKF 81297. Pretreatment with MT-7 (10 nM) failed to affect the response to the DA D<sub>1</sub>-like receptor agonist but completely prevented the Oxo-M-induced potentiation.





**Fig. 6.**  $M_1$  muscarinic receptor stimulation potentiates DA  $D_1$ -like receptor induced CREB phosphorylation in primary neurons of mouse frontal cortex. **a** Cells were preincubated with either vehicle or MT-7 (10 nM) for 10 min and then treated with either vehicle, 1  $\mu$ M SKF 81297 (SKF), 3  $\mu$ M Oxo-M or the indicated combinations for 30 min. Phospho-CREB immunofluorescence (green; colors refer to online color version only) was measured in nuclei of

neuronal cells identified by neurofilament staining (red). The bottom panels are images of primary neuron preparations processed for either neurofilament (NF) or phospho-CREB (pCREB) labeling without the incubation with the corresponding primary antibody. Bar = 50  $\mu$ m. **b** Quantitative analysis of phospho-CREB (pSer-CREB) immunofluorescence expressed as percentage of positive nuclei. Values are the mean  $\pm$  SEM of 4 experiments.

No labeling was detected in samples incubated in the absence of a primary antibody (fig. 6).

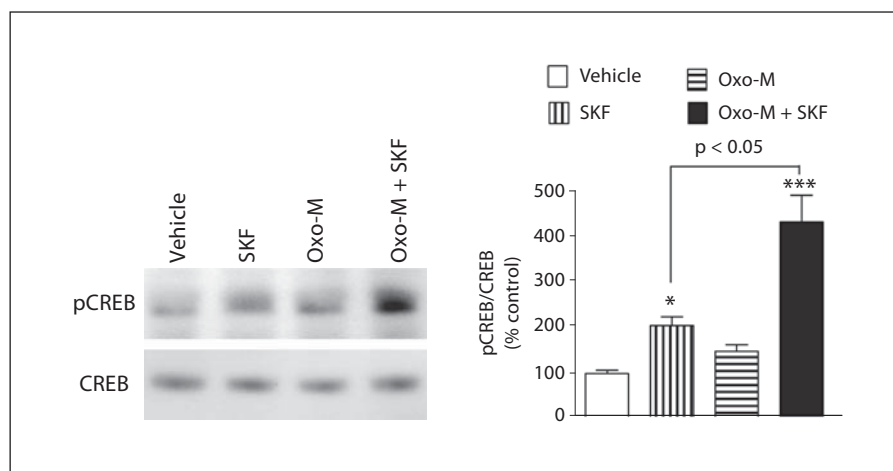
Similar results were obtained by Western blot analysis of phospho-CREB levels. Cells were treated with either SKF 81297 (1  $\mu$ M), Oxo-M (3  $\mu$ M) or the combination of the two receptor agonists. As shown in figure 7, SKF 81297 significantly enhanced phospho-CREB levels, whereas Oxo-M caused a modest and nonsignificant increase. In cells treated with the agonist combination, phospho-CREB immunoreactivity increased to a level

significantly higher than that obtained with SKF 81297 alone. None of the treatments affected the expression of total CREB protein.

#### *Effects of AC2 and AC4 Knockdown on $M_1$ Potentiation of DA $D_1$ -Like Receptor-Induced Cyclic AMP Formation and CREB Phosphorylation*

Western blot analysis of primary cortical cell extracts detected the presence of both AC2 and AC4 immunoreactive bands (fig. 8a). Cell transfection with AC2 siRNA

**Fig. 7.** Western blot analysis of phospho-CREB (pCREB) in mouse cortical neurons. Cells were treated with either vehicle, 1  $\mu$ M SKF 81297 (SKF), 3  $\mu$ M Oxo-M or the combination of the two receptor agonists for 30 min. Thereafter, aliquots of cell extracts containing equal amounts of protein (10  $\mu$ g) were analyzed for pCREB by Western blot. The membranes were then stripped of the antibodies and reprobed for total CREB expression. Densitometric ratios of pCREB/CREB immunoreactivity are reported as percentage of control (vehicle + vehicle). Values are the mean  $\pm$  SEM of 4 experiments performed on 3 separate primary neuron preparations. \*  $p < 0.05$  vs. control; \*\*\*  $p < 0.001$ .



caused a significant decrease of AC2 immunoreactivity without affecting AC4 expression, whereas transfection with AC4 siRNA decreased AC4 but not AC2 immunoreactivity. AC assays of cell homogenates indicated that in AC2 siRNA-treated cells DA-stimulated cyclic AMP formation was not significantly different from that of control cells and CCh was capable of enhancing this response. In contrast, in AC4 siRNA-treated cells DA-stimulated cyclic AMP formation was depressed and CCh did not cause a significant potentiation (fig. 8b). Similarly, the DA D<sub>1</sub>-like receptor stimulation of phospho-CREB expression and its potentiation by Oxo-M were largely preserved in AC2 siRNA-treated neurons but markedly reduced in cells with knockdown of AC4 (fig. 8c).

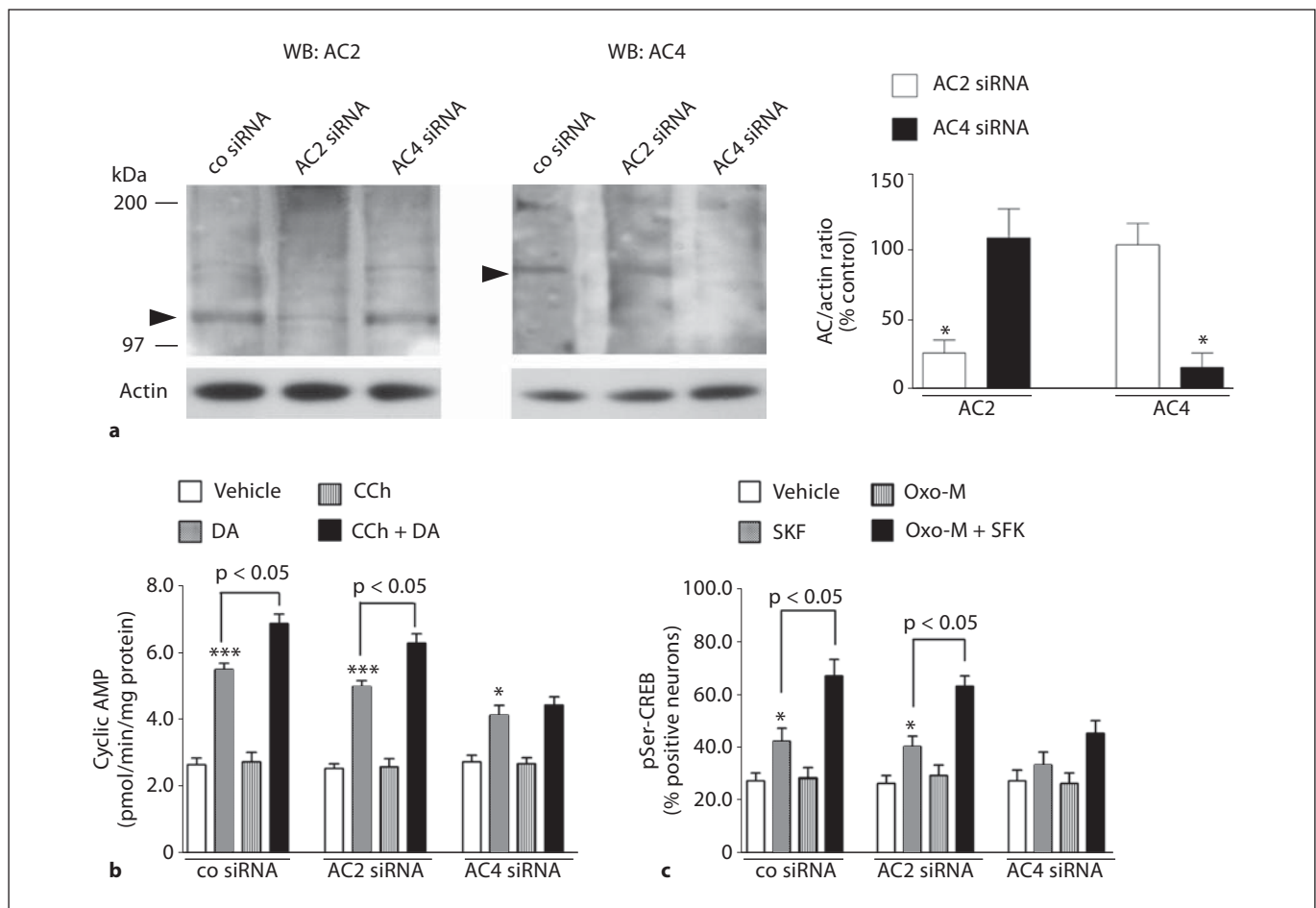
## Discussion

The present study reveals for the first time the occurrence in mouse medial prefrontal cortex of a coincident signaling mechanism whereby concomitant activation of M<sub>1</sub> muscarinic receptors enhance DA D<sub>1</sub>-like receptor-induced stimulation of cyclic AMP formation and CREB phosphorylation. The study also provides evidence indicating that the coincidence signaling involves a specific AC isoform, which integrates the different receptor stimuli.

The signal transduction cross-talk between M<sub>1</sub> and D<sub>1</sub>-like receptors was observed in cell membranes, i.e. in a cell-free system which lessens the possibility of indirect effects mediated by the release of endogenous neurotransmitters. Stimulation of M<sub>1</sub> muscarinic receptors is known to increase intracellular Ca<sup>2+</sup>, which may enhance cyclic

AMP formation by stimulating Ca<sup>2+</sup>-activated AC isoforms, such as AC1, AC3 and AC8 [36, 37]. However, the potentiation of DA D<sub>1</sub>-like receptor stimulation of AC occurred in the absence of added Ca<sup>2+</sup> and in the presence of EGTA, thus ruling out the possible contribution of Ca<sup>2+</sup>-dependent mechanisms. Rather, the findings that the M<sub>1</sub> potentiation was blocked by the G<sub>q/11</sub> antagonist YM-254890, prevented by the G $\beta\gamma$  scavenger  $\alpha_{tGDP}$  and mimicked by the addition of exogenous G $\beta\gamma$  support a membrane-limited mechanism whereby M<sub>1</sub> activation of G<sub>q/11</sub> and the consequent release of G $\beta\gamma$  subunits potentiate the DA D<sub>1</sub>-like receptor-induced stimulation of Ca<sup>2+</sup>-independent/G $\beta\gamma$ -sensitive AC isoforms, such as AC2, AC4 and AC7, which are known to be synergistically activated by G $\beta\gamma$  and the  $\alpha$  subunit of G<sub>s</sub> [36, 37]. The lack of activity of boiled  $\alpha_{tGDP}$  and G $\beta\gamma$  and the purity of the transducin preparations lessen the possibility of unspecific effects on AC responses.

Both AC2 and AC4 have been shown to be expressed in various regions of the brain [37] and their neuronal localization in the hippocampus has been associated with their possible role in the regulation of synaptic plasticity [38]. In the postmortem human brain, a marked decrease of parietal cortex AC2 immunoreactivity has been detected in patients affected by Alzheimer's disease as compared to controls [39]. In the rat olfactory bulb,  $\beta\gamma$ -sensitive AC2 and AC4 isoforms were proposed to mediate the stimulation of cyclic AMP formation by G<sub>i/o</sub>-coupled muscarinic and opioid receptors [40, 41]. With regard to DA D<sub>1</sub>-like receptors of the prefrontal cortex, so far no information has been provided on their association with AC2, AC4 or AC7 and on the possible contribution of these AC isoforms in the regulation of the downstream



**Fig. 8.** Effects of AC2 and AC4 suppression by cognate siRNAs on DA  $D_1$ -like receptor signaling and  $M_1$  potentiation. **a** Primary neurons of mouse frontal cortex were treated with either control siRNA (co siRNA), AC2 siRNA or AC4 siRNA and aliquots of cell extracts containing equal amounts of protein (10  $\mu$ g) were analyzed for the expression of AC2 and AC4 by Western blot (WB). The membranes were then stripped of the antibodies and re-probed with antiactin antibody to control for equal protein loading in each lane. Molecular size markers are indicated on the left. Arrowheads indicate AC immunoreactive bands. Data are the mean  $\pm$  SEM of 5 experiments for AC2 and 4 experiments for AC4. \*  $p < 0.05$  vs. co siRNA. **b** AC activity of membrane preparations of primary neurons treated with either control siRNA (co

siRNA), AC2 siRNA or AC4 siRNA. The enzyme activity was assayed in the presence of vehicle, 30  $\mu$ M DA, 1 mM CCh and the combination of CCh and DA. Values are the mean  $\pm$  SEM of 4 separate primary culture preparations. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , vs. vehicle. **c** Primary cortical neurons were treated with either co siRNA, AC2 siRNA or AC4 siRNA and then exposed to either vehicle, SKF (1  $\mu$ M), Oxo-M (3  $\mu$ M) or the combination of SKF and Oxo-M for 30 min. Thereafter, phospho-CREB-positive neurons were analyzed by double immunofluorescence labeling with phospho-CREB and neurofilament 160/200 antibodies as described in Materials and Methods. Values are the mean  $\pm$  SEM of 4 separate primary culture preparations. \*  $p < 0.05$  vs. the corresponding vehicle.

molecular events triggered by their activation. To study the coexpression of DA  $D_1$ -like receptors with  $\beta\gamma$ -sensitive AC isoforms we employed specific antibodies which have previously been used and validated by several investigators [38, 39, 41–44]. In tissue extracts of mouse medial prefrontal cortex Western blot analysis showed the presence of specific AC2 and AC4 immunoreactive

bands, but no AC7 immunoreactivity, indicating that the expression level of AC7 was too low to be detected by the employed procedure. This finding is, however, in line with previous studies demonstrating that in the mouse brain AC7 mRNA was localized primarily in the cerebellar granule cell layer [45]. By using double immunofluorescence analysis, we found that in mouse prefrontal cor-



tex a fraction of DA D<sub>1</sub> receptor population displayed a colocalization with AC2 and AC4. Both AC isoforms were expressed in fiber-like structures and, predominantly for AC2, in neuronal somata. A similar cellular distribution of AC2 and AC4 immunoreactivities has previously been observed in mouse hippocampus and rat retina [38, 42]. Quantitative colocalization analysis indicated that in different fields a higher fraction of DA D<sub>1</sub> receptor immunoreactivity colocalized with AC4 as compared to AC2. The overlapping of the two signals was particularly evident on fiber-like structures, suggesting the coexpression of D<sub>1</sub> receptors and AC4 on neuronal dendritic processes.

The type of neuronal elements coexpressing DA D<sub>1</sub> receptors, M<sub>1</sub> receptors and AC isoforms remains to be established. In the prefrontal cortex, DA D<sub>1</sub>-like receptors have been shown to be localized on the dendritic spines and shafts of pyramidal neurons and on the dendrites and axon terminals of putative GABAergic interneurons [4, 46, 47]. A recent study on the localization of M<sub>1</sub> muscarinic receptors in the cortex and hippocampus of adult mice has demonstrated the preferential distribution on pyramidal cell dendrites, with low or undetectable expression in various GABAergic interneurons [48]. This observation suggests that the functional interaction between DA D<sub>1</sub>-like and M<sub>1</sub> receptors in the control of  $\beta\gamma$ -sensitive AC may occur at the level of pyramidal cell dendrites. This conclusion is also in line with another recent study showing a convergence of cholinergic and dopaminergic afferents onto pyramidal cells of the rodent prefrontal cortex [49]. Interestingly, both DA D<sub>1</sub>-like and M<sub>1</sub> receptors have been found to be expressed in extrasynaptic membranes of cortical pyramidal cell dendrites and spines [4, 46–48]. As extrasynaptic concentrations of both neurotransmitters are much lower than those reached in the synaptic cleft, the occurrence of a synergistic interaction may operate to increase the strength of subthreshold signals. In this context, the coincidence signaling controlling AC activity may allow the formation of intracellular cyclic AMP only when DA D<sub>1</sub>-like and M<sub>1</sub> muscarinic receptors are concomitantly stimulated. This cooperative interaction may be relevant for optimizing the level of DA D<sub>1</sub>-like receptor stimulation, particularly under conditions of hypodopaminergic transmission, such as those associated with aging and schizophrenia [15, 16]. Indeed, a number of studies in primate and rodents have shown that an optimal DA D<sub>1</sub>-like receptor stimulation is required for the correct performance of working memory tasks [6, 7]. Microdialysis studies in rats have shown that atypical antipsychotics induce a con-

comitant increase of DA and ACh release in the medial prefrontal cortex and this dual effect has been hypothesized to be involved in an antipsychotic-induced improvement of cognitive deficits in schizophrenia [50]. This condition may represent a situation where the two neurotransmitters may cross-talk and generate cyclic AMP signal amplification at the cellular sites of DA D<sub>1</sub>-like and M<sub>1</sub> receptor colocalization.

DA D<sub>1</sub>-like and M<sub>1</sub> receptor synergism in stimulating cyclic AMP formation was associated with an enhanced CREB phosphorylation at Ser133, indicating that the input integration at the AC level translated into a greater activation of DA D<sub>1</sub>-like receptor signaling to the nucleus. Phosphorylated CREB binds to the enhancer element CRE located in the upstream regions of cyclic AMP-responsive genes, thus inducing transcription. CREB-mediated gene transcription is considered as a key factor in long-term memory formation, synaptic plasticity, neuronal differentiation, outgrowth and survival and in certain aspects of drug addiction [12–14]. In the prefrontal cortex, CREB phosphorylation has been found to be associated with DA D<sub>1</sub>-like receptor modulation of memory retrieval performance [51] and is considered to be a major downstream event in the DA D<sub>1</sub>-like receptor signaling cascade regulating long-term potentiation [8]. The M<sub>1</sub> enhancement of DA D<sub>1</sub>-stimulated CREB phosphorylation suggests that a similar potentiation may occur at the level of DA regulation of long-term memory. Although additional studies are required to specifically address this point, it is noteworthy that the administration of DA agonists enhances the positive effects of the muscarinic agonist oxotremorine on memory storage [52].

To investigate the contribution of AC2 and AC4 isoforms to DA D<sub>1</sub>-like receptor signaling, mouse primary cortical cells were transfected with specific siRNAs for AC2 and AC4. Western blot analysis indicated that the siRNA treatment effectively and selectively suppressed the expression of the targeted AC isoform. We found that in AC2 siRNA-treated cells the stimulation of DA D<sub>1</sub>-like receptors was still capable of significantly increasing cyclic AMP formation and CREB phosphorylation and that the concomitant M<sub>1</sub> receptor activation potentiated both responses. Conversely, in AC4 siRNA-treated cells, DA D<sub>1</sub>-like receptor-stimulated cyclic AMP formation and CREB phosphorylation were markedly reduced. Under this condition the M<sub>1</sub> receptor potentiation of both responses was lost, indicating that in cortical neurons AC4, rather than AC2, is critically involved in DA D<sub>1</sub>-like receptor signaling and in mediating the synergistic interaction with M<sub>1</sub> receptors. These functional data are also in

line with the immunofluorescence analysis showing a greater colocalization of the DA D<sub>1</sub> receptor signal with AC4 immunoreactivity.

Studies on the involvement of AC isoforms in DA receptor signaling have shown that in mice lacking AC5, which is highly expressed in basal ganglia [36, 37], the regulation of striatal cyclic AMP by either DA D<sub>1</sub>-like or D<sub>2</sub>-like receptors is impaired and that this AC isoform is essential for the extrapyramidal side effects of antipsychotic drugs [53, 54]. The present study shows the involvement of AC4 in DA D<sub>1</sub>-like receptor signaling in the prefrontal cortex and thus provides additional information on the role of specific AC isoforms in DA receptor function in distinct brain areas.

Previously, the DA D<sub>1</sub>-like and muscarinic receptor interplay has mainly been characterized in the dorsal and ventral striatum, where activation of M<sub>4</sub> muscarinic

receptors inhibits DA D<sub>1</sub>-stimulated cyclic AMP formation [55–57], whereas a blockade of muscarinic receptors enhances DA D<sub>1</sub>-like receptor and amphetamine-stimulated neuropeptide gene expression [58, 59]. It has been speculated that in nucleus accumbens M<sub>4</sub> receptor inhibition could be pharmacologically exploited to treat pathological conditions characterized by a hyperdopaminergic state induced by psychostimulants, such as cocaine, whereas selective M<sub>4</sub> blockade in dorsal striatum may be useful for the treatment of Parkinson's disease [55–57]. The present demonstration that in the medial prefrontal cortex, concomitant activation of M<sub>1</sub> muscarinic receptors potentiates DA D<sub>1</sub> receptor signaling identifies a new aspect of ACh-DA interaction that may constitute a molecular target for the development of new strategies for the treatment of cognitive dysfunctions.

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