

Coincidence Signaling of Dopamine D₁-Like and M₁ Muscarinic Receptors in the Regulation of Cyclic AMP Formation and CREB Phosphorylation in Mouse Prefrontal Cortex

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Key Words

M₁ muscarinic receptors · Dopamine D₁-like receptors · G $\beta\gamma$ -stimulated adenylyl cyclase isoforms · CREB phosphorylation · Mouse prefrontal cortex

Abstract

In the prefrontal cortex, dopamine D₁-like and M₁ 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 M₁ muscarinic receptors potentiated D₁-like receptor-induced cyclic AMP formation through a mechanism involving activation of G_{q/11} 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 D₁-like receptors with a greater association for AC4. In primary cultures of frontal cortex neurons, D₁-like receptor-induced Ser133 phosphorylation of the transcription factor cyclic AMP-responsive element binding protein (CREB) was potentiated by concurrent stimulation of M₁ receptors. Suppression of AC4 expression with small interfering RNA transfection reduced D₁ stimulation of cyclic AMP formation and CREB phosphorylation and abolished the M₁ potentiation, whereas knockdown of AC2 had no significant

effects. These data indicate that in mouse prefrontal cortex G_{q/11}-coupled M₁ receptor and G_s-coupled D₁-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 D₁-like receptors, including D₁ and D₅ subtypes, and the D₂-like receptors, comprising the D₂, D₃ and D₄ 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 D₁-like receptor activity has been shown to be required for working memory function [6, 7]. At the cellular level, DA D₁-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 D₂-like receptors are coupled to inhibition of adenylyl cyclase (AC) activity [9], DA D₁-like receptors transduce their signals via the stimulatory G protein G_s, 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_A receptors and ionotropic glutamate receptors [10, 11]. Moreover, DA D₁-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 D₁ 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 M₁ receptor is currently considered one of the major players in the cholinergic control of cognitive and attentional processes. Behavioral studies have shown that M₁ null mutant mice display selective cognitive dysfunctions, indicating that the M₁ 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 M₁/M₄-preferring antagonist pirenzepine impaired spontaneous working memory in mice, whereas the M₁-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 M₁ 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 M₁ receptor gene with more severe cognitive deficits in schizophrenia, suggesting that a variation in the M₁ receptor sequence may contribute to the core symptom of this disorder [22]. Finally, clinical studies have shown that M₁ 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 M₁ receptor subtype is known to be preferentially coupled to the G proteins G_{q/11} and to regulate phospholipase C β , intracellular Ca²⁺ mobilization and protein kinase C [26]. However, because of the limited availability of selective ligands, the signaling mechanisms mediating the procog-

nitive effects of the M₁ 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 D₁-like and M₁ 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 M₁ muscarinic receptors increases DA D₁-like stimulation of cyclic AMP formation and CREB phosphorylation.

Materials and Methods

Materials

[α -³²P]ATP (30–40 Ci/mmol) and [2,8-³H]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 α subunit of transducin (α_{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). 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 Natissone, 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- μ 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 MgCl₂ (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-L-lysine (Sigma) at a density of 1×10^5 cells and 1×10^6 /well, respectively. The cells were placed in a humidified incubator and maintained at 37°C in 5% CO₂. 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 MgCl₂. 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-Reporeagent (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 μ l) containing 50 mM Hepes-NaOH (pH 7.4), 2.3 mM MgCl₂, 0.3 mM EGTA, 0.2 mM [α -³²P]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 μ M GTP, 50 μ g of BSA, 10 μ g of bacitracin and 10 kIU of aprotinin. The reaction was started by the addition of the tissue preparation (15–20 μ 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 [³²P]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 α_{tGDP} were investigated, 10 μ 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 μ 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 CO₂

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 D₁-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 D₁-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-D₁ 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

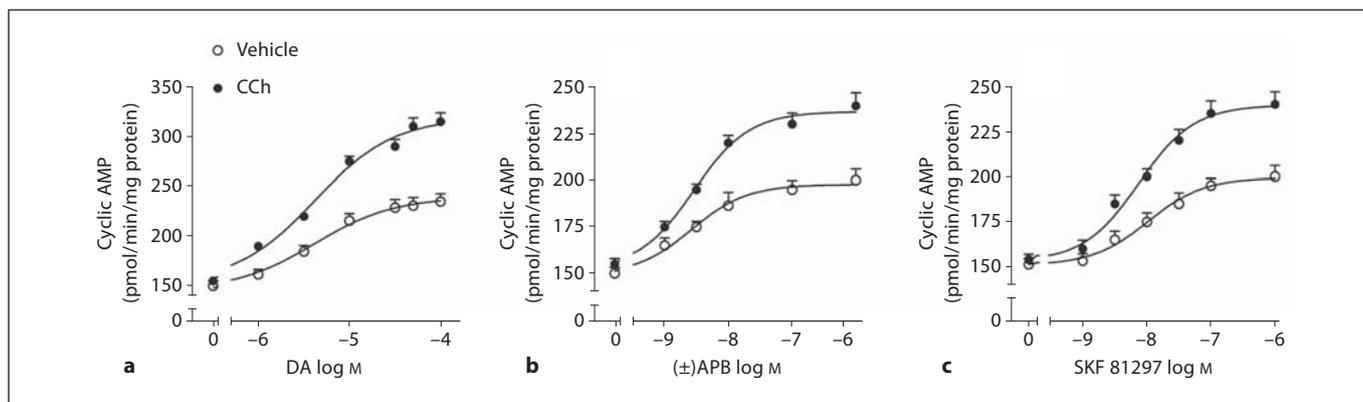


Fig. 1. Potentiation of DA D₁-like receptor-stimulated AC activity by CCh in membranes of mouse medial prefrontal cortex. The enzyme activity was assayed in the presence of the indicated concentrations of either DA (**a**), (±)APB (**b**) or SKF 81297 (**c**) in the absence and in the presence of 1 mM CCh. Values are the mean ± 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 µg/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× 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 D₁-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 ± 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 (EC₅₀ 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.

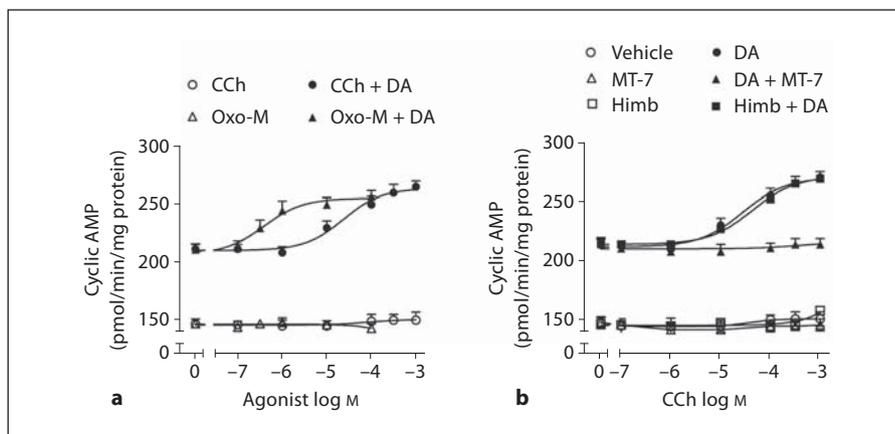
Results

Concomitant Activation of M₁ Muscarinic Receptor Enhances DA D₁-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 EC₅₀ value of $4.1 \pm 0.6 \mu\text{M}$ 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 D₁-like receptors as the selective DA D₁-like receptor antagonist SCH23390 (1 µM) completely blocked the increase of AC activity elicited by a maximal effective concentration (30 µM) 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 EC₅₀ value ($4.0 \pm 0.7 \mu\text{M}$, $p > 0.05$, $n = 4$). CCh (1 mM) also potentiated the stimulatory effects elicited by the selective DA D₁-like receptor agonists (±)APB (fig. 1b) and SKF 81297 (fig. 1c) by 74 ± 5 and $79 \pm 6\%$ ($p < 0.001$, $n = 4$), respectively, without affecting their EC₅₀ values [(±)APB: vehicle $2.6 \pm 0.7 \text{ nM}$, CCh $2.9 \pm 0.6 \text{ nM}$; SKF 81297: vehicle $10.5 \pm 2 \text{ nM}$, CCh $7.5 \pm 1.9 \text{ nM}$, $p > 0.05$, $n = 4$].

Both CCh and Oxo-M, a selective muscarinic receptor agonist, enhanced DA-stimulated cyclic AMP formation in a concentration-dependent manner with EC₅₀ values of $23 \pm 3 \mu\text{M}$ and $382 \pm 30 \text{ nM}$, 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 μ 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 μ 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_{q/11} 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 α 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 ± 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 α_tGDP (500 nM) [35] and assayed for AC activity. The addition of α_tGDP failed to significantly affect DA-stimulated cyclic AMP formation but markedly suppressed the potentiation elicited by CCh (fig. 3b). Heat-inactivated α_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 α_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 α_tGDP , and 36 kDa for $\beta\gamma_t$ (online suppl. fig. 1). These values correspond to the molecular mass of transducin α and β subunits, respectively. The γ 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

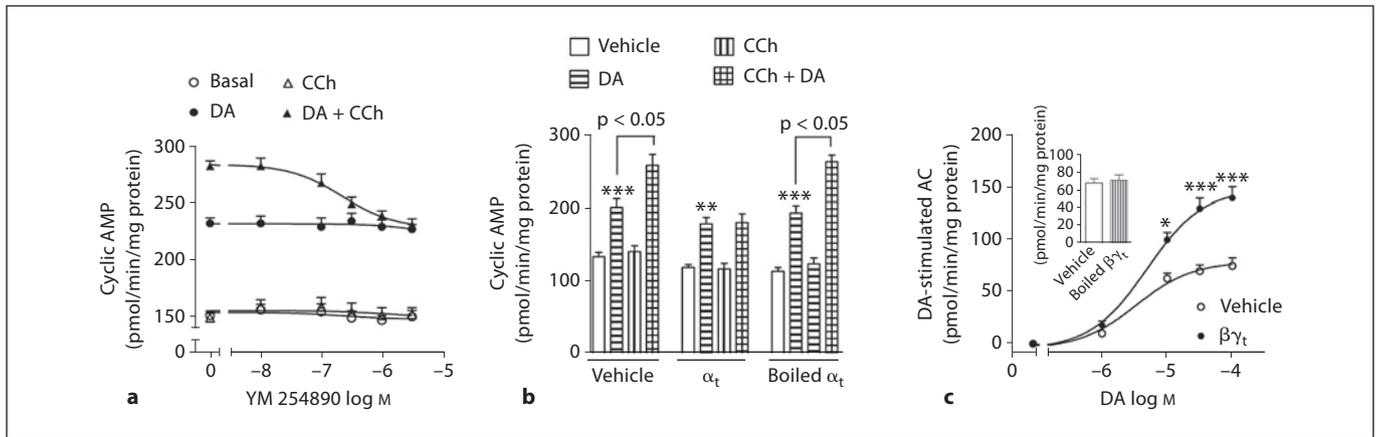


Fig. 3. Involvement of $G_{q/11}$ and $G\beta\gamma$ in M_1 muscarinic potentiation of DA D_1 -like receptor stimulation of cyclic AMP formation. **a** Tissue membranes were incubated with either vehicle or the indicated concentrations of the $G_{\alpha q/11}$ antagonist YM 254890 and AC activity was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

** $p < 0.01$; *** $p < 0.001$ 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 μM 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

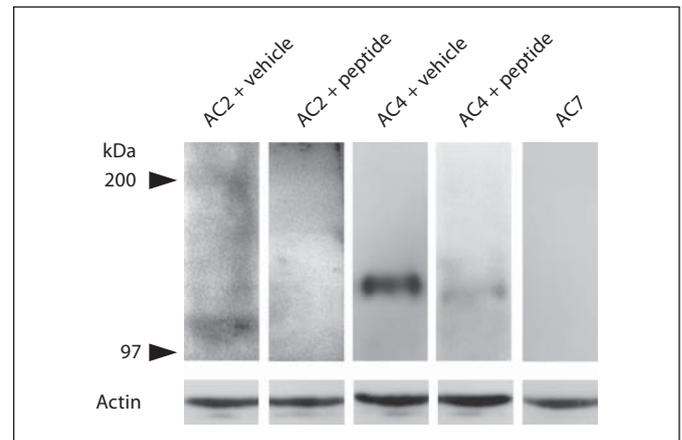


Fig. 4. Western blot analysis of AC2, AC4 and AC7 expression in mouse medial prefrontal cortex. Tissue extracts containing 50 μg of protein were loaded onto 7.5% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes and incubated with anti-AC2 antibody (1:200) pretreated with either 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.

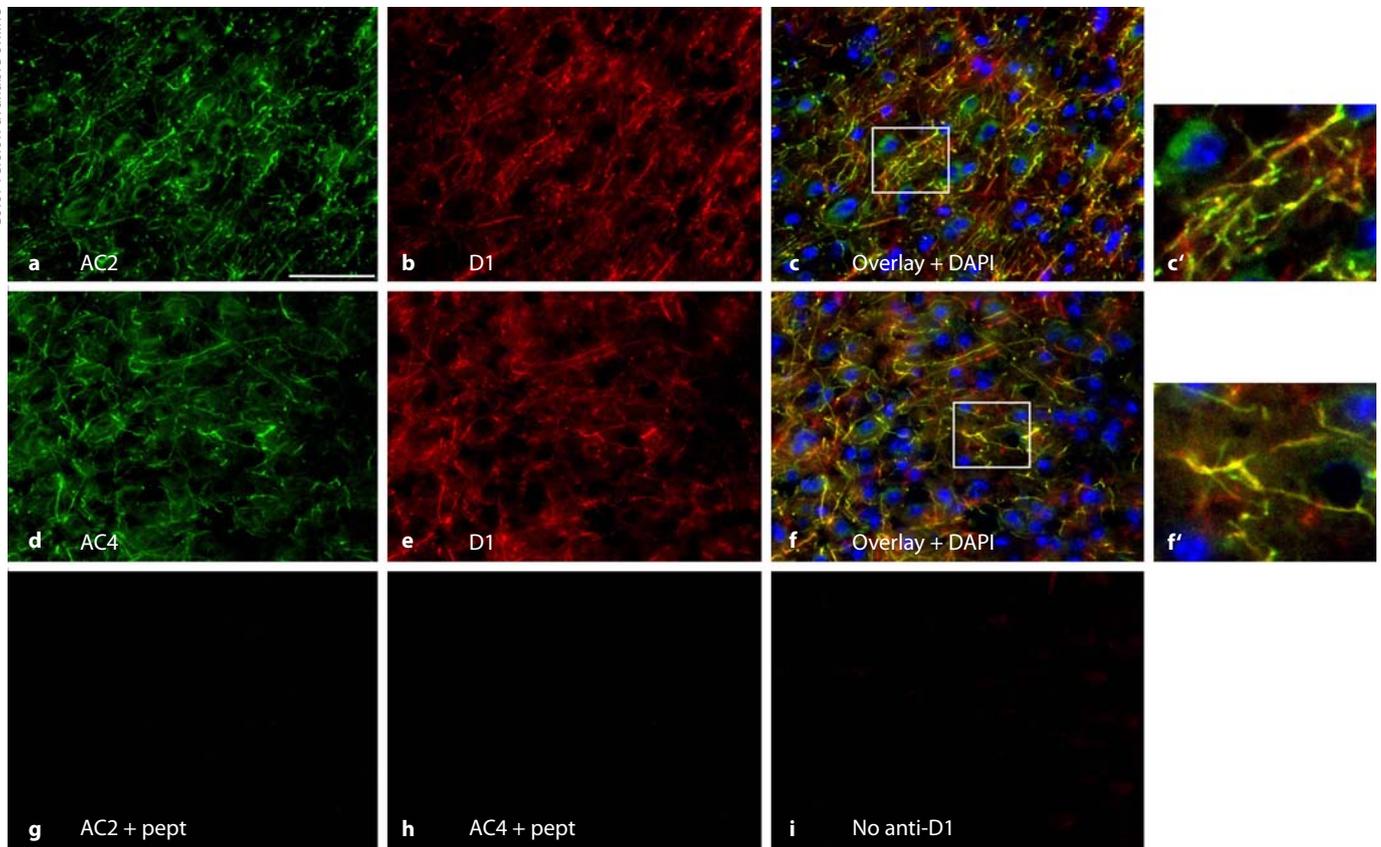


Fig. 5. Double immunofluorescence histochemistry of AC2, AC4 and DA D₁ receptors in the medial prefrontal cortex. Mouse brain coronal sections (10 μm) were labeled with the AC2 and AC4 antibodies each combined with the DA D₁ receptor antibody. **a** AC2 labeling. **b** DA D₁ receptor labeling in the 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 D₁ 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 D₁ receptor immunoreactivities in fiber-like structures. **d** AC4 labeling. **e** DA D₁

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 D₁ 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 D₁ receptor immunolabeling without the addition of a primary antibody. Data are representative of 3 tissue preparations. Bar = 50 μm .

corresponding immune peptide suppressed both AC2 and AC4 immunolabeling (fig. 5g, h). The immunostaining for DA D₁-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 D₁-like receptors displayed a marked AC2 (fig. 5c, c') and AC4 (fig. 5f, f') immunoreactivity. Quantitative analysis indicated that 64.9 ± 6 and $37.0 \pm 10\%$ of DA D₁-like receptor signal colocalized with AC4 and AC2 signals, respectively ($p < 0.05$, $n = 25$).

Activation of M₁ Muscarinic Receptor Enhances DA D₁-Like Receptor-Induced Stimulation of CREB Phosphorylation

Exposure of primary cortical cells to 1 μM SKF 81297 induced a significant increase of the neuronal cells positive for phospho-CREB (fig. 6). The addition of Oxo-M (3 μM), 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 D₁-like receptor agonist but completely prevented the Oxo-M-induced potentiation.

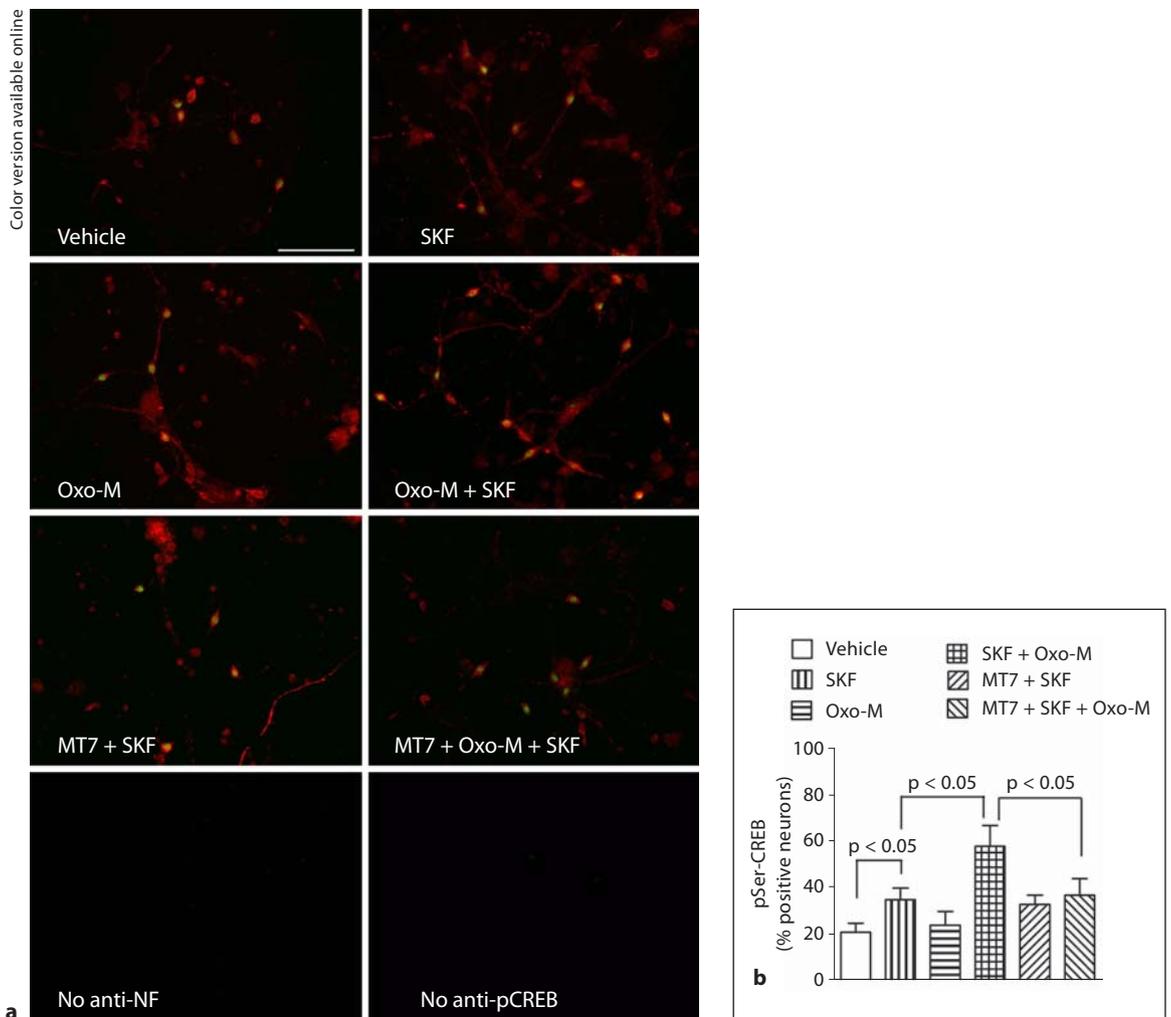


Fig. 6. M_1 muscarinic receptor stimulation potentiates DA D_1 -like receptor induced CREB phosphorylation in primary neurons of mouse frontal cortex. **a** Cells were preincubated with either vehicle or MT-7 (10 nM) for 10 min and then treated with either vehicle, 1 μ M SKF 81297 (SKF), 3 μ M Oxo-M 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 μ 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).

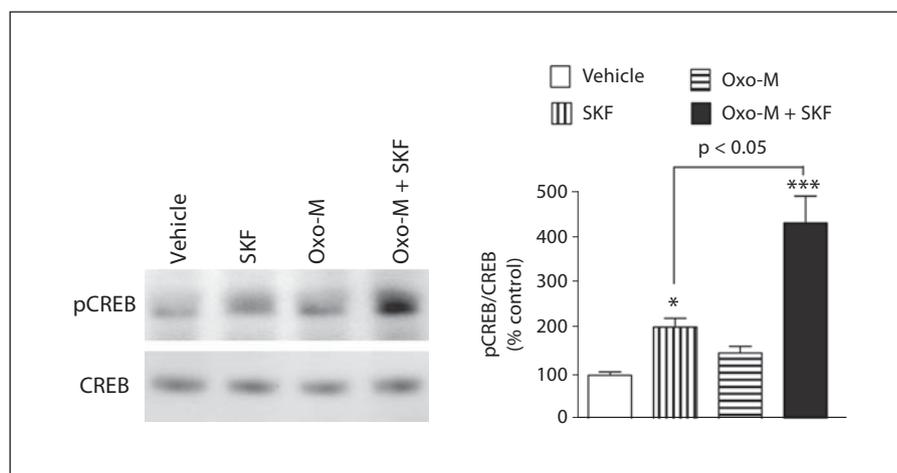
Similar results were obtained by Western blot analysis of phospho-CREB levels. Cells were treated with either SKF 81297 (1 μ M), Oxo-M (3 μ M) or the combination of the two receptor agonists. As shown in figure 7, SKF 81297 significantly enhanced phospho-CREB levels, whereas Oxo-M caused a modest and 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 μ M SKF 81297 (SKF), 3 μ 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 μ g) were analyzed for pCREB by Western blot. The membranes were then stripped of the antibodies and reprobbed 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 D₁-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 M₁ muscarinic receptors enhance DA D₁-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 M₁ and D₁-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 M₁ muscarinic receptors is known to increase intracellular Ca²⁺, which may enhance cyclic

AMP formation by stimulating Ca²⁺-activated AC isoforms, such as AC1, AC3 and AC8 [36, 37]. However, the potentiation of DA D₁-like receptor stimulation of AC occurred in the absence of added Ca²⁺ and in the presence of EGTA, thus ruling out the possible contribution of Ca²⁺-dependent mechanisms. Rather, the findings that the M₁ potentiation was blocked by the G_{q/11} antagonist YM-254890, prevented by the G $\beta\gamma$ scavenger α_{tGDP} and mimicked by the addition of exogenous G $\beta\gamma$ support a membrane-limited mechanism whereby M₁ activation of G_{q/11} and the consequent release of G $\beta\gamma$ subunits potentiate the DA D₁-like receptor-induced stimulation of Ca²⁺-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 α subunit of G_s [36, 37]. The lack of activity of boiled α_{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_{i/o}-coupled muscarinic and opioid receptors [40, 41]. With regard to DA D₁-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

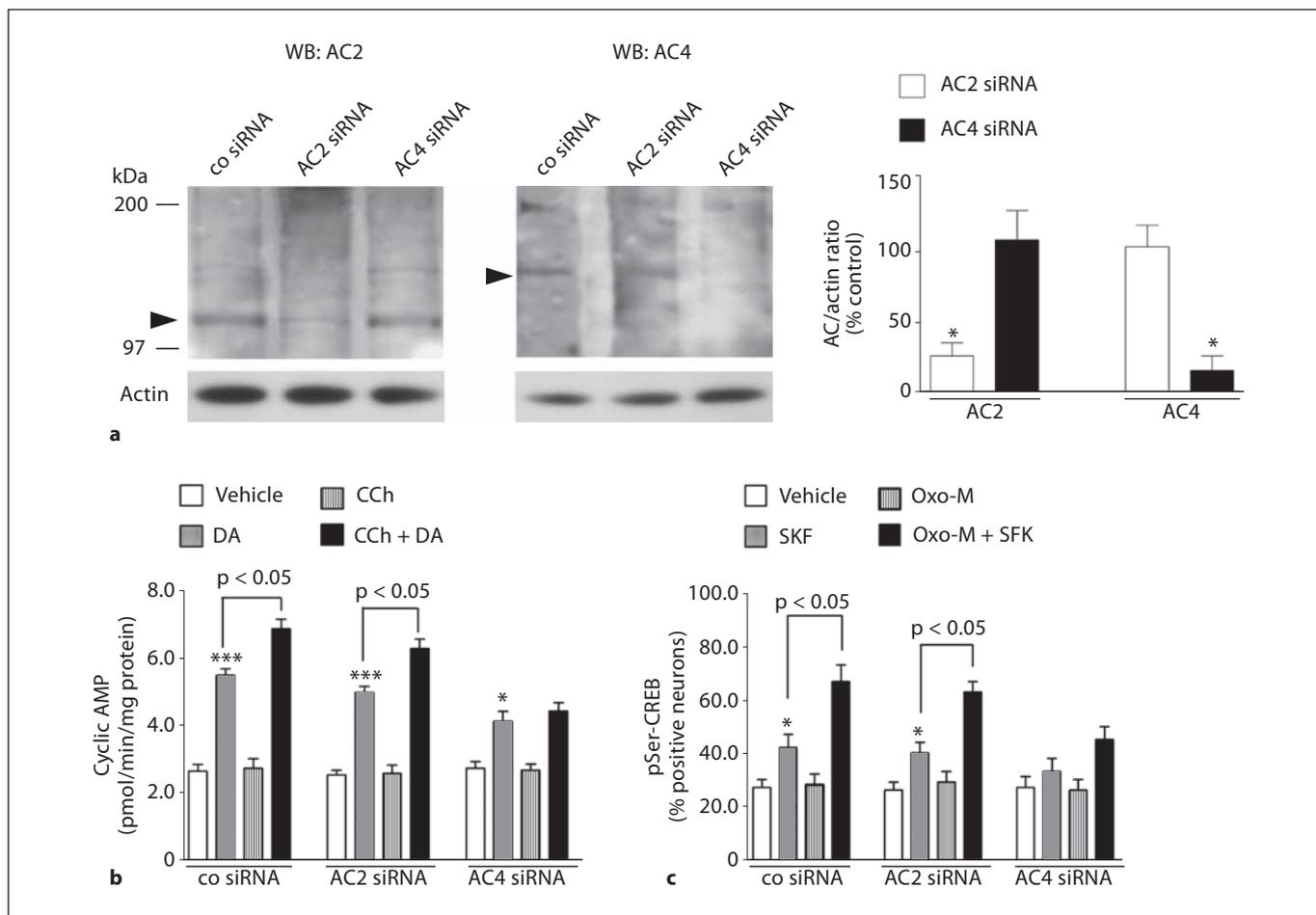


Fig. 8. Effects of AC2 and AC4 suppression by cognate siRNAs on DA D₁-like receptor signaling and M₁ potentiation. **a** Primary neurons of mouse frontal cortex were treated with either control siRNA (co siRNA), AC2 siRNA or AC4 siRNA and aliquots of cell extracts containing equal amounts of protein (10 μg) 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 ± 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 μM DA, 1 mM CCh and the combination of CCh and DA. Values are the mean ± 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 μM), Oxo-M (3 μM) 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 ± 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 D₁-like receptors with βγ-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 D₁ 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 D₁ 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 D₁ receptors and AC4 on neuronal dendritic processes.

The type of neuronal elements coexpressing DA D₁ receptors, M₁ receptors and AC isoforms remains to be established. In the prefrontal cortex, DA D₁-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 M₁ 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 D₁-like and M₁ 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 D₁-like and M₁ 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 D₁-like and M₁ muscarinic receptors are concomitantly stimulated. This cooperative interaction may be relevant for optimizing the level of DA D₁-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 D₁-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 D₁-like and M₁ receptor colocalization.

DA D₁-like and M₁ 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 D₁-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 D₁-like receptor modulation of memory retrieval performance [51] and is considered to be a major downstream event in the DA D₁-like receptor signaling cascade regulating long-term potentiation [8]. The M₁ enhancement of DA D₁-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 D₁-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 D₁-like receptors was still capable of significantly increasing cyclic AMP formation and CREB phosphorylation and that the concomitant M₁ receptor activation potentiated both responses. Conversely, in AC4 siRNA-treated cells, DA D₁-like receptor-stimulated cyclic AMP formation and CREB phosphorylation were markedly reduced. Under this condition the M₁ receptor potentiation of both responses was lost, indicating that in cortical neurons AC4, rather than AC2, is critically involved in DA D₁-like receptor signaling and in mediating the synergistic interaction with M₁ receptors. These functional data are also in

line with the immunofluorescence analysis showing a greater colocalization of the DA D₁ 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 D₁-like or D₂-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 D₁-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 D₁-like and muscarinic receptor interplay has mainly been characterized in the dorsal and ventral striatum, where activation of M₄ muscarinic

receptors inhibits DA D₁-stimulated cyclic AMP formation [55–57], whereas a blockade of muscarinic receptors enhances DA D₁-like receptor and amphetamine-stimulated neuropeptide gene expression [58, 59]. It has been speculated that in nucleus accumbens M₄ receptor inhibition could be pharmacologically exploited to treat pathological conditions characterized by a hyperdopaminergic state induced by psychostimulants, such as cocaine, whereas selective M₄ 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 M₁ muscarinic receptors potentiates DA D₁ 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.

References

- 1 Tzschentke TM: Pharmacology and behavioral pharmacology of the mesocortical dopamine system. *Progr Neurobiol* 2001;63:241–320.
- 2 Seamans JK, Yang CR: The principal features and mechanisms of dopamine modulation in the prefrontal cortex. *Progr Neurobiol* 2004;74:1–57.
- 3 Vincent SL, Khan Y, Benes FM: Cellular distribution of dopamine D₁ and D₂ receptors in rat medial prefrontal cortex. *J Neurosci* 1993;13:2551–2564.
- 4 Smiley JF, Levey AI, Ciliax BJ, Goldman-Rakic PS: D₁ dopamine receptors immunoreactivity in human and monkey cerebral cortex: predominant and extrasynaptic localization in dendritic spines. *Proc Natl Acad Sci USA* 1994;91:5720–5724.
- 5 Gaspar P, Bloch B, Le Moine C: D₁ and D₂ receptor gene expression in the rat frontal cortex: cellular localization in different classes of efferent neurons. *Eur J Neurosci* 1995;7:1050–1063.
- 6 Goldman-Rakic PS, Muly EC 3rd, Williams GV: D₁ receptors in prefrontal cells and circuits. *Brain Res Rev* 2000;31:295–301.
- 7 Vijayraghavan S, Wang M, Birnbaum SG, Williams GV, Arnsten AFT: Inverted-U dopamine D₁ receptor actions on prefrontal neurons engaged in working memory. *Nat Neurosci* 2007;10:376–384.
- 8 Jay TM: Dopamine: a potential substrate for synaptic plasticity and memory mechanisms. *Progr Neurobiol* 2003;69:375–390.
- 9 Onali P, Olanas MC, Gessa GL: Selective blockade of dopamine D₁ receptors by SCH23390 discloses striatal dopamine D₂ receptors mediating the inhibition of adenylyl cyclase in rats. *Eur J Pharmacol* 1984;99:127–128.
- 10 Neve KA, Seamans JK, Trantham-Davidson H: Dopamine receptor signaling. *J Recept Signal Transduct Res* 2004;24:165–205.
- 11 Undieh AS: Pharmacology of signaling induced by dopamine D₁-like receptor activation. *Pharmacol Ther* 2010;128:37–60.
- 12 Silva AJ, Kogan JH, Frankland PW, Kida S: CREB and memory. *Annu Rev Neurosci* 1998;21:127–148.
- 13 Lonze BE, Ginty DD: Function and regulation of CREB family transcription factors in the nervous system. *Neuron* 2002;35:605–623.
- 14 Carlezon WA Jr, Duman RS, Nestler EJ: The many faces of CREB. *Trends Neurosci* 2005;28:436–445.
- 15 Winterer G, Weinberger DR: Genes, dopamine and cortical signal-to-noise ratio in schizophrenia. *Trends Neurosci* 2004;27:683–690.
- 16 Cai JX, Arnsten AFT: Dose-dependent effects of the dopamine D₁ receptor agonists A77636 or SKF81297 on spatial working memory in aged monkeys. *J Pharmacol Exp Ther* 1997;282:1–7.
- 17 Arnsten AFT: Toward a new understanding of attention-deficit hyperactivity disorder pathophysiology: an important role for prefrontal cortex dysfunction. *CNS Drugs* 2009;23:33–41.
- 18 Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnema NP, Nathanson NM, Silva A: Selective cognitive dysfunction in acetylcholine M₁ muscarinic mutant mice. *Nat Neurosci* 2003;6:51–58.
- 19 Wall PM, Flinn J, Messier C: Infralimbic muscarinic M₁ receptors modulate anxiety-like behaviour and spontaneous working memory in mice. *Psychopharmacology* 2001;155:58–68.
- 20 Raedler TJ, Knable MB, Jones DW, Urbina RA, Gorey JG, Lee KS, Egan MF, Coppola R, Weinberger DR: In vivo determination of muscarinic acetylcholine receptor availability in schizophrenia. *Am J Psychiatry* 2003;160:118–127.
- 21 Scarr E, Cowie TF, Kanellakis S, Sundram S, Pantelis C, Dean B: Decreased cortical muscarinic receptors define a subgroup of subject with schizophrenia. *Mol Psychiatry* 2008;14:1017–1023.
- 22 Liao DL, Hong CJ, Chen HM, Chen YE, Lee SM, Chang CY, Chen H, Tsai SJ: Association of muscarinic m₁ receptor genetic polymorphisms with psychiatric symptoms and cognitive function in schizophrenic patients. *Neuropsychobiology* 2003;48:72–76.
- 23 Langmead CJ, Watson J, Reavill C: Muscarinic acetylcholine receptors as CNS drug targets. *Pharmacol Ther* 2008;17:232–243.
- 24 Sellin AK, Shad M, Tamminga C: Muscarinic agonists for the treatment of cognition in schizophrenia. *CNS Spectr* 2008;13:985–996.
- 25 Scarr E: Muscarinic receptors in psychiatric disorders – can we mimic ‘health’? *Neurosignals* 2009;17:298–310.
- 26 Caulfield MP, Birdsall NJ: International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* 1998;50:279–290.

- 27 Ichikawa J, Chung YC, Li Z, Dai J, Meltzer HY: Cholinergic modulation of basal and amphetamine-induced dopamine release in rat medial prefrontal cortex and nucleus accumbens. *Brain Res* 2002;958:176–184.
- 28 Perry KW, Nisenbaum LK, George CA, Shannon HE, Felder CC, Bymaster FP: The muscarinic agonist xanomeline increases monoamine release and immediate early gene expression in the rat prefrontal cortex. *Biol Psychiatry* 2001;49:716–725.
- 29 Yang CR, Mogenson GJ: Dopaminergic modulation of cholinergic responses in rat medial prefrontal cortex: an electrophysiological study. *Brain Res* 1990;524:271–281.
- 30 Franklin KBJ, Paxinos G: *The Mouse Brain in Stereotaxic Coordinates*, ed 2. San Diego, Academic Press, 2001.
- 31 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254.
- 32 Onali P, Adem A, Karlsson E, Olanas MC: The pharmacological action of MT-7. *Life Sci* 2005;76:1547–1552.
- 33 Takasaki J, Saito T, Taniguchi M, Kawasaki T, Moritani Y, Hayashi K, Kobori M: A novel $G\alpha_{q/11}$ -selective inhibitor. *J Biol Chem* 2004;279:47438–47445.
- 34 Nishimura A, Kitano K, Takasaki J, Taniguchi M, Mizuno N, Tago K, Hakoshima T, Itoh H: Structural basis for the specific inhibition of heterotrimeric Gq protein by a small molecule. *Proc Natl Acad Sci USA* 2010;107:13666–13671.
- 35 Federman AD, Conklin BR, Schrader KA, Reed RR, Bourne HR: Hormonal stimulation of adenylyl cyclase through Gi protein $\beta\gamma$ subunits. *Nature* 1992;356:159–161.
- 36 Sunahara RK, Taussig R: Isoforms of mammalian adenylyl cyclase: multiplicities of signaling. *Mol Interv* 2002;2:168–184.
- 37 Chern Y: Regulation of adenylyl cyclase in the central nervous system. *Cell Signal* 2000;12:195–204.
- 38 Baker LP, Nielsen MD, Impey S, Hacker BM, Posser SW, Chan MYM, Storm DR: Regulation and immunohistochemical localization of $\beta\gamma$ -stimulated adenylyl cyclases in mouse hippocampus. *J Neurosci* 1999;19:180–192.
- 39 Yamamoto M, Ozawa H, Saito T, Frölich L, Riederer P, Takahata N: Reduced immunoreactivity of adenylyl cyclase in dementia of the Alzheimer type. *Neuroreport* 1996;7:2965–2970.
- 40 Olanas MC, Ingianni A, Onali P: Role of G protein $\beta\gamma$ subunits in muscarinic receptor-induced stimulation and inhibition of adenylyl cyclase activity in rat olfactory bulb. *J Neurochem* 1998;70:2620–2627.
- 41 Onali P, Ingianni A, Olanas MC: Dual coupling of opioid receptor-like (ORL1) receptors to adenylyl cyclase in the different layers of the rat main olfactory bulb. *J Neurochem* 2001;77:1520–1530.
- 42 Abdel-Majid RM, Tremblay F, Baldrige WH: Localization of adenylyl cyclase proteins in the rodent retina. *Mol Brain Res* 2002;101:62–70.
- 43 Antoni FA, Sosunov AA, Haunso A, Paterson JM, Simpson J: Short-term plasticity of cyclic adenosine 3',5'-monophosphate signaling in anterior pituitary corticotrope cells: the role of adenylyl cyclase isotypes. *Mol Endocrinol* 2003;17:692–703.
- 44 Coté M, Guillon G, Payet MD, Gallo-Payet N: Expression and regulation of adenylyl cyclase isoforms in the human adrenal gland. *J Clin Endocrinol Metab* 2001;86:4495–4503.
- 45 Hellevuo K, Yoshimura M, Mons N, Hoffman PL, Cooper DMF, Tabakoff B: The characterization of a novel human adenylyl cyclase which is present in brain and other tissues. *J Biol Chem* 1995;270:11581–11589.
- 46 Bergson C, Mrzljak L, Smiley JF, Pappy M, Levenson R, Goldman-Rakic PS: Regional, cellular, and subcellular variations in the distribution of D₁ and D₅ dopamine receptors in primate brain. *J Neurosci* 1995;15:7821–7836.
- 47 Muly EC III, Szigeti K, Goldman-Rakic PS: D₁ receptor in interneurons of macaque prefrontal cortex: distribution and subcellular localization. *J Neurosci* 1998;18:10553–10565.
- 48 Yamasaki M, Matsui M, Watanabe M: Preferential localization of muscarinic M₁ receptor on dendritic shaft and spine of cortical pyramidal cells and its anatomical evidence for volume transmission. *J Neurosci* 2010;30:4408–4418.
- 49 Zhang ZW, Burke MW, Calakos N, Beaulieu JM, Vaucher E: Confocal analysis of cholinergic and dopaminergic inputs onto pyramidal cells in the prefrontal cortex of rodents. *Front Neuroanat* 2010;4:21.
- 50 Ichikawa J, Li Z, Dai J, Meltzer HY: Atypical antipsychotic drugs, quetiapine, iloperidone, and melperone, preferentially increase dopamine and acetylcholine release in rat medial prefrontal cortex: role of 5-HT_{1A} receptor agonism. *Brain Res* 2002;956:349–357.
- 51 Hotte M, Thuault S, Lachaise F, Dineley KT, Hemmings HC, Nairn AC, Jay TM: D₁ receptor modulation of memory retrieval performance is associated with changes in pCREB and pDARPP-32 in rat prefrontal cortex. *Behav Brain Res* 2006;171:127–133.
- 52 Castellano C, Cabib S, Puglisi-Allegra S, Gasbarri A, Sulli A, Pacitti C, Introini-Col-lison IB, McGaugh JL: Strain-dependent involvement of D₁ and D₂ dopamine receptors in muscarinic cholinergic influences on memory storage. *Behav Brain Res* 1999;98:17–26.
- 53 Lee KW, Hong JH, Choi IY, Che Y, Lee JK, Yang SD, Song CW, Kang HS, Lee JH, Noh JS, Shin HS, Han PL: Impaired D₂ dopamine receptor function in mice lacking type 5 adenylyl cyclase. *J Neurosci* 2002;22:7931–7940.
- 54 Iwamoto T, Okumura S, Iwatsubo K, Kawabe JI, Ohtsu K, Sakai I, Hashimoto Y, Izumitani A, Sango K, Ajiki K, Toya Y, Umemura S, Goshima Y, Arai N, Vatner SF, Ishikawa Y: Motor dysfunction in type 5 adenylyl cyclase-null mice. *J Biol Chem* 2003;278:16936–16940.
- 55 Olanas MC, Adem A, Karlsson E, Onali P: Rat striatal muscarinic receptors coupled to the inhibition of adenylyl cyclase activity: potent block by the selective M₄ ligand muscarinic toxin 3 (MT3). *Br J Pharmacol* 1996;118:283–288.
- 56 Olanas MC, Onali P: Antagonism of striatal muscarinic receptors inhibiting dopamine D₁ receptor-stimulated adenylyl cyclase activity by cholinergic antagonists used to treat Parkinson's disease. *Br J Pharmacol* 1996;118:827–828.
- 57 Onali P, Olanas MC: Muscarinic M₄ receptor inhibition of dopamine D₁-like receptor signaling in rat nucleus accumbens. *Eur J Pharmacol* 2002;448:105–111.
- 58 Wang JQ, McGinty JF: The full D₁ dopamine agonist SKF-82958 induces neuropeptide mRNA in the normosensitive striatum of rats: regulation of D₁/D₂ interactions by muscarinic receptors. *J Pharmacol Exp Ther* 1997;281:972–982.
- 59 Wang JQ, Jolkkonen M, McGinty JF: The muscarinic toxin 3 augments neuropeptide mRNA in rat striatum in vivo. *Eur J Pharmacol* 1997;334:43–47.