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# Regulation of muscarinic receptor function in developing oligodendrocytes by agonist exposure

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**1** Oligodendrocytes, the myelin forming cells in the CNS, express muscarinic acetylcholine receptors (mAChR), primarily M3, coupled to various signal transduction pathways.

**2** In the present study we have investigated whether mAChR undergo functional agonist-induced regulation in cultured oligodendrocyte progenitors and differentiated oligodendrocytes.

**3** The muscarinic agonist, carbachol (CCh) caused a time-dependent desensitization of phosphoinositide (PI) hydrolysis, and the internalization and down-regulation of receptors. Short-time desensitization (5 min) of PI hydrolysis occurred without receptor internalization and reached 54% by 1 h. The same treatment decreased cell surface receptors labelled with the non-permeable ligand [$^3$H]-NMS by 47%, while total receptor density ([$^3$H]-scopolamine binding) decreased by 30%. Longer CCh treatment down-regulated receptors by 70% and desensitized the PI response by 80%.

**4** Although protein kinase C (PKC) activation desensitized mAChR, CCh-mediated desensitization was independent of PKC.

**5** Inhibition of receptor endocytosis by low temperature during the pre-stimulation period or in the presence of hyperosmotic sucrose (0.5 M) blocked desensitization, receptor internalization and down-regulation.

**6** Recovery of surface mAChR and their functional activity following down-regulation was slow, returning to control levels by 24 h after agonist removal. In progenitor cells, dose-response curves for CCh-mediated PI hydrolysis and c-*fos* mRNA expression showed that newly synthesized mAChR were supersensitive after recovery.

**7** Overall, the present results provide evidence of functional agonist-mediated mAChR regulation in brain oligodendroglial cells.
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**Keywords:** Carbachol; muscarinic receptors; supersensitivity; desensitization; c-*fos*; oligodendrocytes; myelin

**Abbreviations:** bFGF, basic fibroblast growth factor; $[Ca^{2+}]_i$, intracellular calcium; CCh, carbachol; CS, calf serum; CREB, cAMP-response element binding protein; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; G-protein, guanine nucleotide-binding protein; GPCR, G-protein coupled receptor; H7, 1-(5-isoquinolinyl-sulphonyl)-2-methylpiperazine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; IP, total [$^3$H]-inositol phosphates; mAChR, muscarinic acetylcholine receptors; M1–M5 muscarinic receptor subtypes; OP, oligodendrocyte progenitor; OL, oligodendrocyte; PDGF, platelet derived growth factor-AA; PLC, phospholipase C; PI, phosphoinositide; PKC, protein kinase C; [$^3$H]-NMS, [$^3$]-*N*-methylscopolamine; SFM, serum free medium; TPA, 12-O-tetradecanoyl phorbol 13-acetate

## Introduction

In the central nervous system oligodendrocytes form the myelin membrane that insulates axons and facilitates rapid conduction of action potentials. Recent reports have shown that oligodendrocytes are engaged in a complex communication with neurons. Excitatory glutamatergic synapses (Bergles *et al*., 2000), functional neurotransmitter receptors (reviewed in Belachew *et al*., 1999) and ionic channels (see for example, Soliven *et al*., 1988; Sontheimer *et al*., 1989) have been identified in developing and adult brain oligodendrocytes.

In culture, progenitors and mature oligodendrocytes express G-protein-coupled muscarinic acetylcholine receptors (mAChR) which activate intracellular effector pathways (Ritchie *et al*., 1987; Kastritis & McCarthy, 1993; Cohen & Almazan, 1994; Takeda *et al*., 1995). Studies from our laboratory indicated that both progenitors and differentiated oligodendrocytes express the five mAChR mRNAs, with M3 being the predominant subtype expressed in these cells (Ragheb *et al*., 2001). In oligodendrocyte progenitors, binding of the acetylcholine analogue carbachol (CCh) to mAChR decreases $\beta$-adrenergic stimulated cAMP formation, increases inositol-1, 4,5 trisphosphate ($IP_3$) and intracellular calcium ($[Ca^{2+}]_i$) levels (Ritchie *et al*., 1987; Kastritsis & McCarthy, 1993; Cohen & Almazan, 1994) and results in protein kinase C (PKC) activation as well as expression of the immediate-early gene c-*fos* (Cohen *et al*., 1996; Larocca & Almazan, 1997). Stimulation of mAChR with CCh causes activation of extracellular signal-regulated kinases (ERK) (Larocca & Almazan, 1997) and increases the proliferation of oligodendrocyte progenitors (Cohen *et al*., 1996). This effect

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is mediated by M3 receptors and involves the activation of an ERK pathway (Ragheb *et al*., 2001). Moreover, muscarinic stimulation of M3 receptors triggers the phosphorylation of the transcription factor, cAMP response element-binding protein (CREB) (Ragheb *et al*., 2001), which is dependent on PKC and ERK pathways (Pende *et al*., 1997; Sato-Bigbee *et al*., 1999). Together, these data indicate that M3 receptors play a role in muscarinic function in developing oligodendrocytes.

The process of G protein-coupled receptor (GPCR) regulation strongly affects signal transduction and is of fundamental importance for cellular function. One feature of GPCRs is the phenomenon known as desensitization, which is manifested as a reduced responsiveness to subsequent stimulation following short-term agonist occupancy (Hausdorff *et al*., 1990; Lohse, 1993). Uncoupling of a receptor from its G protein as well as receptor internalization to intracellular compartments are mechanisms that underlie an attenuated functional response. This type of desensitization is usually mediated by phosphorylation of the activated receptor by members of the G protein-coupled receptor kinases (GRKs). Phosphorylated receptors then interact with cytoplasmic proteins termed $\beta$-arrestins, which interfere with receptor-G protein interaction, and favour receptor endocytosis (Pitcher *et al*., 1998). Prolonged or repeated exposure to agonists elicits a marked attenuation of cellular responses and a reduction in receptor number, a process referred to as downregulation (reviewed in Tsao & von Zastrow, 2000).

The purpose of the present study was to determine whether endogenously expressed mAChR, primarily M3, undergo agonist-induced regulation in oligodendrocyte progenitor and differentiated oligodendrocyte cultures from rat brain. We examined the effects of short- and long-term treatment of oligodendrocytes with the muscarinic agonist, CCh, on cell surface and total receptor density, on desensitization of PI hydrolysis as well as on the rate of transcription of immediate early gene c-*fos*. The functional activity of receptors after down-regulation was also explored. Finally, we assessed the role of receptor endocytosis in the process of desensitization by prestimulating cells at low temperature or in the presence of hyperosmotic sucrose.

## Methods

### *Materials*

Culture media, foetal calf serum (FCS) and calf serum (CS) were from Gibco-Invitrogen (Burlington, ON, Canada). Progesterone, biotin, sodium selenite, insulin, putrescine, carbachol, atropine sulphate, poly-D-lysine, hydrocortisone-21-P, transferrin, 3,3′,5-triiodo-L-thyronine were purchased from Sigma (Oakville, ON, Canada); while human recombinant platelet derived growth factor-AA (PDGF-AA) and basic fibroblast growth factor (bFGF) were from PeproTech Inc (Rocky Hill, NJ, U.S.A.). [$^3$H]-*N*-methylscopolamine (82 Ci mmol$^{-1}$) and [$^3$H]-scopolamine (83 Ci mmol$^{-1}$) were obtained from Amersham Pharmacia Biotech (Oakville, ON, Canada), analytical-grade Dowex 1-X8 (100–200 mesh formate form) from Bio-Rad (Mississauga, ON, Canada) and myo-[$^3$H]-inositol (12.3 Ci mmol$^{-1}$) from Dupont Co. (Mississauga, ON, Canada). Ammonium formate, formic acid, scintillation fluid and other reagents were from VWR (Mont Royal, QC, Canada) or from Fisher Scientific (Ottawa, ON, Canada).

Serum free medium (SFM) consisted of DMEM: F12 (1 : 1) containing 25 $\mu$g ml$^{-1}$ transferrin, 30 nM triiodothyronine, 20 nM hydrocortisone-21-P, 20 nM progesterone, 10 nM biotin, 30 nM selenium, 5 $\mu$g ml$^{-1}$ insulin, 1 $\mu$g ml$^{-1}$ putrescine, 0.1% BSA, 50 units ml$^{-1}$ penicillin, 50 $\mu$g ml$^{-1}$ streptomycin. Complete medium was composed of DMEM: F12 (1 : 1) containing 50 units ml$^{-1}$ penicillin plus 50 $\mu$g ml$^{-1}$ streptomycin and 12% FCS.

### *Primary cell culture preparation*

Cultures were generated according to the modified technique of McCarthy & de Vellis (1980) as described by Almazan *et al*. (1993). Oligodendrocyte progenitors (OP) were plated on 6-well dishes at a density of 15,000 cells cm$^{-2}$ and were grown in SFM containing 2.5 ng ml$^{-1}$ bFGF and PDGF-AA (SFM + GF) for 4 days in order to expand their numbers and prevent differentiation. Morphological examination established that the progenitor cultures were essentially homogeneous bipolar cells, and acquired ramified processes as they differentiated to mature oligodendrocytes *in vitro*. The cultures were immunocytochemically characterized as previously described (Cohen & Almazan, 1994). More than 95% of the cells reacted positively with the monoclonal antibody A2B5, a marker of oligodendrocyte progenitors. Less than 5% were galactocerebroside (GC) positive oligodendrocytes, glial fibrillary acidic protein (GFAP) positive astrocytes or complement type-3-positive microglia. When progenitors were cultured for 12 additional days in media containing the components of SFM supplemented with 3% CS the cells acquired a complex morphology and the oligodendrocyte markers galactocerebroside and myelin basic protein, and were considered to be mature oligodendrocytes (OL).

### *Homologous mAChR desensitization and resensitization*

In short-term desensitization protocols, progenitor or oligodendrocyte cultures were pre-stimulated at 37°C with 1 mM CCh from 5–60 min in their respective growing media, and rapidly washed three times in warmed HEPES-buffered Hanks balanced salt solution, pH 7.4 (buffer). This was followed by stimulation with 1 mM CCh to determine total [$^3$H]-inositol phosphates (IP) accumulation, to assess agonist-stimulated phospholipase C (PLC) activity or for direct use in binding studies as described later.

In long-term desensitization experiments, cell cultures were pre-stimulated at 37°C for the appropriate time (1–24 h) in media containing 1 mM CCh, washed as described above and used to determine total IP accumulation or muscarinic receptor density. Recovery from CCh-induced desensitization was examined by incubating cells with 1 mM CCh at 37°C for 60 min, after which, cultures were washed three times in warm buffer and allowed to recover for the desired time period in fresh medium (i.e., 1–24 h). The culture medium was then replaced with buffer with or without 1 mM CCh, and total IP accumulation and mAChR density was determined.

To assess the role of mAChR endocytosis in the process of receptor desensitization, cells were pre-exposed to 1 mM CCh

for 30 or 60 min at low temperature (10°C), a manipulation known to prevent receptor translocation (Fisher, 1988; Thompson & Fisher, 1990), washed in warmed buffer and used directly to determine total IP accumulation or receptor density. In addition, to block receptor internalization, cells were preincubated with 0.5 M sucrose in buffer for 20 min, and exposed in the same buffer to 1 mM CCh for 30 or 60 min. This manipulation renders the cells hypertonic and clathrin assembly is disrupted (Heuser & Anderson, 1989; Slowiejko *et al*., 1996). Cells were then washed three times with buffer followed by determination of IP accumulation and mAChR density.

### *Radioligand binding experiments*

Intact cells grown in 6-well dishes (around 100 $\mu$g protein per well for progenitors and 300 $\mu$g for oligodendrocytes) were challenged with CCh as described above, washed and incubated for 16 h at 4°C in 1 ml of buffer containing either 1 nM [$^3$H]-NMS or [$^3$H]-scopolamine (Fisher, 1988). All binding experiments were carried out at 4°C to avoid receptor recycling during the incubation period after CCh treatment. For saturation binding experiments, 0.01–4 nM concentrations of radioligand were used. The binding reactions were terminated by two rapid washes with ice-cold buffer. Cells were solubilized in 250 $\mu$l of 0.2 N NaOH/0.1% Triton X-100, transferred to vials containing 5 ml of Ecolite and radioactivity was determined by liquid scintillation spectrometry. Counting efficiency was 50% and values in d.p.m. were used to calculate fmol of ligand bound. Non-specific binding determined in the presence of 25 $\mu$M atropine (Fisher, 1988) was, at 1 nM concentration of radioligand, 15% for [$^3$H]-NMS and 30% for [$^3$H]-scopolamine in progenitors and 50% for both radioligands in oligodendrocytes.

### *RNA extraction and Northern blot analysis*

Total RNA was extracted from oligodendrocyte progenitors as described previously (Cohen *et al*., 1996). RNA pellets were resuspended in 50% formamide/2.2 M formaldehyde/20 mM MOPS and denatured for 30 min at 65°C. Ten $\mu$g of RNA extracts were electrophoresed on a 1.3% agarose-formaldehyde gel and transferred to Hybond-N membranes. The c-*fos* probe was labelled with $\alpha$-$^{32}$P-dCTP using a random primer kit to a specific activity of $10^8$ c.p.m. $\mu g^{-1}$ DNA. Membranes were hybridized at 42°C for 48 h with $10^6$ c.p.m. of c-*fos* cDNA per ml of hybridization solution (50% formamide, 25 mM sodium phosphate buffer, pH 6.5, 0.8 M NaCl, 0.5% SDS, 1 mM EDTA) and exposed to X-ray films. Autoradiographs were quantified by densitometry. To standardize for equal RNA loading and transfer, the membranes were stripped of radioactive probe and stained with methylene blue.

### *Total [$^3$H]-inositol phosphate measurement*

Cells were incubated for 18 h with 1 $\mu$Ci ml$^{-1}$ of *myo*-[$^3$H]-inositol-free DMEM containing the components found in SFM (labelling medium) plus 2.5 ng ml$^{-1}$ bFGF and PDGF-AA (for progenitors) or labelling medium alone for oligodendrocytes as described (Cohen & Almazan, 1994). In the experiments involving agonist pre-treatment, cells were pre-exposed to 1 mM CCh in labelling medium for various periods of time, washed three times and incubated in 1 ml of buffer containing 10 mM LiCl, with or without 1 mM CCh for another 10 min. After stimulation the reaction was stopped by aspirating the medium, and immediately fixing the cells with 0.5 ml of ice-cold methanol. Total [$^3$H]-inositol phosphates were determined according to the procedure described (Berridge *et al*., 1983). Labelled IPs were collected in 5 ml of 1.2 N ammonium formate in 0.1 N formic acid after free inositol and glycerophosphate fractions were eluted from the column.

### *Data analysis*

Results are presented as the mean $\pm$ s.e.mean of at least three different experiments performed in separate cell preparations, duplicate or triplicate determinations were performed in each experiment. One-way analysis of variance followed by Dunnett's test for multiple comparison was used as indicated in order to examine the statistical significance; $P$ values less than 0.05 were considered significant. The equilibrium binding parameters were estimated using the non-linear iterative algorithm LIGAND (Munson & Rodbard, 1980; McPherson, 1985). Concentration response curves were analysed using non-linear regression (Prism Graph-Pad, San Diego, CA, U.S.A.). Protein content was determined using the Bradford reagent (Bio-Rad).

## Results

### *Characterization of [$^3$H]-NMS and [$^3$H]-scopolamine binding and receptor activity*

To determine the effect of CCh pre-treatment on the density of mAChR, we quantified receptors by the difference in the binding of the hydrophilic ligand [$^3$H]-NMS and the lipophilic ligand [$^3$H]-scopolamine. [$^3$H]-NMS labels receptors located exclusively at the cell surface while [$^3$H]-scopolamine binds to total sites located throughout the plasma membrane plus internalized receptors.

Progenitors and oligodendrocytes expressed mAChRs as determined by the binding of the muscarinic antagonists [$^3$H]-NMS and [$^3$H]-scopolamine. As shown in Table 1 dissociation constants ($K_D$) were similar for both ligands, although they were decreased in mature cells. Maximum binding capacities ($B_{max}$) were around 50 fmol mg$^{-1}$ protein for either [$^3$H]-NMS or [$^3$H]-scopolamine in progenitors and were reduced by $\sim$70% in oligodendrocytes. Exposure of cultures for 10 min to increasing concentrations of CCh (0.1 $\mu$M–1 mM) resulted in a concentration-dependent accumulation of [$^3$H]-IP (Table 1). The $EC_{50}$ was $21 \pm 3$ $\mu$M for progenitors and $24 \pm 2$ $\mu$M for oligodendrocytes. As observed for receptor density, the maximum effect of 1 mM CCh on [$^3$H]-IP production was reduced by 80% in oligodendrocytes ($2.2 \pm 0.19$-fold increase over basal) when compared to progenitors ($11.38 \pm 0.55$-fold increase). The total amount of [$^3$H]-IP accumulated (d.p.m. mg protein) was $159{,}625 \pm 8100$ for progenitors and $29{,}720 \pm 1000$ for oligodendrocytes ($n = 5$).

**Table 1** Parameters of muscarinic binding and phosphoinositide turnover in oligodendroglial cells

| | | *Progenitors* | *Oligodendrocytes* |
|---|---|---|---|
| [$^3$H]-NMS | $K_D$ (pM) | 60±2 | 43±3 |
| | $B_{max}$ (fmol mg$^{-1}$ protein) | 54±2 | 15±1 |
| [$^3$H]-Scop | $K_D$ (pM) | 70±3 | 54±3 |
| | $B_{max}$ (fmol mg$^{-1}$ protein) | 54±1 | 17±3 |
| [$^3$H]-IP | $EC_{50}$ ($\mu$M) | 21±3 | 24±2 |

Binding parameters of [$^3$H]-NMS and [$^3$H]-scopolamine determined in progenitors and oligodendrocytes differentiated for 12 days. Live cells were incubated for 16 h at 4°C with 0.01–4 nM of either radioligand, washed twice with cold buffer and radioactivity was determined as described in Methods. Non-specific binding was determined with 25 $\mu$M atropine. Incubation with increasing concentrations of CCh (0.1 $\mu$M–1 mM) resulted in a concentration-dependent accumulation of [$^3$H]-IP. Results are means±s.e.mean of three independent experiments performed in triplicate.

### *The desensitization and internalization of muscarinic receptors caused by short-term CCh-exposure does not involve PKC*

Pre-exposure of progenitors or oligodendrocytes to 1 mM CCh resulted in a time-dependent loss of cell surface [$^3$H]-NMS binding sites (Figure 1A,B). Receptor sequestration was initially observed after 10 min, and attained a level of ~47% after 60 min, with a half-life of about 15 min. In contrast to [$^3$H]-NMS binding, 30 min exposure to CCh produced an insignificant reduction in total mAChR density monitored by [$^3$H]-scopolamine binding (Figure 1A,B). A 1 h preincubation with agonist resulted in a significant reduction (~30%) in [$^3$H]-scopolamine binding sites, indicating that receptors were down-regulated. The loss of [$^3$H]-NMS binding was concentration-dependent with a half-maximal response ($EC_{50}$) of about 15 $\mu$M after 30 min of agonist preincubation (data not shown).

To determine the functional consequences of agonist pre-treatment of oligodendroglial cells, PI hydrolysis was measured in cells pre-exposed to 1 mM CCh for various time periods (5–60 min) during [$^3$H]-*myo*-inositol labelling. Figure 1C shows the time-dependent CCh-induced inhibition of IP accumulation produced by subsequent stimulation with the same agonist. Pre-exposure to CCh caused a rapid desensitization of mAChR-stimulated PI turnover, but little receptor internalization occurred during the first 5 min of incubation. After 1 h of agonist pre-treatment there was a 56% reduction in response in progenitor cells and a 65% reduction in oligodendrocytes.

To investigate whether PKC activation is involved in CCh-induced functional regulation of mAChR, progenitors and oligodendrocytes were pre-incubated with PKC activators or inhibitors. As shown in Table 2, activation of PKC with the phorbol ester TPA (1 $\mu$M) failed to decrease surface mAChR such as was effected by 1 mM CCh (30 min). However, TPA reduced the subsequent ability of 1 mM CCh to stimulate IP accumulation by 70%. In addition, the decrease in [$^3$H]-NMS binding induced by 30 min exposure to CCh was not blocked by pre-treatment with the PKC inhibitors H7 (10 $\mu$M) or bisindolylmaleimide (2 $\mu$M). To further evaluate the involve-

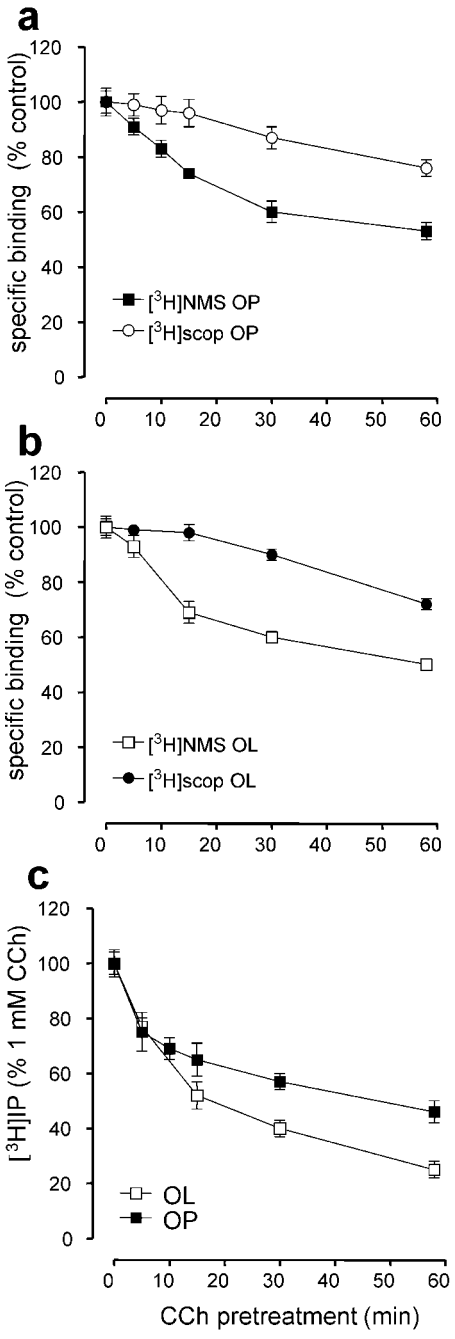


**Figure 1** Effect of short-term CCh pre-treatment on mAChR density and PI hydrolysis. Time course of CCh-induced sequestration of mAChR in progenitors (a) and oligodendrocytes (b). Cells were incubated at 37°C with 1 mM CCh for the indicated times, washed three times with warmed buffer, and mAChRs were measured by radioligand binding at 4°C for 16 h with 1 nM [$^3$H]-NMS or [$^3$H]-scopolamine. Atropine (25 $\mu$M) was used to determine non-specific binding. Data are expressed as percentage of untreated control group. (c) Time course of CCh-induced desensitization of PI hydrolysis. Cells were labelled with 1 $\mu$Ci ml$^{-1}$ myo-[$^3$H]-inositol for 18 h and 1 mM CCh was added during the labelling period to induce receptor desensitization. Following agonist pre-stimulation, cells were washed three times with warmed buffer and challenged with 1 mM CCh (plus 10 mM LiCl) for 10 min at 37°C. IP were determined as described in Methods. Data are expressed as percentage of maximal IP accumulation determined in the absence of CCh pre-treatment. Results are the means±s.e.mean of four independent experiments performed in triplicate.

**Table 2** Role of PKC in homologous muscarinic receptor regulation

| | *Progenitors* | | *Oligodendrocytes* | |
|---|---|---|---|---|
| | *[$^3$H]-IP* | *[$^3$H]-NMS* | *[$^3$H]-IP* | *[$^3$H]-NMS* |
| Control | 100±5 | 100±4 | 100±6 | 100±7 |
| 30 min CCh | 60±3 | 63±3 | 64±6 | 67±7 |
| H7+CCh | 60±4 | 73±4 | 71±5 | 73±5 |
| Bis+CCh | 58±5 | 66±4 | 69±5 | 70±6 |
| TPA | 23±5 | 102±6 | 27±4 | 95±6 |
| TPA+CCh | 19±4 | 66±4 | 18±4 | 64±6 |
| Bis+TPA | 100±3 | 99±5 | 96±5 | 100±6 |

Effect of activation or inhibition of PKC on mAChR sequestration and desensitization. To provoke receptor internalization, cells were treated with 1 mM CCh for 30 min. The PKC inhibitors H7 (10 $\mu$M) and bisindolylmaleinmide (Bis, 2 $\mu$M) were added to the cells 30 min before the addition of 1 mM CCh. A 30 min exposure to 1 $\mu$M TPA was used to activate PKC. Cells were then washed three times with buffer; and [$^3$H]-NMS binding or [$^3$H]-IP were determined as described in Methods. Data for IP are expressed as percentage of maximal accumulation determined in the absence of pre-treatment. [$^3$H]-NMS binding is expressed as percentage of untreated control group. Results are the means±s.e.mean of three independent experiments performed in triplicate.

ment of PKC in receptor desensitization, we tested the ability of H7 and bisindolylmaleimide to prevent CCh-induced desensitization. Both inhibitors blocked the effect of TPA, but not the effect mediated by CCh. Therefore, the data presented thus far suggest that agonist-mediated desensitization of mAChR occurs through a PKC-independent mechanism.

### *Long-term exposure to CCh down-regulates muscarinic receptors*

Pre-treatment of progenitors or oligodendrocytes with 1 mM CCh for 2–24 h significantly decreased surface and total mAChR. Subsequent CCh challenge caused a concomitant reduction in IP accumulation (Figure 2A–C). Carbachol (1 mM) pre-exposure caused a time-dependent reduction in cell surface mAChR labelling by 1 nM [$^3$H]-NMS. Maximal internalization occurred after 6 h CCh prestimulation, with 65 and 79% of surface receptors initially present being lost in progenitors and oligodendrocytes, respectively. Total receptor sites, determined by [$^3$H]-scopolamine binding, decreased at a lower rate after 24 h exposure to CCh.

In progenitors, pre-exposure to CCh produced a rapid desensitization of mAChR-stimulated PI hydrolysis, with an 80% loss of response within 4 h of agonist exposure. Between 4 and 24 h of agonist pre-treatment, receptor desensitization was maintained. In contrast, more than 90% of the response in oligodendrocytes was lost after 2 h of CCh treatment. In addition, CCh-pre-treatment resulted in a time-dependent down-regulation of c-*fos* mRNA expression, after 2 h of pretreatment the levels of c-*fos* mRNA were significantly reduced and disappeared at 6 h (Figure 3). These observations clearly show that while 30% of surface mAChR were still available on the cell surface, the response obtained following agonist stimulation, i.e. IP accumulation and c-*fos* transcription, was considerably reduced.

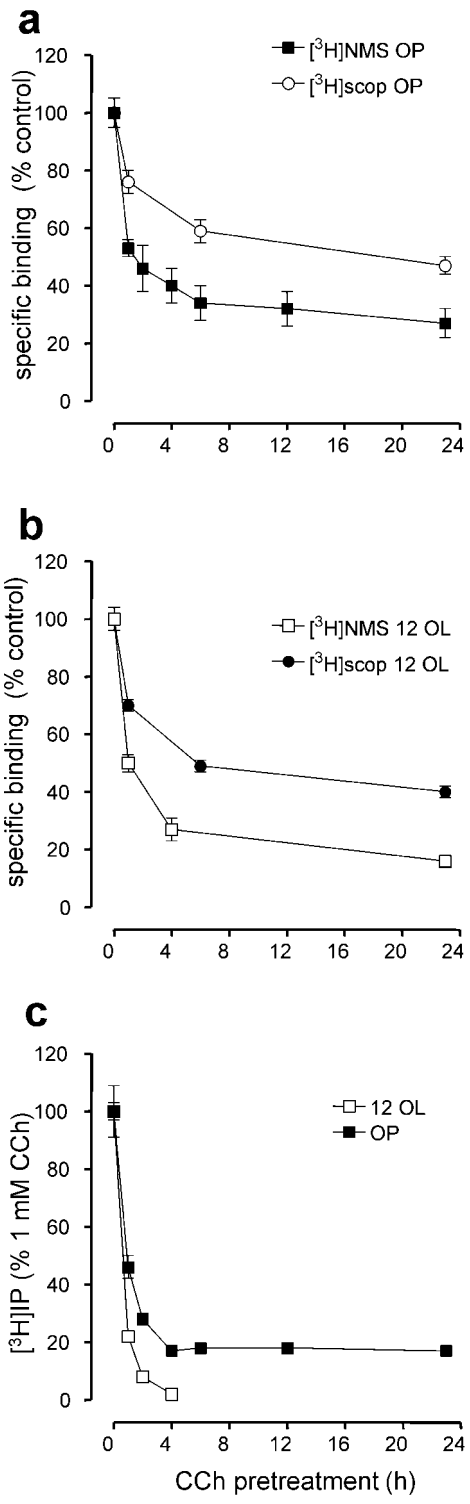


**Figure 2** Effect of long-term CCh pre-treatment on mAChR density and PI hydrolysis. Time courses of CCh-induced reduction of specific [$^3$H]-NMS and [$^3$H]-scopolamine binding in progenitors (a) and oligodendrocytes (b) and desensitization of IP hydrolysis (c). Cells were incubated at 37°C with 1 mM CCh for the indicated times, washed three times with warmed buffer and IP accumulation and mAChRs were measured as described in Methods. Binding data are expressed as percentage of untreated control group. PI hydrolysis data are expressed as percentage of maximal IP accumulation determined in the absence of CCh pre-treatment. Results are the means±s.e.mean of four independent experiments performed in triplicate.

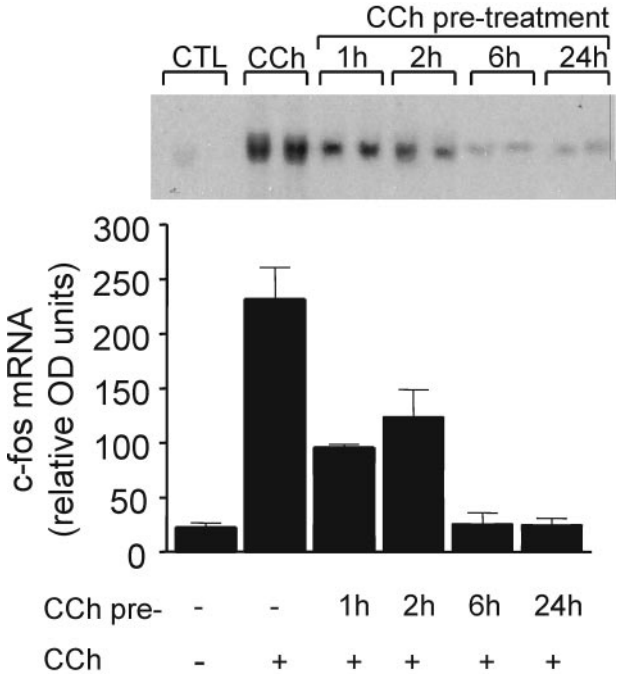


**Figure 3** Time-dependent effect of CCh pre-treatment on mAChR-mediated c-*fos* mRNA expression in oligodendrocyte progenitors. Cells were pre-treated for 1–24 h with 1 mM CCh, washed three times with buffer and maintained for 1 h in buffer before re-stimulation with 100 $\mu$M CCh for 30 min. Such resting period is necessary to decrease c-*fos* levels to control values, after that time cells are again responsive to CCh re-challenge (Cohen *et al*., 1996). Levels of c-*fos* mRNA were detected by Northern blotting as described in Methods. Autoradiographs were analysed by densitometry, and values are expressed as means ± s.e.mean of three independent experiments performed in duplicate.

### *Supersensitive receptors are expressed after down-regulation*

Control levels of receptors and resensitization of CCh-stimulated IP accumulation were found to recover with time after 1 h agonist pre-incubation, although the time-courses for both phenomena were different. Figure 4A shows that [$^3$H]-NMS binding, to surface mAChR returned to control values only after a 24 h recovery. The reappearance of receptors at the cell surface was suppressed by the protein synthesis inhibitor, cycloheximide (5 $\mu$g ml$^{-1}$). With respect to functional recovery, less than 9 h was required to restore a complete agonist evoked IP accumulation (Figure 4B). Interestingly, progenitor cells pre-treated with CCh (1 h) and then allowed to recover for 24 h, exhibited an increased responsiveness to CCh ($P<0.05$, 24 h recovery compared with untreated cells). Since these results could indicate receptor supersensitivity, we next examined the concentration–response relationship of CCh-induced accumulation of IP in cells prestimulated with 1 mM CCh for 1 h after a 24 h recovery (Figure 5). As shown above, in progenitor cells, CCh stimulated a dose-dependent IP accumulation (Figure 5A), with an EC50 of 25 ± 3 $\mu$M and was maximal between 0.1 and 1 mM. In cells pre-treated with CCh and recovered for 24 h there was an increase in the maximal stimulation produced by 1 mM CCh (10 min) plus a shift of the curve to the left (Figure 5A), suggesting that mAChR are in fact supersensitive. The EC50 value was 13 ± 0.6 $\mu$M ($P<0.05$ *versus* non-treated). This response was not observed in differentiated oligodendrocytes (Figure 5B).

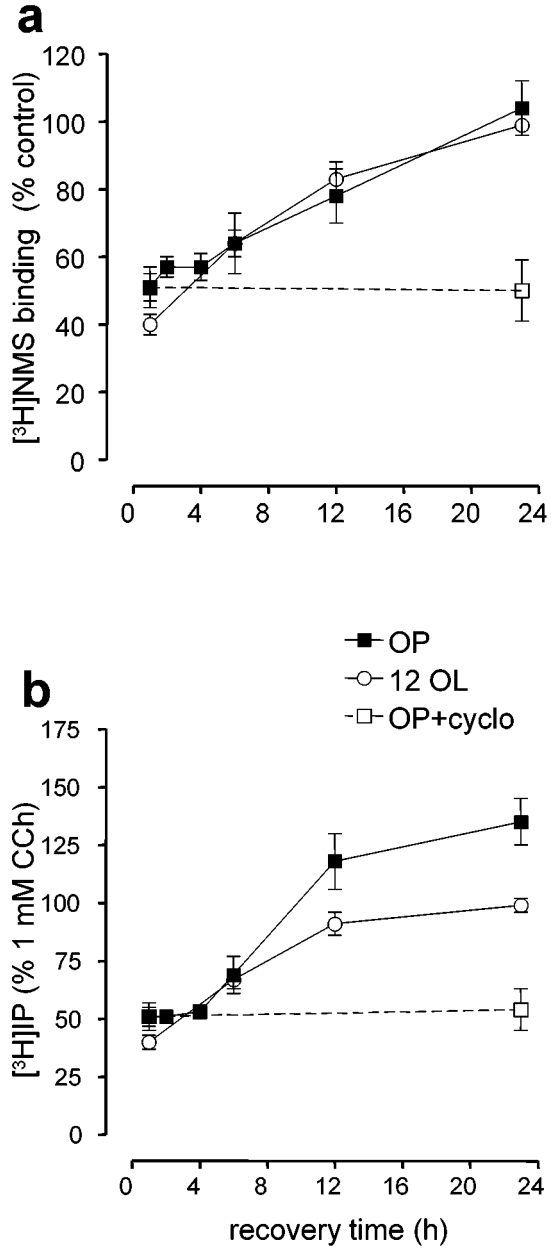


**Figure 4** Time dependence of mAChR resensitization and effect of protein synthesis inhibition by cycloheximide. Progenitors and oligodendrocytes were pre-treated with 1 mM CCh for 1 h, washed three times with buffer and allowed to recover for different times in SFM + GF in the absence or presence of cycloheximide (5 $\mu$g ml$^{-1}$). Following the recovery period, [$^3$H]-NMS binding to surface mAChR (a) and IP accumulation (b) were determined as described in Methods. Binding data are expressed as percentage of untreated control group, and PI hydrolysis as percentage of maximal IP accumulation determined in the absence of CCh pre-treatment. Results are the means ± s.e.mean of four independent experiments performed in triplicate.

To determine whether receptor supersensitivity could have consequences for cell function we measured CCh-stimulated

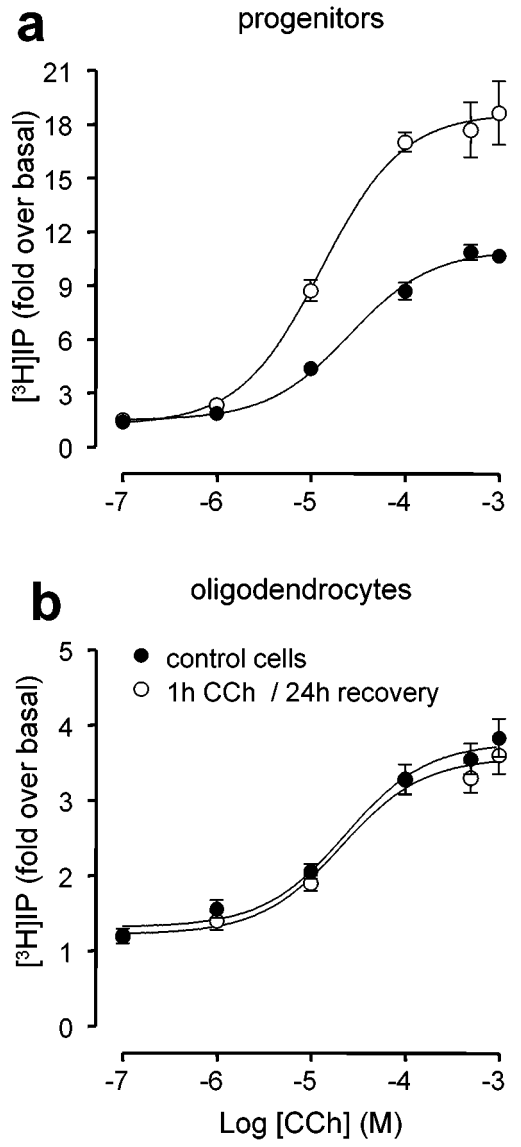


**Figure 5** Dose–response relationship for CCh-stimulated IP accumulation in control, CCh-pre-treated and resensitized progenitors and oligodendrocytes. Cells were pretreated for 1 h with 1 mM CCh, washed three times with buffer and allowed to recover for 24 h in SFM + GF. Following the recovery period IP accumulation was determined as described in Methods. Points represent fold increase over basal levels and are mean ± s.e.mean values for four separate experiments done in triplicate. Basal values of [$^3$H]-IP (d.p.m. mg$^{-1}$ protein) were 13545 ± 1612 for control cells and 13163 ± 822 for CCh-pre-treated and recovered cultures, these data indicate no differences in [$^3$H]-IP labelling.

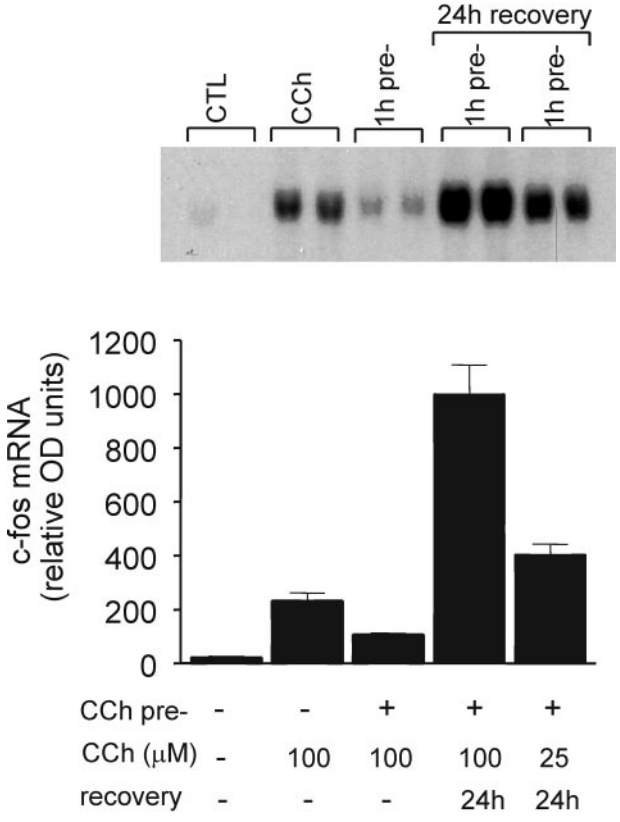


**Figure 6** Activation of supersensitive muscarinic receptors by CCh increases c-*fos* mRNA expression in oligodendrocyte progenitors. Cells were pre-treated for 1 h with 1 mM CCh, washed three times with buffer and allowed to recover for 24 h in SFM + GF. Following the recovery period, cultures were stimulated for 30 min with 25 or 100 $\mu$M CCh. Levels of c-*fos* mRNA were detected by Northern blotting as described in Methods. Autoradiographs were analysed by densitometry, and values are expressed as the means ± s.e.mean of three independent experiments performed in duplicate. Densitometric analysis revealed that 1 h CCh pre-treatment but no rechallenge (22 ± 4) did not significantly modify c-*fos* levels compared to control unstimulated cultures (25 ± 6).

gene transcription in oligodendrocyte progenitors. In agreement with our previous studies (Cohen *et al*., 1996), CCh increased the levels of c-*fos* mRNA by 10 fold above controls (Figure 6) and 1 h CCh-pre-treatment down-regulated c-*fos* mRNA expression by 50%. In contrast, in cells pre-treated with CCh for 1 h and allowed to recover for 24 h, there was a significant increase in CCh-stimulated c-*fos* transcription after a subsequent CCh stimulation (Figure 6). These results indicate that receptor supersensitivity has functional implications.

### *Inhibition of receptor endocytosis blocks desensitization and down-regulation*

To investigate the functional link between mAChR internalization and desensitization, cells were pre-stimulated with 1 mM CCh for 30 or 60 min at 10°C (Fisher, 1988; Thompson & Fisher, 1990). Under these experimental conditions the sequestration, down-regulation and receptor desensitization normally observed after 30 or 60 min CCh treatment at 37°C were blocked (Figure 7A,B). Thus, prestimulation with 1 mM CCh at low temperature prevented the agonist-mediated changes in receptor density and desensitization of PI hydrolysis. To confirm these data, we used another method to inhibit receptor internalization. As previously shown for $\beta$2-adrenergic receptors pre-treating cells with hyperosmotic sucrose concentrations disrupts endocytosis *via* clathrin-coated pits and blocks receptor sequestration (Yu *et al*., 1993; Pippig *et al*., 1995). Therefore, cells were pre-incubated with buffered 0.5 M sucrose for 20 min, and then exposed to 1 mM CCh for 30 or 60 min.

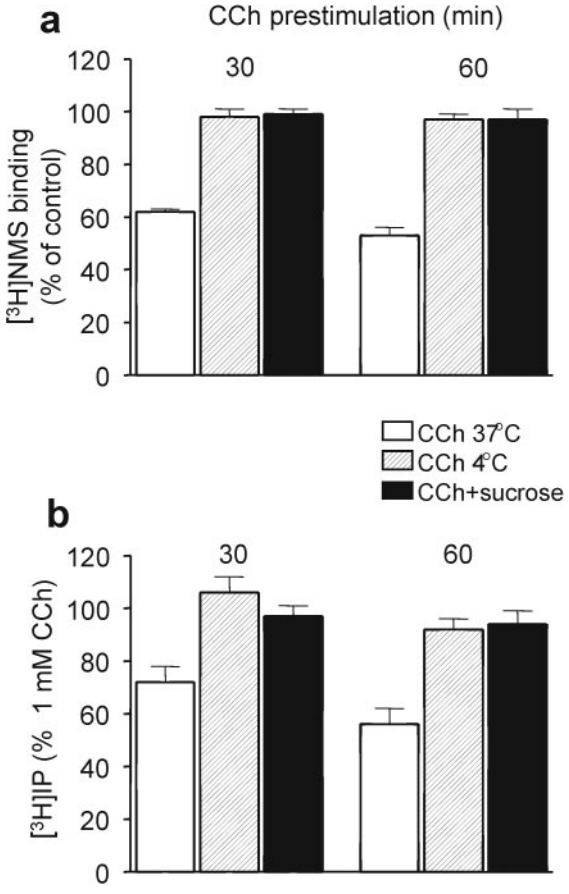


**Figure 7** Effect of pre-stimulation of oligodendrocyte progenitors with 1 mM CCh at low temperature or in the presence of hyperosmotic sucrose. Inhibition of mAChR sequestration (a) and desensitization of PI hydrolysis (b) were produced by decreasing the temperature to 4°C or by addition of buffer containing 0.5 M sucrose during the pre-stimulation period (1 mM CCh for 30 or 60 min). Following CCh pre-exposure, cells were washed three times with buffer and challenged with 1 mM CCh (plus 10 mM LiCl) for 10 min at 37°C to determine IP accumulation or incubated for 16 h at 4°C with 1 nM [$^3$H]-NMS. Binding data are expressed as percentage of untreated control group. The 100% value for [$^3$H]-NMS binding was $42 \pm 2$ fmol mg$^{-1}$ protein. PI hydrolysis data are expressed as percentage of maximal IP accumulation determined in the absence of CCh pre-treatment. The 100% value was $149{,}000 \pm 17{,}740$ d.p.m. mg$^{-1}$ protein. Results are the means $\pm$ s.e.mean of four independent experiments performed in triplicate. Differences with control values: 37°C, 30 or 60 min CCh ($P<0.01$).

Under these conditions the decrease in cell surface mAChR ([$^3$H]-NMS binding) was completely inhibited, after both prestimulation times, as was CCh-mediated receptor-desensitization (Figure 7A,B). These results indicate that inhibition of mAChR sequestration blocks desensitization.

## Discussion

The present study demonstrates that muscarinic receptors expressed in oligodendroglial cell cultures are functionally regulated by pre-exposure to agonist. Results presented herein show differences in receptor turnover in developing oligodendrocytes after down-regulation and when compared to other neural cell types.

The desensitization response in oligodendroglial cells involves reductions in the density of surface mAChR labelled with [$^3$H]-NMS, total receptors labelled with [$^3$H]-scopolamine and CCh-mediated IP accumulation and c-*fos* expression. Pre-exposure to agonist for 5 min caused a significant decrease in CCh-stimulated IP accumulation without alteration of surface receptor levels. Because the onset of receptor sequestration and desensitization are clearly different, it may be suggested that uncoupling of mAChR from G-proteins preceded receptor sequestration. Similar treatment of CHO cells expressing M3 mAChR induced receptor phosphorylation and desensitization without changes in binding (Tobin & Nahorski, 1993). This form of desensitization is mediated by phosphorylation of the activated receptor by G-protein-coupled receptor kinases (GRKs) (Pitcher *et al*., 1998), leading to the binding of arrestins which sterically suppress G protein interaction and terminate the signal (Lohse *et al*., 1990; Gurevich *et al*., 1995). Purified GRKs can phosphorylate M3 receptors (Debburman *et al*., 1995) and recent studies report that both GRK3 and GRK6 enhanced phosphorylation of endogenously expressed M3 and desensitization in SH-SY5Y human neuroblastoma cells (Willets *et al*., 2001), while GRK2 facilitated sequestration of this receptor subtype in COS-7 cells (Tsuga *et al*., 1998). Most interestingly, mAChR in the brain of GRK5-deficient mice were found to be resistant to agonist-induced desensitization and were supersensitive to cholinergic stimulation (Gainetdinov *et al*., 1999).

In progenitors and oligodendrocytes, surface receptors were sequestered after 10 min of agonist pre-treatment, although, total mAChR labelled with the lipophilic ligand [$^3$H]-scopolamine remained unchanged. A significant down-regulation of mAChRs occurred after 60 min of CCh pre-treatment. This indicates that receptor sequestration to a membrane compartment less accessible to hydrophilic ligands such as [$^3$H]-NMS precedes mAChR down-regulation. After prolonged CCh pre-stimulation, receptors were sequestered at a lower rate than the reduction in CCh-stimulated IP formation. Since oligodendrocytes express the five mAChR (Ragheb *et al*., 2001), although M3 predominates, it is possible that receptor subtypes exhibit different rates and degrees of internalization. Similarly, CCh pre-treatment in astrocytes (Pearce *et al*., 1988) or in corticostriatal neurons (Eva *et al*., 1990) reduced both CCh-mediated IP accumulation and [$^3$H]-NMS binding to surface mAChR. However, in cerebral granule cells, 60 min of pre-exposure to CCh caused substantial receptor desensitization without altering mAChR binding (Xu & Chuang, 1987). In contrast, in SK-N-SH neuroblastoma cells, sustained PI hydrolysis could be measured even after 40–50% receptor sequestration, while desensitization and receptor internalization became apparent after 2–4 h exposure to the agonist (Thompson & Fisher, 1990).

One hour of CCh treatment down-regulated [$^3$H]-scopolamine binding by 30%. As illustrated in Figure 5, recovery of receptor function as determined by PI hydrolysis, occurred more rapidly than the appearance of receptors at the cell membrane. Since mAChR were down-regulated, the protein synthesis inhibitor cycloheximide prevented receptor recovery implying that *de novo* protein synthesis is required for the re-

appearance of receptors. The estimated half-life for re-appearance of receptors was 12 h, which is in agreement with data obtained in HEL 299 cells expressing M2 mAChR (Haddad *et al*., 1995).

An important finding in oligodendrocyte progenitors was that newly synthesized mAChR are supersensitive to agonist stimulation after down-regulation. This process occurred without an increase in receptor number. However, receptors displayed an increased affinity for CCh, as determined in concentration-response curves for IP accumulation. Of particular interest was the enhanced ability of CCh to activate mAChR and promote expression of the immediate-early gene c-*fos*. In oligodendrocytes, Fos protein belongs to the AP-1 family of transcription factors, which have been implicated in cell cycle control, cell morphology and apoptosis (Fitzgerald & Barnett, 2000). Receptor supersensitivity may therefore have functional implications for proliferation and differentiation of progenitors following neuronal release of acetylcholine and activation of mAChR in progenitors (Cohen *et al*., 1996). Although the molecular mechanisms underlying receptor supersensitivity remain to be identified it has been proposed that alterations in the conformation of the cytoplasmic domains by phosphorylation stabilize a receptor conformation that activates G proteins more efficiently (review in Chavkin *et al*., 2001). Alternatively, changes in the expression levels of regulatory proteins could be responsible for receptor supersensitivity as was demonstrated in GRK5-deficient mice (Gainetdinov *et al*., 1999).

Our results suggest that mAChR internalization in oligodendrocyte progenitors plays a role in the desensitization of PI hydrolysis and receptor down-regulation. In contrast, previous reports in cells overexpressing $\beta$2-adrenergic receptors demonstrated that receptor internalization is not related to desensitization, but is essential for receptor resensitization (Yu *et al*., 1993; Pippig *et al*., 1995). In addition, mutagenesis of M2 mAChR or overexpression of a dominant-negative allele of GRK2 resulted in reduced agonist-mediated phosphorylation and prevented desensitization, although, receptor internalization occurred normally (Pals-Rylaarsdam *et al*., 1995). We used two different approaches to inhibit receptor internalization (1) preincubation of cells at low temperature, and (2) hypertonic sucrose. Both treatments effectively prevented receptor internalization, and in parallel, blocked down-regulation of receptors and their desensitization. These observations suggest that mAChR internalization is required for desensitization in oligodendrocyte progenitors. Along these lines, evidence obtained in HEK 293 cells transfected with M3 mAChR support the idea that sequestration plays an important role, at least at early times after CCh pre-treatment (Yang *et al*., 1995).

In summary, mAChR endogenously expressed in oligodendrocytes and their progenitors undergo rapid agonist-mediated desensitization and internalization. Because the onsets of such processes are different, it seems that receptor uncoupling after minutes of agonist exposure is responsible for rapid desensitization. Receptor down-regulation is observed after longer periods of agonist treatment, and newly synthesized receptors are supersensitive to further agonist stimulation. In progenitors, receptor internalization seems to be necessary for functional desensitization and receptor down-regulation. In conclusion, modulation of muscarinic receptor sensitivity may have functional implications for the developmental progression, terminal differentiation and survival of oligodendrocyte progenitors in response to acetylcholine released by neurons.

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