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Activation of Phosphatidylinositol-3 Kinase (PI-3K) and Extracellular Regulated Kinases (Erk1/2) Is Involved in Muscarinic Receptor-Mediated DNA Synthesis in Neural Progenitor Cells

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Muscarinic acetylcholine receptor (mAChR), a member of the G-protein-coupled receptors (GPCRs) gene superfamily, has been shown to mediate the effects of acetylcholine on differentiation and proliferation in the CNS. However, the mechanism or mechanisms whereby mAChRs regulate cell proliferation remain poorly understood. Here we show that *in vitro* bFGF-expanded neural progenitor cells dissociated from rat cortical neuroepithelium express muscarinic acetylcholine receptor subtype mRNAs. We demonstrate that stimulation of these mAChRs with carbachol, a muscarinic agonist, activated extracellular-regulated kinases (Erk1/2) and phosphatidylinositol-3 kinase (PI-3K). This, in turn, stimulated DNA synthesis in neural progenitor cells. MEK inhibitor PD98059 and PI-3K inhibitors wortmannin and LY294002 inhibited a carbachol-induced increase in DNA synthesis. These findings indicate that the activation of both PI-3 kinase and MEK signaling pathways via muscarinic receptors is involved in stimulating DNA synthesis in the neural progenitor cells during early neurogenesis.

Key words: progenitor cell; proliferation; muscarinic receptors; phosphorylation; protein kinase-B; MAP kinase

It is well established that, during development, neurotransmitters act as growth regulatory signals to control cell proliferation, differentiation, and gene expression by activating receptors coupled to specific second messenger pathways (Lauder, 1993; Liu et al., 1997). Now there has been strong support for the presence of muscarinic receptors in astrocytes in mammalian CNS (Stephan and Sastry, 1992; Hosli and Hosli, 1993). In these cells the muscarinic receptor agonists caused hydrolysis of phosphoinositides, mobilization of intracellular Ca^{2+}, and activation of phospholipases (A_2 and D), resulting in the inhibition of adenylate cyclase activity and the induction of the immediate early genes c-*fos* and c-*jun* (Trejo and Brown, 1991). Ashkenazi et al. (1989) found that activation of the muscarinic receptors m1, m3, and m5 is involved in proliferation in rat cortical astrocytes. An increase of proliferation in these cells also was observed in the presence of carbachol (Guizzetti et al., 1996). The mitogenic effects of the muscarinic receptor agonist carbachol also have been studied in oligodendrocyte progenitors. Carbachol stimulated DNA synthesis, and this stimulation was prevented by atropine (Cohen et al., 1996). In addition, mAChRs also have been implicated in learning and memory in human and other mammals (Blokland, 1995) via the activation of extracellular-regulated kinases (Erk1/2; Rosenblum et al., 2000). Erk1/2 activation has been correlated with synaptic plasticity (Orban et al., 1999), including long-term potentiation (LTP). mAChRs have been shown to modulate LTP in the cortex and hippocampus (Jerusalinsky et al., 1997). In a recent study atropine was found to attenuate cortical LTP *in vivo* (Jones et al., 1999).

The above studies clearly demonstrate that acetylcholine and its agonist carbachol stimulate muscarinic receptors and promote DNA synthesis and the proliferation of primary astrocytes from prenatal rat brain. Also, in transfected Chinese hamster ovary (CHO) cells expressing recombinant muscarinic receptors (Ashkenazi et al., 1989) and oligodendrocyte progenitors (Cohen et al., 1996), similar effects of the activation of muscarinic receptors have been demonstrated. Little, however, is known about the signal transduction mechanisms involving mAChR activation in regulating the proliferation of neural progenitors during early mammalian brain development. Hence, it is important to understand the role of mAChRs in regulating DNA synthesis and cell proliferation in neural progenitor cells during early neurogenesis.

The muscarinic cholinergic receptor (mAChR) belongs to the superfamily of G-protein-coupled receptor (GPCR) genes and mediates the effects of acetylcholine in the CNS (Hepler and Gilman, 1992; Hadcock and Malbon, 1993; Fraser et al., 1994; Gudermann et al., 1997). Recently, it has been shown that mAChR mediates $G_{\beta\gamma}$-dependent activation of MAP kinase, phosphatidylinositol-3 kinase (PI-3K; Crespo et al., 1994; Wan et al., 1996; Lopez-Ilasaca et al., 1997), and PI-3 kinase-induced activation of Akt (Murga et al., 1998). Akt was implicated in the pathway regulating cell survival in response to growth factors in a variety of cellular systems (Datta et al., 1997; Brunet et al., 1999). Activation of MAP kinases appears to be a critical component of growth-promoting pathways (Davis, 1993). In addition to MAP kinases, PI-3Ks are thought to control DNA synthesis in CHO cells (McIlroy et al., 1997), 3T3 cells (Roche et al., 1994),

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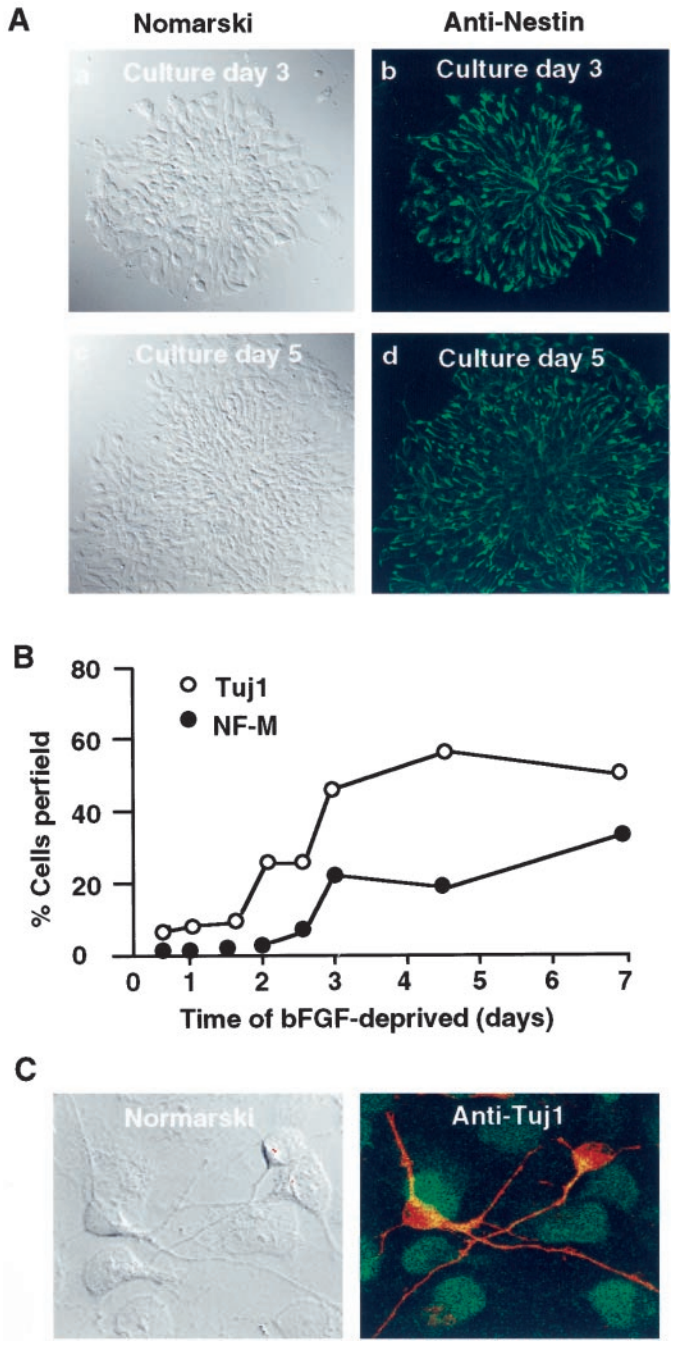

Figure 1. bFGF expansion and differentiation of cortical neuroepithelial cells. *A*, Rat cortical progenitor cells were maintained in serum-free medium containing bFGF for days 1, 3, and 5. The cells on days 3 and 5 were fixed and stained for immunofluorescence with anti-nestin monoclonal antibody. Note the radial morphology of the cells, showing an increase in cell numbers. *B*, Differentiation was initiated by removing bFGF. Rapidly dividing nestin-positive progenitor cells after 5 d in bFGF (10 ng/ml) were labeled with 10 μM BrdU during the last 24 hr of proliferation. Then differentiation was initiated by the withdrawal of bFGF (day 0) and continued for up to 7 d. At indicated times the cells were fixed and stained for BrdU and the neuronal antigens NF-M and TuJ1 or the glial antigen GFAP (data not shown). Ratio of cells double-stained for BrdU and each neuronal antigen to total BrdU$^+$ cells per 20× field are shown. (○), TuJ1$^+$; (●), NF-M$^+$. *C*, Typical clones were immunostained with BrdU and neuron-specific antigen TuJ1 antibody after removal of the bFGF for 7 d.

melanoma cells, T cells (Ahmed et al., 1997; Brennan et al., 1997), and granule neuron progenitor cells (Cui et al., 1998). However, the mechanism or mechanisms whereby PI-3 kinase and MAP kinase signaling from muscarinic receptors regulate neural progenitor cell proliferation remain primarily unknown.

In this study we have identified that the basic fibroblast growth factor (bFGF)-expanded neural progenitor cells dissociated from rat cortical neuroepithelium express m2, m3, and m4 subtype mRNAs. We show that the acetylcholine agonist carbachol, acting via muscarinic receptors, activated PI-3 kinase and extracellular-regulated kinases (Erk1/2). This, in turn, resulted in stimulating DNA synthesis in neural progenitor cells. These findings demonstrate that the PI-3 kinase and MAP kinase signaling pathways via mAChRs are involved in neural progenitor cell proliferation during early neurogenesis.

MATERIALS AND METHODS

Cell culture. Neural progenitor cells were cultured as previously described (Ma et al., 1998). Briefly, pups were removed from pregnant Sprague Dawley rats (Taconic Farms, Germantown, NY). The formative dorsal telencephalic neuroepithelium was dissected from rats of embryonic days 13–13.5 (E13–E13.5). Tissue was dissociated by brief mechanical triturating in HBSS. The dissociated cells were collected by centrifugation and resuspended in a serum-free Neurobasal (NB) medium supplemented with B27, 0.5 mM L-glutamine, and 10 ng/ml recombinant human bFGF (Intergen, Purchase, NY). Cells (25×10^3) were plated in 35 mm plastic dishes precoated with 10 μM poly-L-lysine and 1 μg/ml bovine plasma fibronectin (Life Technologies, Gaithersburg, MD).

Reverse transcriptase-PCR (RT-PCR) analysis. Cortical progenitor cells expanded by bFGF for 1–5 d were harvested before differentiation. For RT-PCR analysis of muscarinic receptor subtype mRNA expression in cultured cortical progenitor cells, total RNA was isolated by a single-step guanidinium-thiocyanate/phenol-chloroform extraction protocol and then reverse-transcribed and amplified. Total RNA amounts in samples were normalized by the amplification of a 441 bp fragment of glyceraldehyde-3-phosphate dehydrogenase mRNA. The primers were as follows: m1, 5′-AGCTCAGAGAGGTCACAG-3′ and 5′-TCGGTCTC-GGCCTTTCTTGGT-3′; m2, 5′-CACGAAACCTCTGACCTACCC-3′ and 5′-TCTGACCCGACGACCCAACTA-3′; m3, 5′-GTGACAACT-GTCAGAAGG-3′ and 5′-CCAGGACCATGATGTTGT-3′; m4, 5′-GAATTCGTTCACAAGCATCGACCTG-3′ and 5′-CTCGAGTGGT-GGCCTCTGCGGTGGAC-3′; m5, 5′-CCCGTAGAAGCACCTCAAC-AACAGG-3′ and 5′-TTTGATGACTGAGGTTGGGATCCGG-3′; GAPDH, 5′-GGACATTGTTGCCATCAACGAC-3′ and 5′-ATGAG-CCCTTCCACGATGCCAAAG-3′. Amplification was performed for 40 cycles at 95°C for 45 sec, at 54°C for 30 sec, and at 72°C for 60 sec.

[^{3}H]thymidine and BrdU incorporation assay. Neural progenitor cells were grown to an approximate density of 1×10^5 cells/cm^2 in 35 mm plastic dishes for 3 d. Before agonist stimulation the cultures were deprived of bFGF for 24 hr to decrease the basal levels of proliferation. After the agonist was added for 24 hr, 2 μCi/ml [methyl-^{3}H]thymidine was incubated for the last 6 hr of the incubation at 37°C under an atmosphere of 5% CO_2/95% air. Cells were washed three times with ice-cold PBS, and 5% trichloroacetic acid (TCA) was added for 20 min at 4°C. The monolayer was washed once with 5% TCA, and a mixture of 0.1N NaOH and 1% SDS was added for 10 min. Samples were transferred to a scintillation vial, and the radioactivity was counted on a Beckman LS3801 scintillation counter. For the BrdU incorporation assay, 4 hr before fixation with 70% ethanol the BrdU (10 μM) was added to the cultures. Cells incorporating BrdU were identified by using the FITC-conjugated monoclonal anti-BrdU (Becton Dickinson, Mountain View, CA).

BrdU labeling of rat embryos. BrdU labeling of cells in the S-phase of the cell cycle was performed according to the protocol described by Hayashi et al. (1988). In brief, BrdU (100 mg/gm of body weight) was injected intraperitoneally into rat pregnant females at E16.5 of gestation. These rats were killed 8 hr after injection, and the uteri were removed; complete deciduas or embryos were fixed in 4% paraformaldehyde at 4°C overnight and processed for immunohistochemistry. The sections were incubated with an anti-BrdU monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) at a 1:10 dilution. Staining was performed according to the protocol described as above. BrdU$^+$ cells were counted

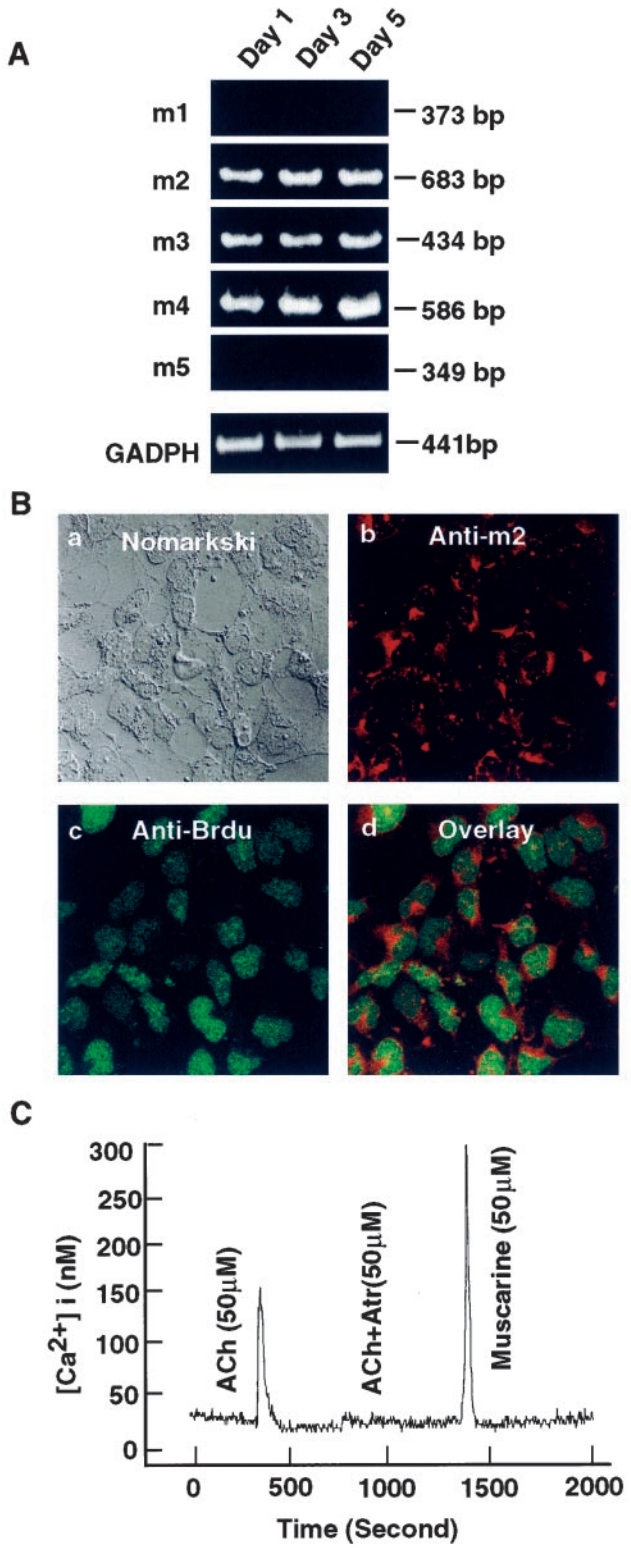

Figure 2. Rat cortical progenitor cells express muscarinic receptor m2, m3, and m4 subtypes. *A*, RT-PCR analysis of mAChR gene expression in rapidly dividing cortical precursor cells. Total RNA isolated from bFGF-expanded for day 1 (*lane 1*), day 3 (*lane 2*), and day 5 (*lane 3*) was reverse-transcribed and analyzed by PCR as described in Materials and Methods. As indicated at *left*, oligonucleotides corresponding to the following gene products were used: m1, m2, m3, m4, m5, and GADPH. The molecular mass of the PCR products is indicated on the *right*. *B*, Cortical progenitor cells were bFGF-expanded for 4 d and then labeled with 10 μM BrdU for an additional 24 hr. Cells were fixed and immunostained with m2 and BrdU antibodies. *C*, mAChR agonists induce an increase in $[Ca^{2+}]_i$ in neural progenitor cells. ACh (50 μM) triggers a $[Ca^{2+}]_i$ transient increase, which was blocked by the muscarinic antagonist atropine (50 μM). Muscarine (50 μM) caused an even larger increase in $[Ca^{2+}]_i$ levels, indicating the presence of mAChRs in neural progenitor cells.

in the 10 different regions of the ventricular and subventricular zones of cerebral cortex. Values are expressed in a percentage of control as the means ± SEM of three independent experiments.

Apoptosis assay. Cortical progenitor cells were grown in 35 mm dishes for 3 d in the presence of bFGF, and then bFGF was deprived for 24 hr. They were treated as indicated for DNA synthesis. In control and CCh-treated cells and cells treated with inhibitor cultures, fragmented DNA was visualized by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) procedure with an *in situ* cell death detection kit (AP) from Boehringer Mannheim, following the manufacturer's instructions. Labeled nuclei and the total number of cells were counted in 10 independent fields.

Immunoprecipitation and in vitro *kinase assay.* For kinase activity assays the progenitor cells were grown in 35 mm dishes for 3 d, deprived of bFGF for 24 hr, and treated as described in the figure legends. After treatment the cells were scraped into 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, and protease and phosphatase inhibitors). Extracts were sonicated and centrifuged for 5 min at 14,000 × *g*. Immunoprecipitations were performed by adding the anti-Akt or anti-Erk1/2 antibodies and incubated overnight at 4°C with constant rotation. Protein G-Sepharose beads, 30 μl of 50% slurry (Amersham Pharmacia Biotech, Piscataway, NJ), were added and incubated under the same conditions for an additional 4 hr. Immunocomplexes were centrifuged for 2 min at 14,000 × *g*, were washed twice in lysis buffer and twice in kinase buffer [containing (in mM) 25 Tris-HCl, pH 7.5, 5 β-glycerol phosphate, 0.1 sodium orthovanadate, 2 dithiothreitol, and 10 magnesium chloride], and were matched for protein content before being used in each specific kinase reaction. Kinase activity assay was performed as described (Li et al., 1999).

Immunoblotting. Neural progenitor cell extracts were prepared by lysing the cells on ice with lysis buffer containing protease and phosphatase inhibitor as previously described (Li et al., 1999). After SDS-PAGE the proteins were transferred to polyvinylidene difluoride membranes. The blots were developed with the enhanced chemiluminescence (ECL) kit from Amersham (Chicago, IL).

Immunofluorescence. Neural progenitor cells or sections were fixed in 4% paraformaldehyde in PBS for 30 min, washed in several changes of PBS for 30 min, and permeabilized in 0.2% Triton X-100 in PBS for 15 min. Monoclonal anti-phospho-independent neurofilament-M antibody (1:200, NN18; Boehringer Mannheim), monoclonal antibody TuJ1 (Lee et al., 1990), BrdU (1:100; Becton Dickinson), or nestin (Rat 401, 1:50; Developmental Studies Hybridoma Bank, IA) incubation was performed overnight at 4°C. After a wash in PBS (three times, 15 min each) the cells or sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and rhodamine-labeled goat anti-rabbit IgG or rhodamine-labeled goat anti-mouse IgG secondary antibody for 1 hr at room temperature. Fluorescent images were obtained with a Zeiss LSM-410 laser-scanning confocal microscope. Colocalization studies were done of fluorescein-labeled BrdU (1:100) or nestin (1:10) and mAChR m2 (1:100 dilution; Alomone Labs, Jerusalem, Israel) or phospho-Akt (T308; 1:200; New England Biolabs, Beverly, MA). Images were processed and merged via Adobe Photoshop software.

$[Ca^{2+}]_i$ measurements. The cytoplasm free Ca^{2+} concentration $[Ca^{2+}]_i$ of nestin and $BrdU^+$ progenitor cells was measured after 3 d of culture as previously described (Ma et al., 1998). Briefly, the cells were loaded with 1 μM fluo-3 AM at room temperature for 30 min in physiological medium containing (in mM): 145 NaCl, 5 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.4, supplemented with 1 mg/ml BSA. Changes in $[Ca^{2+}]_i$ were recorded with the Zeiss Attofluor Ratio Vision workstation (Atto Instruments, Rockville, MD). Intracellular fluo-3 was excited by a 100 W quartz lamp filtered at 488 nm, using a 10 nm bandpass filter into the excitation light path every 500 msec; proper emission wavelengths were monitored with a 520 nm long-pass filter, which was inserted in front of one of the ICDD cameras.

Data analysis. Data are expressed as the means ± SEM. One-way ANOVA, followed by the Newman–Keuls test, was used as indicated in

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the figures to determine the statistical significance; $p < 0.05$ was considered significant.

RESULTS

bFGF maintains rapidly dividing neural progenitor cells expressing nestin

Cells isolated from E13 rat telencephalic neuroepithelium were expanded by the daily addition of bFGF in serum-free medium. A continuous supply of bFGF was important to repress differentiation and to maintain a homogeneous population of rapidly dividing cells expressing nestin, an intermediate filament protein characteristic of CNS precursor cells (Fig. 1*A*). Of the cells, <2% expressed neuronal antigens, the astroglial marker glial fibrillary acidic protein (GFAP), or the oligodendroglial marker O4 (data not shown). Withdrawal of bFGF initiated differentiation, characterized by a progressive increase in the number of cells expressing several well established neuron-specific antigens and glia markers, including β-tubulin type III (TuJ1; Fig. 1*C*), neurofilament-M (NF-M), and GFAP (Ma et al., 1998). It is of interest that withdrawing bFGF initiated the cellular expression of TuJ1 within 36 hr, whereas NF-M expression was delayed for 3 d under the same conditions (Fig. 1*B*). GFAP expression was not seen until day 7 after withdrawal of the bFGF (Ma et al., 1998). These experiments confirm previously established observations that bFGF-expanded neural progenitor cells could divide and, after withdrawal of bFGF, could initiate differentiation of neurons and glia (Ma et al., 1998). Thus, proliferating progenitor cells are providing an *in vitro* cellular model to help define intracellular signal transduction pathways that regulate neural progenitor cell proliferation and differentiation.

Cortical progenitor cells express muscarinic receptor subtypes

We determined the expression of five subtype mRNAs by using an RT-PCR technique in bFGF-expanded neural progenitor cells and found that m2, m3, and m4 mAChR mRNAs were expressed in neural progenitor cells, whereas the m1 and m5 were not (Fig. 2*A*). To confirm whether mAChR proteins were expressed in dividing neural progenitor cells, we examined m2 subcellular localization by immunofluorescence analysis. We found that m2 protein was localized almost within the membrane in the $BrdU^+$ cells (Fig. 2*B*). Because increased intracellular Ca^{2+} is a critical signal in determining muscarinic receptor function activity, we loaded precursor cells with 1 μM fluo-3 and monitored the changes in acetylcholine and muscarine-induced $[Ca^{2+}]$ increases as changes in relative fluorescence. The transient $[Ca^{2+}]$ elevation induced by acetylcholine was blocked by the muscarinic receptor antagonist atropine (Fig. 2*C*). Subsequently, it was shown that the recorded cells were $BrdU^+$. This analysis of $[Ca^{2+}]$ responses to mAChRs agonists further indicates the presence of functional mAChRs in neural progenitor cells.

Muscarinic receptor agonist-induced increase in DNA synthesis in the cortical progenitor cells

Because proliferative signaling mediated by GPCRs has been implicated in embryogenesis and growth stimulation (Olashaw and Pledger, 1988; Nagata et al., 1996), we investigated whether the stimulation of muscarinic receptors might affect the proliferation of neural progenitor cells. We used [^{3}H]thymidine incorporation into bFGF-expanded progenitor cells to evaluate DNA synthesis as a measure of cell proliferation *in vitro*. Carbachol (CCh), the mAChR agonist, significantly increased DNA synthesis indicated by [^{3}H]thymidine incorporation, and this effect was blocked by atropine, a selective muscarinic antagonist and PTX (Fig. 3*B*). We also showed that CCh produced a dose-dependent increase in [^{3}H]thymidine incorporation, with 100 μM carbachol stimulating DNA synthesis the most (Fig. 3*A*).

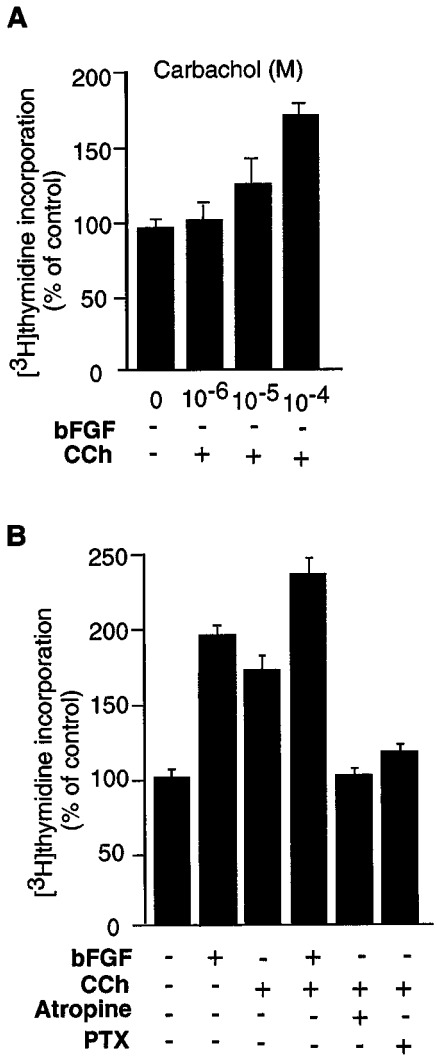

Figure 3. Effect of carbachol on the incorporation of [^{3}H]thymidine in cultures of neural progenitor cells. Cortical progenitor cells were bFGF-expanded for 3 d. Before the addition of mAChR agonists and antagonists, bFGF was removed for 24 hr, and the cells were subjected to stimulation with carbachol (100 μM), carbachol plus atropine (50 μM), or PTX (100 ng/ml). [^{3}H]thymidine (2.5 μCi/ml) was added during the last 6 hr of culture. *A*, Dose-dependent analysis of carbachol stimulation of proliferation in neural progenitor cells. Cells were treated with different concentrations of carbachol; [^{3}H]thymidine incorporation was measured as an index of DNA synthesis. Results are the means ± SEM of four independent experiments. *B*, Effects of carbachol and carbachol plus atropine or PTX on [^{3}H]thymidine incorporation in neural progenitor cells. Results are the means ± SEM of four independent experiments.

Role of mAChRs on progenitor cell proliferation *in vivo*

Recently, there have been numerous studies to investigate the role of trophic factors, e.g., epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), on neural progenitor cell proliferation *in vitro* (Ray and Gage, 1994; Santa-Olalla and Covarrubias, 1999) and *in vivo* (Kuhn et al., 1997; Gritti et al., 1999); however, there has been no information about the role of mAChRs on neural progenitor cell proliferation *in vivo*. To in-

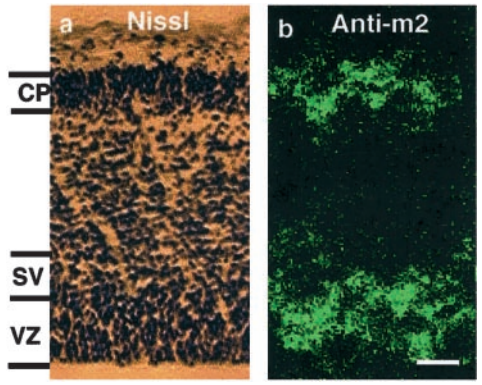

Figure 4. Muscarnic acetylcholine receptor expressed in the ventricular (*vz*) and subventricular (*sv*) zones of the embryonic cortex. Nissl staining (*a*) and immunofluorescence that uses an anti-m2 antibody (*b*) show a widespread distribution of m2 AChR-immunoreactive cells in the vz and sv zones and cortical plate (*CP*) of coronal sections of E17 rat cortex. Scale bar, 150 μm.

vestigate that mAChRs may play roles in the proliferation of neural progenitor cells *in vivo*, we first determined whether mAChRs are expressed in progenitors during the appropriate times during development. We showed that the prominent m2 AChR-immunoreactive cells were detected in the ventricular (VZ) and subventricular (SV) zones of the cerebral cortex of E17 embryos (Fig. 4). Because the most proliferating neuroepithelial cells are in the VZ zone (Bayer and Altman, 1991), the indication is that proliferating cells express m2 AChR. We next demonstrated the effects of carbachol and carbachol plus atropine administration on proliferating cells in the ventricular and subventricular zones. We found that carbachol increased BrdU incorporation in the VZ and SV zones (Fig. 5). This effect was inhibited by the administration of atropine (Fig. 5), supporting proliferative roles for mAChRs in stem or progenitor cells *in vivo*.

DNA synthesis mediated by muscarinic receptors via activation of MAP kinase pathway in the cortical progenitor cells

The question arises as to how the signals at muscarinic receptors are translated into the stimulation of DNA synthesis and cell proliferation. Several signal transduction pathways downstream from G-protein-coupled receptors have been implicated in different cell systems (Olashaw and Pledger, 1988; Nagata et al., 1996). Among these are the mitogen-activated protein kinase (MAPK) or extracellular-regulated kinases (Erk1/2) cascade and PI-3K pathways (Crespo et al., 1994; Larocca and Almazan, 1997; Lopez-Ilasaca et al., 1997). The latter, by virtue of its effect on the phosphorylation of phosphoinositides, seems to be involved in many aspects of cell behavior from cell adhesion to cell survival (Toker and Cantley, 1997). To determine whether Erk1 and Erk2 are involved in the downstream pathway from CCh-stimulated muscarinic receptors, we investigated CCh-induced DNA synthesis, Erk1/2 phosphorylation, and kinase activity in the neural progenitor cells. We found that 100 μM CCh significantly increased Erk1/2 phosphorylation, kinase activity (Fig. 6*A,B*), and [^{3}H]thymidine (Fig. 6*C*) or BrdU (data not shown) incorporation into neural progenitor cells. These effects were reduced significantly in the presence of PD98059 (25 μM), a MEK-selective inhibitor (Fig. 6), implying that the G-protein-coupled muscarinic receptor appears to regulate neural progenitor cell proliferation via the MEK–Erk1/2 signaling pathway.

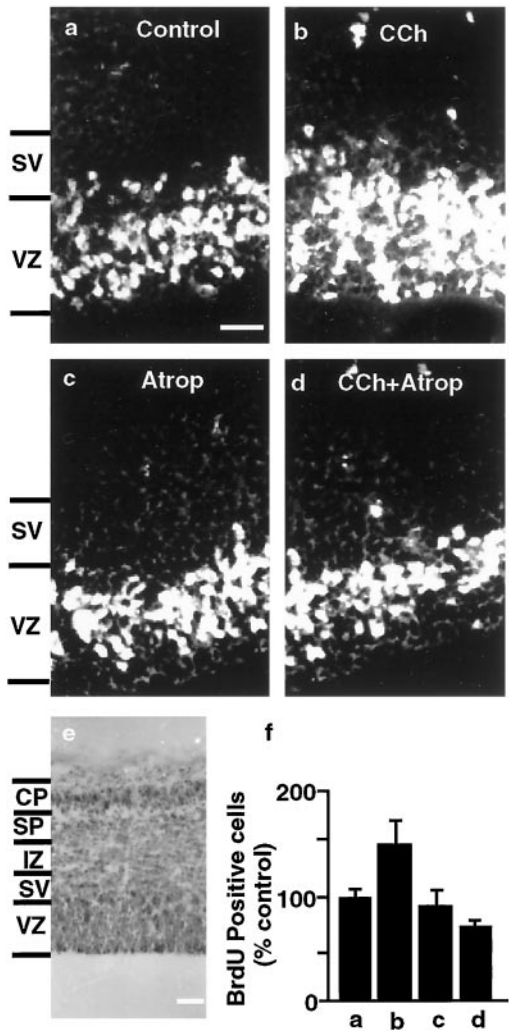

Figure 5. Effect of carbachol administration on proliferating cells in the ventricular and subventricular zones. Pregnant female rats at E16.5 of gestation were injected intraperitoneally with BrdU (100 mg/gm of body weight), and cortices from pups were used for BrdU staining 8 hr after injection, as described in Materials and Methods. *e*, A light micrograph of coronal-sectioned rat brain at E17 stained with Nissl showing normal lamination of the cerebral cortical wall consisting of cortical plate (*CP*), cortical subplate (*SP*), intermediate zone (*IZ*), subventricular zone (*SV*), and ventricular zone (*VZ*). Scale bar, 150 μm. Strong BrdU$^+$ cells can be seen with the administration of carbachol sections throughout all ventricular and some subventricular zones (*b*) as compared with control sections (*a*). These effects can be reduced by the administration of atropine alone (*c*) or with atropine and carbachol (*d*). *f*, Quantitative analysis of BrdU$^+$ cells after the administration of CCh and atropine. Values were expressed in a percentage of control as the means ± SEM of three independent experiments.

PI-3 kinase pathway is involved in carbachol-induced increases in DNA synthesis in the cortical progenitor cells

Akt was identified as one of the directed targets of PI-3 kinase activation (Dudek et al., 1997; Franke et al., 1997). To assess CCh-induced PI-3 kinase activity, we investigated the ability of the Akt phosphorylation and kinase activation affecting DNA synthesis in the neural progenitor cells. CCh increased Akt phosphorylation. This is demonstrated in Figure 7*A*, using the antibody specific to phospho-Akt (Thr^{308}) or Ser^{473}. Wortmannin (50 nM), a PI-3 kinase-selective inhibitor, and atropine, an mAChR

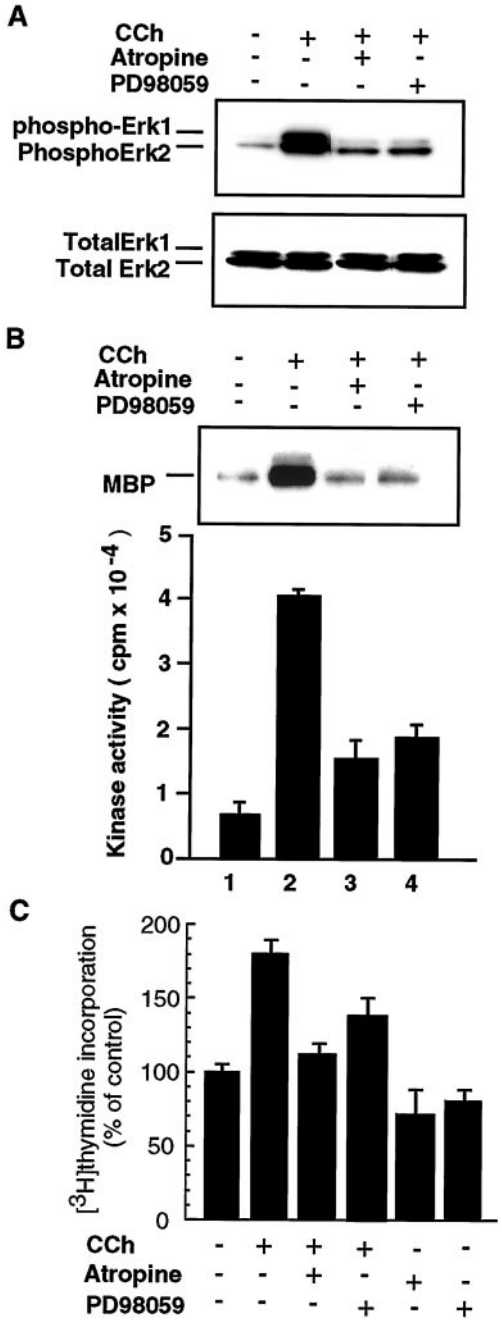

Figure 6. Erk1 and Erk2 regulate the proliferation of neural progenitor cells mediated by muscarinic receptor. *A*, Effect of atropine and PD98059 on carbachol-induced Erk1/2 phosphorylation in bFGF-deprived neural precursor cells. Cortical progenitor cells were bFGF-expanded for 3 d; then bFGF was removed for 24 hr, and the cells were treated with atropine (50 μM) or PD98059 (25 μM) for 1 hr, followed by carbachol (100 μM) for 30 min. Total Erk1/2 protein and phosphorylated protein were analyzed by Western blot with anti-Erk1/2 and anti-phospho-dependent Erk1/2 antibodies. *B*, Effect of atropine and PD98059 on carbachol-induced Erk1/2 kinase activity in bFGF-deprived neural progenitor cells. Cortical precursor cells were bFGF-expanded for 3 d; then bFGF was removed for 24 hr, and the cells were treated with atropine (50 μM) or PD98059 (25 μM) for 1 hr, followed by carbachol (100 μM) for 30 min. Immunoprecipitates of Erk1/2 with Erk1/2 antibody were used in a kinase assay in the presence of myelin basic protein (*MBP*) and [γ^{32}P]ATP. Phosphorylated MBP was separated on an 8–16% SDS polyacrylamide gel. The graph represents the means ± SEM of three independent experiments. A representative autoradiograph is shown in Figure 4*b*. *C*, Effect of atropine and PD98059 on carbachol-induced DNA synthesis. Cortical progenitor cells were bFGF-expanded for 3 d. After bFGF was removed for 24 hr, carbachol (100 μM), carbachol plus atropine (50 μM), or PD98059 (25 μM) was added. [^{3}H]thymidine (2.5 μCi/ml) was added during the last 4 hr of culture. [^{3}H]thymidine incorporation was measured as an index of DNA synthesis. Results are the means ± SEM of four independent experiments.

antagonist, significantly inhibited CCh-induced Akt phosphorylation (Fig. 7*A*). The CCh-induced Akt phosphorylation was confirmed by immunofluorescence analysis that used the same phospho-dependent Akt antibody (Fig. 7*B*). Nestin-expressing cells in the presence of CCh showed a significant increase in phospho-Akt expression, which was decreased significantly in the presence of wortmannin and atropine (Fig. 7*B*). We next examined whether the CCh-induced increase in Akt phospho-kinase was accompanied by increased Akt kinase activity; we measured kinase activity in Akt immunoprecipitates of CCh-stimulated cortical progenitor cells pretreated with wortmannin and atropine, using histone 2B as a substrate. We found that CCh induced Akt activity, and this effect was inhibited by wortmannin and atropine (Fig. 7*C*). Finally, we determine whether CCh-induced PI-3 kinase activity could result in an increase in DNA synthesis. Neural progenitor cells were treated with carbachol only, with PI-3 kinase inhibitor, or with atropine in the presence of carbachol. BrdU and [^{3}H]thymidine incorporation into DNA was assessed. Carbachol significantly increased [^{3}H]thymidine (Fig. 7*D*) and BrdU incorporation (data not shown) as compared with control. The carbachol-stimulated [^{3}H]thymidine incorporation into DNA was inhibited partially by the inhibitors of PI-3 kinase, wortmannin and LY294002 (Fig. 7*D*).

Carbachol stimulation of DNA synthesis via both MAP kinase and PI-3 kinase pathways in the cortical progenitor cells

It has been reported that the activation of MAPK (Erk1/2) by carbachol was inhibited by the PI-3 kinase inhibitor wortmannin in transfected COS-7 cells, suggesting a crosstalk between these kinase systems in these cells (Lopez-Ilasaca et al., 1997). Therefore, we investigated whether CCh-induced activation of MAP kinase affects the stimulation of PI-3 kinase. The neural progenitor cells were treated with PD98059 (25 μM) or wortmannin (50 nM) or LY294002 (10 μM) alone or together in the presence of carbachol; the increased phosphorylation of Erk1/2 and Akt was examined by Western blot analysis. We found that the PI-3 kinase inhibitor LY294002 or wortmannin (data not shown) did not affect the activation of Erk1/2 (Fig. 8*A*), and the MEK inhibitor PD98059 did not affect the CCh-induced Akt activation (Fig. 8*B*). On the other hand, a CCh-induced increase in DNA synthesis was inhibited completely in the presence of both PD98059 (25 μM) and wortmannin (50 nM) or PD98059 and LY294002 (10 μM; Fig. 8*C*). Wortmannin or PD98059 each produced an almost 50% reduction in [^{3}H]thymidine incorporation into DNA (Figs. 6*C*, 7*D*). These data suggest that G-protein-coupled muscarinic receptors appear to regulate DNA synthesis in neural progenitor cells via both MAP kinases and PI-3 kinase pathways.

Carbachol stimulation of DNA synthesis and survival of neural progenitor cells

Because the PI-3K and MEK–Erk1/2 pathways are thought to promote cell survival (Datta et al., 1997; Toker and Cantley, 1997; Brunet et al., 1999), it is possible that CCh stimulation of thymidine incorporation (Fig. 3) reflected a greater survival of neural progenitor cells rather than a stimulation of DNA synthe-

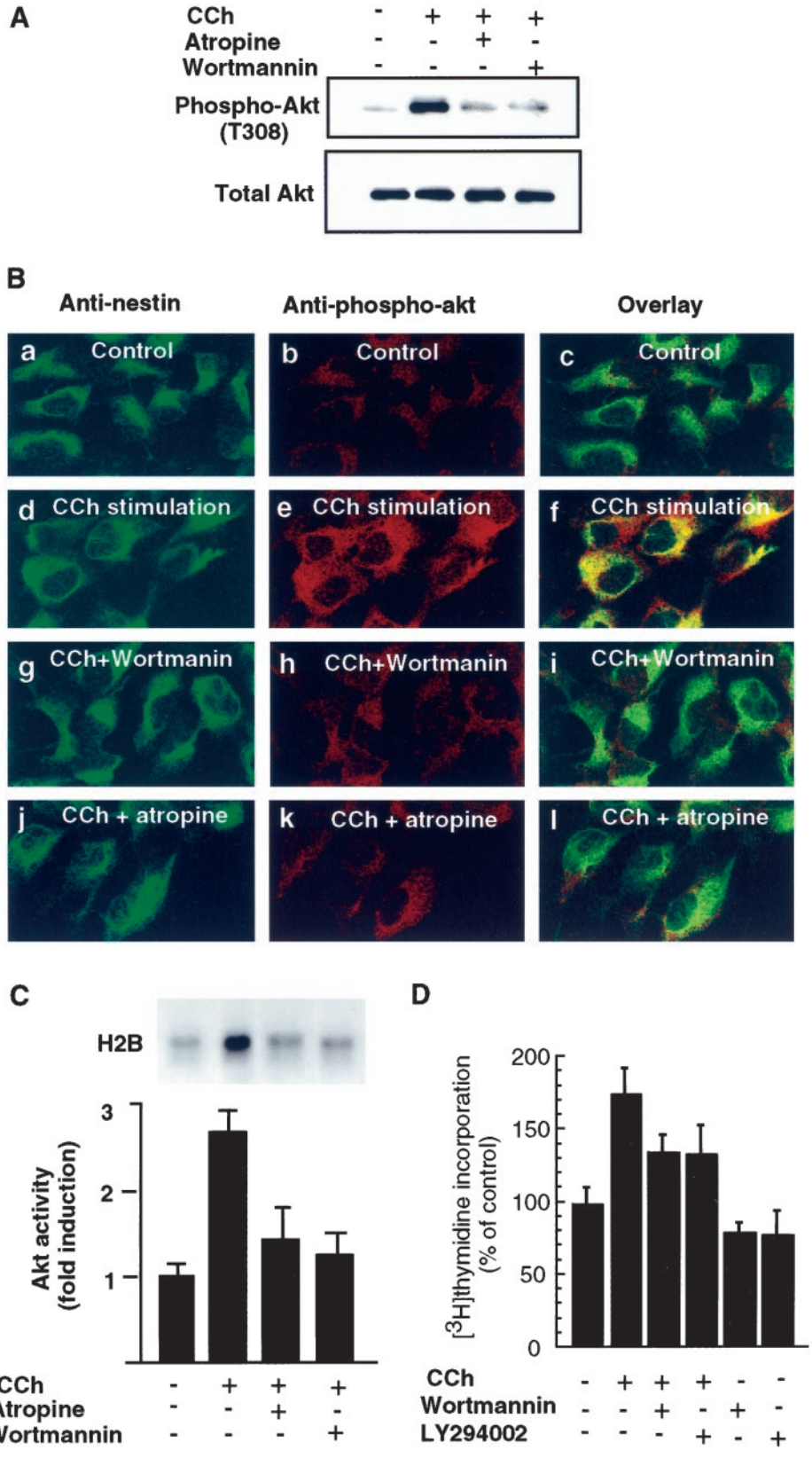

Figure 7. Carbachol activates Akt in neural progenitor cells mediated by muscarinic receptor in a wortmannin-sensitive manner. *A*, Carbachol induced Akt phosphorylation in a wortmannin-sensitive manner. Cortical progenitor cells were bFGF-expanded for 3 d. After bFGF was removed for 24 hr, carbachol (100 μM), carbachol plus wortmannin (50 nM), or carbachol plus atropine (50 μM) was added for 30 min. Cell lysates were analyzed by immunoblotting with phospho-dependent T308 and phospho-independent Akt antibodies. Blots were visualized by enhanced chemiluminescence. *B*, Analysis of CCh-induced Akt phosphorylation in neural progenitor cells by immunofluorescence that used a phospho-dependent Akt T308 antibody. Cells were fixed and double-stained with nestin and phospho-Akt T308 antibodies, followed by a secondary layer of fluorescein-conjugated goat antibody to mouse IgG and Texas Red-conjugated goat antibody to rabbit IgG. Photographs are representative confocal microscopic images depicting the cellular distribution of nestin (*left column*) and Akt T308-phosphorylated Akt (*center column*). Overlay images (*right column*) depict the colocalization of phospho-Akt and nestin in nestin-positive neural precursor cells. *C*, Effect of wortmannin (50 nM) and atropine (50 μM) on carbachol-induced Akt kinase activity in neural progenitor cells. The cells were treated as described in *A*. Cell lysate (50 μg) was immunoprecipitated with an anti-Akt antibody. Immunoprecipitates were used in an immunocomplex kinase assay in the presence of histone B2 (*H2B*) and [γ^{32}P]ATP. Phosphorylated H2B was separated on an 8–16% SDS polyacrylamide gel. The graph represents the means ± SEM of three independent experiments. A representative autoradiograph is shown in Figure 5*C*. *D*, Effect of atropine and wortmannin on carbachol-induced DNA synthesis. Cortical progenitor cells were bFGF-expanded for 3 d. After bFGF was removed for 24 hr, carbachol (100 μM), carbachol plus atropine (50 μM), wortmannin (50 nM), or LY294002 (10 μM) was added. [^{3}H]thymidine (2.5 μCi/ml) was added during the last 4 hr of culture. [^{3}H]thymidine incorporation was measured as an index of DNA synthesis. Results are the means ± SEM of four independent experiments.

sis. To examine this possibility, we compared cell death in CCh alone, and in treatment with PD98059 or wortmannin (inhibitors of MEK and PI-3K) alone and in the presence of carbachol by using a TUNEL-staining apoptotic assay procedure. Under an identical experimental paradigm of bFGF treatment, carbachol stimulation, and treatment with inhibitors (see Materials and Methods) we found that <12% of control, carbachol stimulation, and carbachol with PD98059 or wortmannin-treated cortical progenitor cell cultures displayed an apoptotic phenotype and were labeled by the TUNEL reaction 2 d after withdrawal of bFGF

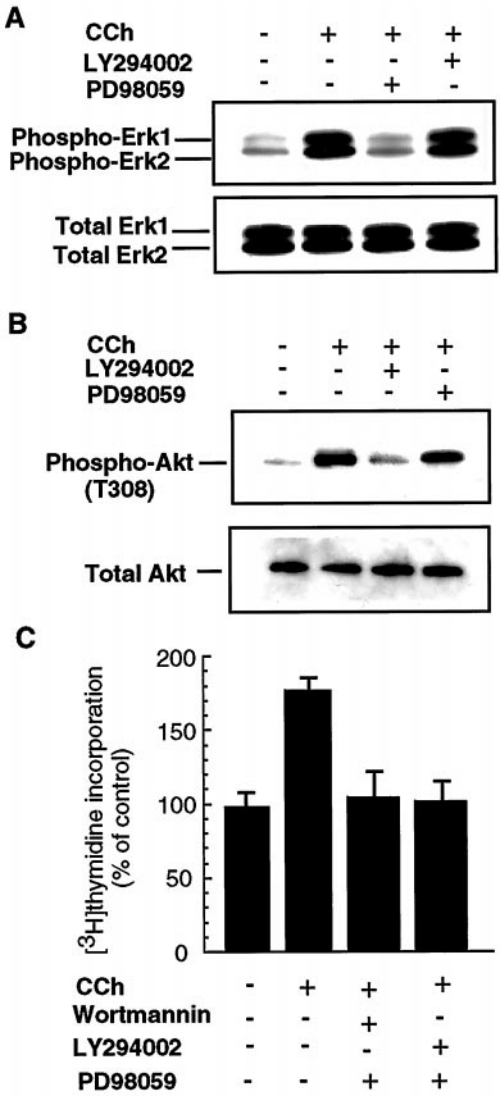

Figure 8. Carbachol stimulation of DNA synthesis in neural progenitor cells via both MAP kinase and PI-3 kinase pathways. *A*, Effect of LY294002 and PD98059 on carbachol-induced Erk1/2 phosphorylation in bFGF-deprived neural precursor cells. Cortical progenitor cells were treated as described above. Phosphorylated Erk1/2 and total Erk1/2 protein were analyzed by Western blot with anti-phospho-dependent Erk1/2 and anti-Erk1/2 antibodies. *B*, Effect of LY294002 and PD98059 on carbachol-induced Akt kinase activity in bFGF-deprived neural precursor cells. Cell lysates were analyzed by immunoblotting with phospho-dependent T308 and Akt antibodies. *C*, Effect of wortmannin, LY294002, and PD98059 on carbachol-induced DNA synthesis. Cortical progenitor cells were bFGF-expanded for 3 d. After bFGF was removed for 24 hr, carbachol (100 μM) or carbachol plus wortmannin (50 nM) and PD98059 (25 μM) or LY294002 (10 μM) and PD98059 (25 μM) were added. [^{3}H]thymidine (2.5 μCi/ml) was added during the last 4 hr of culture. [^{3}H]thymidine incorporation was measured as an index of DNA synthesis. Results are the means ± SEM of four independent experiments.

(Fig. 9*A,B*). At longer periods there was a significant increase of apoptotic cells. In addition, we also found that carbachol caused a decreased rate of cell death (Fig. 9*B*) and displayed a delay of differentiation (Fig. 9*C*) as compared with the control cells 2 d after withdrawal of bFGF. These results suggest that the extent of cell death in treated and control cultures is similar after the withdrawal of bFGF for 2 d and that the greater levels of thymidine incorporation in CCh-treated cultures were correlated with CCh-induced Erk1/2 and PI-3 kinase activity to stimulate DNA synthesis and to maintain survival in the cortical progenitor cells.

DISCUSSION

During CNS development it is essential that appropriate numbers of neurons and glia are produced to establish normal brain functions. It is well established that the regulation of both progenitor cell proliferation and neuronal cell death depends on an interaction of diverse extracellular signal molecules linked to a network of intracellular signal transduction pathways. Elucidating the regulatory mechanisms that control neural progenitor cell proliferation is important for understanding how intracellular signal transduction pathways regulate proliferation during early neurogenesis. In the present study we show that the acetylcholine agonist carbachol, acting via mAChR, activates MAPK and PI-3 kinase, resulting in increases in DNA synthesis in the neural precursor cells. These data suggest that acetylcholine acting via mAChR functions as a mitogen that activates MAPK and PI-3K and is involved in DNA synthesis during early neurogenesis.

Accumulating evidence indicates that GPCRs and their signaling molecules are important for growth stimulation (Rozengurt, 1986; Gutkind, 1998). Many ligands acting via GPCRs elicit a mitogenic response in a variety of cell types (Rozengurt, 1986; Pages et al., 1993); recent studies suggest that certain GPCRs are essential for cell growth under physiological conditions (Burstein et al., 1998). Although the mechanism or mechanisms whereby GPCRs regulate cell proliferation remain poorly understood, several lines of investigation have implicated the family of extracellular signal-regulated kinases (Erk1 and Erk2) or MAP kinases as a critical component of mitogenesis in promoting cell growth (Davis, 1993; Schlessinger, 1993). Consistent with the proposed role of MAPKs and the results of our experiments showing Erk1/2 activation inducing increases in [^{3}H]thymidine uptake as a result of CCh stimulation in neural progenitor cells, we propose signaling from mAChR to Ras, thereby initiating a cascade of events leading to MAP kinase kinase such as MEK1 and MEK2 activation. MEKs ultimately phosphorylate Erk1 and Erk2 on both threonine and tyrosine residues. In turn, Erk1/2 phosphorylate and regulate the expression of genes, such as transcription factors, which are essential for neural progenitor cell proliferation.

The results of the experiment (Fig. 6) showed that PD98059 significantly reduced carbachol-induced Erk1/2 activation and DNA synthesis; however, PD98059 only partially inhibited CCh-induced DNA synthesis, thus suggesting that an alternate mechanism to the MEK-Erk1/2 pathway is involved in regulating DNA synthesis in the neural progenitor cells. PI-3K recently was shown to play a central role in promoting the survival of a wide range of cell types (Datta et al., 1997; Brunet et al., 1999). There has also been the recent suggestion that, at least in IL-2 signaling, PI-3 kinase with its downstream target Akt may be important for cytokine-driven proliferation (Roche et al., 1994; Brennan et al., 1997). These data prompted us to explore whether the activation of PI-3 kinase by muscarinic receptors also might participate in regulating neural progenitor cell proliferation. To test this hypothesis, we investigated CCh-induced PI-3 kinase activity in neural progenitor cells. Potential links between GPCRs and the PI-3K signaling pathway also have been identified recently (Murga et al., 1998). Data presented in Figure 7 demonstrated that the CCh-induced increased in [^{3}H]thymidine incorporation

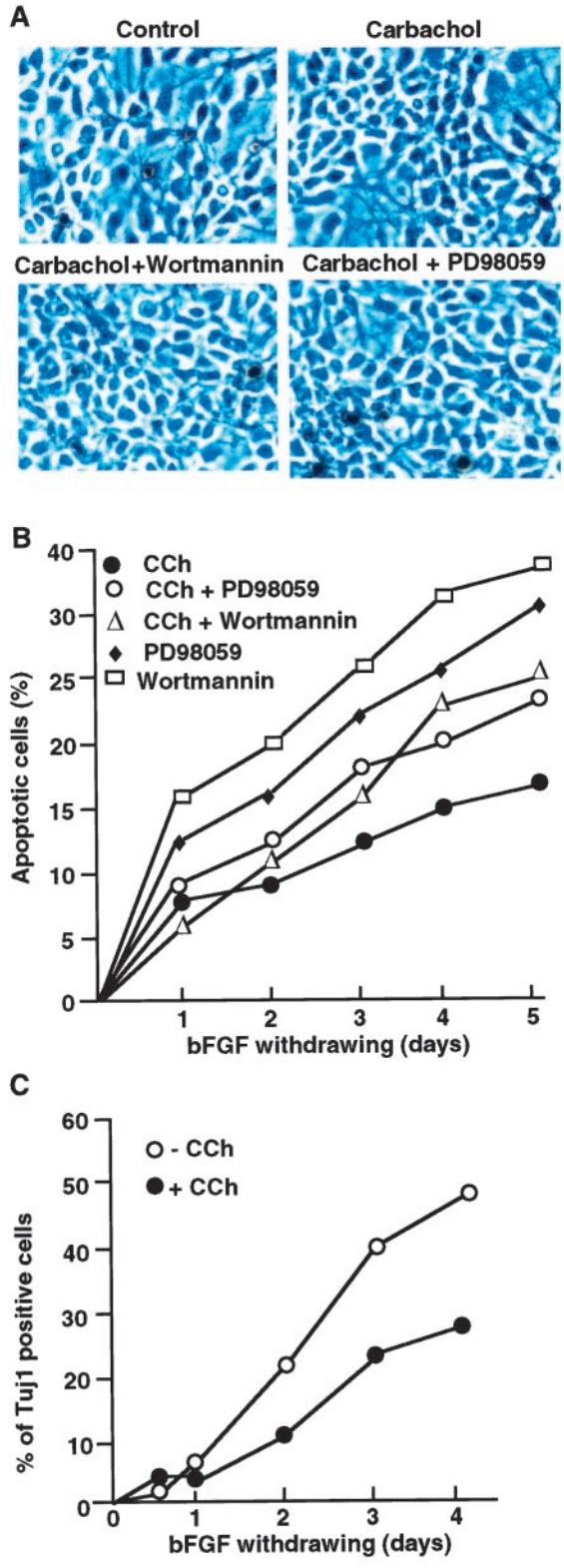

Figure 9. Carbachol stimulation and its effect on neural progenitor cell proliferation and survival. *A*, A representative field TUNEL staining of control cells and carbachol alone or cells treated with inhibitors 2 d after the withdrawal of bFGF. Cells were photographed in a light microscope. *B*, Neural progenitor cells were grown to an approximate density of 1×10^5 cells/cm^2 in 35 mm plastic dishes for 3 d. Cultures were bFGF-deprived and treated in the presence of carbachol alone, or inhibitors were added up to 5 d; the percentage of apoptosis was determined by counting labeled nuclei from different fields. Results from four independent experiments are shown as the means ± SEM. Day 0 indicates the day before bFGF withdrawal (control). The numbers of apoptotic cells at day 0 were normalized as 0% for comparing the apoptotic cells under different treatment conditions. *C*, Cells were treated as described in *B*. Immunostaining for the neuronal marker Tuj1 and cell counting were performed up to 4 d after the withdrawal of bFGF. The percentage of Tuj1$^+$ cells was counted from different fields. Results from four independent experiments are shown as the means ± SEM.

in neural progenitor cells was inhibited almost equally by both PD98059, an MEK inhibitor, and PI-3 kinase inhibitors (wortmannin or LY294002). In the presence of both kinase (MEK and PI-3 kinase) inhibitors, the increase in [^{3}H]thymidine incorporation produced by carbachol was inhibited completely to basal level (Fig. 8*C*). These results are consistent with a role for PI-3K and MAPK signaling in CCh stimulation of neural precursor cell proliferation. Recently, Lopez-Ilasaca et al. (1997) observed that a novel PI-3K isotype, termed PI-3Kγ, was found to link G-protein-coupled receptors and activated MAP kinase. This suggests a potential mechanism whereby mAChR can regulate crosstalk between PI-3 kinase and MAP kinase. In the present study we explored this possibility by using specific inhibitors of these kinases (Fig. 8). We found that carbachol stimulated both MAPK (Erk1/2) and PI-3 kinase, but inhibition of either one did not affect the activity of other kinase.

PI-3 kinase has many targets, including Akt, PDK1, and ILK, which can regulate PKC isoforms, p70S6 kinase, GSK-3, and PKA (Monfar et al., 1995; Batty et al., 1997; Delcommenne et al., 1998; Le Good et al., 1998; Cass et al., 1999; Wu, 1999). In addition to other kinases, GSK-3 is a critical downstream element of Akt (Pap and Cooper, 1998). GSK-3 has been shown to phosphorylate several other proteins, including β-catenin and the transcription factors c-Jun, c-Myc, c-Myb, and CREB; several of these substrates are implicated in oncogenesis and cell proliferation (Plyte et al., 1992; Dickinson et al., 1994; Miller and Moon, 1996). Recently, it has been shown that GSK-3β catalyzes cyclin D1 phosphorylation on Thr^{286}, thereby regulating cyclin D1 turnover. This results in a redistribution of cyclin D1 from the cell nucleus to the cytoplasm with proteosomal degradation during cell proliferation. Cyclin D1 turnover can be stabilized by overexpression of a constitutively active isoform of Akt (Diehl et al., 1998). The present data support the hypothesis that the G-protein-coupled mAChR activation of the PI-3K pathway phosphorylates GSK-3β and results in the inhibition of cyclin D1 phosphorylation, turnover, and redistribution. This may lead to an increase in DNA synthesis and cell proliferation.

Proliferation requires cell survival, but survival can occur without proliferation. The MEK–Erk1/2 and PI-3 kinase are thought to promote cell survival (Datta et al., 1997; Songyang et al., 1997; Toker and Cantley, 1997; Brunet et al., 1999). Therefore, it is important to measure carbachol-induced cell proliferation and survival. Our data indicate that the extent of cell death in CCh-treated cells or those cells treated together with PD98059 or wortmannin and in control cells is similar 2 d after the withdrawal of bFGF. Thus, we excluded the possibility that CCh stimulation of thymidine incorporation reflected a greater survival of neural progenitor cells rather than a stimulation of DNA synthesis (at least under these conditions), suggesting that mAChR-mediated MAP kinase and PI-3 kinase activity may play a key role in promoting neural progen-

itor cell proliferation. These findings raise the possibility that, in addition to promoting cell survival, PI-3K and MAP kinase signal transduction pathways regulate the proliferation of neural progenitor cells during early neurogenesis.

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