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Research report

Amyloid precursor protein and membrane phospholipids in primary cortical neurons increase with development, or after exposure to nerve growth factor or $A\beta_{1-40}$

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Abstract

We examined the relationships between membrane phospholipid levels, the secretion and expression of the amyloid precursor protein (APP), and the responses of both to nerve growth factor (NGF), $A\beta_{1-40}$ or $A\beta_{40-1}$ in developing cortical neurons cultured from rat embryos. Neuronal membrane phospholipid levels per cell, and phosphatidylcholine, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine increased individually between the first and seventh days of culturing. The amounts of APP holoprotein and APP mRNAs in the cells, as well as the amounts of soluble APP (APPs) secreted by them, also increased during neuronal development in vitro. The increases in APPs exceeded the increases in APP which, in turn, exceed those in phospholipid levels. The levels of APP holoprotein, but not of phospholipids, increased when neurons were grown in a choline-free medium, suggesting that increases in APP are not sufficient to stimulate changes in membrane phospholipids. Treatment of neuron cultures for four days with NGF or $A\beta_{1-40}$, but not with $A\beta_{40-1}$, dose-dependently increased membrane phospholipids, tau and GAP-43, as well as APP holoprotein and secreted APPs. These results indicate that agents, like NGF or $A\beta_{1-40}$, which enhance membrane phospholipid levels may promote neurite formation, APP expression and APPs secretion in primary neuronal cultures.

Themes: Disorders of the nervous system

Topics: Degenerative disease: Alzheimer's – other

Keywords: Alzheimer's disease; Neurite outgrowth; Amyloid; Neurotrophic; Lipid

1. Introduction

Amyloid plaques that accumulate in the brains of individuals with Alzheimer's disease are derived from the amyloid precursor protein (APP, ~110–130 kD). The beta amyloid (Aβ) domain of the APP lies between its long, extracellular N-terminus and its short, cytoplasmic C terminal region [10]. The first 28 amino acids of Aβ reside extracellularly, but the remaining 11–14 amino acids are embedded within the cell membrane. Cleavage of APP at the extracellular N-terminus and the transmembrane C-terminus of the Aβ domain liberates potentially amyloidogenic or neurotoxic Aβ peptides. In contrast, the formation of Aβ is prevented by cleavage of APP within the amyloid domain; thereafter APPs, which lacks an intact Aβ domain, is secreted into the extracellular space.

Membrane phospholipids or their metabolic products are associated with the synthesis and secretion of APP. Second messengers generated by phospholipid hydrolysis, such as arachidonic acid, diacylglycerol and inositol trisphosphate, increase APPs secretion and simultaneously reduce Aβ production [4,8,16,27,35]. The production of prostaglan-

Abbreviations: APP, amyloid precursor protein; APPs, soluble APP; Aβ, beta amyloid; DPH, 1,6-diphenyl-2,3,5-hexatriene; GAP-43, growth associated protein-43; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MEM, minimum essential medium; NGF, nerve growth factor; PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Rf, relative mobility; SEM, standard error of the mean; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with 0.15% Tween 20; TLC, thin layer chromatography

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dins by the cycloxygenation of arachidonic acid is also associated with increased syntheses of both APP mRNA and APP holoprotein [15]. Analysis of postmortem brains revealed that individuals with Alzheimer's disease had significantly thinner lipid membranes than those of age-matched controls [21]. Additionally, levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and of their respective precursors choline and ethanolamine, were found to be decreased in the Alzheimer disease brains, but not in brain samples from patients with Huntington disease, Parkinson's disease or Down's syndrome [27]. Destabilization of membrane phospholipids may expose abnormal cleavage sites on the APP transmembrane region, thereby promoting formation of amyloidogenic fragments [32]. Thus alterations in the phospholipid composition of cellular membranes during aging or neuronal injury could influence the pathogenesis of Alzheimer's disease [45].

The relationships between physiologic changes in membrane phospholipid levels and the synthesis or secretion of APP have not been established, even though major increases in membrane phospholipid levels are known to occur during neurite outgrowth [1]. Therefore we measured the levels of membrane phospholipids, cellular APP, and APPs secretion in primary cultured neurons undergoing spontaneous or NGF-stimulated neurite outgrowth. Additionally, because amyloidogenic peptides have been shown to increase lipid peroxidation and to damage cellular membranes [20], we also examined the effects of $A\beta_{1-40}$ or its reverse sequence, $A\beta_{40-1}$, on membrane phospholipid levels and APP expression and secretion. Parts of this work have previously been published in abstract form [41].

2. Materials and methods

2.1. Neuronal cultures and measurement of neuronal processes

Dissociated cortical neurons were cultured from fetal Sprague–Dawley rat pups at embryonic day 18–19, as previously described [6] with minor modifications [16]. Briefly, cortices were incubated for approximately 60 min in minimum essential medium (MEM; GIBCO) containing 0.25% Trypsin–0.1% DNAse. The cell pellet obtained by centrifugation was resuspended in MEM containing 5% fetal bovine serum and plated onto poly-L-lysine coated dishes (10^4–10^5 cells/cm^2). Neurons were grown on 35 mm culture dishes for Western blot or phospholipid analysis, or on 100 mm culture dishes for Northern blot analysis. One hour after plating, unattached cells and debris were removed by replacing the initial medium with fresh Neurobasal medium containing B27 supplements (GIBCO), Glutamine (0.5 mM), cytosine arabinoside (5 μM), glutamate (25 μM) and fetal bovine serum (5%). Glial and fibroblast proliferation was prevented by the presence of cytosine arabinoside (5 μM) in the medium. Neurons were maintained at 37°C in a humidified 5% CO_2–95% air incubator. The length of neuronal processes were measured on days 1, 3, 5 and 7.

2.2. Northern blot analysis

Total RNA was extracted from cultured neurons on days 1, 3, 5 and 7 using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) following the procedures recommended by the manufacturer. Briefly, the medium was aspirated and neurons were scraped in 1 ml of TRI Reagent; this mixture was then incubated for 15 min at room temperature after which 0.2 ml chloroform was added and the contents of each tube were mixed vigorously and stored for 15 min at room temperature. After centrifugation at 12,000 *g* for 15 min, 0.5 ml isopropanol was added to the aqueous phase to precipitate RNA. The RNA pellet was collected by centrifugation (12,000 *g*, 15 min at 4°C); washed once with 70% ethanol; and solubilized in an appropriate amount of Formazol (Molecular Research Center, Cincinnati, Ohio). RNA samples were denatured by heating for 15 min at 60°C, after which equal amounts of RNA (~10 μg) were loaded onto 1.2% agarose–formaldehyde gels for electrophoresis. RNA was blotted onto Hybond polyvinyl membranes by overnight downward capillary transfer using Turboblotter (Schleicher and Schuell) and fixed onto the membranes by baking (80°C) and UV light illumination. Membranes were prehybridized with Amersham Rapid-hyb buffer (Amersham Lab, Arlington Heights, IL, USA) for 2 h at 50°C. Human APP695 full-length cDNA was used to recognize all forms of APP transcripts in cultured neurons [25]. Rat tau cDNA was used to hybridize with all transcripts of the tau gene [24]. APP695, tau (gifts of Dr. Rachael Neve, McLean Hospital, Harvard Medical School, Belmont, MA, USA) or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNAs (Clontech) were labeled with ^{32}P-dCTP (Du Pont-New England Nuclear, Boston, MA, USA) using random primed extension (Amersham Megaprime DNA labeling kit). After a series of washes with 2X, 1X and 0.1X SSC–0.1% SDS to remove excess or unbound radioactive cDNA, the membranes were dried and exposed to Kodak X-ray film at −80°C for 48–72 h with an Amersham enhancer sheet. The relative amounts of mRNA obtained by hybridization were estimated using densitometric analyses of autoradiographs. The levels of APP or tau mRNA were normalized to the amounts of G3PDH mRNA and expressed as a ratio to their corresponding levels on day 1.

2.3. Western blot analysis

Cell-associated APP, tau and GAP-43 proteins were isolated from cultured neurons on days 1, 3, 5 and 7 respectively. The media were aspirated, and neurons were

scraped into 60 μl lysis buffer (60 mM Tris–HCl, 4%SDS, 20% glycerol, 1 mM dithiotreitol) and collected in Eppendorf tubes. To inhibit protease activity, the samples were boiled for 10 min. The concentration of protein was estimated by the bicinchoninic acid (Sigma) assay.

To detect APPs, the culture media from neurons grown on 35 mm dishes were collected on days 1, 3, 5 or 7 in vitro. After centrifugation (13 K rpm for 10 min) to remove cell debris, the media were desalted by filtration through Sephadex PD-10 columns (Pharmacia) and eluted with distilled water. Column elutes were frozen on dry ice and dried by vacuum centrifugation. The lyophilized proteins were reconstituted in 60 μl lysis buffer and boiled for 10 min. Prior to electrophoresis, 1 μl of 5% bromphenol blue solution was added to each sample. Equal amounts of medium or cell protein were loaded for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10–20% SDS–PAGE; Bio-Rad). Proteins (equivalent to ~100 μg cell protein/lane) were separated by electrophoresis, and then electroblotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) that had been incubated in Tris-buffered saline with 0.15% Tween 20 (TBST)-containing 5% powdered milk for 30 min to block nonspecific binding. After rinses in TBST, the membranes were incubated overnight in TBST containing one of the following antibodies: mAb 22C11 (Boehringer-Mannheim) which recognizes the amyloid precursor-like protein 2 (APLP-2) and the extracellular N-terminus of APP; mAb 4G8 (Senetek) which recognizes the Aβ domain of APP; GAP-43 (Boehringer-Mannheim) or 5E2 (Sigma) which respectively recognizes the growth associated protein-43 and tau in cell lysates. Membranes were rinsed (4×15 min) in TBST and incubated for 1 h with a peroxidase-linked secondary antibody; protein bands were visualized on Kodak X-AR films by an enhanced chemiluminescence method (Amersham). Optical densities of the protein bands were quantitated by laser scanning densitometry (LKB, Bromma, Sweden), and normalized to the density of bands generated by neurons assayed on day 1 or untreated, control neurons.

2.4. Phospholipid assay

To measure the total amounts of phospholipids in neurons on days 1 and 7 in vitro, they were rinsed with cold PBS (4°C, 2 ml) after media had been aspirated, and collected in 1 ml cold methanol. Each sample was sonicated and a 0.1 ml suspension was taken for DNA assay using the Hoechst 33258 reagent. Chloroform (1.8 ml) and distilled water (0.9 ml) were added to extract lipids using the method of Van Veldhoven and Bell [40]. The mixture was vortexed, centrifuged at 3500 *g* 15 min, and separated into two phases. The aqueous phase was aspirated and the organic phase was dried using a speed vacuum concentrator (Savant Instruments, Farmingdale, NY, USA). The dried samples were reconstituted in a mixed solution (30 μl) of chloroform and methanol (1:1, by volume). An aliquot (20 μl) of the phospholipid extract was purified by one dimensional thin layer chromatography (TLC) for about 150 min, on a pre-absorbent silica gel G plate (Analtech, Newark, DE, USA), using a mobile phase containing chloroform–triethylamine–ethanol–water (30:30:34:8, by volume). Phospholipid standards were used to identify the corresponding bands for PC, phosphatidylserine (PS), phosphatidylinositol (PI) and PE under long wave UV light after spraying the plates with 0.1% 1,6-diphenyl-2,3,5-hexatriene (DPH) in petroleum ether. The relative mobility (Rf) values were 0.09, 0.54, 0.30 and 0.42, respectively. The samples scraped from individual phospholipid bands were digested with 70% perchloric acid at 150°C for at least 2 h, and phosphate levels were assayed according to the method of Svanborg and Svennerholm [38], using dipalmitoyl PC as standard.

Cultured neurons were treated with NGF (50 ng/ml), $A\beta_{1-40}$ (BACHEM Torrance, CA; 10 or 100nM, 1 or 10 μM) or $A\beta_{40-1}$ (Quality Controlled Biochemicals, Hopkinton, MA,USA, 10 nm, 1 or 10 μM,) on day 1 in vitro for 4 days, or in choline-free medium for 24 h. Neurons in the control group were incubated in a test medium consisting of Neurobasal medium, B27 (1X), Glutamine (0.5 mM) and cytosine arabinoside (5 μM). To measure treatment-induced changes in phospholipid levels, ^{3}H-oleic acid (Du Pont-New England Nuclear, Boston, MA, 0.5 μCi/ml) was used to label neuronal PC, PS, PI and PE for 24 h before cell collections. TLC was used to separate individual phospholipid bands on silica gel G plates. The bands were identified under long wave UV light and scraped into scintillation vials containing ultrafluor (15 ml), after which their radioactivities were counted by liquid scintillation spectrometry. The concentrations of individual phospholipid were calculated.

2.5. Data analysis

Measurements of APP mRNA, APP, APPs, tau, GAP-43 and individual phospholipid on days 3, 5 or 7 in vitro were normalized against those on day 1. The levels of APP, APPs, tau or GAP-43, and the concentrations of various phospholipid in neurons treated with NGF, $A\beta_{1-40}$, or $A\beta_{40-1}$ were compared with those in untreated, control neurons. Analyses of variance (ANOVA) and *t*-tests were used to evaluate differences between days or between treatment groups (significance level, $P<0.05$).

3. Results

3.1. Changes in APP expression and secretion, and in phospholipid levels, with neuronal development

Morphological analysis revealed that the cultured neurons undergo extensive neurite outgrowth between day 1

and day 7 (Fig. 1). The axons of cultured neurons are prominent a few hours after plating and conspicuous growth cones are visible at the tips of the axons; dendrites appear after the axons have sprouted from the soma. Measurements of the axonal and dendritic lengths confirmed these morphological observations. The mean axon length on day 1, 3, 5 and 7 were 23.6, 47.4, 81.6 and 106.3 microns respectively (Fig. 2A) ($P<0.05$ day 3 vs. day 1; $P<0.05$ day 7 vs. days 3 or 5). The mean dendrite length on day 1, 3, 5 and 7 were 13.6, 21.1, 46.6 and 62.3 microns respectively ($P<0.05$ day 3 vs. day 1; $P<0.05$ day 7 vs. days 3 or 5) (Fig. 2B).

The levels of the cytoskeletal protein tau and of the growth associated protein GAP-43 increased linearly between days 1 and 7 of incubation (Fig. 2C). Tau levels on days 3, 5 and 7 were approximately 2.1-, 3.1- and 6.2-fold, respectively, those present on day 1 (Fig. 2C and D) ($P<0.05$ day 3 vs. day 1; $P<0.05$ day 7 vs. days 3 or 5). Similarly, GAP-43 protein levels on days 3, 5 and 7 were approximately 5.9-, 9.3-, and 21.7-fold, respectively, those on day 1 (Fig. 2C and E) ($P<0.05$ day 3 vs. day 1; $P<0.05$ day 7 vs. days 3 or 5).

APP mRNA and APP holoprotein levels in the cultured neurons were increased significantly between days 1 and 7. APP mRNA levels on days 3, 5 and 7 were approximately 1.4-, 2.6- and 2.9-fold, respectively, those on day 1 ($P<0.05$ day 1 vs. days 3, 5, or 7). The levels of tau mRNA also increased between days 1 and 7 (Fig. 3A, C), while those of G3PDH mRNA, which were used to control for the amounts of mRNA loaded for gel electrophoresis, remained unchanged ($P>0.05$). The development-related increase in APP mRNA was associated with parallel

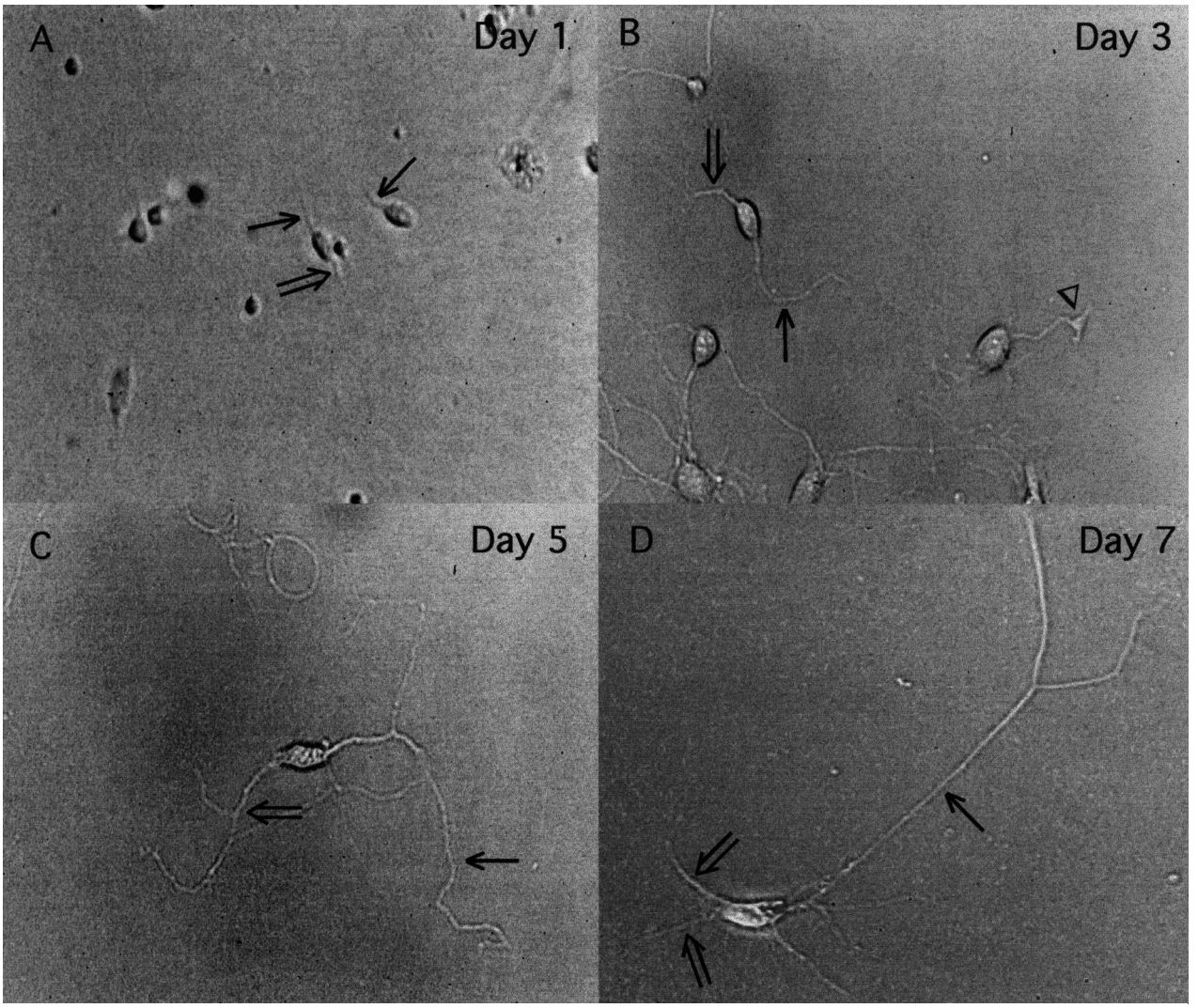

Fig. 1. Neurite outgrowth in primary cortical neurons between day 1 and day 7 in vitro. The photomicrographs show that the length of primary neurites increased between day 1 and day 7 (Fig. 1A, B, C and D). The axons (arrows) of cultured neurons are typically longer and more prominent than the dendrites (open arrows) at day 1 in vitro (Fig. 1A). At later stages of development, conspicuous growth cones (arrowhead, Fig. 1B) are visible at the tips of growing axons.

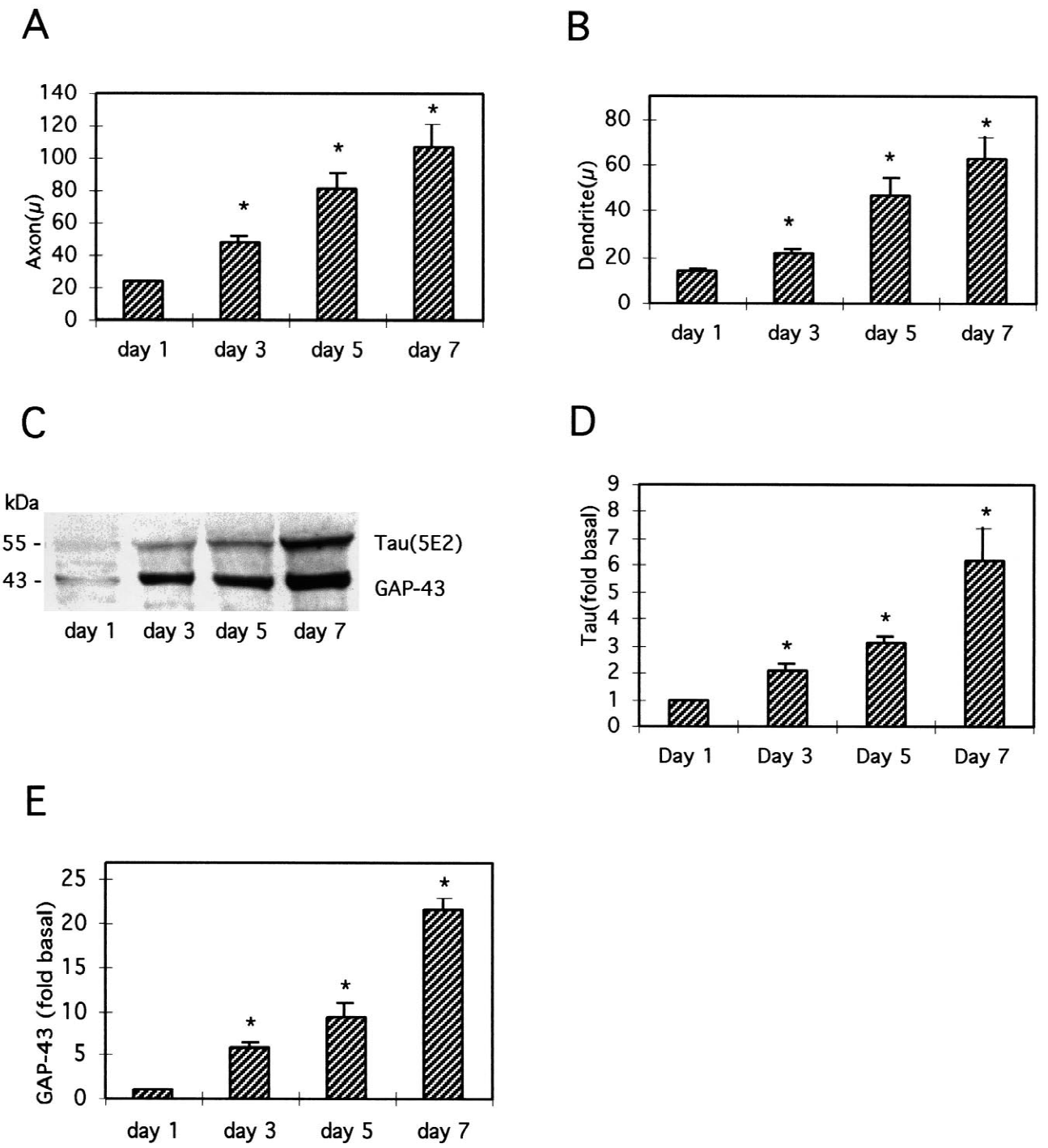

Fig. 2. Measurement of neurite length between day 1 and day 7, and the effect of neuronal development on tau and GAP-43 expression. (A) The length of axons increased significantly between day 1 and day 7 ($n=20$). (B) The length of dendrites increased significantly between day 1 and day 7 ($n=20$). (C) A representative Western blot shows that the levels of total tau and GAP-43 are both increased between 1 and 7 days during the development of cultured neurons. The levels of tau and GAP-43 were measured by mAb 5E2 and anti-GAP-43, respectively. (D, E) The graphs represent the means and SEM of tau (D) and GAP-43 (E) levels obtained from triplicate dishes of cultured neurons at various stages of development (*$P<0.05$; significantly different from the lengths and levels of day 1 in vitro).

increases in the levels of APP holoprotein and APPs. The levels of APP holoprotein on days 3, 5 and 7 were 2-, 2.5- and 4.2-fold those on day 1, and APPs levels in the culture media on days 3, 5 and 7 were 2.4-, 3.7- and 6.5-fold those on day 1 (Fig. 3D, E, F) as revealed by immunodetection with mAb 22C11. Similar increases in cellular APP

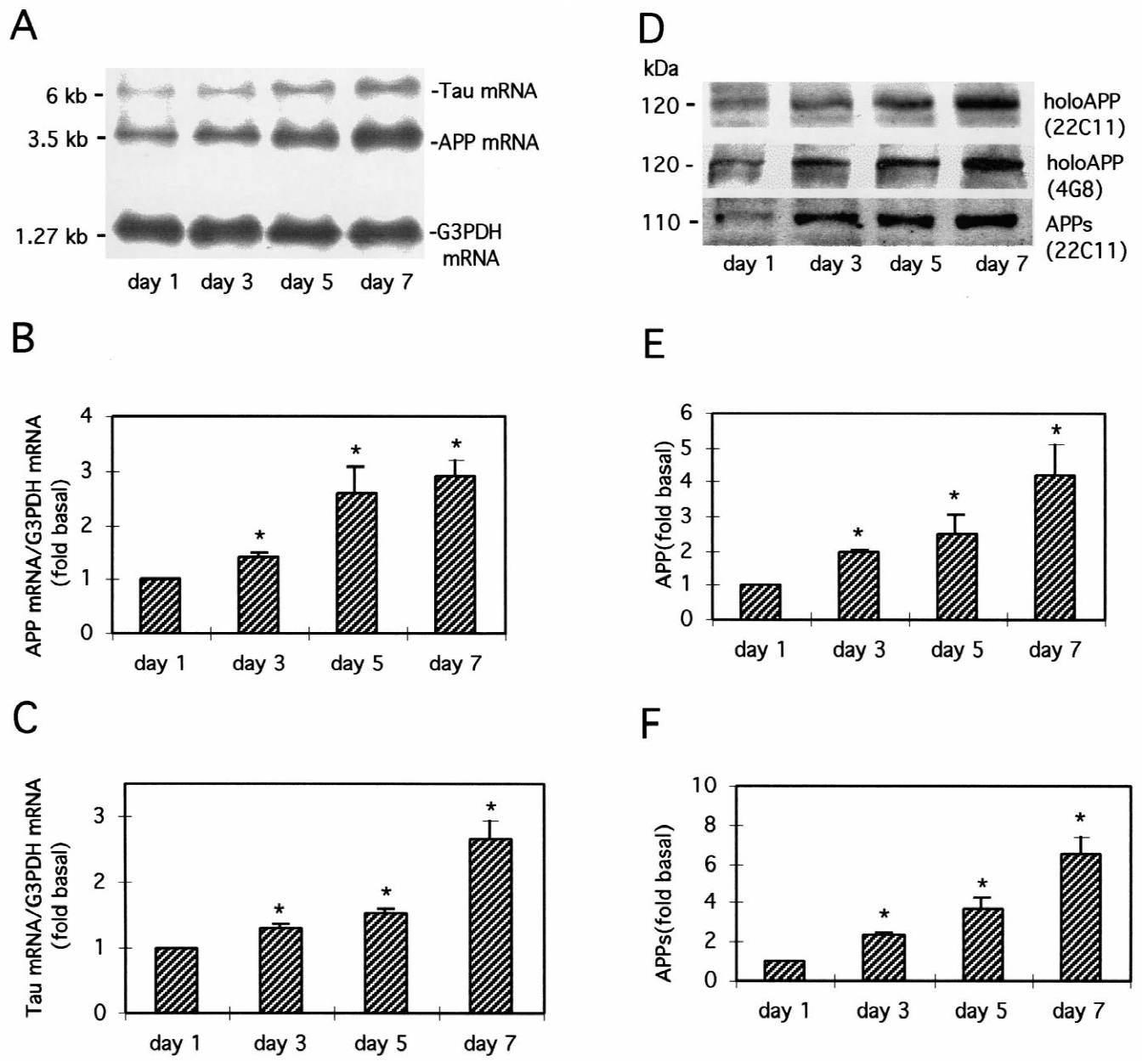

Fig. 3. Effect of neuronal development on APP mRNA, tau mRNA, APP holoprotein and APPs secretion. (A) A representative Northern blot shows that APP mRNA and tau mRNA are both increased in cultured neurons between 1 and 7 days in vitro. The levels of G3PDH mRNA, which were used as a measure of the amount of RNA loaded per lane, did not differ between 1 and 7 days in vitro. (B, C) The graphs represent the means and SEM of APP mRNA:G3PDH mRNA (B) and tau mRNA:G3PDH mRNA (C) ratios from 3 independent experiments (*$P<0.05$; significantly different from the levels measured on day 1 in vitro. (D) Representative Western blots show that both APP holoprotein (holo APP) and secreted APPs (APPs), as measured by mAb 22C11 or 4G8, are increased between 1 and 7 days in vitro. (E, F) The graphs represent the means and SEM of the APP holoprotein (E) and secreted APP (F) levels obtained from triplicate dishes of cultured neurons at various stages of development, as measured by mAb 22C11 (*$P<0.05$; significantly different from the levels measured on day 1 in vitro).

holoprotein between day1 and day 7 were detected by mAb 4G8 (Fig. 3D).

Phospholipid levels increased significantly from day 1 in vitro (828 nmol/mg DNA) to day 7 (1607 nmol/mg DNA) ($t=10.3$, $P<0.01$). Levels of PC, PE, PS and PI were 545, 204, 48 and 32 nmol/mg DNA respectively on day 1, and 1011, 448, 98 and 50 nmol/mg DNA respectively on day 7 (Fig. 4). The ratios of PC:PS:PI:PE did not change between days 1(65:6:4:25) and 7 (63:6:3:28).

3.2. Effects of choline-free medium on phospholipid levels and APP expression

Primary cortical neurons exposed to choline-free

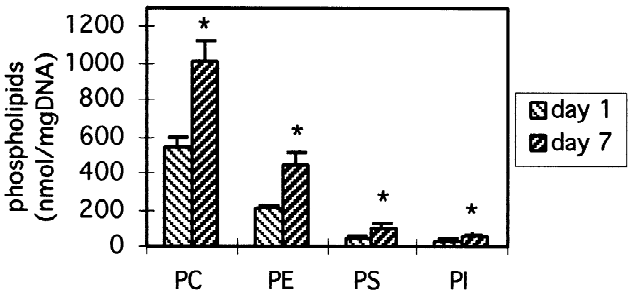

Fig. 4. Effect of neuronal development on the cellular phospholipids PC, PE, PS and PI. The levels of PC, PE, PS and PI are increased from day 1 to day 7 in cultured neurons. The graph represents the means and SEM of PC, PE, PS and PI levels obtained from triplicate dishes of cultured neurons between 1 and 7 days in vitro (*$P<0.05$; significantly different from the levels observed at day 1).

medium for 24 h had significantly increased levels of APP (1.8-fold±0.1 SEM, $P<0.05$) but not of membrane phospholipids relative to control neurons grown in medium containing 2 μM choline.

3.3. Effects of NGF or Aβ$_{1-40}$ on phospholipid levels, APP expression and secretion

As shown previously [1,18], NGF increased the levels of phospholipids during neuronal development. After treatment with 50 ng/ml NGF for 4 days, phospholipid levels (74 pmol/μg DNA) were significantly higher ($P<0.05$) than in untreated, control neurons (58 pmol/μg DNA). Similarly, treatment with 1 μM and 100 nM Aβ$_{1-40}$ also significantly increased phospholipid levels to 78 and 70 pmol/μg DNA relative to those of untreated, control neurons ($P<0.05$). By contrast, Aβ$_{40-1}$ had no significant effect on phospholipid levels (Fig. 5A).

The increases in phospholipid levels stimulated by NGF or Aβ$_{1-40}$ were associated with increases in cellular APP holoprotein (Fig. 5B). NGF (50 ng/ml) increased the APP levels by 60% ($P<0.05$) relative to these in untreated, control neurons; Aβ$_{1-40}$ (0.01, 0.1, 1 or 10 μM) increased APP levels to 1.6-, 1.6-, 1.8- and 1.9-fold those in untreated, control neurons (Fig. 5D)($P<0.05$). Treatment with either 10 nM or 10 μM Aβ$_{40-1}$ did not affect basal APP holoprotein levels.

NGF and Aβ$_{1-40}$, but not Aβ$_{40-1,}$ also increased the levels of APPs secreted into the media. Treatment of neurons for 4 days with 50 ng/ml NGF elicited APPs level that were 1.3-fold those in media from untreated, control cells ($P<0.05$). Treatment with 10, 100 nM or 1 μM Aβ$_{1-40}$ resulted in secreted APPs levels that were 1.3-, 1.3- and 1.5-fold ($P<0.05$) those in media of untreated, control neurons (Fig. 5 C, E). NGF or Aβ$_{1-40}$ (10, 100 nM or 1 μM), and also increased cellular levels of both tau and GAP-43 (Fig. 6); Aβ$_{40-1}$ failed to affect any of these proteins. The levels of tau in neurons treated with 10, 100 nM or 1 μM Aβ$_{1-40}$, or with NGF (50 ng/ml) were 1.6-, 1.8-, 2.7- and 2.9-fold, respectively, those in untreated, control neurons ($P<0.05$). Levels of GAP-43 in neurons treated with 0.01, 0.1 or 1 μM Aβ$_{1-40}$, or with NGF (50 ng/ml) were 2.3-, 2.4-, 4.0- and 3.9-fold respectively, those in untreated, control neurons ($P<0.05$).

4. Discussion

These data show that membrane phospholipid levels, as well as those of the APP holoprotein and the amounts of APPs secreted from primary neurons, increase as primary cultured neurons develop. Although the levels of PC, PS, PE and PI all increase between 1 and 7 days (Fig. 4), the ratios of each phospholipid to the others remain unchanged. Between day 1 and day 7 in vitro, the levels of neuronal membrane phospholipids increased by about 1-fold, whereas those of APP holoprotein and APPs levels increased by approximately 3.2-fold and 5.5-fold respectively. These data indicate that the increases in APPs exceeded those in APP, and that the ratio of APP or APPs to phospholipids increased between day 1 and day 7 of neuronal development in vitro. Moreover, exposing the neurons to NGF or Aβ$_{1-40}$, but not to Aβ$_{40-1}$, further increased the levels of phospholipids, of APP holoprotein and secreted APPs. Monoclonal antibody 22C11, used to detect secreted and cell-associated APP, also shows a low affinity for APLP-2 [36], raising the possibility that the production and secretion APLP-2 is also increased during the development of the neuronal cultures. Monoclonal antibody 4G8 recognizes the Aβ domain that is harbored within APP but that is lacking in APLP-2. Since mAb 4G8 also detected increases in the levels of APP between day 1 and day 7, the principal polypeptide detected in our experiments was primarily APP.

The increase in the levels of membrane phospholipids between day 1 and day 7 in vitro were associated with increases in axonal and dendritic lengths, as well as with increases in the levels of tau and GAP-43 proteins. Tau, a microtubule-associated protein required for neuritogenesis, which is known to be upregulated during axonal outgrowth [5,34,39], and GAP-43, a protein associated with growth cone formation that also modulates neurite outgrowth [22,37], also increased substantially between days 1 and 7, tau rising by about 6-fold and GAP-43 by 20-fold. These measurements show that increases in membrane phospholipids parallel the increases in the cellular proteins tau and GAP-43 that are associated with neurite outgrowth. These data also suggest that the elevations we observed in membrane phospholipid and APP levels were indeed associated with neurite outgrowth.

APP appears to mediate NGF-induced neurite outgrowth [23] suggesting that APP may stimulate the increased levels of phospholipids that is associated with neurite outgrowth. However, our studies suggest that increases in

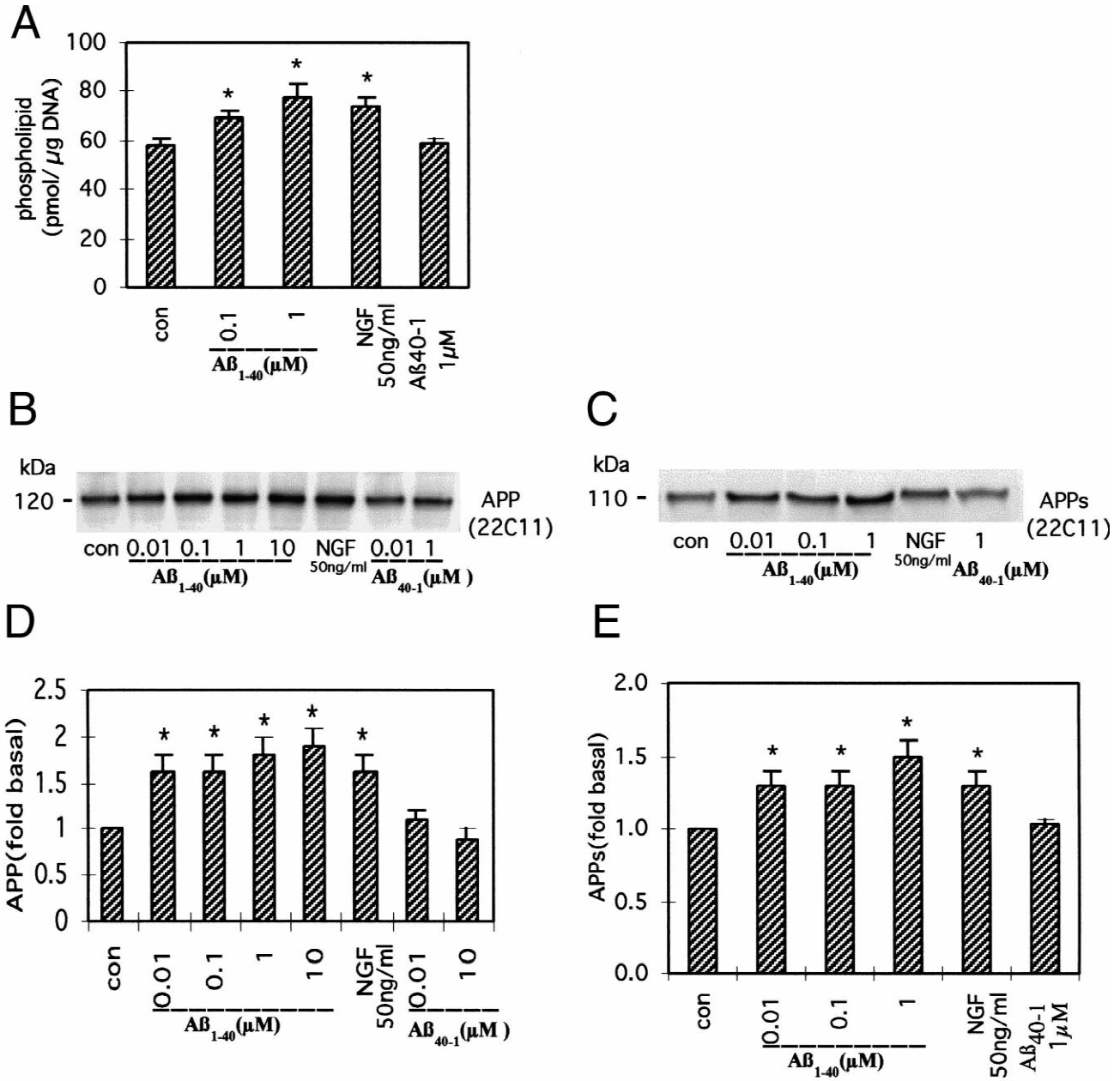

Fig. 5. Effect of NGF or $A\beta_{1-40}$ on phospholipid levels, APP expression and APPs secretion. (A) NGF or $A\beta_{1-40}$ treatment significantly increased the level of phospholipids in neuronal cultures (*$P<0.05$). By contrast, $A\beta_{40-1}$ had no significant effect on the level of phospholipids relative to the untreated, control group. The means and SEM were obtained from nine independent experiments. (B) A representative Western blot shows that APP expression in cultured neurons, as detected by mAb 22C11, is increased by $A\beta_{1-40}$(0.01, 0.1, 1 or 10 μM) or by NGF. By contrast, $A\beta_{40-1}$ (10 nM or 10 μM) had no significant effect on APP expression relative to untreated, control neurons (Con). (C) A representative Western blot indicates that the levels of secreted APPs, as detected by mAb 22C11, is increased by NGF or $A\beta_{1-40}$ treatment. $A\beta_{40-1}$ had no significant effect on secreted APPs levels relative to untreated, control neurons (Con). (D) The graph represents the means and SEM of APP holoprotein levels stimulated by NGF or $A\beta_{1-40}$; $A\beta_{40-1}$ had no significant effect on the levels of APP relative to untreated, control neurons. These data were obtained from three independent experiments (*$P<0.05$). (E) The graphs represents the means and SEM of secreted APPs levels stimulated by NGF or $A\beta_{1-40}$; $A\beta_{40-1}$ had no significant effect on the levels of secreted APPs relative to untreated, control neurons. The results reflect the data obtained from 3 independent experiments (*$P<0.05$).

APP do not always stimulate increased membrane phospholipids. Neurons that upregulate APP holoprotein levels in the absence of choline do not show increased levels of membrane phospholipids. Moreover, treatment of astrocytes with prostaglandin E_2 increases APP mRNA and holoprotein [15] but do not increase membrane phospholipid levels (unpublished data). These data suggest that agents that enhance APP production do not always stimulate increases in membrane phospholipids. Instead, agents that stimulate increases in membrane phospholipids may promote APP expression, APPs secretion and possibly neurite outgrowth in primary neuronal cultures.

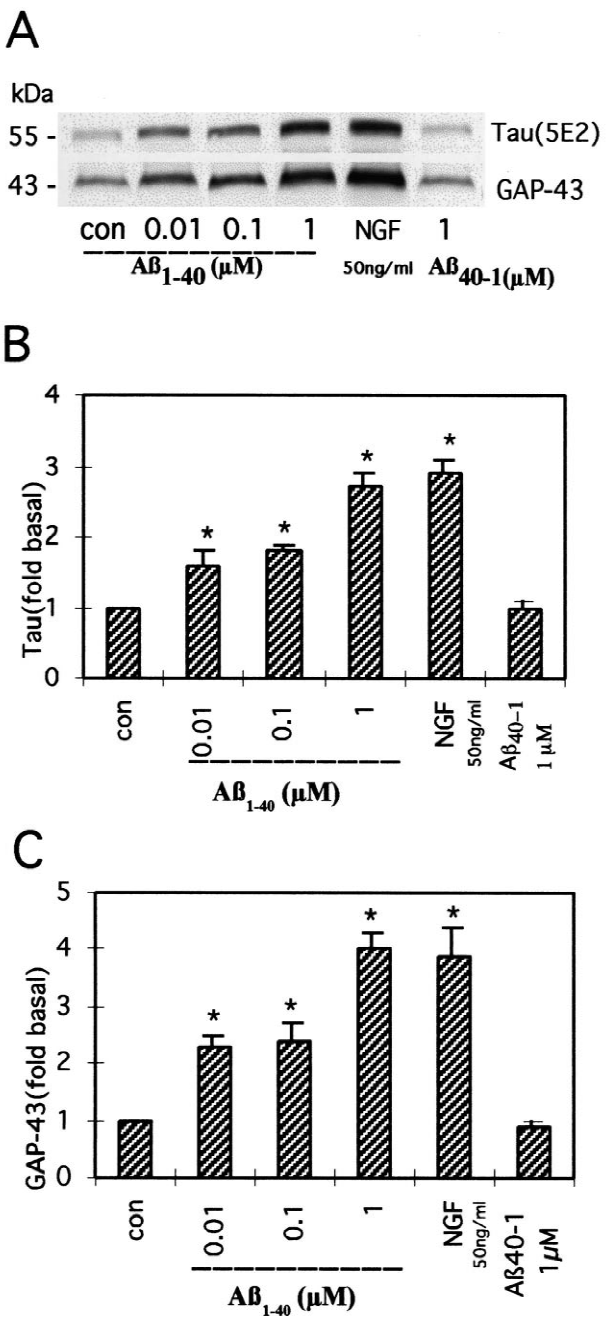

Fig. 6. Effect of NGF or $A\beta_{1-40}$ treatment on total tau or GAP-43 levels in cultured neurons. (A) A representative Western blot indicates that the levels of cellular tau (detected by mAb 5E2) and GAP-43 are increased by NGF or $A\beta_{1-40}$ treatment. $A\beta_{40-1}$ had no significant effect on total tau or GAP-43 levels relative to untreated, control neurons (Con). (B,C) The graphs represents the means and SEM of (B) total tau or (C) GAP-43 levels stimulated by NGF or $A\beta_{1-40}$; $A\beta_{40-1}$ had no significant effect on the levels of total tau or GAP-43 levels relative to untreated, control neurons. The results reflect the data obtained from three independent experiments (*$P<0.05$).

The increases in APP mRNA and APP holoprotein levels that occurred when our primary cultured neurons underwent neurite outgrowth are consistent with the prior finding that the induction of process formation in P19 carcinoma cells by retinoic acid was also associated with increased APP mRNA and holoprotein [9]. Although these effects were not associated with increased APPs secretion [9], we did find in our primary neuronal cultures both increased APP holoprotein and APPs secretion. These data are also consistent with the observation that APP is highly expressed in fetal neurons of the cerebral cortex and that the levels of APPs are increased during in vitro development [14].

Exposing the neurons to NGF or $A\beta_{1-40}$, but not to $A\beta_{40-1}$, further increased the levels of APP holoprotein and secreted APPs by about 0.5-fold, and phospholipids only by about 0.3 fold, compared with control neurons. These data suggest the density of APP in the newly-formed membrane phospholipid bilayer is greater than in the overall initial membrane. Similarly, tau and GAP-43 levels rose by about 1.5-fold in neurons treated with NGF, and by 3-fold in neurons treated with $A\beta_{1-40}$. Although Aβ is known to induce lipid peroxidation, it is unlikely that the increased APP expression caused by Aβ treatment is mediated by increased peroxidation since the levels of membrane phospholipids were not decreased but were, in fact, increased, by Aβ treatment.

Aβ can be either neurotoxic or neurotrophic [46]. Non-aggregated forms of Aβ used in our study did not appear to be neurotoxic but, instead, increased the levels of membrane phospholipids, of tau and of GAP-43. Non-aggregated Aβ also increased the levels of APP holoprotein and secreted APPs in cultured neurons, an effect that has also been demonstrated in PC12 cells [31]. Interestingly, in aged amyloid precursor protein transgenic mice, amyloid plaques appear to have neurotrophic effects and appear to be the main cause of aberrant axon sprouting and increased levels of GAP-43 immunostaining [28]. Our results are also consistent with those of [12,28,43,44] demonstrating that amyloid peptides can induce neurite outgrowth in neurons, and with previous demonstrations that amyloid peptides such as $A\beta_{1-40}$ or $A\beta_{1-42}$ can increase the expression of APP mRNA and holoprotein in smooth muscle cells or neuronal hybrid cells [3,17].

NGF elevated the levels of membrane phospholipids in primary cultured neurons, as has been shown in PC12 cells [1,18] and also increased the levels of GAP-43 and tau proteins. Although NGF appeared to stimulate APPs secretion in PC12 cells [2,7,29] it appeared not to do so in rat brains [26]. However, our results indicate that NGF did increase both APPs secretion and APP expression in primary neuronal cultures derived from rat cortices, and suggest that agents that increase membrane phospholipids may also promote APP expression and APPs secretion. Consistent with this suggestion, we recently demonstrated that treatment of PC12 cells or neurons with drugs such as cytidine, choline or cytidine diphosphocholine that specifically increase membrane phospholipid composition [19,33] also can increase APP expression and secretion [42].

The neurotrophic effects of NGF are mediated by p75 neurotrophin ($p75^{NTR}$) or tyrosine kinase (Trk) A receptors [11,30] and Aβ has been shown to bind to $p75^{NTR}$ [13]. Recently, the stimulatory effect of NGF on increased APP expression and its secretion was shown to be mediated by $p75^{NTR}$, whereas activation of Trk A receptors alone increased APPs secretion without affecting APP expression in PC12 cells [31]. These studies suggest that the increases in membrane phospholipids, as well as the increases in APP holoprotein and APP secretion observed in cultured neurons treated with NGF or $A\beta_{1-40}$ may be mediated by p75NTR or Trk A receptors.

Elevations in APP holoprotein during development, or with $A\beta_{1-40}$ or NGF treatment of cultured neurons appear to be associated with increased APPs secretion. Similarly, increased APP synthesis in astrocytes stimulated by prostaglandin E_2 is also associated with increased APPs [15]. It might be expected that pathologic events, such as reduced phosphatidylinositol signaling, that reduce APPs secretion when APP expression is increased might accelerate the production of amyloidogenic Aβ fragments.

In summary, we show that neuronal development in vitro is associated with both increased APP expression, APPs secretion and increased membrane phospholipid synthesis; the increases in APPs exceed the increases in APP; the increases in APP exceed the increases in phospholipids. The ratios of the APPs or APP to the lipids also increased. NGF, like soluble Aβ peptides, increases membrane phospholipids. Additionally, both APP expression and APPs secretion are increased by NGF or soluble Aβ peptides. We suggest that agents that regulate membrane biosynthesis may be used to modulate APP expression and secretion, and that such compounds may useful for ameliorating some of the brain pathologies associated with Alzheimer's disease.

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