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NGF PROMOTES AMYLOID PRECURSOR PROTEIN SECRETION VIA MUSCARINIC RECEPTOR ACTIVATION

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Summary: Processing of β-amyloid precursor protein (APP) is coupled to several neurotransmitter receptors, including m1 muscarinic (m1AChR), and is associated with decreased amyloid deposition. Muscarinic agonist-stimulated APP secretion and membrane APP were measured in control and in NGF-differentiated PC12 cells stably transfected with m1AChR. This secretion was markedly enhanced following treatment with 50 ng/ml NGF for 3 days, and was observed using either carbachol or the M1-selective agonist AF102B. The effects of NGF were reflected by larger reductions in membrane-associated APP levels following muscarinic stimulation. These observations imply that M1 muscarinic receptors may act in concert with NGF to boost APP processing, and M1-selective agonists may thus be beneficial for reducing amyloid deposition by NGF-responsive neurons.

Amyloid β protein (βA4) is a major component of senile plaques, a distinct neuropathological lesion observed in the brains of Alzheimer's disease (AD) patients. Deposition of aggregated βA4 amyloid deposits in plaques, and the neurotoxicity that is associated with such deposits, suggest an involvement in the etiology of AD (1). The βA4 peptide is a proteolytic product of the amyloid precursor protein (APP), a membrane-associated glycoprotein whose processing may be followed by secretion of non-amyloidogenic APP isoforms. Such secreted APP isoforms may participate in neuroprotective mechanisms (2), and it has been proposed that APP secretion participates in NGF-mediated neurite outgrowth (3). APP secretion involves proteolytic cleavage by an unidentified protease ("α-secretase") at residue 687 of the fully mature APP_{770} (4), which corresponds to residue 16 of the βA4.

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Hence, APP secretion hampers amyloid generation. In human HEK293 cells transfected with the m1 muscarinic cholinergic receptor (m1AChR) subtype (5), as well as in m1AChR-transfected rat pheochromocytoma PC12 cells (6), the agonist carbachol stimulated the secretion of APP. These observations suggested a connection between the well-documented cholinergic deficiencies and amyloid deposition in the brains of AD patients, and implied an additional possible benefit for cholinergic replacement treatment in this disease.

AF102B is an M1-selective muscarinic agonist under evaluation for AD (7-10). This agonist exhibits a distinctive signaling pattern via m1AChR: it elevates intra-cellular Ca^{2+} levels to the same extent as the non-selective agonist carbachol, but unlike carbachol, only weakly stimulates phosphoinositide hydrolysis or arachidonic acid release. In addition, it potently antagonizes carbachol-activated adenylyl cyclase (9). In a recent study employing m1AChR-transfected PC12 cells (PC12M1 cells), we demonstrated that AF102B lowered membrane-associated APP levels to a similar extent as the full agonist carbachol, in spite of its relatively weak m1AChR-activated PI hydrolysis signal (10). This may be related to the unique signaling pattern of this partial agonist (9). In addition, muscarinic agonists are capable of inducing neurite-outgrowth in PC12M1 cells, a response which is markedly augmented by NGF (11). In the same cell line AF102B exhibits neurite-inducing activity which is highly dependent on the presence of NGF (12), implicating the unique property of this M1-selective partial agonist.

The "α-secretase" pathway for stimulation of APP secretion involves activation of protein kinase C (PKC), a family of Ca^{2+} and diacylglycerol activated kinases (13). PKC activity, in turn, may be affected *via* G protein-coupled receptors such as the m1AChR (5,6), secondary to receptor-mediated phosphoinositide hydrolysis which elevates both cytosolic Ca^{2+} and diacylglycerol levels. NGF treatment of PC12 cells was recently shown to increase the α, β, and γ-isoforms of PKC (14), as well as the calcium-insensitive PKC-ε isoform (15). In addition, NGF treatment affects the ratio between various APP isoforms arising in PC12 cells from alternate splicing of the APP gene (16,17). Indeed, phorbol-ester stimulated APP secretion, which reflects PKC activation, was increased in NGF-treated PC12 cells (18,19). PKC-governed events may also be affected by steps taking place down-stream from activation of growth-factor activated receptor tyrosine-kinases, such as *trk A*, the high-affinity NGF receptor (20). It is therefore plausible that NGF treatment may improve the ability of m1AChR-mediated signals to increase APP processing in cells that are responsive to both NGF and M1 agonists. PC12 cells are an excellent model for studying NGF-mediated events (21). The present studies therefore address these possibilities by measuring APP secretion and cell-associated APP levels in m1AChR-transfected PC12 cells (PC12M1).

MATERIALS AND METHODS

Transfected cells: The preparation and culture conditions of PC12 cells stably transfected with the m1AChR (PC12M1 cells) are described in detail elsewhere (11). Cells were plated in 6-well plates (Corning, NY) in complete RPMI medium (Beth Haemek, Israel) and used at the age of 4 - 5 days after plating.

APP secretion studies: For studies of APP secretion, cells were washed twice in serum-free RPMI medium supplemented with 20 mM HEPES and 0.2 mg/ml BSA. Incubations with the muscarinic ligands were carried out in this medium at 37 °C for the indicated periods and terminated by collecting the conditioned medium samples into tubes containing protease inhibitor cocktail (0.1 mM PMSF; 5 µg/ml leupeptin and pepstatin; 5 units/ml aprotinin) and concentrating with Centricon tubes (Amicon, Beverly, MA). Following protein determination (Bio-Rad protein assay), equal protein amounts were loaded on 10% PAGE-SDS gels; gels were subsequently blotted to nitrocellulose membranes (Hoefer Scientific Instruments, San Francisco, CA) and probed with mAb 22C11 (Boehringer Mannheim, FRG), and subsequently with a peroxidase-linked rabbit anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) followed by 4-chloro-1-naphtol staining. Densitometric scanning of the stained bands was performed by video-imaging densitometry (Biological Detection Systems, Pittsburgh, PA). Data of APP secretion are expressed as percentage of control values. NGF-treated and non-treated PC12M1 cultures were always grown and assayed in parallel. This enabled combination of data from separate experiments.

Cell-associated APP levels: Determination of cell-associated APP was done similarly to the APP secretion studies, with the exception that for 24 h stimulation with agonists, complete medium was employed to avoid apoptotic cell death which takes place when PC12 cells are grown without serum. Incubations were terminated by washing the cells three times in serum-free RPMI medium followed by scrapping in lysis buffer (20 mM TRIS, 150 mM NaCl, 5 mM EDTA, 1 % Triton-X-100, and protease inhibitor cocktail). Following 5 sec sonication, samples were centrifuged (5 min x 10,000 g) and equal amounts of supernatant proteins were loaded on SDS-PAGE and analyzed for cell-associated APP similarly to the secreted APP studies.

Materials: AF102B was synthesized as described (9). Mouse submaxillary gland NGF (2.5S) was from Sigma (St. Louis, MO cat. # N-6009) Tissue culture reagents and antibiotics were from Beth Haemek, Israel. Molecular weight standards were from Bio-Rad (Hercules, CA). All other chemicals were from Sigma.

RESULTS AND DISCUSSION

NGF enhances m1AChR-mediated APP secretion: Measurements of the M1 muscarinic receptor-mediated APP secretion to the culture medium of PC12M1 cells were made by immunoblotting with mAb 22C11, as described previously (10) and detailed in the Methods. We have recently reported that m1AChR-mediated APP secretion in the PC12M1 cell line was maximal at 1 h following agonist stimulation (10). We therefore selected these assay conditions for analyzing the effects of long-term NGF treatment on carbachol and AF102B-mediated APP secretion. Data presented in Figure 1 indicated that a 3 day treatment of PC12M1 cells with NGF (50 ng/ml) resulted in an enhanced m1AChR-mediated APP secretion. This was evident for both the full agonist carbachol or the M1-selective agonist AF102B (Figure 1A). The extent of the NGF enhancement of the muscarinic response varied

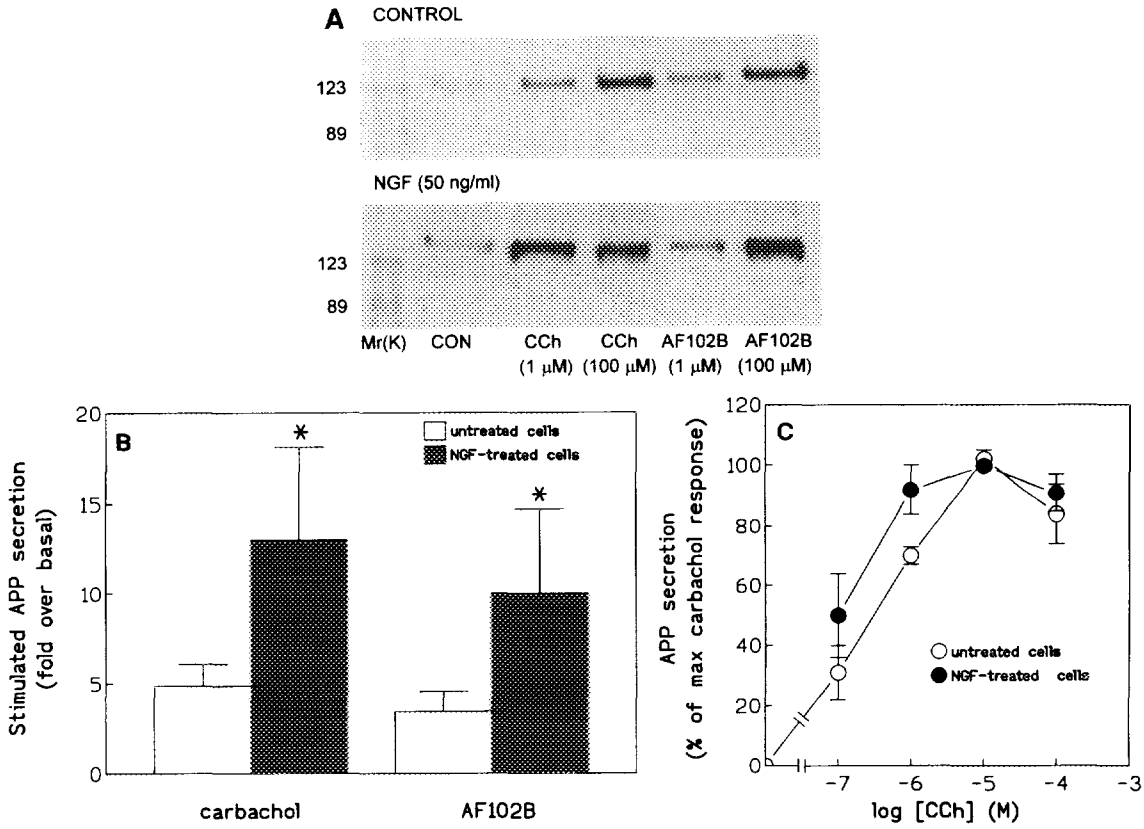

Figure 1. Enhanced m1AChR-stimulated APP secretion from PC12M1 cells following NGF treatment.

A. Representative immunoblot of secreted APP. Cells were stimulated for 1 h in serum-free medium with carbachol (CCh) or AF102B (both at 1 μM or 100 μM, as indicated). NGF (50 ng/ml) was added 3 days earlier in complete medium, and was not present during the 1 h stimulation period. The numbers on the left represent the molecular weight markers used on the gels.

B. Video-imaging densitometry data (mean±SEM from 5 independent experiments) for APP secretion with carbachol and AF102B (both at 100 μM; 1 h) in control and NGF-treated cells (same as in Fig. 1A). Data are presented as fold over basal, determined separately in each experiment for control and for NGF-treated cells. NGF treatment resulted in significant enhancement of APP release by both ligands (*$p<0.05$; two-tailed Student's *t*-test). Basal APP secretion (1 h in serum-free medium) was not significantly affected in NGF-treated PC12M1 cells and was 1.05±0.32 vs. control for the depicted experiment.

C. Video-imaging densitometry data (mean±SEM from 3 independent experiments) for APP secretion (1 h) with varying carbachol concentrations in control and NGF-treated cells (same as in Fig. 1A). Data are presented percent of maximal carbachol response, determined separately in each experiment for control and for NGF-treated cells. Calculated EC_{50} values were 0.3 and 0.1 μM, respectively, for control and NGF-treated PC12M1 cells.

among experiments, probably reflecting variable levels of NGF-mediated differentiation, also evident from the extent of neurite outgrowth (not shown). This was not related to muscarinic receptor density (3.1±0.4 pmol/mg protein) or muscarinic signaling (phosphoinositide hydrolysis) which were both consistent (10). Video-imaging densitometry data of immunoblots

from 5 independent experiments (Figure 1B) demonstrated that NGF significantly increased muscarinic agonist-stimulated APP secretion from 4.8±1.2 to 13±5, and from 3.4±1 to 10±4.7 fold over basal, respectively, for carbachol and AF102B (both at 0.1 mM). Concentration-dependence studies of the carbachol-stimulated APP secretion indicated that following NGF treatment the EC_{50} was decreased from 0.3 to 0.1 μM, so that the effect of NGF was more evident at lower carbachol concentrations (Figure 1C). Similar observations were made using AF102B (not shown).

NGF augments decreases in membrane APP: PC12M1 cells exhibit persistent basal APP secretion at incubation periods exceeding 1 h (10). In contrast, muscarinic agonist-stimulated APP secretion is progressively decreased with time, probably reflecting increased desensitization of m1AChR-mediated signals (10). To study APP processing following longer incubation periods with agonists, we therefore resorted to measuring the levels of cell-associated APP, which were previously shown to decrease in association with increased APP secretion (4-6). Fig. 2A (top panel) demonstrates that following stimulation for 3 h with carbachol, the levels of cell-associated APP were decreased more markedly than when AF102B was employed (both at 0.1 mM). This was also found for a stimulation period of 1 h (Fig. 2B). Stimulation for 24 h (Fig. 2A, middle panel) resulted in similarly decreased cell-associated APP levels using either agonist. The muscarinic agonist-mediated decreases in cell-associated APP levels were more pronounced in NGF-treated PC12M1 cells (Fig. 2A, bottom panel). Video-imaging densitometry measurements of total cell-associated APP yielded values of 66±6% and 72±10% *vs.*control for carbachol and AF102B, respectively, and these values were further decreased to 45±6% and 39±14% *vs.*control in NGF-treated PC12M1 cells (Fig. 2C). Total cell-associated APP-immunoreactivity did not significantly change following NGF treatment. For example, in a typical experiment total cell-associated APP immunoreactivity following NGF treatment was 92±4% *vs.*control (calculated per mg of cell protein).

Association with phorbol-ester-mediated APP secretion: APP secretion probably requires activation of PKC (13). It is not clear whether m1AChR-mediated APP secretion acts exclusively via this signaling pathway (5,6). Moreover, Buxbaum *et al.* have shown that APP processing may also involve increased cytoplasmic calcium ion levels (22). This is of particular interest in lieu of our observations on independent activation of several signaling pathways by agonist-activated m1AChR (9). We therefore studied the effects of NGF treatment on phorbol-ester mediated APP secretion, which represents activation of PKC alone, without changes in intracellular calcium levels. We observed a robust increase in PMA-mediated APP secretion following a 3 day exposure of PC12M1 cells to NGF (Fig. 3). Comparing the NGF effect in the same experiment for carbachol and for PMA (both at 1 μM) indicated that the effect of NGF treatment was about twice as large for PMA compared with carbachol (Fig. 3).

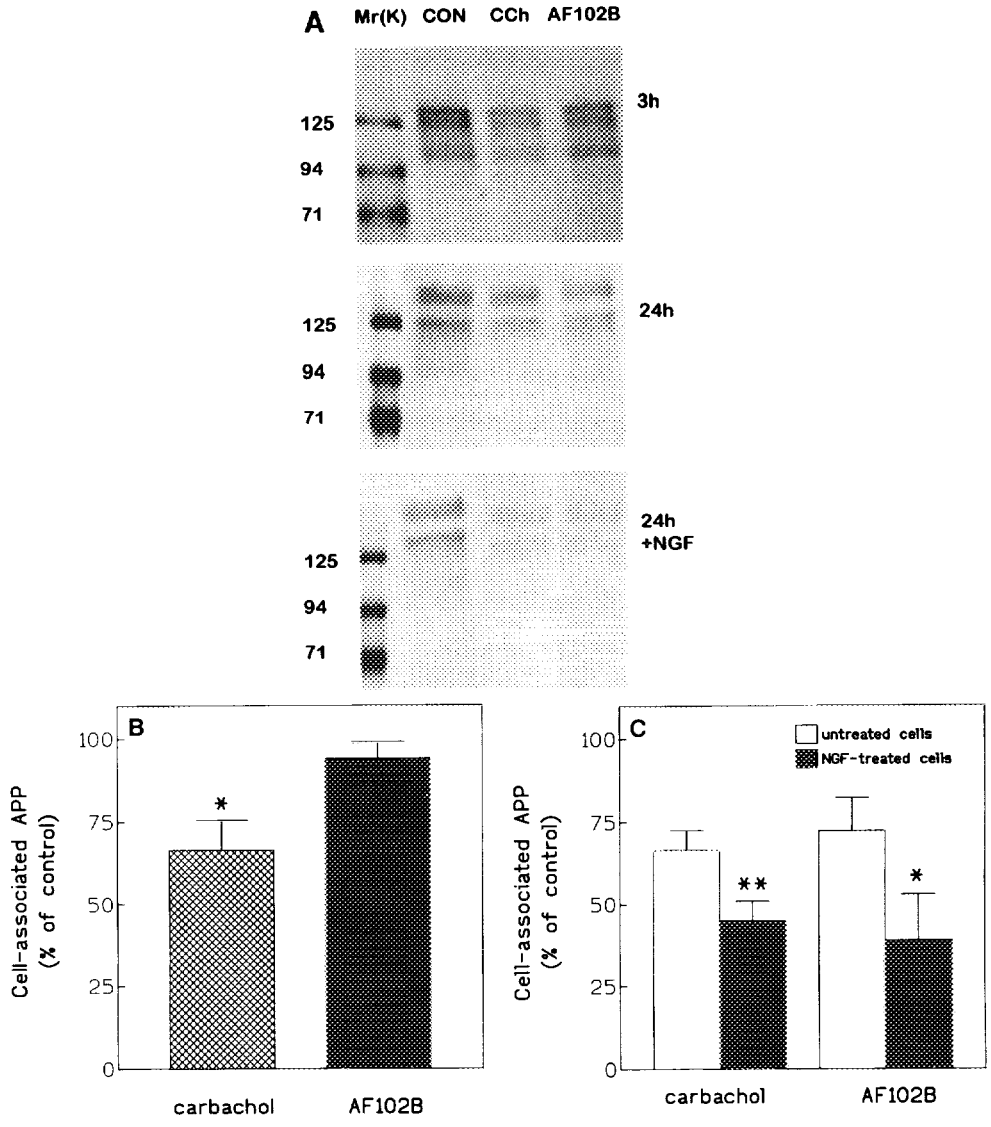

Figure 2. Cell-associated APP levels following 24 h incubations with carbachol or AF102B in control and NGF-treated PC12M1 cells.

A. Representative immonoblot. Cells were stimulated for 3 h (top panel) or for 24 h (middle and bottom panels) in complete medium with the indicated additions (both at 100 μM). NGF (50 ng/ml) was added 2 days earlier and was present for the entire 3 day period, including the stimulation period with the muscarinic agonists. Note that decreased membrane-associated APP is evident following 24 h (but not 3 h) with AF102B.

B. Video densitometry data for a 1 h stimulation period (mean±SEM for 3 independent experiments; carbachol and AF102B, 100 μM). Two tailed Student's t-test indicated that the reduction from basal levels was significant for 1 h stimulation with carbachol (*$p<0.05$), but not for AF102B.

C. Video densitometry data for a 24 h stimulation period (mean±SEM for 7 independent experiments; carbachol and AF102B, 100 μM). NGF (50 ng/ml) was added 2 days earlier and was present for the entire 3 day period. Two tailed Student's t-test indicated that the reduction from basal levels was significant for all combinations ($p<0.05$). In addition, NGF treatment resulted in enhanced reduction, compared with control cells, in membrane-associated APP levels following carbachol (**$p<0.01$) or AF102B (*$p<0.05$) stimulations.

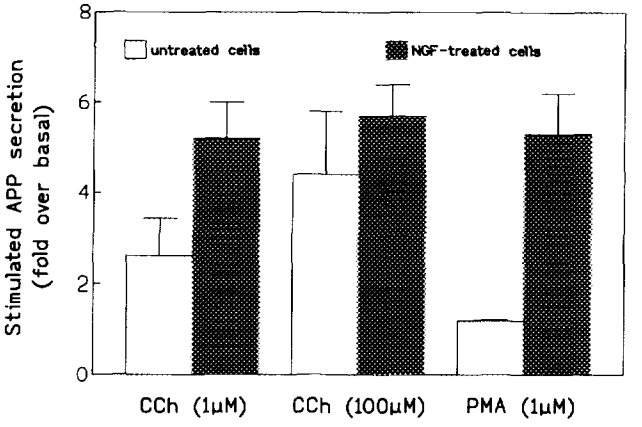

Figure 3. NGF enhances both carbachol and PMA-induced APP secretion. Video densitometry data for APP secretion following 1 h incubations with the indicated concentrations of carbachol or phorbol 12-myristate 13-acetate (PMA) in control or NGF-treated PC12M1 cells. Data are mean±SEM for triplicate plates from a representative experiment (similar observations were made in a second experiment). NGF (50 ng/ml) was added 3 days earlier in complete medium and was not present during the 1 h stimulation period.

This suggests that the m1AChR-stimulated APP secretion is not mediated exclusively via PKC, and may involve other pathway(s), e.g., elevated cytoplasmic calcium ion levels (9). These observations may also indicate that the other pathway(s) are probably less affected by NGF treatment, compared with the PKC pathway.

To address this issue, we studied carbachol and PMA-mediated APP secretion following down-regulation of PKC by a prolonged incubation with PMA (13). Following such treatment only PMA-mediated APP secretion was reduced (Fig. 4). Thus, the role played by PKC in m1AChR-mediated APP secretion may be relatively minor. Notably, m1AChR-stimulated arachidonic acid release (which reflects activation of phospholipase A_2) is markedly reduced following PKC down-regulation (10). Therefore, phospholipase A_2 may not be crucial for m1AChR-mediated APP secretion, in contrast with earlier suggestions (23).

AF102B and other M1-selective muscarinic agonists are being developed for cholinergic replacement therapy in Alzheimer's disease patients (7-10). Our new findings on NGF-induced augmentation of m1AChR-mediated APP processing imply that M1-selective muscarinic agonists, such as AF102B, may be capable of reducing cell-associated APP levels in concert with NGF, whose availability and/or function may be limiting in this disease (24). Possibly, such agonists may also act in concert with other neurotrophines which share signaling events with NGF. Muscarinic agonists were recently shown to exhibit NGF-dependent neurotrophic actions for cultured rat cerebellar Purkinje cells (25) and for PC12M1 cells (26). Lindner *et al.* reported on reduced cortical mRNAs levels for both NGF and APP in

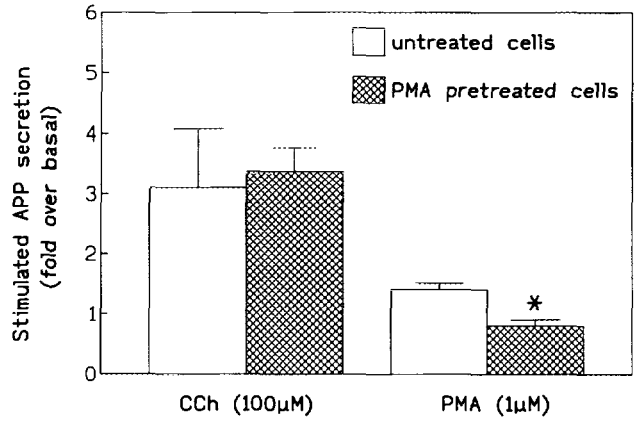

Figure 4. Down-regulation of PKC by PMA attenuates PMA- but not carbachol-induced APP secretion.
Video densitometry data for APP secretion following 1 h incubations of PC12M1 cells with the indicated concentrations of carbachol or PMA. Down-regulation of PKC was performed by incubating the cells for 18 h with 1 μM PMA, followed by extensive washout. Data are mean±SEM for triplicate plates from a representative experiment (similar observations were made in two additional experiments). Two tailed Student's t-test indicated that following 18 h PMA, carbachol-stimulated APP secretion was unchanged, while PMA-stimulated APP secretion was reduced (*$p<0.05$).

aged, cognition-deficient rats (27). Studies employing hippocampal explant cultures (28) indicated that both membrane-associated and secreted APP levels were elevated following NGF treatment, and were involved in the neurotrophic action of NGF in these explants. The possibilities for involvement of altered APP processing in these neurotrophic events and in AD are intriguing, and should be examined in future studies.

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