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Cortico-hippocampal APP and NGF levels are dynamically altered by cholinergic muscarinic antagonist or M1 agonist treatment in normal mice

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Abstract

To determine whether altered cholinergic neurotransmission can modify the long-term secretion of amyloid precursor protein (APP), endogenous levels of APP and nerve growth factor (NGF), we administered a selective M1 muscarinic receptor agonist (RS86) or the muscarinic antagonist, atropine, for 7 days *in vivo* into young adult mice (C57BL/6j). The levels of NGF and total APP in the hippocampus, frontal cortex, striatum, parietal cortex and cerebrospinal fluid (CSF) were examined by ELISA and Western blot. We found that this repeated i.m. administration of M1 receptor agonist resulted in decreased total APP levels in the hippocampus, frontal cortex and parietal cortex, and increased secreted α-APPs levels in the CSF. M1 agonist treatment also resulted in decreased NGF levels in the hippocampus and CSF. These effects of the M1 muscarinic agonist could be blocked by atropine, which by itself elevated tissue levels of total APP. Interestingly, we found that the decrease of total APP in the hippocampus and striatum after M1 agonist treatment inversely correlated with the change in NGF levels. These data suggest that a sustained increased cholinergic, M1-mediated neurotransmission will enhance secretion of α-APPs in CSF and adaptively reduce the levels of total APP and NGF in the corticohippocampal regions of normal mice. The dynamic and adaptive regulation linking total APP and NGF levels in normal adult mice is relevant for understanding the pathophysiology of conditions with cholinergic and APP related pathologies, like Alzheimer's disease and Down's syndrome.

Introduction

Alzheimer's disease (AD) is a severe cognitive disorder associated with degenerative changes in the cholinergic system (Bartus *et al*., 1982; Coyle *et al*., 1983). The loss of cholinergic neurons in the basal forebrain contributes to the progressive memory loss in AD patients. One of the major neuropathological features of AD is extracellular amyloid-beta (Aβ) deposits in senile neuritic plaques. The major component of neuritic plaques is a 39–43 amino acid, Aβ peptide (Glenner *et al*., 1984; Masters *et al*., 1985), derived from the proteolytic cleavage of the membrane bound glycoprotein, amyloid precursor protein (APP) (Kang *et al*., 1987; Tanzi *et al*., 1987). Several alternative proteolytic mechanisms have been identified: in the secretory pathway (nonamyloidogenic pathway), cleavage of APP at position 16–17 or the Aβ segment generates a large soluble ectodomain of APP (α-APPs) and a 10-kDa membrane associated C-terminal fragment (Esch *et al*., 1990); in the amyloidogenic pathway, cleavage of APP at both the N-terminus (β-secretase) and C-terminus (γ-secretase) of the Aβ domain, yields the Aβ peptide which easily aggregate and form amyloid and β-APPs (Estus *et al*., 1992; Golde *et al*., 1992; Haass *et al*., 1992; Shoji *et al*., 1992; Haass *et al*., 1993).

A majority of reports demonstrate that increased α-APPs secretion is associated with a reduction in Aβ generation, suggesting a reciprocal regulation of processing between α-APPs and Aβ formation (Busciglio *et al*., 1993a; Busciglio *et al*., 1993b; Gabuzda *et al*., 1993). A number of biological functions for α-APPs have been reported such as neuroprotective and neurotrophic effects (Whitson *et al*., 1989; Mattson *et al*., 1993), neurite outgrowth (Saiton *et al*., 1989; Milward *et al*., 1992; Clarris *et al*., 1994), cell proliferation (Saiton *et al*., 1989), promotion of cell–substratum adhesion (Schubert *et al*., 1989; Breen *et al*., 1991; Chen & Yankner, 1991), and the prevention of intracellular calcium accumulation and cell death (Mattson *et al*., 1993). The mechanisms that regulate this pathway of APP processing and the physiological actions of α-APPs are not well understood.

Nitsch *et al*., (1992), found that the stimulation of phosphoinositide-linked M1 and M3 mAChR, stably transfected into human embryonic kidney cells, increases the α-APPs, which is controlled by protein kinase C (PKC)-coupled mAChR activation (Nitsch *et al*., 1992; Buxbaum *et al*., 1993; Hung *et al*., 1993; Slack *et al*., 1993; Nitsch & Growdon, 1994; Caputi *et al*., 1997). Elevated release of α-APPs by receptor stimulation is not affected by protein synthesis inhibitors, indicating that pre-existing APP is cleaved (Nitsch *et al*., 1998). However, increased APP processing by PKC activation is independent of direct APP phosphorylation, indicating that other proteins may be involved in APP processing (Hung & Selkoe, 1994).

Nerve growth factor (NGF) regulates the survival, differentiation, and phenotypic maintenance of cholinergic basal forebrain neurons and also neuronal plasticity after injury (Korsching *et al*., 1985; Whittemore *et al*., 1986; Whittemore *et al*., 1988; Conner *et al*., 1992). Nerve growth factor is highly expressed in major targets of the ascending projection from cholinergic basal forebrain neurons (Bigl *et al*., 1982; Mesulam *et al*., 1983) that transport (retrogradely) NGF

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from these areas to the cholinergic cell bodies of the basal forebrain (Seiler & Schwab, 1984). After cortical/hippocampal release, NGF mediates its actions on cholinergic basal forebrain neurons via the low-affinity $p75^{NTR}$ and the NGF-specific TrkA (tyrosine receptor kinase A) receptor (Bickel & Kewitz, 1990; Lauterborn *et al*., 1991; Holtzman *et al*., 1992; Gibs & Pfaff, 1994).

NGF treatment *in vitro* increases total APP mRNA levels, APP expression levels, and secretion of α-APPs (Smith *et al*., 1991; Fukuyama *et al*., 1993). Exogenous NGF treatment increases choline acetyltransferase (ChAT) activity and choline uptake activities, enhances acetylcholine synthesis and also basal and depolarization-induced acetylcholine release in adult rat (Rylett *et al*., 1993). Conversely, there are several data suggesting a cholinergic mechanism in the regulation of cortical/hippocampal NGF synthesis and release (Yu *et al*., 1995; Yu *et al*., 1996; Rossner *et al*., 1997). Recent studies provide a better understanding of the cholinergic effects and adaptive regulation of NGF levels and APP in postsynaptic target areas of the hippocampus and cortical cortex (Isacson *et al*. 2002).

Curiously, in AD, hippocampal NGF levels have variably been shown to be either increased or not significantly changed (Goedert *et al*., 1986; Crutcher *et al*., 1993; Mufson *et al*., 1995; Fahnestock *et al*., 1996). This is explained by a disease progression dependent response at later stages of cholinergic synapse loss. At some AD-stages and ageing stages, there is potentially a reduced retrograde transport of NGF leading to accumulation of NGF in the hippocampal tissue (Cooper *et al*., 1994; Mufson *et al*., 1995; Cooper *et al*. 2001). To determine whether increased or decreased cholinergic muscarinic function can modify α-APPs secretion and the levels of APP and NGF *in vivo*, we examined the effects of chronic treatment with an M1 muscarinic agonist, RS86, compared with a general muscarinic antagonist, atropine, in the hippocampus, frontal cortex, striatum, parietal cortex brain regions and CSF of normal mice.

Materials and methods

All experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC), McLean Hospital, guidelines.

Experimental design

Experiment 1

A total of 16 young adult female C57BL/6J mice (18–21 g, Jackson Laboratories, Bar Harbor, ME) were treated with the selective M1 agonist, RS86, for 1 week. A total of 16 adult female C57BL/6J mice were treated with saline as a control group.

Experiment 2

Normal adult female C57BL/6J mice were divided into four balanced groups for pretreatment with a muscarinic receptor antagonist, atropine, or vehicle before treatment with the M1 agonist; saline plus M1 agonist (n = 16), atropine plus M1 agonist (n = 16), atropine (n = 16), saline (n = 16). After 7 days of drug treatment, CSF was collected and mice were killed for brain dissection.

Muscarinic receptor agonist treatment

RS86 (gift from Novartis, Switzerland) is a selective muscarinic M1 receptor agonist (Palacios *et al*., 1986; Wanibuchi *et al*., 1990; Rupniak *et al*., 1992). Based on our previous studies (Lin *et al*., 1999), animals in all experiments reported here were injected twice daily with the following doses: 1.5 mg/kg i.m. at approximately 09.00 h and 2.0 mg/kg i.m. 8 h later, for seven consecutive days. Control animals received an equal volume of saline (i.m.) at the same time intervals as the M1 agonist-treated groups. All injections were performed at the same time points every day throughout the experiments.

Muscarinic receptor antagonist treatment

Animals were preinjected with the muscarinic receptor antagonist, atropine (Sigma, St. Louis, MO), 20 min prior to RS86 injection (1.5 mg/kg, i.m. at 09.00 h and 2 mg/kg, i.m. 8 h later), twice daily with the following doses: 8 mg/kg s.c. at approximately 08.40 h and 12 mg/kg s.c. 8 h later, for seven consecutive days (modified from Lin *et al*., 1999). Control animals received an equal volume of saline (i.m.) at the same time intervals as the antagonist and agonist-treated groups. All injections were performed at the same time points every day throughout the experiments.

Post-mortem procedures

Immediately prior to being killed, mice from all groups were placed in a mouse stereotaxic apparatus under anaesthesia with sodium pentobarbital (50 mg/kg i.p., Sigma Chemicals, St Louis, MO). The occipital foramen was exposed and CSF was collected from the cisterna magna with a 20-μL pipette with a long flexible tip. The collected CSF (~20 μL) was frozen on dry ice and stored at –70 °C for use in Western blot and NGF ELISA assays. The brains were then removed and dissected on ice. Tissue samples from the hippocampus, frontal cortex, striatum and parietal cortex were taken for Western blot and NGF ELISA assays.

Western blots for APP protein detection

The antibody, 22C11 (Boehringer Mannheim, Indianapolis, IN), raised against an N-terminal epitope of APP was used to determine the total APP in protein extracts obtained from the brain tissues and the α-APPs level in the CSF (Lin *et al*., 1999). In some cases (see Results), a monoclonal antibody, 'Jonas', directed against residues 643–695 in the C-terminus of APP (Boehringer Mannheim, Indianapolis, IN), was used to demonstrate the absence of C-terminus in α-APPs in CSF. The tissue was homogenized using a hand-held homogenizer with cell lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 μg/mL Aprotinin, 25 μg/mL Leupeptin, 10 μg/mL Pepstatin, 1 mM PMSF; all protease inhibitors purchased from Sigma Chemicals, St Louis, MO) and then sonicated. Homogenates were centrifuged at 14 000 × g for 30 min at 4 °C. The supernatant was collected and aliquots were stored at –70 °C. The protein levels were determined using Bio-Rad Protein Assay (BIO-RAD, Hercules, CA). Samples containing equal amounts of total protein were boiled with SDS sample buffer and electrophoresed on 10% SDS–polyacrylamide gels. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (BIO-RAD, Hercules, CA). Membranes were blocked with 2.5% nonfat dried milk in 0.05 M Tris-buffered saline (pH 7.4) with 0.1% Tween 20 (TBS-T) and then incubated with the 22C11 antibody (1 : 500) in 1% nonfat dried milk in TBST overnight at 4 °C. After the incubation with the secondary horseradish peroxidase (HRP)-linked anti-mouse IgG antibody (dilution 1 : 6000, Jackson Laboratory, Bar Harbor, ME) in 0.25% nonfat dried milk in TBST, the membranes were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) using Kodak X-omat films (Kodak, Rochester, NY).

Enzyme linked immunosorbent assay (ELISA) for NGF

Nerve growth factor levels in several tissues and the CSF were determined using NGF EmaxTM Immunoassay System [Promega,

TABLE 1. Total APP and NGF levels: the effects of 7-days treatment of RS86, atropine, and RS86 plus atropine in normal mice

	Treatment					
	RS86 (M1 agonist)		Atropine		RS86 + atropine	
	Total APP	NGF	Total APP	NGF	Total APP	NGF
Regions						
Hippocampus	84 ± 5.1*	54 ± 6.4*	202 ± 11.1**	92 ± 5.4	144 ± 16.7*†	101 ± 4.1
Frontal Cortex	82 ± 5.8*	109 ± 15.5	129 ± 2.5**	117 ± 15	127 ± 5.1	110 ± 6.9
Striatum	115 ± 7.2	107 ± 6.8	93 ± 16.1	86 ± 13	116 ± 6.6	88 ± 17
Parietal Cortex	91 ± 3.1*	88.5 ± 3.5	119 ± 11.5**	104 ± 13	73 ± 3.3**†	93 ± 15

Data are presented as mean ± SEM APP OD values in different region of the brain and are expressed as percentage of APP and NGF levels in normal, vehicle-treated (saline) control group. *P-value ⩽0.05; **P-value < 0.001;
†P-value < 0.05

Madison, WI (Zettler *et al*., 1996)]. Briefly, a polystyrene coated ELISA plate was incubated overnight at 4 °C with the polyclonal anti-NGF antibody (1 : 1000 dilution). Non-specific binding was blocked by incubating the plate with serum albumin for 1 h at room temperature and washed with 0.1% TBS-T once. A standard curve was created from serial dilutions of known concentrations of NGF. Samples were dispensed into the remaining wells and the plate was incubated for 6 h at room temperature. The plate was then washed with 0.1% TBS-T five times, followed by incubation with the monoclonal anti-NGF antibody (1 : 4000 dilution) overnight. After rinsing the plate five times, samples were incubated in the HRP conjugated tertiary anti-rat antibody (1 : 2000 dilution) solution for 2.5 h at room temperature. After rinsing the plate five times, a TMB solution/peroxidase substrate mixture was added to the wells. The reaction was allowed to proceed for 10 min and stopped with 1 M phosphoric acid. The ELISA plate was then read using a Labsystem Multiscan Plus plate reader set at 450 nm wavelength.

Densitometric analysis

Quantification of the APP immunoreactive bands was performed using densitometry. Films of the Western blots were scanned (Scanner UMAX ASTRA 1200S) using Adobe Photoshop (version 5.5, Adobe Systems) and the optical density (OD) of the APP bands was measured using NIH Image (Version 1.61). The relative APP values were calculated by subtracting the background OD-value from the measured OD of the APP bands. The results were confirmed by duplicate measurements of the same sample.

Statistical analysis

All statistical analyses were carried out using JMP (version 3.1.6, SAS Institute). Data from Western blots and ELISAs were compared between different groups for each brain region with an unpaired student's *t*-test and differences between groups were considered statistically significant when $P < 0.05$. Regression analyses were performed with a linear fit for two independent variables (JMP, version 3.1.6, SAS Institute).

Results

Effects of a selective M1 muscarinic receptor agonist (RS86) on the levels of APP and NGF

To determine the effects of a selective M1 muscarinic receptor agonist on the levels and secretory processing of APP and NGF, a group of young normal mice was treated with the muscarinic receptor agonist (RS86, 3.5 mg/kg per day, i.m. for 7 days). Western blot analysis revealed that total APP levels were significantly reduced to 84% ($P = 0.04$) in the hippocampus, 82% ($P = 0.02$) in the frontal cortex, and 91% ($P = 0.03$) in the parietal cortex of normal mice treated with M1 agonist compared to those treated with vehicle (saline) (Table 1). No such change was found in the striatum (Table 1). The levels of α-APPs in the CSF in animals treated with vehicle or M1 muscarinic receptor agonist were detected using Western blot analysis with the 22C11 antibody. The levels of α-APPs in the CSF in animals treated with M1 agonist were significantly increased to 180% ($P = 0.01$) compared to α-APPs levels in the CSF in vehicle treated control animals (Fig. 1). In order to confirm that the α-APPs detected with the 22C11 antibody in the CSF was the secreted N-terminal derivative of APP, a C-terminal (643–695) specific antibody 'Jonas' was used to examine full-length APP levels in the CSF. No obvious immunoreactive bands were found in the CSF with this antibody [data not shown, and (Lin *et al*., 1999)], demonstrating that α-APPs in the CSF lacks the C-terminus, as described previously (Palmert *et al*., 1989).

In the same group of normal mice treated with the M1 muscarinic agonist, ELISA analysis revealed that NGF protein levels were reduced to 54% ($P = 0.02$) in hippocampus (Table 1) and 73% ($P = 0.04$) in CSF (Fig. 1) compared to levels seen in vehicle-treated controls. In this experiment, NGF protein levels in the hippocampus of normal mice was approximately 8 ng/g of wet tissue for the value of 100% in hippocampus in Table 1. No significant changes in NGF levels were found in the frontal cortex, parietal cortex or striatum in any of three experimental groups (Table 1).

Effects of a muscarinic receptor antagonist (atropine) on the levels and secretion of APP and NGF in normal mice

To investigate the effects of a broad muscarinic receptor antagonist on total APP and NGF levels, atropine was given to normal mice (20 mg/kg per day, s.c. for 7 days, Lin *et al*., 1999). Following atropine treatment, total APP levels were significantly increased to 202% ($P < 0.0001$) in the hippocampus, 129% ($P < 0.0001$) in the frontal cortex, and 119% ($P < 0.001$) in the parietal cortex compared to vehicle-treated controls (Table 1). No such changes in total APP levels were found in the striatum (Table 1). The levels of α-APPs in the CSF were decreased to 38% ($P = 0.05$) of control (Fig. 1) by atropine treatment. Atropine treatment did not significantly alter NGF protein levels in any of the four brain tissue regions or CSF (Fig. 1).

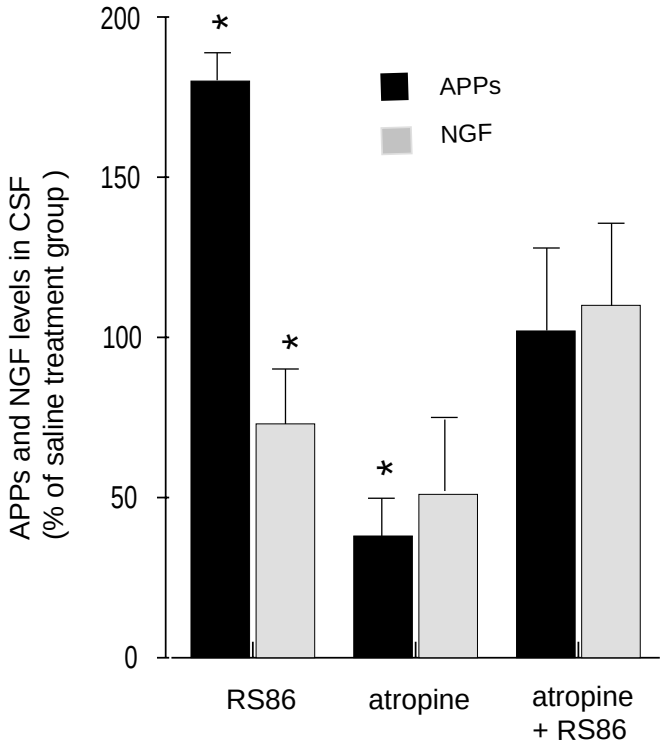

FIG. 1. The effect of RS86, atropine and atropine plus RS86 on α-APPs and NGF levels in the CSF of normal mice. The treatment with RS86 increased α-APPs level in the CSF to 180% of control ($P = 0.01$) and decreased NGF levels to 73% of control ($P = 0.04$) in the CSF. Atropine treatment decreased α-APPs levels by 38% ($P = 0.05$) compared to α-APPs levels in the saline treated group. When normal mice were treated with atropine plus RS86, changes in α-APPs levels were not detectable. NGF levels in the CSF were not significantly changed by either atropine treatment or atropine plus RS86 treatment.

Atropine blocks the effect of the M1 muscarinic receptor agonist (RS86)

To further study the interactions of muscarinic activation or blockade on total APP and NGF levels, normal mice were treated concomitantly with atropine and RS86. Comparing atropine plus M1 agonist treatment to saline plus RS86 treatment showed that atropine significantly reversed the reduction of total APP levels in hippocampus caused by M1 agonist (Table 1). The change in total APP levels by treatment with M1 agonist plus atropine was also significant when compared to atropine treatment alone. In the frontal cortex and striatum, no significant changes in total APP levels were found between the atropine plus M1 agonist and saline plus M1 agonist treatment groups (Table 1). Atropine plus M1 agonist treatment did not significantly change NGF levels in any of the four tissue regions or CSF (Table 1 and Fig. 1).

Relative changes of APP and NGF levels by muscarinic agonist treatment in individual mice

To determine whether the effects of the selective M1 agonist on total APP and NGF levels were interrelated in individual animals, we did a regression analysis on the data for total APP and NGF levels. In the hippocampus of normal mice treated with the M1 agonist, the percentage decrease in total APP levels was inversely related to the

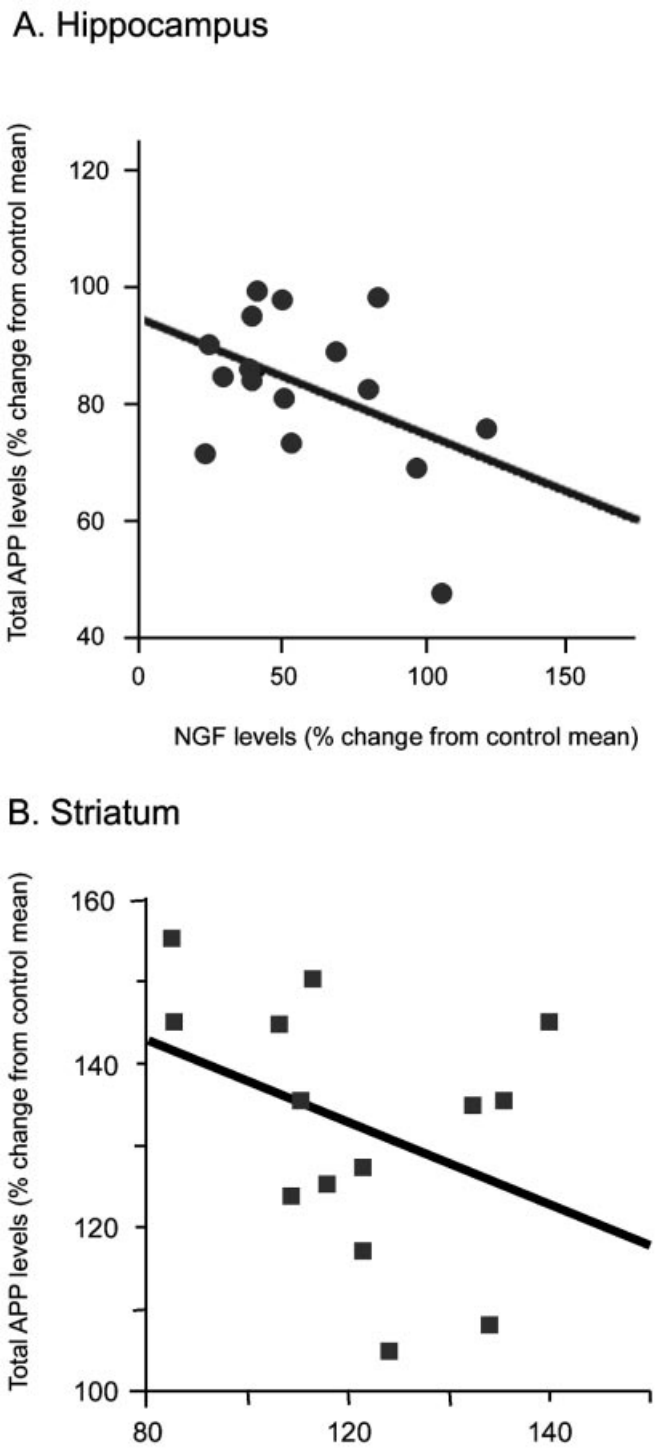

FIG. 2. Correlation of the changes in total APP levels and NGF levels by M1 muscarinic agonist (RS86) treatment in the hippocampus from individual mice (regression analysis: $n = 16$, $P = 0.01$ in the hippocampus; $n = 14$, $P < 0.05$ in the striatum). The changes in total APP levels were inversely related to the changes in NGF levels in the hippocampus and striatum of normal mice treated with an M1 agonist. The total APP and NGF values are indicated as percentage decrease from control mean value.

percentage decrease in NGF levels (Fig. 2A, regression analysis: $n = 16$; $P = 0.01$), suggesting a stable relationship between the regulation of APP and NGF levels by M1 muscarinic receptor

stimulation. These changes were also observed in the striatum (Fig. 2B, regression analysis: $n = 14$; $P < 0.05$), but not in the parietal or frontal cortex of the same animal. We could not detect a correlation between the levels of α-APPs and NGF in the CSF.

Discussion

Muscarinic receptor agonist or antagonist can modify APP levels and APP secretion

The administration of a muscarinic receptor agonist resulted in significant decreases in total APP levels in the hippocampus and frontal cortex with parallel increases in α-APPs levels in the CSF, indicating that treatment with M1 receptor agonist enhances the α-secretase pathway of APP in normal adult mice. This finding is consistent with previous reports in normal adult rats and rats with selective degeneration of cholinergic basal forebrain neurons (Farber *et al*., 1995; Lin *et al*., 1999). Although we couldn't determine Aβ levels directly in these mice, α-APPs is closely and inversely related to the formation of the Aβ peptide and this data may be relevant for the understanding of amyloidogenic processing of APP (Lin *et al*., 1999).

Other groups have reported effects of other selective M1 agonists on APP expression and processing *in vitro*. M1 agonists increase α-APPs release from PC12 cells (Buxbaum *et al*., 1992; Lee *et al*., 1995), 293 cells, hippocampal slices and M1 transfected CHO cells (Muller *et al*., 1997; Muller *et al*., 1998). Selective M1 agonists have been reported to decrease Aβ levels in CHO cells (Wolf *et al*., 1995; DeLapp *et al*., 1998). Recently, it was reported that total Aβ is decreased in the CSF of Alzheimer's disease (AD) patients after chronic M1 agonist treatment (Hock *et al*. 2000; Nitsch *et al*. 2000). In our study, treatment with a muscarinic antagonist (atropine) resulted in significant increases in total APP levels in the hippocampus and frontal cortex as well as decreases in α-APPs levels in the CSF. A combined treatment with atropine and the M1 agonist significantly reversed the APP changes induced by either the M1 agonist or atropine alone in these normal mice (Table 1 and Fig. 1). However, the effect of atropine was not fully reversed by the M1 agonist, possibly due to differences in ligand specificity for subtypes of muscarinic receptors; as RS86 is a selective M1 agonist and atropine is a general muscarinic antagonist (M1–M5) (Palacios *et al*., 1986; Wanibuchi *et al*., 1990; Rupniak *et al*., 1992). This interpretation is supported by our CSF data, as in the atropine plus M1 agonist treatment group there were no changes in α-APPs levels, suggesting that the opposing effects of these cholinergic agents cancelled each other out. Consistent with our results, Beach *et al*., (1996) found that atropine treatment increased the levels of APP mRNA in rat cerebral cortex, dentate gyrus and CA1 region. Similarly, a series of experiments in brain slice cultures indicated that atropine treatment decreases α-APPs levels (Muller *et al*., 1997). Thus, it appears that the expression, proteolytic processing and/or release of APP can be regulated by muscarinic receptor stimulation or blockade.

Nerve growth factor levels are reduced by chronic M1 muscarinic receptor agonist treatment in normal mice

During early postnatal development, it has been reported that cholinergic activity in basal forebrain neurons may initially stimulate NGF synthesis in appropriate target areas (Whittemore *et al*., 1988; dePenha-Berxahi *et al*., 1993). However, in the mature CNS, NGF effects are primarily seen on phenotypic maintenance of cholinergic basal forebrain neurons and also neuronal plasticity after injury (Korsching *et al*., 1985; Whittemore *et al*., 1986; Whittemore *et al*., 1988; Sofroniew, 1991; Conner *et al*., 1992; Sofroniew *et al*. 2001). In this study of adult mice, administration of M1 agonist decreased the levels of NGF in the hippocampus and CSF. Importantly, these changes in NGF protein levels observed after chronic 7-days treatment (long-term) with an M1 agonist do not mirror the NGF mRNA or BDNF (brain-derived neurotrophic factor) changes seen after singular acute administrations (dePenha-Berxahi *et al*., 1993; Lapchak, 1993; French *et al*., 1999), with effects seen within hours (short-term), of antagonist, muscarinic agonist or acetylcholine modifying drugs (Knipper *et al*., 1994; French *et al*., 1999). The most likely explanation is that long-term M1 agonist delivery artificially and chronically overstimulates the postsynaptic cholinergic receptors thereby causing adaptive synaptic changes and compensatory decreased NGF levels. Short-term agonist treatment may elicit a neutral or positive trophic response while long-term (7 day) overactivation induces corrective reduced trophic signals in the septohippocampal cholinergic circuitry.

Recent data showing LTP (long-term potentiation) dependence on NGF, NGF/TrkA and the muscarinic cholinergic system is consistent with the interpretation of our APP and NGF findings as adaptive responses of the corticohippocampal cholinergic synaptic system (Pesavento *et al*. 2000; Isacson *et al*. 2002).

Interaction between the cholinergic system, APP and NGF: does neurotransmission regulate trophic homeostasis?

In the present study, the percentage decrease in the total APP levels were inversely related to the change in NGF levels in the hippocampus and striatum of the normal mouse suggesting a homeostatic feedback regulation of these proteins (Fig. 2). Interestingly, α-APPs levels were increased in the CSF after cholinergic activation, which may indicate a lack of receptor complex binding of α-APPs in the hippocampal system, consistent with decreased neurotrophic tone. One possible postulate from these findings is the existence of an equilibrium of NGF and APP levels, normally regulated by the cholinergic synaptic complex. Generally, NGF levels in the hippocampus are dependent both on expression and release of NGF by hippocampal neurons in the dentate gyrus, CA1–4 regions. For example, blockade of transport in dysfunctional cholinergic neurons leads to markedly increased NGF protein levels in the hippocampus in ageing, AD and possibly Down's syndrome (Crutcher *et al*., 1993; Mufson *et al*., 1995; Fahnestock *et al*., 1996; Calamandrei *et al*. 2000). Thus, in our study, the chronic over-stimulation of the M1 muscarinic receptor was negatively regulated by a compensatory reduction in trophic signals (NGF and α-APPs) to cholinergic afferent terminals in hippocampal and cortical fields. Conversely, atropine mediated cholinergic receptor blockade for 7 days elicited a positive compensatory trophic stimulation by a combined increase in total APP plus NGF levels (Table 1). Indeed, in the atropine treated group, the combined total APP plus NGF protein levels of the hippocampus showed a twofold increase compared to those levels in control group. One possible interpretation of this elevation within the tissue is an increased α-APPs binding and internalization by cholinergic hippocampal terminals as an adapted positive trophic response (Mufson *et al*., 1995).

It has been reported that *in vitro*, NGF initially promotes α-APPs secretion by M1 agonist treatment in M1 receptor transfected PC12 cell lines (Haring *et al*., 1995). After exposure to NGF, primary cortical neuronal cultures showed increased levels of membrane phospholipids that may promote neurite formation, APP expression and secretion of α-APPs (Wang *et al*. 2000). Acutely, in a growing cell culture system, APP gene expression is up-regulated by NGF by

transactivating its promoter as shown in transient transfection experiments (Lahiri & Nall, 1995). Treatment of PC12 cells with NGF also caused an increase in the steady-state levels of endogenous presenilin-1 (PS1) (Counts *et al*. 2001), which is required for γ-secretase activity to produce α-APPs. During trophic factor withdrawal-induced death of PC12 cells, APP expression was increased (Araki & Wurtman, 1998). In agreement with the interpretation of our *in vivo* data, α-APPs synergistically potentiates the tyrosine phosphorylation of TrkA and modulates the interaction of NGF with the $p75^{NTR}$ receptor (Akar & Wallace, 1998). Conversely, the activation of TrkA (high-affinity receptor for NGF) initially results in increased α-APPs in PC12 cell lines (Rossner *et al*., 1999). Mobley *et al*., (1988) observed an acute increase in APP mRNA levels in the developing hamster brain after NGF administration. However, others have shown that NGF administration has no effect on APP mRNA or APP expression levels in the rat brain (Forloni *et al*., 1993; Neve *et al*., 1996).

Muscarinic M1 stimulation could induce the expression of APP and/or secretion of α-APPs through the protein kinase C (PKC)-independent pathway activated by the NGF receptor. The retrograde transport of NGF is mediated by $p75^{NTR}$ and plays a role in the recruitment of NGF and its presentation to high-affinity receptors that modulate Trk signalling (Hantzopoulos *et al*., 1994; Verdi *et al*., 1994; MacPhee & Barker, 1997). Stimulation of the $p75^{NTR}$ induces expression of APP mRNA through activation of sphingomyelinase and via subsequent generation of ceramide and activation of NFκB (MacPhee & Barker, 1997). Conversely, NGF binding to TrkA leads to suppression of APP mRNA expression through p21Ras activated signalling pathways involving MAP kinases and phosphatidylinositol 3-kinase. Secretory processing of APP is activated via a phospholipase Cγ (PLCγ) transduction cascade (Mills *et al*., 1997; Rossner *et al*., 1998).

The regulation of APP and NGF protein levels in the corticohippocampal system: relevance to Alzheimer's disease

α-APPs and NGF have important functions not only in trophic mechanisms but also neural transmission. α-APPs modulate neuronal excitability, counteract the effects of glutamate on growth cone behaviour and increase synaptic complexity (Mattson & Furukawa, 1998; Rossner *et al*., 1998) illustrating the functions of α-APPs as a trophic and modulatory factor. Nerve growth factor regulates the survival, differentiation and phenotypic maintenance of cholinergic basal forebrain neurons and also plays a role in neuronal plasticity after injury. Nerve growth factor treatment resulted in a drastic reduction in synaptophysin release in PC12 cells (Marquez-Sterling *et al*., 1997; Chapman *et al*., 1999). In APP transgenic mice, mutant APP causes age-dependent learning impairment through disruption of synaptic plasticity (Chapman *et al*., 1999). As learning and memory are thought to depend on changes in synaptic efficacy in certain key brain structures, including the hippocampus (Bliss & Collingridge, 1993; Chapman *et al*., 1999), better understanding of the interaction of trophic factors, like α-APPs and NGF, with afferent hippocampal synapses will be of importance.

The alterations of APP expression, processing and secretion are closely related to the production of the Aβ peptide, which is the principal constituent of senile plaques and cerebrovascular deposits in AD (Mattson & Furukawa, 1998; Rossner *et al*., 1998). Recently, it has been reported that aged anti-NGF transgenic mice display pathological symptoms similar to AD, including amyloid plaques, insoluble and hyperphosphorylated tau, neurofibrillary tangles in cortical and hippocampal neurons, neuronal loss, cholinergic deficits and behavioural deficits (Capsoni *et al*. 2000). Alzheimer's disease patients show less TrkA NGF receptor mRNA levels than normal control subjects (Mufson *et al*., 1996), reduced TrkA protein levels (Mufson *et al*., 1997; Salehi *et al*., 1998), no change in NGF mRNA levels (Massaro *et al*., 1994; Hock *et al*., 1998) and an increase in hippocampal NGF protein levels (Crutcher *et al*., 1993; Fahnestock *et al*., 1996).

Cortical cholinergic activation may also alter APP processing in favour of the generation of neurotrophic α-APPs and decreased production of amyloidogenic APP fragments. Recently, it was reported that M1 agonist treatment decreased total Aβ levels in the CSF of AD patients (Hock *et al*. 2000; Nitsch *et al*. 2000). The observed reduction in total APP levels in the normal hippocampus, increase in α-APPs levels in the CSF and reduction in NGF levels in the hippocampus and CSF after muscarinic agonist treatment demonstrates the normal regulation and interdependence of these factors. This may be relevant for our understanding of the appropriate physiological function of ACh, APP and NGF and their abnormal regulation and levels seen in AD and Down's syndrome.

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Abbreviations

α-APPs, secreted form of amyloid precursor protein; Aβ, amyloid β; AD, Alzheimer's disease; APP, amyloid precursor protein; CSF, cerebrospinal fluid; ELISA, enzyme linked immunosorbent assay; LTP, long-term potentiation; NGF, nerve growth factor, TrkA, tyrosine receptor kinase A.

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