Regulation of Biochemical Pathways Involved in Neurodegeneration

by

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by cognitive decline and memory loss. Although much is known about how AD affects the brain, the cause of this disease remains elusive. Current AD treatments target symptoms of the disease but do not prevent or slow the underlying neurodegeneration. Therefore, research into the biochemical mechanisms of AD is necessary in order to develop a better understanding of how to treat it.

Misprocessing of the amyloid precursor protein (APP) in the brains of AD patients leads to accumulation of the amyloidogenic peptide Aβ. A soluble APP fragment (APP_s) is formed when APP is cleaved within the Aβ region, thereby preventing Aβ formation. Activation of 5-HT_{2A} or 5-HT_{2C} receptors has been shown to increase APP_s secretion *in vitro*; therefore, we determined whether activation of these receptors might have a similar effect *in vivo*. We found that a 5-$HT_{2A/2C}$ agonist affected brain APP metabolism in guinea pigs by increasing CSF levels of APP_s and, following chronic treatment, by decreasing levels of Aβ. Our data indicate that activation of brain 5-HT_{2C} receptors may be useful for treating AD by reducing Aβ production.

Traumatic brain injury is a risk factor for AD, although the reason is unknown. To explore this relationship, we examined the effect of the inflammatory mediator PGE_2 on production of APP in cultured microglia. We found that PGE_2 treatment stimulated APP overexpression and that this effect was likely mediated by the prostaglandin EP2 receptor and the cAMP signaling cascade. Therefore, EP2 receptor antagonists may constitute an additional target for prevention of AD following brain injury.

The neuropathology associated with AD includes neuritic dystrophy and degeneration. Therefore, restoration of neuritic growth and repair of phospolipid membranes may be important for treating AD. We found that treatment of NGF-differentiated PC12 cells with the phospholipid precursor uridine enhanced neurite outgrowth by both enhancing phosphatide biosynthesis and by stimulating a G-protein receptor-coupled signaling pathway. Subsequently, we found that the HMG-CoA reductase inhibitor pravastatin enhanced neurite outgrowth in rat hippocampal neurons, not by affecting cholesterol synthesis, but by inhibition of isoprenoid formation. Stimulation of neurite growth by either uridine or statins may reduce AD risk by averting neuritic dystrophy and degeneration. However, further studies must be conducted to determine whether they are able to affect neuritic processes *in vivo*.

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Finally, I dedicate this thesis to my late father Edwin, whose encouragement and love were, and are, invaluable to me.

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Chapter 1:

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by cognitive decline and memory loss. The major neuropathological hallmarks of AD are neurofibrillary tangles and extracellular amyloid plaques. Amyloid β (Aβ), the principal component of such plaques, is derived by proteolytic cleavage of the amyloid precursor protein (APP) by the β- and γ-secretases. APP may alternatively undergo a single cleavage by the α secretase within the Aβ region, causing the formation of a soluble APP fragment and thereby preventing the formation of Aβ (Esch et al., 1990; Sisodia et al., 1990). Misprocessing of APP occurs early in the development of AD and is associated with activation of numerous biochemical pathways, many of which lead to neuronal degeneration and cell death (St George-Hyslop and Petit, 2005). Although Aβ plaques are a prominent feature of AD, both the underlying cause of plaque formation and its effect on cellular pathways remain elusive. Furthermore, it is not known whether plaque accumulation is the sole source of the memory deficits observed in AD patients, or whether additional causes exist. Therefore, potential treatments for preventing or ameliorating AD have many possible mechanisms of action. The set of studies presented in this thesis explore biochemical pathways that may be involved in AD with the goal of better understanding development of the disease and possible ways to inhibit its progression.

Alzheimer's disease is associated with dysregulation of APP expression and processing

Misprocessing of APP in the brains of AD patients causes widespread accumulation of Aβ-enriched plaques. Reducing production of brain Aβ prevents plaque formation, and concomitantly slows memory loss (Hock et al., 2003). Cleavage of full-length APP by the α-secretase prevents formation of Aβ and produces a non-amyloidogenic, soluble APP fragment (APP_s); therefore, treatments that increase activity of the α-secretase may be useful in preventing plaque accumulation. We determined whether cleavage of APP (and subsequent formation of Aβ and APP_s) can be regulated by serotonin receptors. We found that, in guinea pigs, stimulation of 5-$HT_{2A/2C}$ receptors by dexnorfenfluramine increased cerebrospinal fluid levels of APP_s, while levels of Aβ were decreased (Arjona et al., 2002; see Chapter 1). These data suggest that serotoninergic compounds like dexnorfenfluoramine that activate brain 5-HT_{2C} receptors may be useful for treating AD by increasing APP_s formation and decreasing production of Aβ.

Elevations in Aβ levels result not only from alterations in secretase activity, but also from overexpression of full-length APP. For example, traumatic brain injury, which is associated with APP overexpression in the brain, may be a major risk factor for developing AD (Basha et al., 2005). However, it is not known how brain injury leads to APP overexpression. To investigate this question, we incubated cultured rat microglia with the inflammatory mediator prostaglandin E2 (PGE_2), and found that PGE_2 treatment significantly increased levels of APP expression compared to levels in untreated cells. Since PGE_2 also enhanced levels of the second messanger cyclic AMP (cAMP), we

studied whether PGE_2 might affect APP via receptors coupled to the cAMP signaling pathway. We found that stimulation of cAMP-coupled EP2 receptors by a specific receptor agonist also increased APP expression, and that incubation of the cells with an EP2 receptor antagonist completely inhibited the APP overexpression caused by PGE_2. These findings suggest that, during brain inflammation, APP overexpression may occur due to activation of the cAMP signaling cascade by the binding of PGE_2 to the prostaglandin receptor EP2 (Pooler et al., 2004; see Chapter 2). In addition, our data support the hypothesis that glial cells may significantly contribute to brain amyloid accumulation and thus the development of AD.

Alzheimer's disease causes abnormal phospholipid metabolism and neuritic deterioration

Alzheimer's disease is associated with more than simply misprocessing of APP; brains of AD patients also display abnormal metabolism of membrane phospholipids. Alterations in the metabolism of choline-containing phospholipids have been detected in the cerebrospinal fluid of AD patients (Mulder et al., 2003). Moreover, cortices of AD patients have decreased levels of the phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine, compared with age-matched controls (Nitsch et al., 1992). PC synthesis is regulated by levels of its precursors (Savci and Wurtman, 1995; Araki and Wurtman, 1997); therefore, stimulation of PC synthesis by increasing precursor levels may, in part, prevent the disruption in normal phospholipid metabolism caused by AD. Furthermore, increasing cell membrane synthesis may have morphological consequences for the cell. Since dendritic atrophy and loss occur in mouse models of AD (Calon et al., 2004; Moolman et al., 2004) and dystrophic neurites are observed in human

cases of AD (McKee et al., 1991), treatments that increase neuritic growth may be useful for preventing progression of AD . In transgenic mice, it is possible to reverse neuritic dystrophy through infusion of anti-Aβ antibody (Brendza et al., 2005). Therefore, restoration of normal neuritic growth may be possible in humans, and might be efficacious in repairing the underlying neurodegeneration caused by AD.

To determine whether increasing levels of phospholipid precursors might affect cell morphology, we treated NGF-differentiated PC12 cells with the PC precursor uridine and quantified neurite outgrowth. We found that four-day uridine treatment significantly enhanced the number of neurites produced per cell, as well as the mean number of branches per neurite (Pooler et. al. 2005; see Chapter 3). The increase in neurite outgrowth was accompanied by a concomitant enhancement of neurite-enriched neurofilament proteins. Differentiated PC12 cells express pyrimidine-sensitive (UTP-sensitive) nucleotide receptors (P2Y). Therefore uridine, by increasing uridine triphosphate (UTP) formation, may also stimulate neuritogenesis by activating these receptors, in addition to enhancing phospholipid formation. The increase in neurite outgrowth by uridine was mimicked by exposing the cells to UTP, and could be blocked by various drugs known to antagonize P2Y receptors. Treatment of the cells with uridine or UTP stimulated their accumulation of inositol phosphates, and this effect was also blocked by a P2Y antagonist. Moreover, degradation of nucleotides by apyrase blocked the stimulatory effect of uridine on neurite outgrowth. Taken together these data indicate that uridine can regulate neuritogenesis in differentiating PC12 cells, and suggest that it does so in two ways, i.e., both by acting as a precursor for PC biosynthesis and, via conversion to UTP, as an agonist for P2Y receptors.

Epidemiological studies revealed a link between usage of cholesterol-lowering drugs known as statins and reduced risk of developing AD (Wolozin et al., 2000; Rockwood et al., 2002). Although this discovery led to speculation that elevated cholesterol levels may cause AD, further studies yielded mixed results, suggesting that reduction of cholesterol levels may or may not correlate with reduced risk of AD (Wood et al., 2003; Wolozin, 2004). Furthermore, statin usage is associated with a decreased risk of depression and anxiety; this effect is not correlated with plasma cholesterol levels (Young-Xu et al., 2003). Oral administration of statins, in addition to inhibiting cholesterol synthesis, also affects gene expression the mouse brain (Johnson-Anuna et al., 2005). Thus, statins may protect the brain from AD by a mechanism independent of their effect on cholesterol. In addition to inhibition of cholesterol synthesis, statins block mevalonate formation and subsquently prevent formation of isoprenoids such as farnesylpyrophosphate and geranylgeranylpyrophosphate. Statins inhibit isoprenylation of proteins, including the Rho family of small GTPases, in neuronal cells (Meske et al., 2003; Pedrini et al., 2005) and cultured microglia (Bi et al., 2004; Cordle and Landreth, 2005). RhoA is a monomeric G-protein that is negatively coupled to cell growth; prevention of RhoA isoprenylation increases neurite extension (Sebok et al., 1999). However, it is not known whether statins can affect neurite outgrowth, and whether such an effect might be mediated by regulation of Rho activity. We investigated whether the HMG-CoA reductase inihibitor pravastatin might affect neurite growth in primary cultured rat hippocampal neurons. We found that treatment of neurons with pravastatin enhanced neurite number, length and branching, and that this effect is likely mediated by inhibition of mevalonate synthesis and subsequent inhibition of protein isoprenylation

(Pooler et al., see Chapter 4). Therefore, the ability of statins to reduce AD risk may be due, at least in part, to inhibition of isoprenylation of Rho GTPases and subsequent prevention of neuritic dystrophy and deterioration.

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Chapter 2:

Effect of a 5-HT(2C) serotonin agonist, dexnorfenfluramine, on amyloid precursor protein metabolism in guinea pigs

Effect of a 5-HT(2C) serotonin agonist, dexnorfenfluramine, on amyloid precursor protein metabolism in guinea pigs

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Abstract

Stimulation of serotonin receptor subtypes 5-HT_{2A} or 5-HT_{2C} in stably transfected 3T3 cells by dexnorfenfluramine (DEXNOR) or serotonin increases secretion of the APP metabolite APP_s. It is not known whether activation of these receptors can also affect APP metabolism in vivo. We examined the effects of a single intraperitoneal (i.p.) injection of DEXNOR on APP_s levels in cerebrospinal fluid (CSF) of guinea pigs. These levels were significantly ($P<0.05$) increased by a single dose of DEXNOR (1–4 mg/kg); those of the APP metabolites $A\beta_{1-40}$ and $A\beta_{1-42}$ were unaffected. The DEXNOR-induced (1 mg/kg) increases in CSF APP_s were suppressed by ritanserin (1 mg/kg) but not by ketanserin (2 mg/kg). When given alone, ritanserin did not affect CSF levels of APP_s, $A\beta_{1-40}$, or $A\beta_{1-42}$. Chronic treatment with DEXNOR for 9 days (1 mg/kg bid, i.p.) increased CSF APP_s levels, measured 2 h after the last injection ($P<0.05$), and decreased those of CSF $A\beta_{1-42}$ ($P<0.05$). Neither hippocampal nor cortical levels of the APP holoprotein (APP_h), nor body weight, were affected by DEXNOR. Chronic administration of mCPP (1-(*m*-chlorophenyl)piperazine) (2 mg/kg bid, i.p.), a 5-$HT_{2B/2C}$ agonist, for 9 days also increased CSF APP_s levels ($P<0.05$) when measured 2 h after the drug's last administration; hippocampal and cortical APP_h levels were unaffected. However, mCPP also caused a significant decrease in body weight gain. These data indicate that the pharmacological activation of 5-HT_{2C} receptors can stimulate CSF APP_s secretion and reduce Aβ production in vivo. Hence 5-HT_{2C} receptors, which apparently are localized to the brain, may represent useful targets for the development of treatments for Alzheimer's disease.

Author Keywords: Dexnorfenfluramine; mCPP; Guinea pig; Alzheimer's disease; APP; Aβ; Serotonin agonist; Ritanserin; Ketanserin

Neuroscience classification codes: Neurotransmitters, modulators, transporters, and receptors, Serotonin receptors

1. Introduction

Therapeutic agents currently prescribed for Alzheimer's disease (AD) are cholinomimetic substances, i.e., acetylcholinesterase inhibitors. Although dysfunction of long-axon cholinergic brain neurons is an early manifestation of AD, attempts to slow the progression of AD with these drugs have had only modest success, perhaps because the doses that can be administered are limited by peripheral cholinergic side effects. *In vitro* activation of muscarinic m_1 or m_3 receptors in human embryonic kidney cells increases APP_s secretion [16] by releasing diacylglycerol (DAG) from membrane phosphatides, and concurrently decreases Aβ production [5]. *In vivo*, selective m_1 agonists have also been shown to decrease cerebrospinal fluid Aβ concentrations in rabbits and humans [1 and 18]. However, the muscarinic receptors exist in both the central nervous system and peripheral tissues, hence their activation can cause adverse peripheral side effects. Moreover, muscarinic agonists have not clearly been shown to improve cognition in AD [7 and 8].

Activation of 5-HT_{2A} or 5-HT_{2C} receptors in transfected 3T3 cells by serotonin or DEXNOR has been shown to increase APP_s secretion, also by liberating DAG [17]. We have examined the effects of 5-HT-2 agonists in vivo by measuring APP_s (and, for DEXNOR, Aβ) in cerebrospinal fluid of guinea pigs treated with DEXNOR or mCPP. We chose this species because guinea pig and human APP exhibit 98% sequence homology [3], the proteins are processed similarly [2], and their Aβ peptide sequences are identical [6].

2. Materials and methods

2.1. Cerebrospinal fluid withdrawal

Male Dunkin Hartley guinea pigs (Charles River, Wilmington, MA, USA) (300 g, 28 days of age) were given access to feed (Prolab RMH 3000, PMI Nutrition International, St. Louis, MO, USA; 22% protein) and water ad libitum, and exposed to a 12:12 light:dark cycle. Animals were allowed to habituate to the environment for 1 week prior to being used in an experiment. All treatment groups contained six or more animals.

CSF collection was carried out in fully anesthetized animals (ketamine/xylazine, 40 and 5 mg/kg, respectively) as described by Kusumi and Plouffe [9] with minor modifications. Briefly, the cervical area was shaved and cleansed with alcohol. A 23 gauge needle without its hub was attached to a length of PE60 tubing (I.D. 0.76 mm, O.D. 1.22 mm; Becton Dickinson, Sparks, MD, USA) connected to a 1 cc syringe. The needle was then used to penetrate the cisterna magna at the atlanto–occipital joint. CSF (<100 μl) was withdrawn over 10 s. CSF was collected once per session, and no more than once per week. Samples contaminated with blood were discarded. The entire procedure was completed within 4 min.

2.2. Dose response studies

The effects on CSF APP_s, Aβ 1–40 and Aβ 1–42 levels of acute administration of varying concentrations of DEXNOR were determined as follows. Guinea pigs randomly assigned to each treatment group were injected (i.p.) with DEXNOR (0.5, 1.0, 2.0 or 4.0 mg/kg) or buffer. The DEXNOR was generously provided by Technology Servier (Paris, France) or synthesized by Professor Timothy Maher (Massachusetts College of

Pharmacy, Boston, MA, USA). DEXNOR and all other compounds administered were dissolved in dimethyl sulfoxide (DMSO). CSF was collected after 2 h and processed as described below.

2.3. Time course studies

Guinea pigs randomly assigned to each treatment group received DEXNOR (1 mg/kg, i.p.) or DMSO. CSF was collected after 1, 2, 4 or 8 h.

2.4. Antagonists

To examine the effects of the serotonin antagonists ritanserin (1 mg/kg) and ketanserin (2 mg/kg) (RBI–Sigma, St. Louis, MO, USA) on DEXNOR-induced increases in CSF APP_s, animals were injected (i.p.) with one of the antagonists, or DMSO, 2 h prior to receiving DEXNOR (1 mg/kg, i.p.), and CSF samples were collected after 2 h.

2.5. Chronic administration studies

2.5.1. Dexnorfenfluramine

Animals received daily injections of DEXNOR (1 mg/kg bid, i.p.) for 9 consecutive days, and body weights were determined every 2 days. On the ninth day, some animals in the DEXNOR-treated groups received their last injection 2 h prior to CSF collection, while the remaining animals in the treated group received their last injection 24 h prior to CSF collection. After CSF withdrawal the guinea pigs were sacrificed, and the hippocampus and cortex were dissected and processed for analysis of APP_h.

2.5.2. mCPP

To assess the effects of chronic mCPP (Tocris, Ballwin, MO, USA) administration on APP metabolism and body weight, animals received mCPP (2 mg/kg bid, i.p.) for 9 consecutive days, and were weighed every 2 days. On the ninth treatment day, the mCPP

group received its last injection 2 h prior to CSF collection for APP_s assay. After CSF withdrawal, the guinea pigs were sacrificed and the hippocampus and cortex were dissected and processed for APP_h analysis.

Guinea pigs were used repeatedly and were randomly assigned to the various treatment groups for the dose–response, time-course, and receptor–antagonist studies. A minimum of 1 week was allowed to pass between studies. Body weights were determined weekly for the duration of the studies unless otherwise stated. Naïve guinea pigs were used for studies involving chronic DEXNOR and mCPP administration.

2.6. Western blot analysis and ELISA

An aliquot of CSF was diluted 1:1 with 2x sample buffer (125 mM Tris, pH 6.8, 8% SDS, 40% glycerol, 10% -mercaptoethanol, 1.4% bromophenol) prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis. APP_h concentrations were determined using approximately 10 mg of temporal cortex or hippocampal tissue. Each sample was placed in a microfuge tube containing 200 µl lysis buffer (60 mM Tris–HCl, 20% glycerol, 1 mM dithiothreitol, 1 mM AEBSF, 8 µM Aprotinin, 500 µM Bestatin, 15 µM E64, 200 µM Leupeptin, 10 µM Pepstatin A). The samples were then sonicated, boiled (10 min), and centrifuged (14,000 x *g*/1 min/room temperature). The supernatant fluid was transferred to a clean microfuge tube and total protein concentrations in this fluid and in CSF samples were determined using the Bicinchoninic Acid (Sigma, St. Louis, MO, USA) assay.

Equal amounts of protein were loaded for electrophoretic analysis (4–20% gradient) (BioRad, Hercules, CA, USA). Proteins were then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) and incubated in 5% bovine serum

albumin (Tris-buffered saline/0.15% Tween 20) for 1 h. Monoclonal antibodies 22C11 (Roche, Indianapolis, IN, USA) and APP-KPI (Chemicon, Temecula, CA, USA) were used to detect the N-terminus and KPI-domain of APP, respectively. In addition, an antibody against glial fibrillary acidic acid (GFAP) (Chemicon) was used. Protein–antibody complexes were detected and visualized using the ECL system (Amersham, Piscataway, NJ, USA) and Kodak X-AR film, respectively, as suggested by the manufacturer. Films were digitized using a Supervista S-12 scanner with a transparency adapter (UMAX Technologies, Freemont, CA, USA). Analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institute of Health) and available on the internet at http://rsb.info.NIH.gov/NIH-IMAGE/.

2.7. $A\beta_{1-40}$ and $A\beta_{1-42}$ measurements

CSF $A\beta_{1-40}$ or $A\beta_{1-42}$ levels were determined using commercially available ELISA kits for human Aβ (BioSource International, Camarillo, CA, USA). CSF samples were diluted 1:2 using sample diluent prior to analysis and processed as described in the manufacturer's instructions. Total protein in the CSF was determined using the Bicinchoninic Acid (Sigma) assay. Aβ levels were corrected for protein content.

2.8. Data analysis

Measurements of cellular and secreted proteins were normalized against those of control groups. Analysis of variance (ANOVA) was used to determine differences between groups (significance level, $P \leq 0.05$), using drug treatments as the independent variable. When differences were detected, the means were separated using Dunnett's test. Data are presented as mean±S.E.M.

3. Results

A single intraperitoneal injection of DEXNOR (1, 2 or 4 mg/kg) caused a significant increase in CSF APP_s levels (1.51±0.13-, 1.87±0.18- and 1.75±0.13-fold control values, respectively; $P<0.05$) of guinea pigs 2 h after its administration (Fig. 1). CSF $A\beta_{1-42}$ levels, however, were not significantly different from those of controls (Fig. 1).

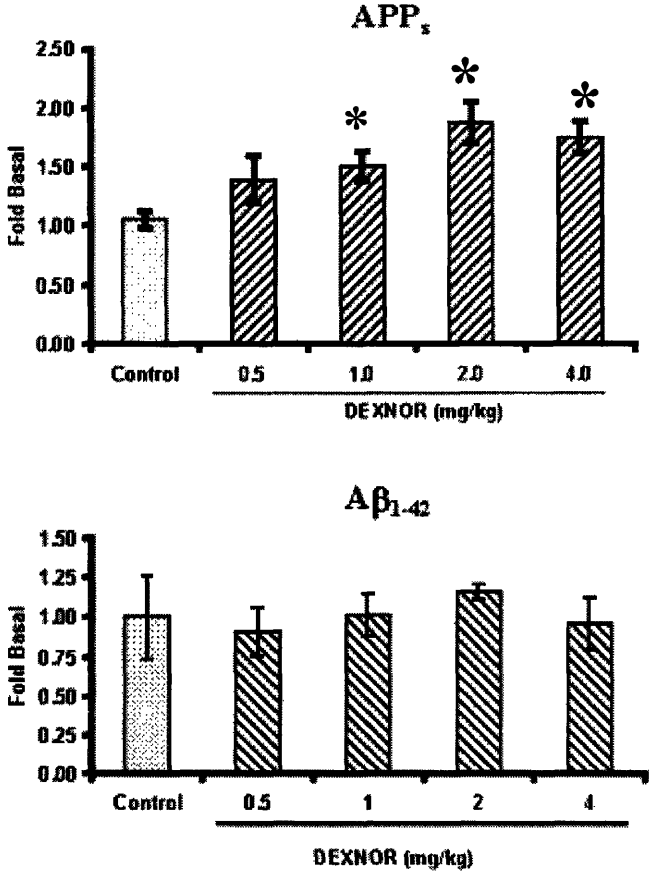

Figure 1. DEXNOR-induced increases in CSF APP_s levels of guinea pigs. A single intraperitoneal injection of DEXNOR (1, 2 or 4 mg/kg) caused a significant (*$P<0.05$) increase in CSF APP_s levels 2 h after its administration. CSF $A\beta_{1-42}$ levels were not different from those of controls. Values represent mean±S.E.M.

Ritanserin (1 mg/kg) blocked the DEXNOR-induced (1.60±0.14) increase in CSF APP_s levels when compared with the increases seen in control animals or in guinea pigs treated with ritanserin alone (1.03±0.067). Ketanserin failed to do so (2 mg/kg) (1.33±0.193) (P>0.05) (Fig. 2). When administered alone, ritanserin did not have a significant effect on CSF APP_s levels (Fig. 2).

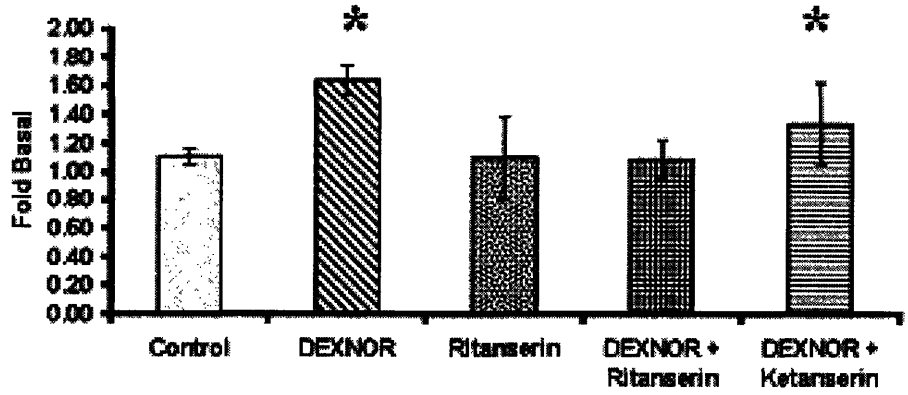

Figure 2. Effect of the serotonin antagonists ritanserin and ketanserin on DEXNOR-induced increases in CSF APP_s levels. When compared to control, ritanserin (1 mg/kg) but not ketanserin (2 mg/kg) (* P<0.05) suppressed the DEXNOR-induced rise in CSF APP_s. When administered alone, ritanserin did not have an effect on APP_s CSF levels. Values represent mean±S.E.M.

Time-course (1, 2, 4 and 8 h) studies showed that, following a single intraperitoneal injection of DEXNOR, APP_s levels peaked after 2 h and gradually decreased thereafter (0.929±0.15-, 1.64±0.103-, 1.26±0.216- and 0.890±0.197-fold, respectively) (Fig. 3).

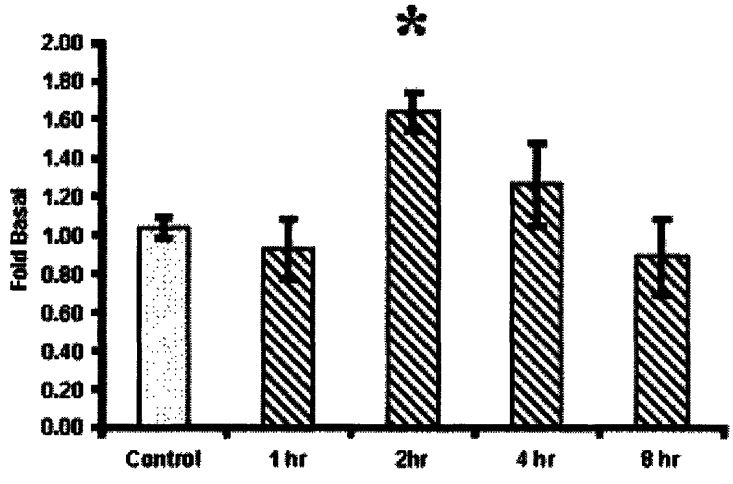

Figure 3. Time-course of DEXNOR-induced changes in APP_s levels. Animals received a single intraperitoneal injection of DEXNOR (1 mg/kg), and CSF was collected 2 h later. CSF APP_s levels peaked (* $P<0.05$) at 2 h and gradually decreased thereafter. Values represent mean±S.E.M.

Chronic administration of DEXNOR (1 mg/kg) significantly ($P<0.05$) increased CSF APP_s levels, relative to those of controls, 2 h following the last injection of DEXNOR (2.27±0.48-fold) but not 24 h after that injection (Fig. 4). CSF $A\beta_{1-42}$ levels were significantly decreased ($P<0.05$) by chronic DEXNOR treatment at both 2 and 24 h following the last injection of DEXNOR (0.72±0.05 and 0.61±0.07 ng/mg protein, respectively) (Fig. 4). A decrease in CSF $A\beta_{1-40}$ levels occurred 24 h following DEXNOR administration. This decrease, however, was not statistically significant.

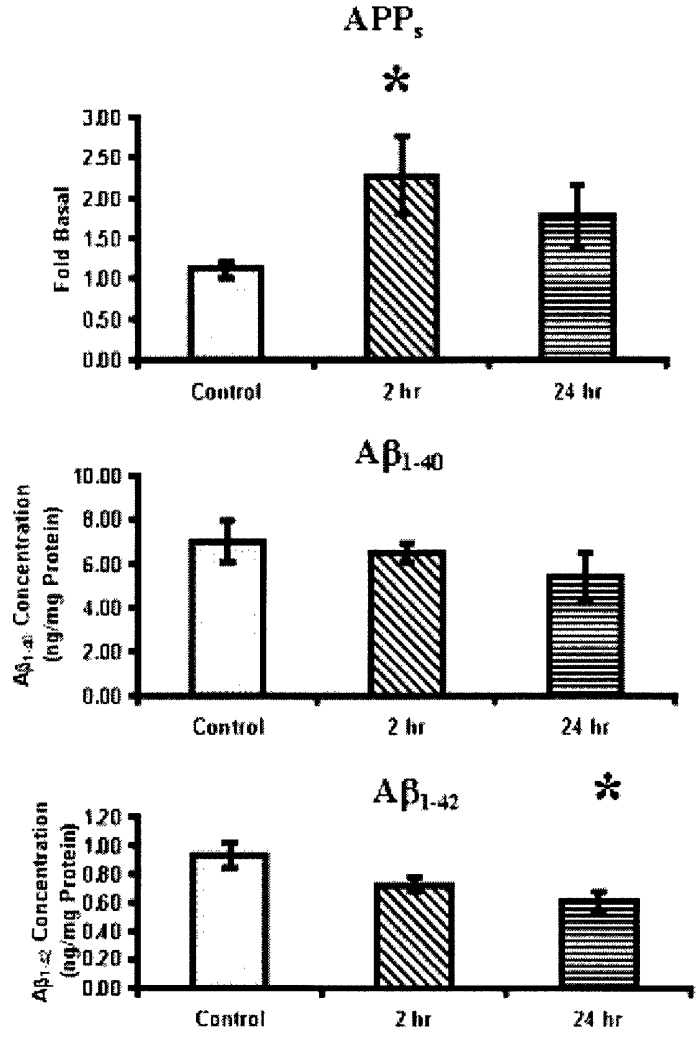

Figure 4. Effect of chronic administration of DEXNOR (1 mg/kg, bid) on CSF levels of APP_s, $A\beta_{1-40}$ and $A\beta_{1-42}$. Samples were collected 2 and 24 h after DEXNOR administration. APP_s levels were increased (* $P<0.05$) 2 and 24 h after the last injection, while levels of $A\beta_{1-42}$ were decreased (*$P<0.05$) after 24 h. However, $A\beta_{1-40}$ levels were not significantly decreased by DEXNOR administration. Values represent mean±S.E.M.

Neither hippocampal nor cortical APP_h levels (Fig. 5), nor body weight (Fig. 6), were significantly affected by chronic DEXNOR treatment at the single dosage (1 mg/kg) tested (Fig. 5).

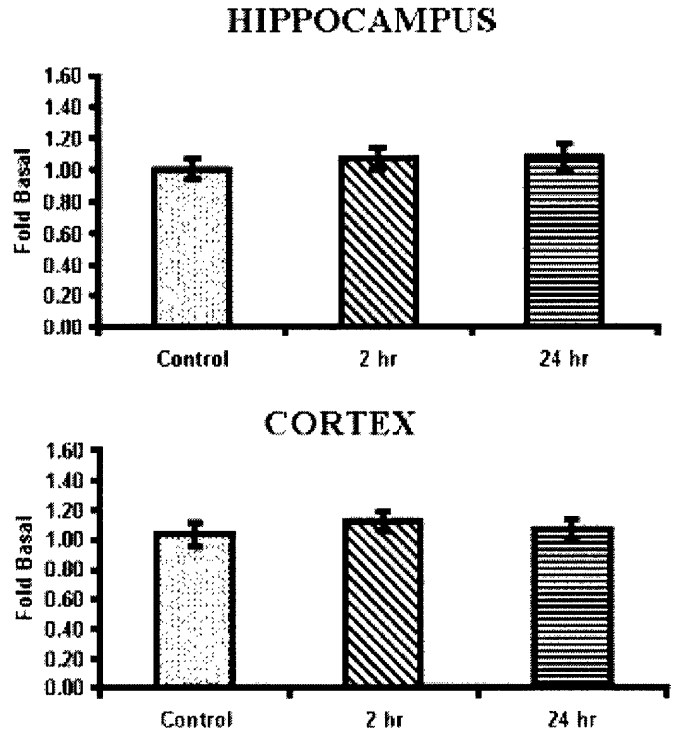

Figure 5. Hippocampal and cortical APP_h levels in guinea pigs which received DEXNOR chronically, as described in Fig. 4. No significant changes were observed. Values represent mean±S.E.M.

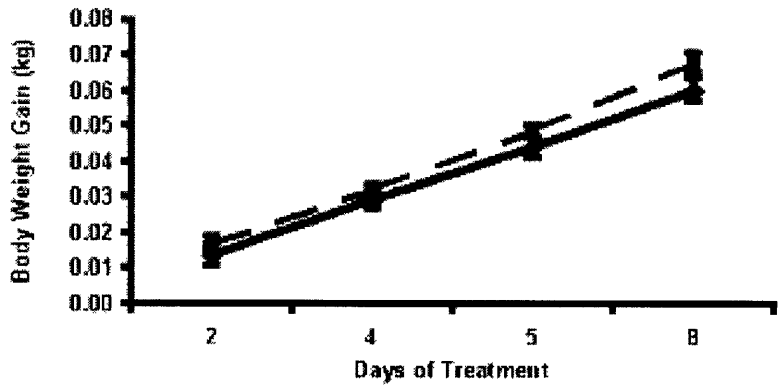

Figure 6. Body weight of guinea pigs injected with DEXNOR chronically, as described in Fig. 4. No significant changes were observed on body weight. Treated (hatched); control (solid). Values represent mean±S.E.M.

GFAP levels in cortex or hippocampus also were unaffected by chronic DEXNOR treatment (results not shown), suggesting that this treatment was not neurotoxic. Chronic treatment with mCPP also increased CSF APP_s levels relative to those in controls ($P<0.05$) when measured 2 h after the last dose (1.81±0.28-fold basal) (Fig. 7), but failed to affect hippocampal or cortical APP_h levels (1.03±0.07- and 1.05±0.11-fold basal, respectively) (Fig. 7B). Unlike DEXNOR, mCPP administration decreased ($P<0.05$) body weight gain (Fig. 7C).

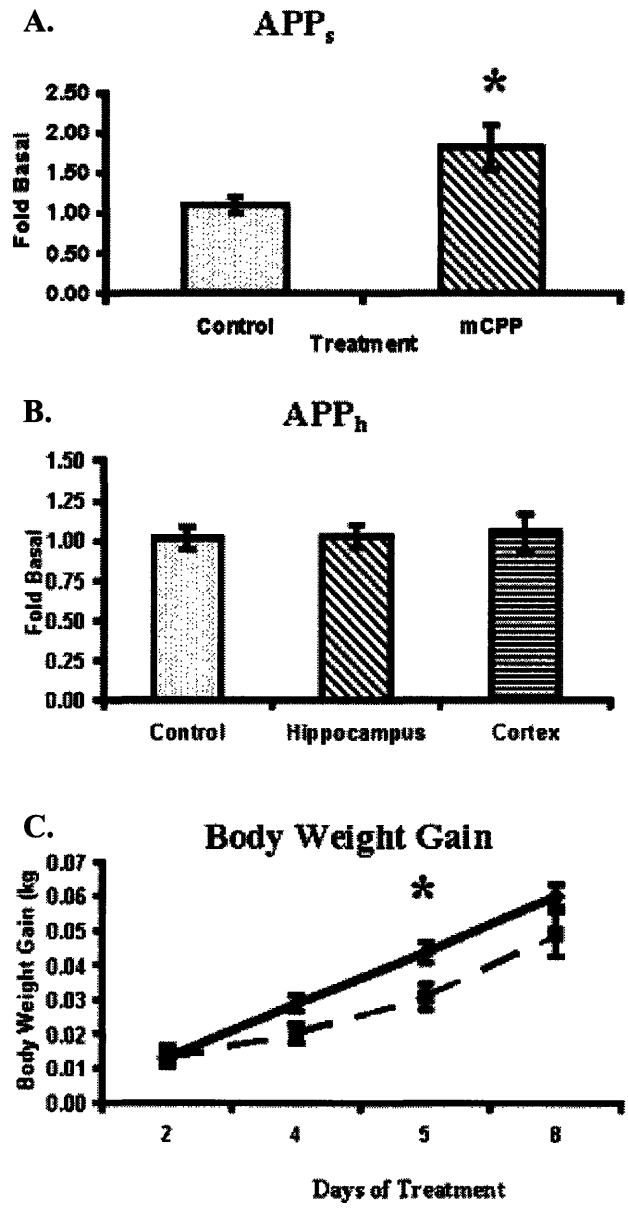

Figure 7. Effect of chronic administration of mCPP (2 mg/kg, bid) on CSF APP_s, cortical and hippocampal APP_h levels, and body weight gain. The mCPP treatment increased (* $P<0.05$) CSF APP_s levels relative to controls when measured 2 h after administration (1.81±0.28-fold basal). Neither hippocampal nor cortical APP_h levels were significantly altered by chronic mCPP treatment. mCPP caused a significant (*$P<0.05$) decrease in body weight gain. Treated (hatched); control (solid). Values represent mean±S.E.M.

4. Discussion

These data demonstrate that DEXNOR administration affects brain APP metabolism *in vivo*, increasing CSF APP_s levels (Fig. 1 and Fig. 4), probably by activating 5-HT_{2C} receptors. Although CSF APP_s levels increased 2 h after a single dose of the drug, CSF $A\beta_{1-42}$ levels failed to change at this time. However, chronic treatment with DEXNOR (1 mg/kg, bid) for 9 consecutive days increased CSF APP_s levels and concurrently decreased those of $A\beta_{1-42}$ (Fig. 4). These changes were not associated with changes in body weight (Fig. 4), nor with neurochemical evidence of neurotoxicity (i.e. elevated GFAP).

The rapid (i.e. within 2 h) increase in CSF APP_s levels caused by DEXNOR most likely is mediated by the same mechanism as that previously shown to operate in 3T3 cells [17], namely, activation of phospholipase C, metabolism of membrane phospholinositol (PI) to yield DAG, which activates PKC, and PKC-related activation of α-secretase activity. That the rise in CSF APP_s was not caused by enhanced APP_h synthesis is suggested by the speed of this response, and by the failure of chronic DEXNOR treatment to affect hippocampal or cortical APP_h levels (Fig. 5).

In vitro, agonists that stimulate intracellular cAMP formation can cause APP overexpression [10 and 11], while those like DEXNOR which are coupled to PI hydrolysis do not have this effect [16]. Cultured rat astrocytes treated with DEXNOR increased both PI hydrolysis and APP_s secretion, but did not increase cAMP, APP mRNA nor APP_h levels. In contrast, treatment of cultured rat astrocytes with prostaglandin E2 or norepinephrine induced the formation of cAMP, APP mRNA, and APP_h and of GFAP, a

marker for astrocytic activation. DEXNOR also failed to increase GFAP or APP_h levels in the present study, suggesting that DEXNOR is not coupled to cAMP formation *in vivo*.

Besides activating 5-HT_{2A} and 5-HT_{2C} receptors, DEXNOR also enhances serotonin release and is a potent inhibitor of serotonin re-uptake [4 and 13]. DEXNOR is the major metabolite of dexfenfluramine, and the activation of 5-HT-2 receptors that follows dexnorfenfluramine administration mediates dexfenfluramine's anorexic effects [23].

In 3T3 cells transfected with 5-HT_{2A} or 5-HT_{2C} receptors, the stimulatory effect of DEXNOR on APP_s secretion was inhibited by the serotonin antagonists ritanserin or ketanserin [17]. In our *in vivo* study, the stimulatory effect of DEXNOR on CSF APP_s was completely inhibited by ritanserin, a 5-$HT_{2A/2C}$ antagonist, but not affected by ketanserin, a 5-HT_{2A} antagonist (Fig. 2). Furthermore, chronic administration (2 mg/kg bid) of mCPP, a 5-$HT_{2B/2C}$ receptor agonist that is structurally unrelated to DEXNOR [20], caused similar increases in CSF APP_s (Fig. 7). Chronic administration of mCPP at this dosage, however, caused a significant reduction in body weight. These results suggest that, *in vivo*, the main effect of DEXNOR is mediated via 5-HT_{2C} receptors.

APP overexpression and Aβ production are potentially neurotoxic, while APP_s has neurotrophic and neuroprotective functions *in vitro* [12 and 21]. In rats, infusion of APP_s improved cognition and synaptic density [22] and enhanced memory retention in a variety of learning tasks [14]. Our data indicate that serotoninergic compounds like DEXNOR that activate brain 5-HT_{2C} receptors may be useful for treating AD by increasing APP_s formation and decreasing that of $A\beta_{1-42}$. Because 5-HT_{2C} receptors apparently are localized to the brain [7,15 and 19] 5-HT_{2C} agonists may not produce

peripheral side effects as seen with other drugs (e.g. muscarinic receptor agonists) that similarly promote non-amyloidogenic APP cleavage.

Acknowledgements

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Chapter 3:

Prostaglandin E_2 regulates amyloid precursor protein expression via the EP2 receptor in cultured rat microglia

Prostaglandin E 2regulates amyloid precursor protein expression via the EP2 receptor in cultured rat microglia

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Abstract

We investigated the effects of prostaglandin E_2 (PGE_2) on amyloid precursor protein (APP) expression in cultured rat microglia. PGE_2 treatment significantly increased the expression of APP holoprotein and was associated with an elevation in cyclic AMP (cAMP). Direct activation of adenylate cyclase with forskolin also increased APP expression. Co-treatment of microglia with PGE_2 and the PKA inhibitor H-89 suppressed the overexpression of APP caused by PGE_2 alone. The prostaglandin EP2 receptor is known to be positively coupled to cAMP production. Stimulation of the EP2 receptor with butaprost increased APP holoprotein, whereas co-incubation of the cells with PGE_2 and the EP2 receptor antagonist AH-6809 blocked the effect of PGE_2 on APP expression. These data suggest that PGE_2 is able to regulate the expression of APP, and that this effect may be mediated by the EP2 receptor and the cAMP signaling cascade.

Keywords: Amyloid; Microglia; Prostaglandin; Inflammation; Alzheimer's disease; Cyclic AMP

The neurodegenerative disorder Alzheimer's disease (AD) is characterized by cognitive decline and memory loss. The accumulation of extracellular amyloid plaques is a hallmark of AD. Amyloid (Aβ), the principal component of such plaques, is derived by proteolytic cleavage of the amyloid precursor protein (APP) by the β- and γ-secretases. APP may alternatively undergo a single cleavage by the α-secretase within the Aβ region, causing the formation of the non-amyloidogenic soluble APP fragment and thereby preventing the formation of Aβ [3 and 18].

Brain injury, a major risk factor for AD [4,12 and 22], is associated with APP overexpression [19] and an increase in cerebrospinal fluid (CSF) Aβ levels [15]. Trauma to the brain can activate an inflammatory pathway that involves the release of arachidonic acid from cellular phospholipids by cytosolic phospholipase A_2 (PLA_2) [17], and its subsequent cyclooxygenation to form prostaglandin E_2 (PGE_2). In brains of AD patients, the activation of such components of this pathway as PLA_2 [21] and cyclooxygenase-2 (COX-2), the inducible enzyme responsible for prostaglandin production in response to injury or inflammation, is enhanced [14]. Moreover, PGE_2 levels are elevated in the CSF of patients thought to have AD [11].

In rat astrocytes, PGE_2 stimulates the overexpression of APP. This effect is probably mediated by the cyclic AMP (cAMP) signaling pathway [8]. Moreover, studies have shown that treatments that increase cAMP levels may enhance the formation of amyloidogenic Aβ [6and 9]. PGE_2 can interact with the cAMP pathway by stimulating the G protein-coupled EP2 receptor, which activates adenylate cyclase and increases cAMP production [1]. EP2 receptors are expressed by microglia [2], the primary mediators of brain inflammation. Microglia are activated by neuronal damage [7 and 13]

and therefore may be involved in the overexpression of APP observed following brain trauma. In the present study, we examined the relationship of the inflammatory mediator PGE_2 to production of APP in rat microglia.

Microglial cells were prepared by agitating primary cultured astrocytes and replating the detached microglia onto fresh, uncoated culture dishes using procedures similar to those described by Giulian and Baker [5]. Briefly, dissociated astrocytes were cultured from cortices of rat pups (postnatal day 1–2) as previously described [10]. Cells from dissociated cortices were plated onto poly-l-lysine-coated 35 mm culture dishes. The initial culture medium, Minimal Essential Medium (MEM, Invitrogen, Carlsbad, CA) containing 5% horse serum (Cambrex, Rockland, ME), was aspirated 2–5 h after plating to remove unattached cells and debris, and replaced with MEM containing 5% fetal bovine serum (FBS, Cambrex, Rockland, ME). Half the medium was replaced with MEM/10% FBS twice weekly. The cells were kept at 37 °C in a humidified 5%CO_2/95% air incubator for 10–14 days, by which time the cultures were confluent.

Floating microglia were harvested from the underlying monolayer of astrocytes by gentle shaking of the cell culture dishes, seeded onto uncoated 75 cm^2 culture flasks and maintained in MEM/5% FBS for 2 weeks. When the microglia were 60–90% confluent, they were detached by trypsin and harvested. Cells were then plated onto six-well plates and maintained in MEM/5% FBS for approximately 2 weeks; by this time the cultures were confluent and ready for use in experiments.

The following drugs were used: PGE_2, cycloheximide and forskolin (Calbiochem, San Diego, CA); H-89 dihydrochloride, Sp-cAMP triethylamine, butaprost and AH6809 (Sigma, St. Louis, MO). Frozen aliquots of these drugs were diluted in serum-free MEM

(37 °C) to appropriate concentrations. Experiments were conducted in triplicate unless otherwise stated.

Cell-associated APP levels were measured in cultured microglia grown on six-well plates. The medium was aspirated, and microglia were scraped in 100 μl lysis buffer (60 mM Tris–HCl, 4% SDS, 20% glycerol, 1 mM dithiothreitol, 1 mM AEBSF, 8 μM aprotinin, 500 μM bestatin, 15 μM E64, 200 μM leupeptin, 10 μM pepstatin A). The total amount of protein in each sample, estimated by the bicinchoninic acid assay (Sigma, St. Louis, MO), was not altered by pharmacological treatments. Prior to gel electrophoresis, bromphenol blue solution (0.07%) was added to each sample. The amount of cell protein loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4–20% SDS PAGE; Bio-Rad, Hercules, CA) was normalized to the amount of protein per sample. Proteins (equivalent to approximately 50 μg cell protein/lane) were separated by electrophoresis, electroblotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA), and blocked in bovine serum albumin for 1 h. After 3x10 min rinses in Tris-buffered saline (TBST), the membranes were incubated in TBST containing an appropriate antibody. Monoclonal antibody 22C11 (Roche, Indianapolis, IN) was used to detect the N-terminus of APP. Antibodies against glial fibrillary acid protein (GFAP; Roche, Indianapolis, IN) and CD11b/membrane attack complex 1 (MAC-1; Serotec, Raleigh, NC) were used to confirm that the cells were microglial. Cells used in this study did not express the astrocytic marker GFAP, but did express, when activated by PGE_2 or lipopolysaccharide, the microglial marker CD11b/MAC-1 (data not shown). Protein–antibody complexes were detected and visualized using the ECL system (Amersham, Piscataway, NJ) and Kodak X-AR film, respectively, as suggested by the

manufacturer. Films were digitized using a Supervista S-12 scanner with a transparency adapter (UMAX Technologies, Freemont, CA). Analysis was performed using the public domain NIH Image program (developed at the U.S. National Institute of Health), available on the internet at http://rsb.info.NIH.gov/NIH-IMAGE/. Levels of cAMP were measured in rat microglia grown on 35 mm dishes using an [8-^{3}H]cAMP assay kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Data were analyzed using analysis of variance (ANOVA), and the Newman–Keuls test was used to evaluate differences between groups (significance level, $P<0.05$), with drug treatments as the independent variable. Data are presented as means ± SEM.

Microglia were treated for 24 h with 0.1, 1 or 10 μM PGE_2, which produced dose-dependent and significant increases in levels of APP holoprotein, relative to those in controls (Fig. 1a,b).

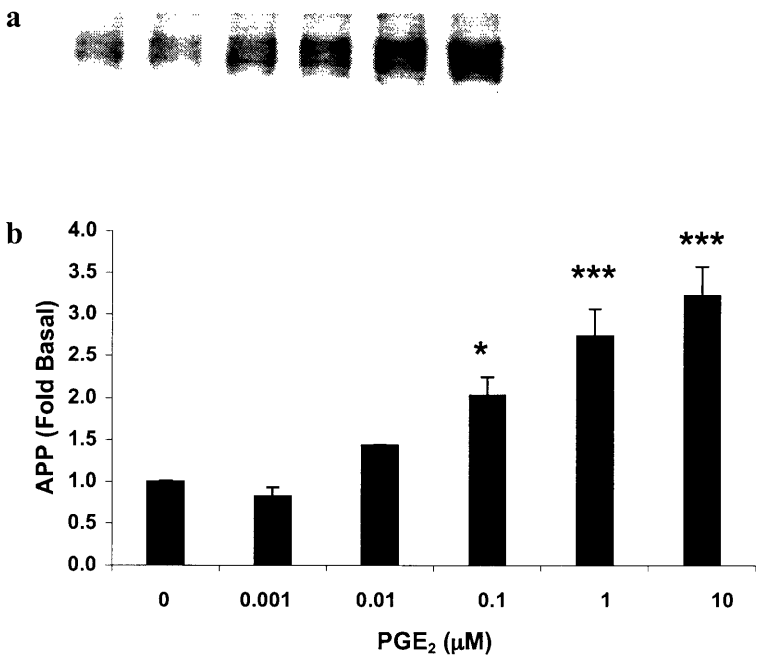

Figure 1. PGE_2 treatment increases cellular APP levels in rat microglia. (a) APP expression was determined by western blotting using the antibody 22C11. (b) Treatment with 0.1, 1 and 10 μM PGE_2 for 24 h stimulated significant increases in levels of cellular APP holoprotein. Values represent means ± S.E.M. * $p < 0.05$; *** $p < 0.001$ *vs* control.

Cycloheximide (1 μM), a protein synthesis inhibitor, suppressed the increase in APP holoprotein stimulated by PGE_2 (data not shown). In order to determine how PGE_2 affects APP production, we next assessed the effect of PGE_2 on the second messenger cAMP.

PGE_2 treatment (1 or 10 μM) significantly increased cellular cAMP levels in the cultured microglia (Fig. 2), suggesting that PGE_2 may exert its effect on APP via the cAMP signaling pathway.

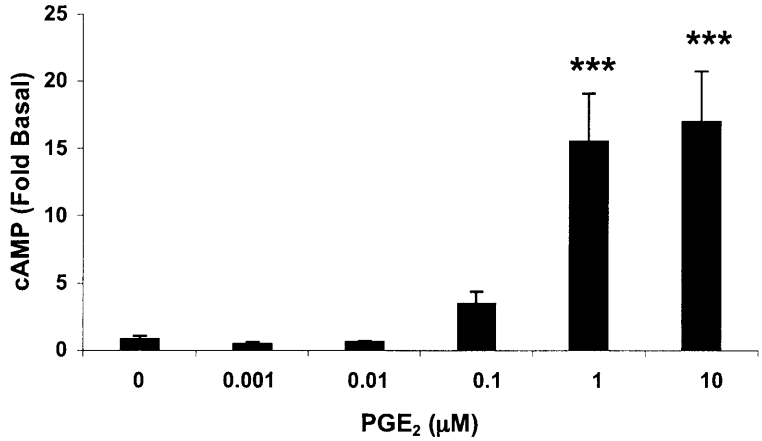

Figure 2. PGE_2 treatment increases cAMP production in cultured rat microglia. Intracellular cAMP levels were significantly enhanced after one hour treatment with 1 and 10 μM PGE_2. Values represent means ± S.E.M. *** $p < 0.001$ *vs* control.

The effect of PGE_2 on APP was mimicked by direct activation of the cAMP pathway: forskolin (1 or 10 μM), an activator of adenylate cyclase, significantly increased APP expression in the microglia (Fig. 3). Activation of protein kinase A (PKA) by sp-cAMP triethylamine also significantly increased APP levels relative to those in control cells (data not shown). Furthermore, the increase in APP levels caused by PGE_2 treatment was significantly inhibited by co-treatment with the PKA inhibitor H-89 (10 μM); treatment with H-89 alone had no effect on basal APP levels (data not shown). Taken together,

these results suggest that PGE_2 may increase APP production by stimulating the cAMP signaling pathway.

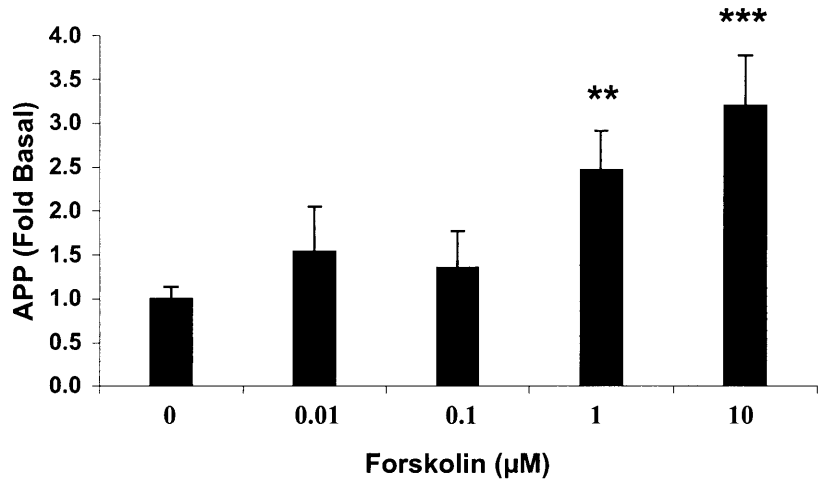

Figure 3. APP expression in cultured rat microglia is increased following treatment with the protein kinase A-activator forskolin. Treatment with 1 and 10 μM forskolin for 24 h stimulated significant increases in intracellular APP levels. Values represent means ± S.E.M. ** $p < 0.01$, *** $p < 0.001$ *vs* control.

To determine how PGE_2 may be affecting cAMP levels, we examined the role of prostaglandin EP2 receptors, which are known to be positively coupled to cAMP formation [13].

We found that stimulation of microglial EP2 receptors for 24 h by the EP2 receptor-specific agonist butaprost significantly and dose-dependently increased APP expression (Fig. 4a). Moreover, co-incubation of microglial cells with PGE_2 and the EP2 receptor antagonist AH6809 dose-dependently suppressed the effect of PGE_2 on APP overexpression (Fig. 4b). Both of these findings support the hypothesis that EP2 receptors may mediate PGE_2-induced APP overexpression.

These data show that the inflammatory mediator PGE_2 increases APP production in primary cultured rat microglia possibly via activation of the cAMP signaling pathway.

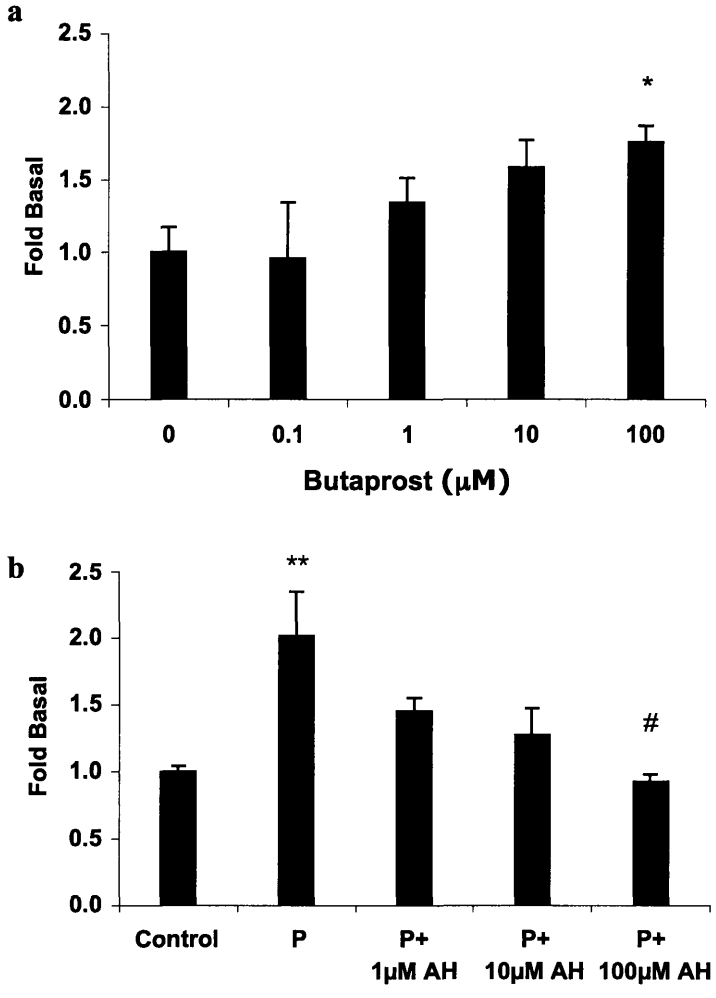

Figure 4. The EP2 receptor can modulate APP expression. (a) Cells were treated for 24 h the EP2 receptor agonist butaprost. Treatment with 100 μM butaprost significantly increased APP expression. (b) Co-treatment of the cells with PGE_2 (P) plus the EP2 receptor antagonist AH6809 (AH) inhibited the overexpression of APP caused by PGE_2 treatment alone. Values represent means ± S.E.M. * $p < 0.05$, ** $p < 0.01$ *vs* control; # $p < 0.05$ *vs* PGE_2 treated.

The stimulatory effect of PGE_2 on APP overexpression was suppressed by the PKA inhibitor H-89. Since H-89 did not fully inhibit the effect of PGE_2, it is possible that the cAMP produced following PGE_2 stimulation may also increase the expression of APP via a PKA-independent mechanism [20]. One such pathway could involve cAMP binding to, and activation of, the guanine nucleotide exchange factor Epac [16]. The unavailability of

selective Epac inhibitors at this time makes exploring this possibility difficult.

These data suggest that the effect of PGE_2 on APP production involves the cAMP signaling pathway, at least in part, and that the prostaglandin receptor EP2, which is coupled to cAMP activation, might mediate the effect of PGE_2 on APP expression. Treatment of microglia with butaprost, an EP2 receptor agonist, increased APP levels, which suggests that stimulation of the EP2 receptor is sufficient to affect APP expression. Incubation of the cells with AH6809, an EP2 receptor antagonist, fully blocked the APP overexpression caused by PGE_2. These findings provide the first evidence that the stimulatory effect of PGE_2 on APP levels may involve activation of the cAMP signaling cascade by the binding of PGE_2 to the prostaglandin receptor EP2. Our results are consistent with the idea that microglia may contribute to the neuropathology of AD, possibly by increasing APP expression in response to inflammatory prostaglandins acting at the microglial EP2 receptor.

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Chapter 4:

Uridine enhances neurite outgrowth in NGF-differentiated PC12 cells

Uridine enhances neurite outgrowth in NGF-differentiated PC12 cells

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Abbreviations

CDP-choline, 5'-cytidine diphosphocholine; CTP, cytidine triphosphate; DAG, diacylglycerol; IP, inositol phosphates; IP3, inositol triphosphate; NGF, nerve growth factor; PC, phosphatidylcholine; PI, phosphatidylinositol; PPADS, pyridoxal-phosphate-6-azophenyl-2',4' disulfonic acid; RB-2, reactive blue 2; UTP, uridine triphosphate.

Abstract

During rapid cell growth the availability of phospholipid precursors like cytidine triphosphate (CTP) and diacylglycerol (DAG) can become limiting in the formation of key membrane constituents like phosphatidylcholine (PC). Uridine, a normal plasma constituent, can be converted to CTP in PC-12 cells and intact brain, and has been shown to produce a resulting increase in PC synthesis. To determine whether treatments that elevate uridine availability also thereby augment membrane production, we exposed PC-12 cells which had been differentiated by NGF to various concentrations of uridine, and measured the numbers of neurites the cells produced. After 4 but not 2 days uridine significantly and dose-dependently increased the number of neurites per cell. This increase was accompanied by increases in neurite branching and in levels of the neurite proteins neurofilaments M and neurofilament 70. Uridine treatment also increased intracellular levels of CTP, which suggests that uridine may affect neurite outgrowth by enhancing PC synthesis. Uridine may also stimulate neuritogenesis by a second mechanism, since the increase in neurite outgrowth was mimicked by exposing the cells to uridine triphosphate (UTP), and could be blocked by various drugs known to antagonize P2Y receptors (suramin; reactive blue 2; pyridoxal-phosphate-6-azophenyl-2',4' disulfonic acid [PPADS]). Treatment of the cells with uridine or UTP stimulated their accumulation of inositol phosphates, and this effect was also blocked by PPADS. Moreover, degradation of nucleotides by apyrase blocked the stimulatory effect of uridine on neuritogenesis. Taken together these data indicate that uridine can regulate the output of neurites from differentiating PC-12 cells, and suggest that it does so in two ways, i.e.,

both by acting through CTP as a precursor for PC biosynthesis and through UTP as an agonist for P2Y receptors.

Keywords: Phospholipid, Nucleotide, Receptors, Neurofilaments, Membrane

Introduction

The principal constituents of mammalian cell membranes are phosphatides, the most abundant of which is phosphatidylcholine (PC). PC biosynthesis is initiated by the phosphorylation of choline to form phosphocholine, which then combines with cytidine triphosphate (CTP) to form 5'-cytidine diphosphocholine (CDP-choline); this compound then reacts with diacylglycerol (DAG) to produce PC (Kennedy & Weiss, 1956). The rate at which cells form PC is affected by the availability of its precursors. Thus, uridine or cytidine increase CTP levels (Richardson et al. 2003); availability of CTP levels in turn can be rate-limiting in the syntheses of CDP-choline (Choy et al., 1980) and PC (Savci & Wurtman, 1995); and DAG levels can control the conversion of CDP-choline to PC (Araki & Wurtman, 1997).

CTP can be synthesized both from the phosphorylation of cytidine and from the pyrimidine nucleotide uridine triphosphate (UTP). Intracellular levels of UTP depend on those of free uridine (Wurtman et al., 2000). Recently, our laboratory demonstrated that treating undifferentiated rat pheochromocytoma (PC12) cells with uridine could increase intracellular levels of CTP, UTP and CDP-choline (Richardson et al., 2003). Hence, it seemed possible that uridine would also enhance the production of cellular membranes. Since exposing PC12 cells to nerve growth factor (NGF) stimulates both PC synthesis (Araki & Wurtman, 1997) and the extension of membrane-rich neurites, we hypothesized that increasing the availability of uridine might further promote neurite formation in these cells.

UTP produced in response to uridine treatment could also promote neuritogenesis by an additional mechanism unrelated to its role as a CTP precursor. Extracellular

nucleotides can act as trophic factors for glial and neuronal cells (Rathbone, 1999), and UTP can stimulate intracellular signaling pathways by activating the pyrimidine-sensitive, G-protein-coupled P2Y nucleotide receptors (Gallagher & Salter, 2003; Moskvina et al., 2003). Antagonism of these P2Y receptors inhibits NGF-induced neurite outgrowth in PC12 cells (Arslan et al., 2000); however, it has not been determined whether their stimulation enhances neurite formation. Thus, we also investigated whether, if uridine was found to stimulate neurite outgrowth, this effect might also depend on activation of P2Y receptors.

The present study shows that uridine treatment significantly increases neurite outgrowth and branching in NGF-differentiated PC12 cells and that this effect apparently involves both the CTP-mediated enhancement of PC synthesis and the activation of P2Y receptors.

Experimental Procedures

Cell culture. PC12 cells were maintained in Minimal Essential Medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37 °C. Cells were differentiated for 2 or 4 days in medium containing 50 ng/ml mouse 2.5S NGF, and 1% fetal bovine serum, with or without test compounds. When test compounds were dissolved in a vehicle, the same amount of that vehicle was also added to the control group. PC12 cells that were not exposed to NGF are called undifferentiated. NGF and fetal bovine sera were obtained from Invitrogen.

Neurite outgrowth studies. PC12 cells were sparsely plated on collagen-coated 60 mm culture dishes in MEM containing 1% fetal bovine serum. The experimental

treatments were as follows: uridine, uridine triphosphate, cytidine, reactive blue 2, suramin, PPADS and apyrase Grade VII (Sigma, St. Louis, MO). All treatments were added 24 hours after plating. At the end of the treatment period, images were obtained with a phase-contrast Zeiss Axioplan 2 microscope, using OpenLab software. Six digital images were captured for each dish, for a total of 18 to 24 images per treatment group. Approximately 300 cells were quantified for each treatment group for each experiment. Experiments were performed in triplicate unless otherwise noted. Quantification of neurites, including neurite branching and neurite length, was performed by one or usually more researchers blind to experimental treatments. Neurite length was measured using public domain NIH software Image J. Processes longer than the diameter of the cell body were counted as neurites, and only process-bearing cells were analyzed.

Western blot analysis. Following experimental treatments, the media were aspirated and cells were scraped in 100 µl lysis buffer which included the appropriate protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem, San Diego, CA). The total amount of protein in each sample was determined using the bicinchoninic acid assay (Sigma, St. Louis, MO). Prior to gel electrophoresis, bromphenol blue (0.07%) was added to each sample. Equal amounts of protein were loaded and separated using SDS-PAGE (4–20%; Bio-Rad, Hercules, CA). Proteins were then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA), which were blocked in 5% bovine serum albumin and incubated overnight with the antibody of interest (rabbit anti-neurofilament M and mouse anti-neurofilament 70 [both Chemicon, Temecula, CA]; rabbit anti-P2Y2 and anti-P2Y4 [Calbiochem]; or rabbit anti-P2Y6 [Novus Biologicals, Littleton, CO]). Following this incubation, membranes were

incubated for 1 hr with the appropriate peroxidase-linked secondary antibody (Sigma, St. Louis, MO). Protein–antibody complexes were detected and visualized using the ECL system (Amersham Biosciences, Piscataway, NJ) and Kodak X-AR film. Films were digitized using a Supervista S-12 scanner with a transparency adapter (UMAX Technologies, Freemont, CA). Analysis was performed using the public domain NIH Image program available on the internet at http://rsb.info.NIH.gov/nih-image/.

Analysis of nucleotides. Levels of intracellular UTP and CTP were analyzed by HPLC using an anion-exchange column, as previously described (Richardson et al., 2003).

Immunocytochemistry. PC12 cells were treated as described above, except they were grown on 12mm glass coverslips (A. Daigger & Co., Vernon Hills, IL) coated with collagen. Proteins were visualized using immunofluorescence. Briefly, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, blocked in 10% normal goat serum, and incubated overnight in the appropriate antibodies (mouse anti-neurofilament 70, and either rabbit anti-P2Y2, rabbit anti-P2Y4 or rabbit anti-P2Y6). Cells were then incubated in fluorochrome-conjugated secondary antibodies for 1 hour (goat anti-rabbit ALEXA 488 and goat anti-mouse ALEXA 568; Molecular Probes, Eugene, OR) and mounted on glass slides with mounting media with or without DAPI (Vector Laboratories, Burlingame, CA). Control antigens provided with the primary antibodies were used to ensure that immunostaining was specific. Digital images were obtained on a Zeiss (Oberkochen, Germany) Axioplan microscope with OpenLab software, using a Zeiss Plan-Neofluor 40x oil-immersion objective.

Metabolic labeling and PI turnover analysis. Analysis of phosphatidylinositol (PI) turnover was performed as described by Nitsch et al. (1997). Briefly, cells were labeled metabolically for 36h with 1.25 μCi/dish of myo-[2-^{3}H]inositol (17.0 Ci/mmol; Amersham Biosciences) in serum-free MEM, washed twice with Hank's balanced salt solution (HBSS), and treated for 15 min with 10 mM lithium chloride in HBSS. Drugs were added in the presence of 10 mM lithium for 60 min at 37°C. Cells were lysed with ice-cold methanol, and lipids were removed by extraction with chloroform/methanol/water (2:2:1; by volume). Labeled water-soluble inositol phosphates were separated from free [^{3}H]inositol by ion-exchange chromatography, using AG 1-X8 columns (Bio-Rad), and 1M ammonium formate and 0.1M formic acid as eluent. Radioactivity was quantified by liquid scintillation spectrometry.

Data Analysis. Data are presented as mean ± S.E.M. Analysis of variance (ANOVA) was used to determine differences between groups (significance level, $p<0.05$). When differences were detected, means were separated using the Newman-Keuls multiple range test.

Results

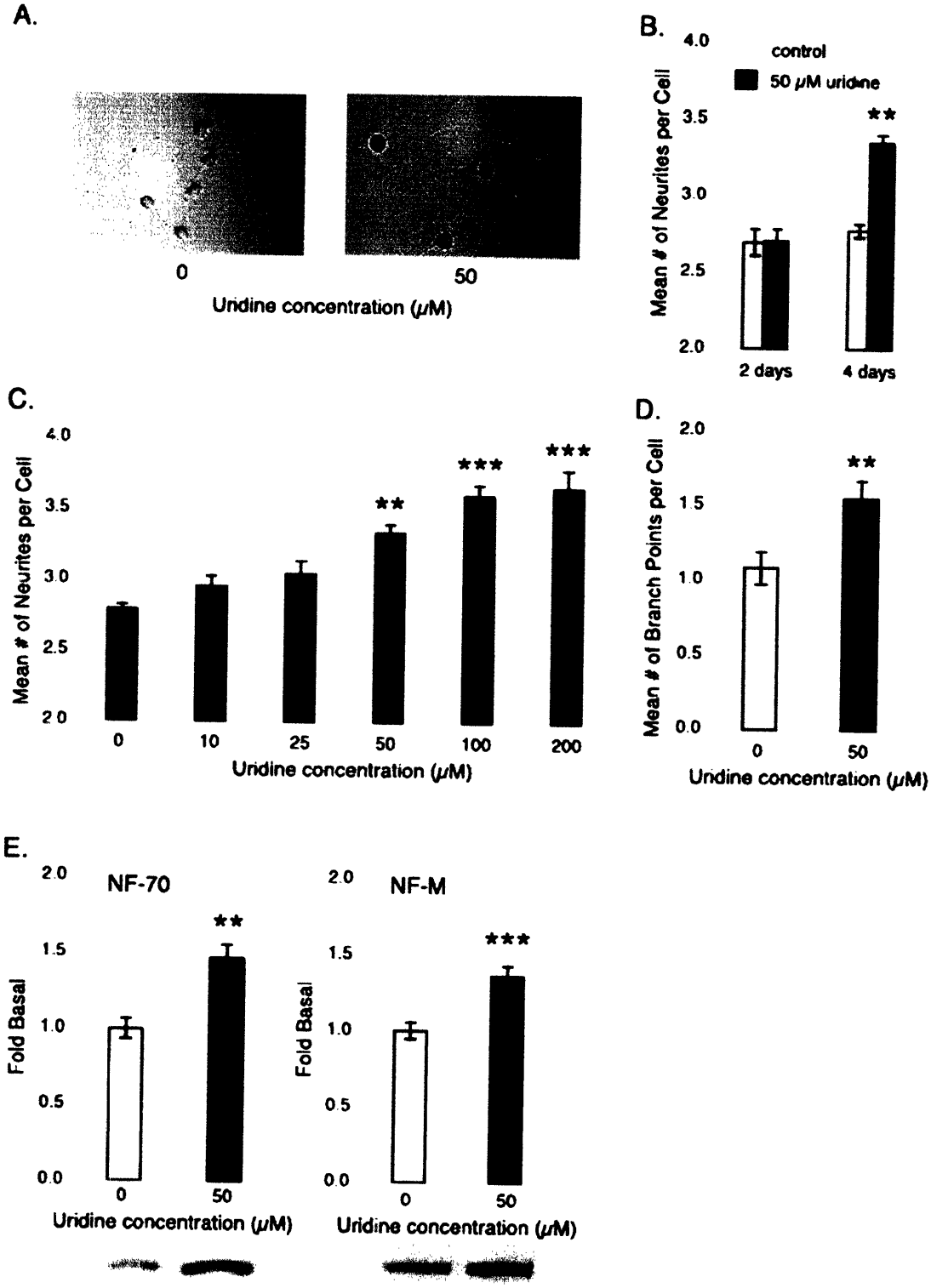
Extracellular uridine increases neurite outgrowth and neurite branching in NGF-differentiated PC12 cells.

Undifferentiated PC12 cells did not sprout neurites (fewer than 1%), whether or not they were exposed to uridine (50 μM; data not shown). In contrast, NGF-differentiated cells both produced neurites and exhibited a significant enhancement in neurite number when exposed to uridine (50 μM, $p<0.01$; 100 or 200 μM, $p<0.001$) for four days (Fig. 1 A-C). Shorter treatments (2 days) or lower uridine concentrations (10 μM or 25 μM) were ineffective, as was treatment with cytidine.

Exposure to uridine (50 μM for 4 days) significantly increased neurite branching ($p<0.01$; Fig. 1D), but had no effect on the average length of neurites (data not shown).

Uridine treatment enhances neurofilament expression in differentiated PC12 cells.

Neurofilament proteins are highly enriched within neurites (Lee et al. 1982); hence an increase in neurite number might be expected to be associated with increased expression of neurofilament proteins. We measured neurofilament 70 (70kD) and neurofilament M (145 kD) levels in differentiated PC12 cells using Western blotting, following 4 day treatment with uridine (50 μM) (Fig. 1E). Both neurofilament M and neurofilament 70 expression were significantly ($p<0.01$, $p<0.001$, respectively) increased following uridine treatment, compared with their levels in cells treated only with NGF. In undifferentiated cells, uridine treatment had no effect on levels of either neurofilament protein (data not shown).


A.
0
50
Uridine concentration (μM)
B.
control
50 μM uridine
Mean # of Neurites per Cell
4.0
3.5
3.0
2.5
2.0
**
2 days
4 days
C.
Mean # of Neurites per Cell
4.0
3.5
3.0
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2.0
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0
10
25
50
100
200
Uridine concentration (μM)
D.
Mean # of Branch Points per Cell
2.0
1.5
1.0
0.5
0.0
**
0
50
Uridine concentration (μM)
E.
NF-70
Fold Basal
2.0
1.5
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0
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Uridine concentration (μM)
NF-M
Fold Basal
2.0
1.5
1.0
0.5
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0
50
Uridine concentration (μM)

Figure 1. Uridine treatment enhanced neurite outgrowth in differentiated PC12 cells. PC12 cells were cultured in MEM supplemented with 1% fetal bovine serum, with 50 ng/mL NGF and in the presence of different concentrations of uridine. *A,* Differentiated PC12 cells in the absence (left image) or presence (right image) of uridine (50 μM, 4 day treatment). *B,* After 2 or 4 days of treatment, the number of neurites per cell was scored. Uridine treatment significantly enhanced neurite formation after 4 days, but no effect was observed after 2 days. *C,* Four-day uridine treatment (50, 100 and 200 μM) significantly increased the number of neurites produced during differentiation. *D,* The number of branch points was quantified for each cell. Uridine treatment significantly increased the amount of neurite branching after 4 days, compared with branching observed in control cells. *E,* Levels of the structural proteins neurofilament 70 and neurofilament M were determined using Western blotting. Treatment of differentiated PC12 cells with uridine (50 μM) for 4 days significantly increased the expression of these proteins relative to control. Values represent means ± SEM. ** $p < 0.01$, *** $p < 0.001$ vs. control.

Uridine increased intracellular UTP and CTP levels in differentiated PC12 cells.

In undifferentiated PC12 cells, the addition of exogenous uridine increases intracellular UTP and CDP-choline levels. (Richardson et al., 2003). To determine whether uridine also affects UTP or CTP levels in differentiated PC12 cells, we treated the cells for 2 days with NGF, with or without uridine, cytidine or UTP. Uridine (50 μM) significantly ($p<0.05$) increased both intracellular UTP and CTP levels (Figs. 2A, B, respectively) compared with levels in control cells. Exposure to UTP (100 μM) or cytidine (50 μM) had no effect.

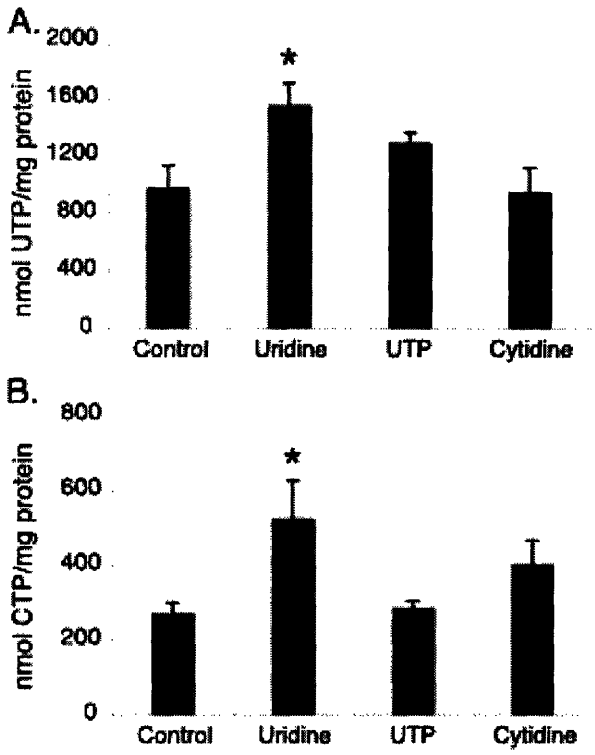

Figure 2. Uridine treatment increased intracellular levels of UTP and CTP in PC12 cells differentiated with NGF (50 ng/mL). Levels were determined using HPLC. Two-day uridine treatment (50 μM) significantly increased *A*, intracellular UTP levels and *B*, intracellular CTP levels. Neither UTP (100 μM) nor cytidine (50 μM) had a significant affect on either UTP or CTP levels within the cells. Values represent means ± SEM.
* $p < 0.05$ vs. control.

UTP treatment increased neurite outgrowth in differentiated PC12 cells.

Since uridine treatment increased UTP levels, and since PC-12 cells reportedly contain UTP-sensitive G-protein-coupled receptors (Arslan et al., 2000), we determined whether exogenous uridine may also modulate neuritogenesis via a direct, receptor-mediated action of UTP, in addition to acting via CTP. Differentiated PC12 cells were exposed to various concentrations of UTP (Fig. 3), for 4 days. UTP (10 and 50 μM) was found to be more effective than uridine itself inasmuch as both enhanced neurite outgrowth.

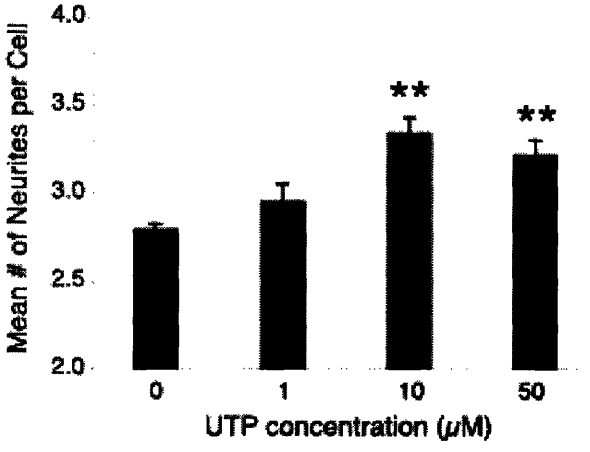

Figure 3. UTP treatment increased neurite outgrowth. Treatment of PC12 cells for 4 days with NGF (50 ng/mL) and different concentrations of UTP significantly enhanced the number of neurites produced per cell, compared to control. Values represent means ± SEM. ** $p < 0.01$ vs. control.

P2Y2, P2Y4 and P2Y6 receptors were expressed in differentiated PC12 cells.

Next, we investigated the mechanism by which extracellular UTP affects neurite outgrowth. UTP is an agonist of the pyrimidine-activated class of P2Y receptors, namely, P2Y2, P2Y4 and P2Y6 receptors. Previously, Arslan et al. (2000) demonstrated that differentiated PC12 cells contain mRNA for these P2Y receptors. To determine whether UTP-responsive P2Y receptor proteins are expressed in PC12 cells, we measured the levels of P2Y2, P2Y4 and P2Y6 receptors in undifferentiated cells, and cells exposed to NGF for 0 – 7 days. Expression of the P2Y2 receptor reached maximal levels after exposure to NGF for 3 days; these were significantly ($p<0.001$) higher than the levels observed in cells exposed for shorter periods. To visualize the expression and localization of the P2Y2, as well as the P2Y4 and P2Y6, receptors, we grew cells in the presence or absence of NGF for 4 days and then immunostained them for the neuritic marker neurofilament 70, and for P2Y2, P2Y4 or P2Y6 (Fig. 4B, left to right, respectively). For P2Y2 and P2Y4 visualization, control cultures were incubated with

primary antibody plus a control antigen in order to ensure that the immunostaining would be specific. Control antigen was not available for the P2Y6 receptor. All three receptors were highly expressed in differentiated PC12 cells (Fig 4B), but undetectable in the undifferentiated cells (data not shown).

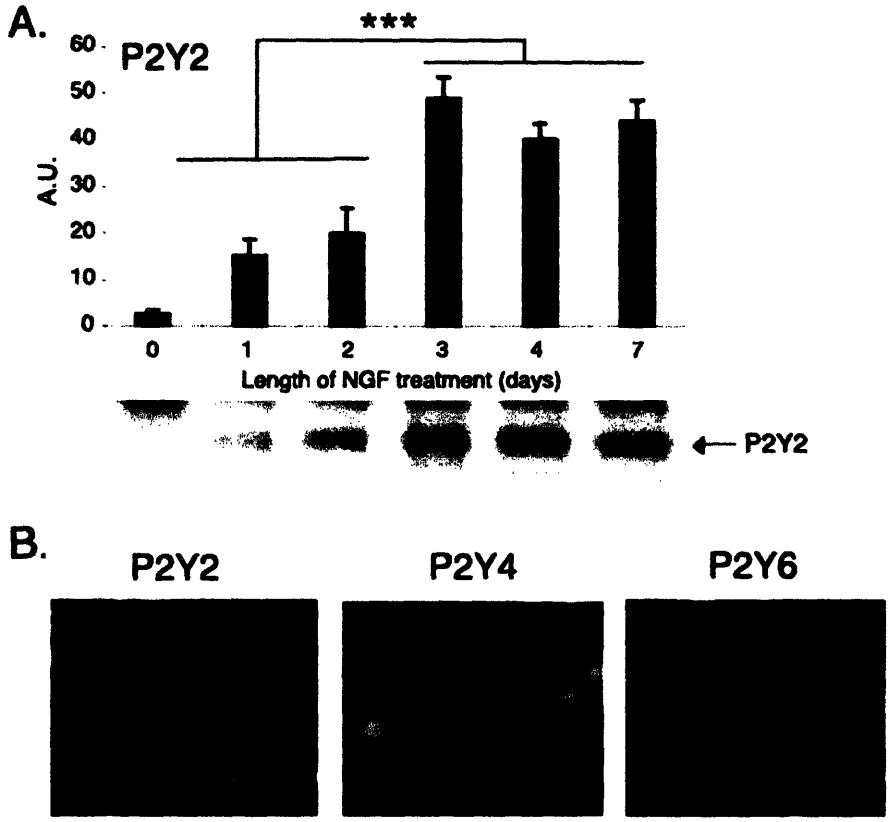

Figure 4. NGF-differentiated PC12 cells express pyrimidine-sensitive P2Y receptors. *A*, Cells were differentiated for various lengths of time. P2Y2 receptor expression was measured using Western blotting. After 3 days of differentiation, levels of P2Y2 receptor protein were significantly enhanced compared to fewer than 3 days of differentiation. *B*, Following 4 days of NGF differentiation, cells were fixed and neurofilament 70 (red) and P2Y receptor (green) proteins were visualized using immunofluorescence. From left to right: P2Y2, P2Y4 and P2Y6. Values represent means $\pm$ SEM. *** $p < 0.001$ vs. cells receiving fewer than 3 days of NGF treatment.

Antagonism of P2Y receptors inhibited the effect of uridine on NGF-induced neurite outgrowth.

To determine whether P2Y2, P2Y4 or P2Y6 receptors participate in the stimulatory effect of uridine on neurite outgrowth, we incubated differentiated PC12 cells with uridine (100 μM) and a P2Y receptor antagonist, chosen from suramin (30 μM), pyridoxal-phosphate-6-azophenyl-2',4' disulfonic acid (PPADS; 30 μM) or reactive blue 2 (RB-2; 10 μM) for 4 days. As before, uridine enhanced neurite outgrowth; each of the P2Y antagonists significantly ($p<0.05$ or 0.001) blocked this response (Fig. 5). It was not possible to determine which P2Y receptor or receptors (e.g. P2Y2, P2Y4 or P2Y6) mediates this effect, since no potent and selective antagonists exist at this time for individual pyrimidine-activated P2Y receptors. None of the P2Y receptor antagonists inhibited the uptake of uridine into the PC12 cells (data not shown).

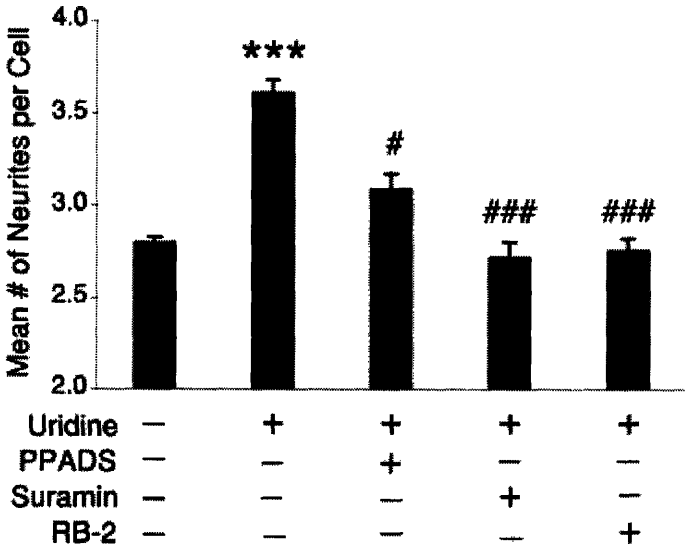

Figure 5. P2Y receptor antagonists inhibited the effect of uridine on neurite outgrowth. Cells were incubated for four days in MEM containing 50 ng/mL NGF and with or without uridine (100 μM), the P2Y receptor antagonists pyridoxal-phosphate-6-azophenyl-2',4' disulfonic acid (PPADS; 30 μM), suramin (30 μM) or reactive blue 2 (RB-2; 10 μM). All three antagonists significantly blocked the stimulatory effect of uridine on neurite outgrowth. Values represent means ± SEM. *** $p < 0.001$ vs. control; # $p < 0.05$, ### $p < 0.001$ vs. uridine treatment.

Uridine and UTP stimulated inositol phosphate formation.

P2Y2, P2Y4 and P2Y6 receptors are positively coupled to the phospholipase C/diacylglycerol/inositol triphosphate (PLC/DAG/IP3) signaling pathway. To determine whether concentrations of uridine or UTP that promote neurite outgrowth also are able to activate these receptors, we exposed differentiated PC12 cells labeled with [^{3}H]-inositol to uridine (50 μM) or UTP (10, 100 μM) for 1 hour, and then measured radiolabeled inositol phosphates (IP) formed as a result of [^{3}H]-phosphatidylinositol breakdown (Fig. 6). IP formation was significantly increased by 100 μM UTP ($p<0.05$) or 50 μM uridine ($p<0.01$) treatment. The P2Y receptor antagonist PPADS (100 μM) significantly ($p<0.05$) blocked the effect of UTP on IP formation.

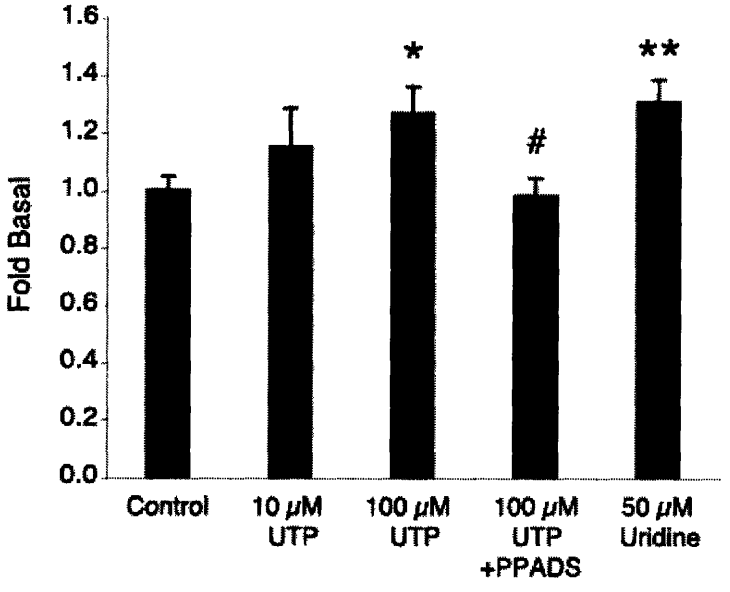

Figure 6. Phosphatidylinositol (PI) turnover is stimulated by UTP and uridine. Differentiated cells were metabolically labeled with [^{3}H]inositol overnight, stimulated with UTP (10, 100 μM), uridine (50 μM) or UTP plus PPADS (100 μM) in the presence of 10 μM lithium, and radiolabeled inositol phosphates derived from PI breakdown were measured by scintillation counting. Both UTP (100 μM) and uridine (50 μM) significantly increased inositol phosphate formation, and the effect of UTP was significantly blocked by co-incubation with the P2Y receptor antagonist PPADS. Values represent means ± SEM. * $p < 0.05$, ** $p < 0.01$ vs. control; # $p < 0.05$ vs. 100 μM UTP treatment.

Apyrase inhibited the effect of uridine on neurite outgrowth.

To determine whether uridine nucleotides are involved in uridine's effect on neurite outgrowth, we treated differentiated PC12 cells with uridine (50 μM) and apyrase (1 U/mL), which cleaves both tri- and diphosphate nucleotides. Degradation of nucleotides by apyrase blocked stimulation of neurite outgrowth by uridine ($p<0.05$); apyrase alone had no effect (Fig. 7).

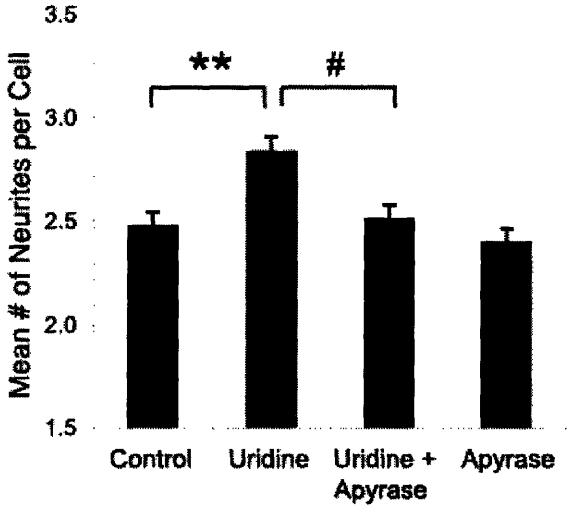

Figure 7. Apyrase treatment significantly inhibited the effect of uridine on neurite outgrowth. PC12 cells were incubated for four days in MEM containing 50 ng/mL NGF and with or without uridine (50 μM) or apyrase (1 U/mL). Values represent means ± SEM. ** $p < 0.001$ vs. control; # $p < 0.05$ vs. uridine treatment.

Discussion

These data show that uridine (50 – 200 μM) is able to enhance neurite outgrowth (Figs. 1B,C) and neurite branching (Fig 1D) in NGF-differentiated PC12 cells. The increase in neuritogenesis is accompanied by increases in cellular levels of the proteins neurofilament M and neurofilament 70, which are enriched in neurites. Uridine treatment increases cellular levels of CTP and UTP in the differentiated PC12 cells (Fig. 2), suggesting that one mechanism by which it enhances neurite outgrowth involves promoting membrane biosynthesis. Treatment of the PC12 cells with low concentrations of exogenous UTP, which is thought not to enter cells, also significantly increases neuritogenesis (Fig. 3), suggesting that cell-surface UTP-sensitive receptors are also involved in uridine's enhancement of neurite outgrowth. Since the pyrimidine-sensitive P2Y2, P2Y4 and P2Y6 receptors were found to be present on differentiated PC12 cells (Fig. 4), and since several P2Y receptor antagonists blocked the stimulatory effect of uridine on neurite outgrowth (Fig. 5), this set of receptors might be the site at which UTP

acts, a hypothesis supported by the finding that UTP (or uridine) also stimulated the formation of IP (Fig. 6), and that UTP's effect was blocked by a P2Y receptor antagonist. Furthermore, degradation of nucleotides (such as UTP) by apyrase abolished uridine's stimulatory effect on neuritogenesis (Fig. 7). Taken together, our data suggest that uridine promotes neurite outgrowth by two distinct mechanisms, 1) by increasing the availability of CTP, a limiting precursor in phosphatide biosynthesis (Savci & Wurtman 1995), and 2) by stimulating a P2Y receptor-coupled signaling pathway. Uridine is not unique in playing multiple roles in the cell; for example, intracellular DAG, can control PC synthesis both by combining with the enzyme that couples it to CDP-choline (Araki & Wurtman, 1997) and by acting as a second messenger in the DAG/IP3 signaling pathway (Li & Wurtman, 1998). It is not presently possible to determine the relative contributions of each of uridine's two actions to neurite outgrowth.

Previous studies in our laboratory have demonstrated that the rate of formation of new cellular membrane is dependent upon the availability of such membrane precursors as UTP, CTP, CDP-choline and DAG. CTP and DAG levels can, respectively, regulate PC synthesis in brain slices (Savci & Wurtman, 1995) and in cell culture (Araki & Wurtman, 1997). Degradation of nucleotides by apyrase inhibited the stimulation of neurite outgrowth by uridine (Fig. 7), indicating that nucleotides, such as UTP and CTP, are necessary for uridine's effect on neuritogenesis. The present study suggests that increasing the availability of UTP and CTP can also increase PC synthesis, and thus can increase the formation of new cell membrane in the form of neurites.

A growing body of evidence suggests that nucleotides and receptors for these compounds constitute signaling pathways in the brain. The P2 family of nucleotide

receptors includes ionotropic P2X receptors and G-protein-coupled P2Y receptors (Communi et al., 2000). A subset of the P2Y receptors, namely the P2Y2, P2Y4 and P2Y6, are pyrimidine-sensitive (Ralevic & Burnstock, 1998), and their activation releases calcium from intracellular stores via the diacylglycerol/inositol triphosphate (DAG/IP3) pathway (Arslan et al., 2000; Bofill-Cardona et al., 2000). The nucleotides and their receptors may have trophic effects in neurons (Liu et al., 2000), perhaps mediated by P2Y receptors, since increases in intracellular calcium levels have been associated with neuritogenesis (Gysbers et al., 2000). In NGF-differentiated PC12 cells, the addition of ATP or GTP enhances the percentage of cells that form neurites (D'Ambrosi et al., 2001; Gysbers et al., 2000), whereas inhibition of P2 receptors by antagonists prevents neuritogenesis (D'Ambrosi et al., 2000). Furthermore, a previous study suggested that UTP treatment also increases neurite sprouting (Gysbers et al., 1996), although the mechanism by which this occurred was not investigated. Another study (Silei et al., 2000) reported that uridine treatment increased the differentiation of neuroblastoma cells, but did not assess whether P2Y receptors were involved. In order to determine whether P2Y receptors participate in uridine's stimulation of neurite outgrowth in the present study, we treated differentiated PC12 cells with uridine and P2Y receptor antagonists, and found that the antagonists blocked uridine's effect (Fig. 5). Furthermore, uridine and UTP stimulated both neurite outgrowth (Figs. 1, 3) and inositol phosphate (IP) formation (Fig. 6), and these effects could also be inhibited by P2Y receptor antagonists. Hence our data suggest that some proportion of the effects of uridine and UTP on neurite production is mediated by activating pyrimidine-sensitive P2Y receptors. It is not presently possible to determine which specific receptor (P2Y2, P2Y4 or P2Y6) or combination of receptors

mediates this effect, since highly specific antagonists for individual P2Y receptors are not available.

Uridine has not been shown to bind directly to P2Y receptors, and its effects on these receptors probably require its conversion to extracellular UTP. The UTP could reach the receptors by release from the PC12 cells, or, alternatively, extracellular nucleotide diphosphokinases could form nucleotide triphosphates from uridine in the extracellular space (Lazarowski et al., 2000). Neurons, during normal cellular activity (Zhang et al., 2003), and astrocytes, following cell damage (Lazarowski et al., 1997), have been shown to release nucleotides (e.g. ATP) which can activate cell surface receptors (Koizumi et al., 2003). Therefore uridine could be taken up into the cell and converted to UTP, which would then be released into extracellular space where it could stimulate P2Y receptors. Since UTP is rapidly broken down following its release from the cell, its extracellular concentration is thus difficult to measure accurately (Lazarowski & Boucher, 2001). Regardless, the stimulation of IP accumulation caused by uridine (Fig. 6) suggests that, following uridine treatment, either one or both of the above mechanisms could operate to increase extracellular UTP levels, and thus to activate P2Y receptors. Moreover, the formation of IP3 requires activation of phospholipase C, an enzyme that may be involved in NGF-induced differentiation in PC12 cells (Rong et al., 2003).

When considering the possible role of uridine in neuron growth *in vivo*, it may be important to determine whether brain levels of uridine actually fluctuate, and the range of such fluctuations. In humans, consumption of CDP-choline causes dose-related increases in plasma uridine levels (Wurtman et al., 2000), suggesting that uridine levels, at least in

blood, can be modulated *in vivo*. In gerbils, increases in blood uridine produced by consuming uridine monophosphate causes parallel changes in brain uridine levels (M. Cansev, C.J. Watkins, personal communication). Currently, no data are available on possible effects of short-term (e.g. eating) or long-term (e.g. development; aging) processes on brain uridine levels.

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Chapter 5:

The HMG-CoA reductase inhibitor pravastatin enhances neurite outgrowth in hippocampal neurons

The HMG-CoA reductase inhibitor pravastatin enhances neurite outgrowth in hippocampal neurons

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ABSTRACT

Epidemiological studies demonstrate a relationship between statin (HMG-CoA reductase inhibitor) usage and a reduced risk of developing Alzheimer's disease. However, reduction in cholesterol levels does not reliably correlate with disease development, which suggests that statins may exert their beneficial effect via a cholesterol-independent mechanism. To determine whether statins affect the development of neurons in the hippocampus, we treated cultured rat hippocampal neurons with pravastatin, a potent HMG-CoA reductase inhibitor. After 4 – 48 hours of treatment, pravastatin significantly increased the number of neurites produced by each cell, and caused a corresponding increase in the levels of the membrane phospholipid phosphatidylcholine (PC). Pravastatin treatment also significantly increased neurite length and branching. Co-incubation with cholesterol did not inhibit the stimulatory effect of pravastatin on neurite outgrowth; by contrast, co-incubation with mevalonate abolished the effect. Statins inhibit isoprenoid formation in addition to inhibiting cholesterol synthesis. Members of the Rho family of small GTPases are activated by isoprenylation, and regulate cell growth and neurite extension. We found that treatment of the neurons with the isoprenoids FPP or GGPP abolished the effect of pravastatin on neurite growth; furthermore, a specific inhibitor of geranylgeranylation mimicked the stimulatory effect of pravastatin on neuritogenesis. Pravastatin-treated neurons also contained significantly lower membrane-associated and total RhoA levels, compared with levels in control cells. Co-incubation with cholesterol did not prevent the reduction in RhoA levels caused by pravastatin, whereas mevalonate, FPP and GGPP prevented this effect. These data suggest that pravastatin treatment increases neurite outgrowth and may do so via inhibition of RhoA.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that results in memory loss and cognitive decline. In addition to their characteristic Aβ-enriched amyloid plaques and neurofibrillary tangles, the brains of AD patients display pathologies such as synapses loss and neuritic dystrophy (McKee et al., 1991). *In vitro*, the presence of Aβ peptide alone can cause retraction and deterioration of neuritic processes (Tohda et al., 2004). In transgenic mice, it is possible to reverse neuritic dystrophy through infusion of anti-Aβ antibody (Brendza et al., 2005). However, the mechanism by which Aβ may cause neuritic deterioration is not known. This finding suggests that treatments targeting the restoration of neuritic growth might be efficacious in repairing or preventing the underlying neurodegeneration caused by AD.

Epidemiological studies have found an inverse relationship between usage of the cholesterol-lowering drugs and risk of developing AD (Wolozin et al., 2000; Rockwood et al., 2002; Zamrini et al., 2004). Statins are inhibitors of the enzyme HMG-CoA reductase, which converts HMG-CoA into mevalonate; this is the rate-limiting step in cholesterol biosynthesis (Hamelin and Turgeon, 1998). However, reduction of cholesterol levels may or may not correlate with reduced risk of AD in patients taking statin drugs (Wood et al., 2003; Wolozin, 2004; Eckert et al., 2005). Furthermore, statin usage is associated with a decreased risk of depression and anxiety, which is not correlated with plasma cholesterol levels (Young-Xu et al., 2003). Oral administration of statins, in addition to inhibiting cholesterol synthesis, also affects gene expression in the mouse brain (Johnson-Anuna et al., 2005). Thus, statins might prevent onset of AD by a mechanism independent of their effect on cholesterol.

Mevalonate is required not only for cholesterol synthesis, but also for formation of the isoprenoids geranylgeranylpyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) (reviewed by (Rando, 1996)). Members of the Rho family of small GTPases are activated when isoprenylated by these compounds (Zhang and Casey, 1996), and regulate cell growth and neurite extension by reorganizing the actin cytoskeleton (reviewed by (Takai et al., 2001)). RhoA is negatively coupled to cell growth; thus, prevention of RhoA isoprenylation increases neurite extension (Sebok et al., 1999). Statins inhibit isoprenylation of Rho proteins in neuronal cells (Meske et al., 2003; Pedrini et al., 2005) and cultured microglia (Bi et al., 2004; Cordle and Landreth, 2005). However, it is not known whether statins can affect neurite outgrowth, and whether such an effect might be mediated by regulation of Rho activition.

In the present study, we investigated the effect of the HMG-CoA reductase inihibitor pravastatin on neurite growth in primary cultured hippocampal neurons. We found that treatment of neurons with pravastatin enhances neurite number, length and branching, and that this effect is probably mediated by inhibition of Rho isoprenylation. These data suggest that the ability of statins to reduce AD risk may be due, in part, to inhibition of isoprenoid formation and subsequent prevention of neuritic dystrophy and deterioration.

MATERIALS AND METHODS

Preparation of Hippocampal Cultures Cultures of rat hippocampal neurons were prepared as described by (Malgaroli and Tsien, 1992), with a few modifications. Rat

pups, ages P1 to P3, were decapitated. The brains were placed in a dish containing Hank's Balanced Salt Solution Ca^{++} and Mg^{++} free (HBSS; Sigma, St. Louis, MO) with Fetal Bovine Serum (FBS; 20%) and the hippocampi dissected. The hippocampi were then incubated with digestion solution (0.5 % trypsin/0.05% Dnase/137 mM NaCl/5 mM KCl/7 mM Na_2HPO_4/25 mM HEPES) for 5 min at 37 °C. The supernatant was removed, then the cell pellet was resuspended in 0.05% Dnase/3% $MgSO_4$/HBSS and triturated using a flame-narrowed pasteur pipette. After centrifugation, the supernatant fluid was aspirated and the cell pellet resuspended in Minimum Essential Medium (MEM; Gibco; Rockville, MD) containing glutamine (3 mg/ml), insulin (0.25 mg/ml) and FBS (20%). The cells were then plated onto 35mm cell culture dishes, coated with Matrigel (Collaborative Research, Inc; Bedford, MA). After 48 hours of incubation, the media were removed and replaced with MEM containing glutamine (3 mg/ml), B-27 (1%) (Gibco) and cytosine arabinoside (Ara-C; 0.3%) (Sigma), to arrest proliferation of non-neuronal cells. After 7 days in culture, the cells were incubated for various lengths of time in media containing pravastatin (0.01 – 200 μM; LKT, St. Paul, MN), cyclodextrin-bound cholesterol (10 or 20 μg/ml), mevalonate (1 mM), geranylgeranylpyrophosphate (20 μM), farnesylpyrophosphate (20 μM), GGTI-286 (50 μM), or methyl-β-cyclodextrin (MβCD; 50 μM). Once a compund was added to the culture it was not withdrawn, with the exception of MβCD. To acutely extract cholesterol from the cultures, we incubated the neurons for 30 min in media containing 50 μM MβCD. After the incubation, we aspirated the treatment, replaced it with fresh media, and analyzed neurite growth 24 hours later. All treatments longer than 48 hours were refreshed every 48 hours. All compounds from Sigma unless indicated otherwise.

Neurite morphology For experiments examining the effect of a treatment on neurite development, neurons were incubated with media containing the appropriate test compound for 2 to 48 hours. Following the treatment period, phase-contrast digital images of the cells were taken using a Zeiss Axioplan 2 Fluorescent/Phase- Contrast microscope (Carl Zeiss, Germany). Images were captured using a Photometrics digital camera attached to the microscope and controlled through an Apple G4 computer running OpenLab software. Digital images of neurons were analyzed for neurite number, length, and branching. A neurite was defined as a process that is longer than the width of the cell body. These measurements were taken by an assistant blind to experimental treatment. Four to six images were taken per dish; approximately 100 neurons were quantified per treatment group, per experiment. Each experiment was performed in triplicate, unless otherwise noted.

Phospholipid quantification Following experimental treatment, the media were removed, and the cells harvested in methanol and disrupted using a sonicator (Ultrasonics Inc.). An aliquot of homogenate was removed for total protein determination. The remaining homogenate was extracted with a methanol:chloroform:water (1:2:1) mixture. After centrifugation, the aqueous phase was aspirated and the organic phase dried in a vacuum centrifuge. The pellets were resuspended in a methanol:chloroform solution (1:1) and loaded onto a prechanneled silica plate (Adsorbasil Plus 1, 250 mm; Alltech, Nicholasville, KY). The lipids were separated by running the plate for 2 hours using a chloroform/ethanol/triethylamine/ water (30:34:30:8) mobile phase. After separation, the bands were visualized under UV light by spraying the plate with a diphenyl-

hexatriene/ether solution. The bands of interest were scraped into glass tubes and subjected to perchloric acid digestion (3 hours; 160 °C) followed by determination of total phosphate content using a colorimetric assay. Phosphate values were normalized for total protein content.

Cholesterol determination Plasma-membrane cholesterol was visualized using the cholesterol-binding antibiotic filipin (Sigma). Briefly, hippocampal neurons grown on 12mm glass coverslips were treated with either cholesterol or pravastatin for 24 hours, then fixed with 4% paraformaldehyde + sucrose for 30 min at room temperature. After washing with PBS, the cells were incubated in filipin solution (50μg/ml) for 30 min at room temperature in the dark. The coverslips were washed with PBS and mounted on slides. Images were acquired with Zeiss Axioplan 2 Fluorescent/Phase-Contrast microscope (Carl Zeiss) using a DAPI filter set. Digital images were obtained as described above and were analyzed with the NIH software ImageJ.

Cell fractionation and immunoblotting For whole-cell lysates, neurons were scraped into 100 μl lysis buffer (60 mM Tris–HCl, 20% glycerol, 1 mM dithiothreitol, plus a protease inhibitor cocktail containing: 1 mM AEBSF, 8 μM Aprotinin, 500 μM Bestatin, 15 μM E64, 200 μM Leupeptin, 10 μM Pepstatin A) and boiled (10 min). For subcellular fractionation, cells were processed as described in (Tanaka et al., 2000), with a few modifications. Briefly, neurons were harvested in hypotonic buffer (5 mM Tris-HCl, 5 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 1 mM $MgCl_2$, 2 mM DTT) including the protease inhibitor cocktail described above. The cell suspension was disrupted using a stroke

sonicator (approximately 20 strokes) and separated into membrane and cytosolic fractions by centrifugation (100,000 x *g*, 30 min). The pellet containing the membrane fraction was resuspended in 50 μl lysis buffer and boiled (10 min). For immunoblotting, the total amounts of protein in each sample were determined by the Bicinchoninic Acid Assay (Smith et al., 1985) (Sigma; St. Louis, MO). Equal amounts of protein were loaded and separated using SDS-PAGE (4–20%) (Cambrex, Rockland, ME). Proteins were then transferred onto polyvinylidene difluoride membranes (Millipore; Bedford, MA). The membranes were then blocked in 5% bovine serum albumin/Tris-buffered saline/0.15% Tween 20 (TBST) for 2 hr at room temperature. The membranes were washed briefly in TBST and incubated overnight (4°C) in rabbit polyclonal RhoA primary antibody or mouse monoclonal β-tubulin primary antibody (Santa Cruz Biotechnology; Santa Cruz, CA). Following this incubation, membranes were washed with TBST (3X; 10 min) and incubated for 1 hour with a peroxidase-linked secondary antibody. Protein–antibody complexes were detected and visualized using the ECL system (Amersham, Piscataway, NJ) and Kodak X-AR film as described by the manufacturer. Specificity of RhoA staining was confirmed using a blocking peptide: when the primary RhoA antibody was combined with the commercially available blocking peptide, no band was visible. Films were digitized using a Supervista S-12 scanner with a transparency adapter (UMAX Technologies, Freemont, CA) and analyzed using the public domain NIH Image program (developed at the U.S. National Institute of Health) and available on the internet at http://rsb.info.NIH.gov/NIH-IMAGE/. The optical densities of the experimental groups are normalized to the average optical density of the control group. All values are expressed as fold-basal increases or decreases compared with controls.

Data analysis Data were analyzed using Analysis of Variance (ANOVA) using treatments as the independent variable. When differences were detected ($P \leq 0.05$), the means were separated using the Newman-Keuls test. Values represent mean ± S.E.M.

RESULTS

The HMG-CoA reductase inhibitor pravastatin reduces cellular cholesterol levels

Primary cultured rat hippocampal neurons (7 days *in vitro*) were incubated for 24 hours in media containing either the HMG-CoA reductase inhibitor pravastatin (50 or 200 μM) or cholesterol (10 μg/mL). We used cyclodextrin-bound cholesterol, since it is water-soluble and has been demonstrated to increase cellular cholesterol levels (Christian et al., 1997; Kenworthy et al., 2004). Following incubation, the cells were fixed and stained with the fluorescent cholesterol-binding antibiotic filipin (Fig. 1). Cholesterol treatment increased the intensity of filipin staining (Fig. 1A), whereas pravastatin treatment decreased the filipin signal (Figs. 1C and 1D) compared to the fluorescence intensity observed in control cells (Fig. 1B).

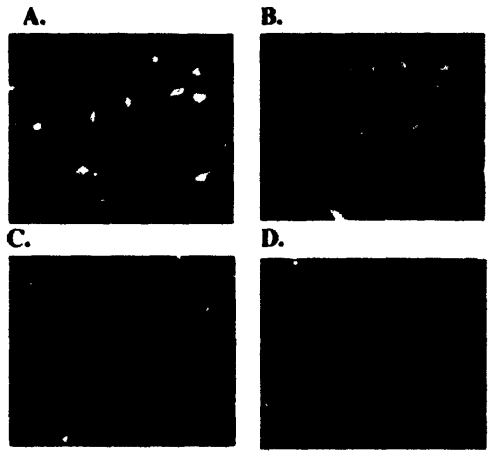

Figure 1. Hippocampal neurons were treated with either cholesterol or the cholesterol inhibitor pravastatin and stained with the cholesterol-binding antibiotic filipin. Brighter staining corresponds to higher cholesterol concentration. **A.** After 24 hour cholesterol (10 μg/ml) treatment, neuronal membrane levels of cholesterol appear noticeably higher than in control cells (**B**). Membrane cholesterol is visibly reduced following 24 hour treatment with **C.** 50 μM pravastatin or **D.** 200 μM pravastatin.

Pravastatin increases neurite outgrowth in hippocampal neurons

Pravastatin treatment significantly enhanced neurite outgrowth in rat hippocampal neurons (7 DIV). Twenty-four hour treatment with pravastatin (50 – 100 μM) significantly increased the number of neurites produced per cell (Figs 2A and B). Pravastatin (100 μM) visibly enhanced neuritogenesis as early as four hours after the start of treatment (Fig 2C), relative to neurite outgrowth observed in control cells. Neuritic processes are highly enriched with membrane phospholipids; hence an increase in neurite number might be associated with increased levels of phosphatidylcholine, a major component of the cell membrane. We found that 24 hour treatment of neurons with pravastatin (50 μM) significantly increased phosphatidylcholine levels (Fig. 2D).

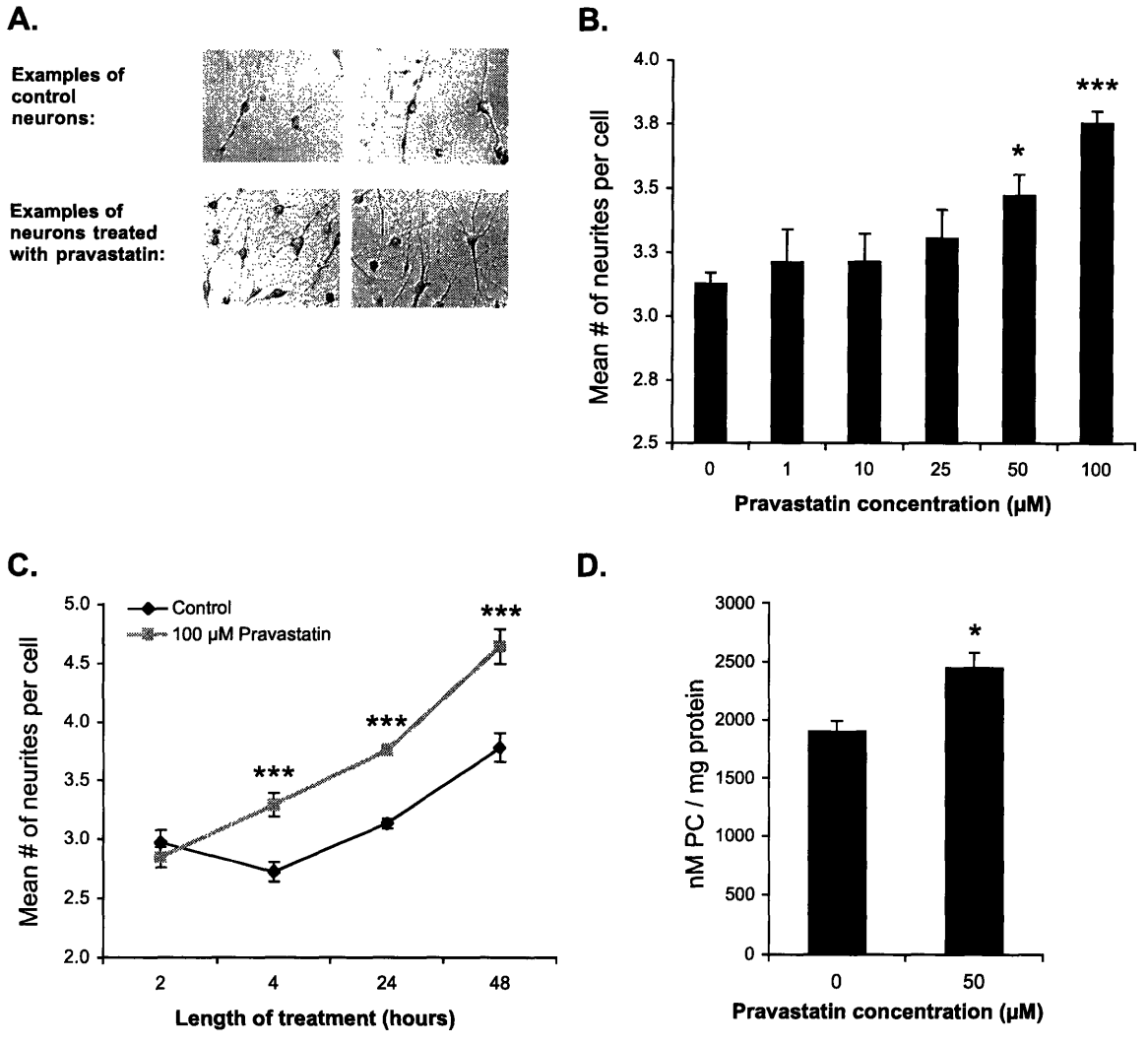

Figure 2. The HMG-CoA reductase inhibitor pravastatin enhances neurite outgrowth in rat hippocampal neurons (7 DIV). **A.** Examples of neurons grown for 24 hours either in the absence (top two panels) or presence of 200 μM pravastatin (bottom two panels). **B.** After 24 hours of pravastatin treatment, phase contrast digital images of the neurons were taken at fixed points along the dish. Pravastatin treatment (50, 100 μM) significantly increased the number of neurites produced per cell, relative to the number of neurites observed on control cells. **C.** Pravastatin (100 μM) significantly enhanced neurite outgrowth after 4, 24 and 48 hours of treatment. Treatments began when neurons were 7 DIV. **D.** Levels of the membane phospholipid phosphatidycholine were determined to confirm pravastatin-induced changes in cell area. Twenty-four hour pravastatin treatment (50 μM) enhanced PC levels relative to levels in control cells.
Values represent means +/- S.E.M.; * $p<0.05$; ***$p<0.001$ vs control.

Pravastatin increases neurite length and branching

Besides quantifying the number of neurites produced per cell, we also measured the effect of pravastatin on neurite length and branching. Following 24 hour pravastatin treatment (100 μM), both the number of branches per neurite and neurite length were significantly enhanced (Fig. 3A and B, respectively), compared with branching and length observed in untreated cells.

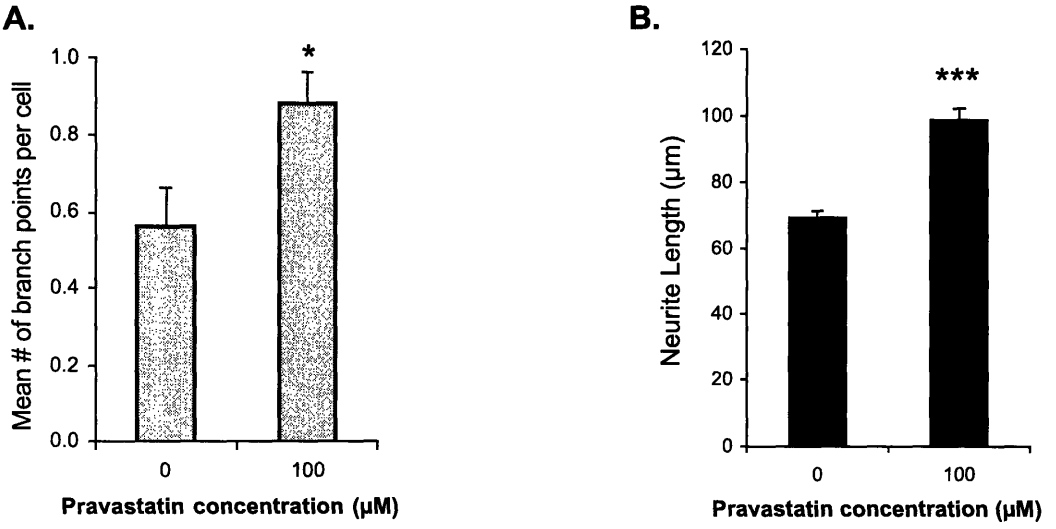

Figure 3. Pravastatin significantly increases neurite length and branching. Hippocampal neurons were treated with the HMG-CoA reductase inhibitor pravastatin for 24 hours. **A.** Pravastatin (100 μM) significantly increased the mean number of branch points per neurite relative to the number of branch points observed in control cells. **B.** Pravastatin (50 or 100 μM) also significantly enhanced neurite length.
Values represent means +/- S.E.M.; * $p<0.05$; ***$p<0.001$ vs control.

The effect of pravastatin on neuritogenesis is not prevented by cholesterol treatment

Pravastatin, as an HMG-CoA reductase inhibitor, blocks formation of mevalonate, which is required not only for cholesterol synthesis, but also for formation of isoprenoids (Fig. 4).

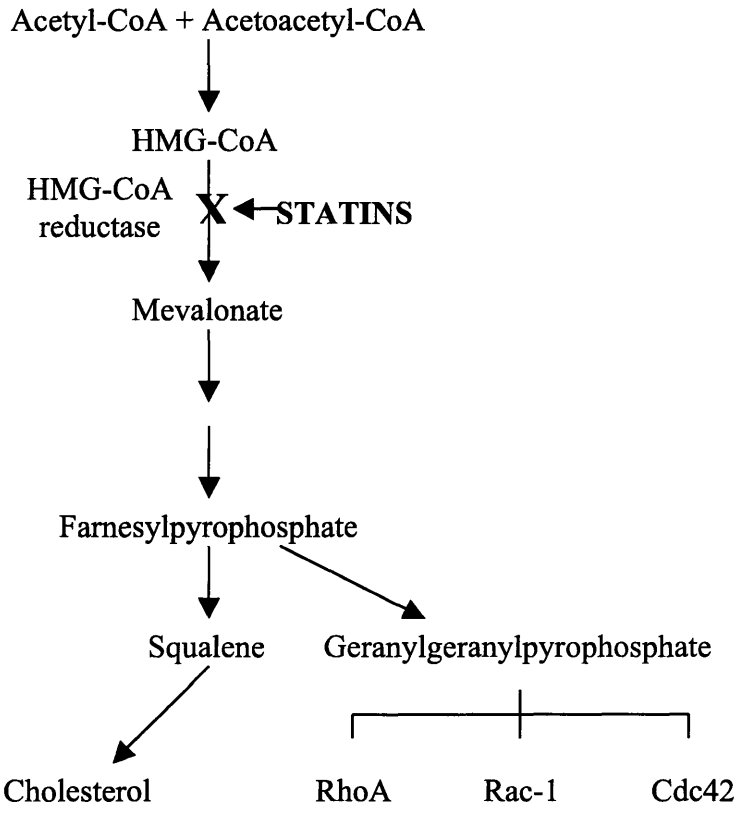

Figure 4. Statins, such as pravastatin, inhibit HMG-CoA reductase activity and therefore prevent mevalonate formation. In addition to reducing cholesterol synthesis, statins inhibit formation of the isoprenoids farnesylpyrophosphate and geranylgeranylpyrophosphate. (Adapted from Stamatakis et al., 2002).

In order to determine whether pravastatin might be stimulating neurite outgrowth by inhibiting cholesterol synthesis, we co-incubated neurons with pravastatin (100 μM) and cholesterol (20 μg/mL). We found that cholesterol did not block the increase in neurite number or length caused by pravastatin (Figs. 5A and C); cholesterol alone had no effect on neuritogenesis. Furthermore, depletion of cholesterol by methyl-β-cyclodextrin (MβCD; 30 minute treatment) did not mimic the effect of pravastatin on

neurite outgrowth; instead, it significantly inhibited neurite outgrowth relative to growth from control cells (Fig. 5A). However, co-incubation of neurons with mevalonate (1 mM) prevented stimulation of neurite growth by pravastatin (Figs. 5B and C), suggesting that the effect of pravastatin is due to specific inhibition of mevalonate synthesis, and not to inhibition of cholesterol synthesis.

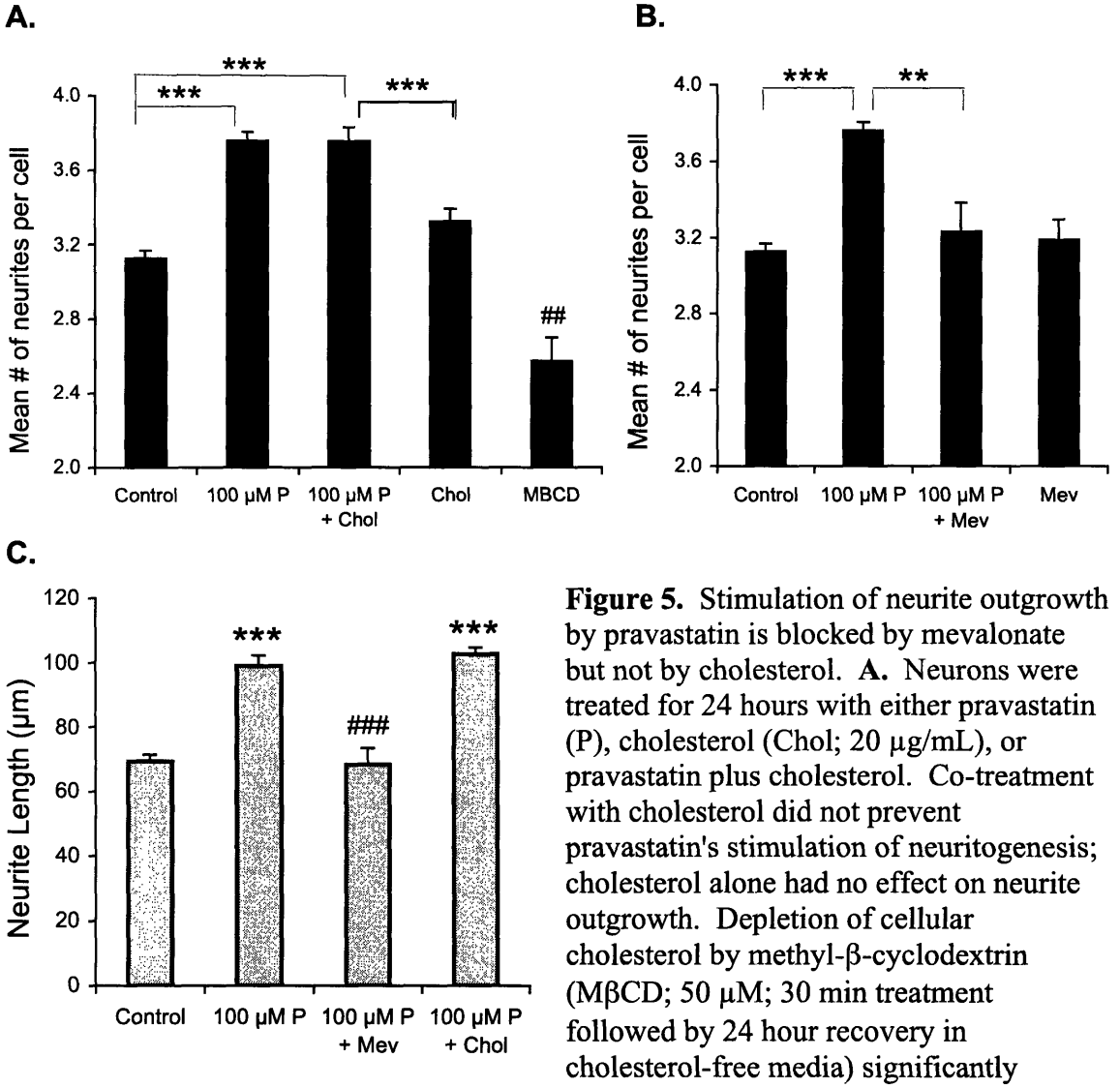

Figure 5. Stimulation of neurite outgrowth by pravastatin is blocked by mevalonate but not by cholesterol. **A.** Neurons were treated for 24 hours with either pravastatin (P), cholesterol (Chol; 20 µg/mL), or pravastatin plus cholesterol. Co-treatment with cholesterol did not prevent pravastatin's stimulation of neuritogenesis; cholesterol alone had no effect on neurite outgrowth. Depletion of cellular cholesterol by methyl-β-cyclodextrin (MβCD; 50 µM; 30 min treatment followed by 24 hour recovery in cholesterol-free media) significantly inhibited neurite outgrowth, compared to outgrowth in control cells. **B.** Incubation of neurons with mevalonate (Mev; 1 mM) significantly inhibited the stimulatory effect of pravastatin on neurite outgrowth; mevalonate alone had no effect. **C.** Mevalonate, but not cholesterol, significantly blocked the increase in neurite length stimulated by pravastatin.
Values represent means +/- S.E.M.; ** $p<0.01$; ***$p<0.001$. ##$p<0.01$ vs control, ###$p<0.001$ vs pravastatin-treated group.

Pravastatin enhances neuritogenesis via inhibition of isoprenylation

Mevalonate is required for synthesis of isoprenoid lipids like farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Rho-GTPases are activated when FPP or GGPP attaches to their C-terminus, allowing the GTP-ase to associate with the cell membrane. In order to determine whether pravastatin's stimulation of neuritogenesis involves inhibition of isoprenylation, we co-incubated neurons with pravastatin (100 μM) and either FPP (20 μM) or GGPP (20 μM). Both FPP and GGPP blocked the effect of pravastatin on neurite outgrowth (Fig. 6A). Furthermore, treatment of neurons with GGTI-286, a specific inhibitor of geranylgeranylation, mimicked the effect of pravastatin and significantly enhanced both neurite number (Fig. 6B) and neurite length (data not shown).

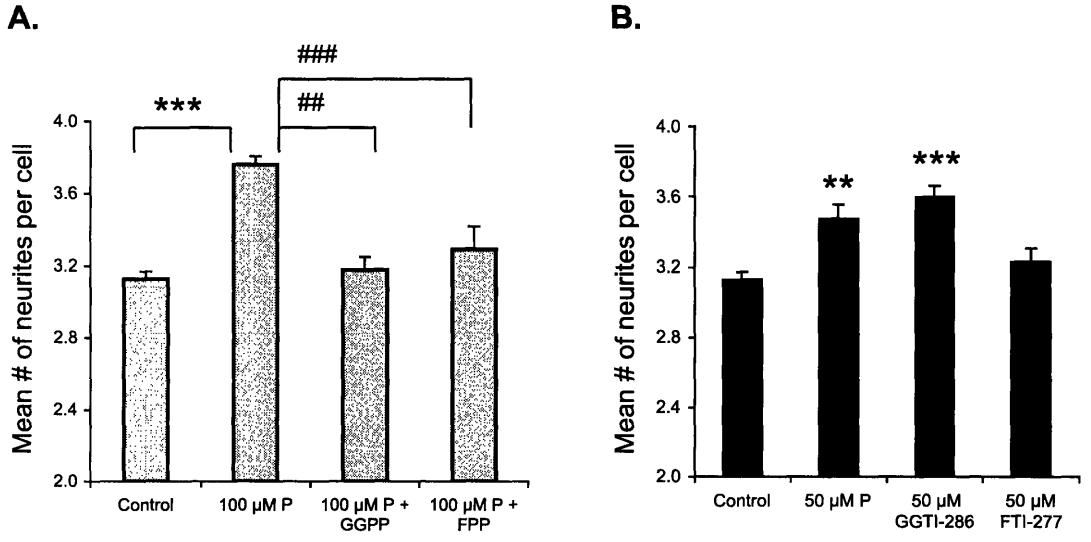

Figure 6. Pravastatin may stimulate neurite outgrowth via inhibition of isoprenoid formation. **A.** Co-treatment of hippocampal neurons with pravastatin and geranylgeranylpyrophosphate (GGPP; 20 μM) or farnesylpyrophosphate (FPP; 20 μM) blocked the stimulatory effect of pravastatin on neurite outgrowth. **B.** Incubation of neurons with GGTI-286, a highly specific inhibitor of geranylgeranylation, significantly increased neurite outgrowth and neurite length (data not shown), whereas FTI-277, a farnesyltransferase inhibitor, had no effect on either of these measures.
Values represent means +/- S.E.M.; **p<0.01, ***p<0.001 vs control. ##p<0.01, ###p<0.001 vs pravastatin-treated group.

Pravastatin affects RhoA translocation and expression

RhoA is an active GTPase when bound to the cell membrane, a process that requires isoprenylation by gernaylgeranylpyrophosphate. Following 24-hour pravastatin treatment, cell membranes were separated from total cell lysates by centrifugation. Levels of membrane-bound RhoA were significantly decreased by 24-hour pravastatin treatment (Fig. 7A). Whole-cell lysates were also subjected to separation by SDS-PAGE after neurons were treated with various compounds for 24 hours. Total RhoA expression was decreased by either pravastatin (100 μM) or GGTI-286 (50 μM) (24 hour treatment) relative to levels in control cells. In pravastatin-treated cells, co-incubation with either mevalonate (1 mM), GGPP (20 μM) or FPP (20 μM) prevented the reduction in RhoA levels; co-incubation with cholesterol (20 μg/mL) had no effect (Fig. 7B).

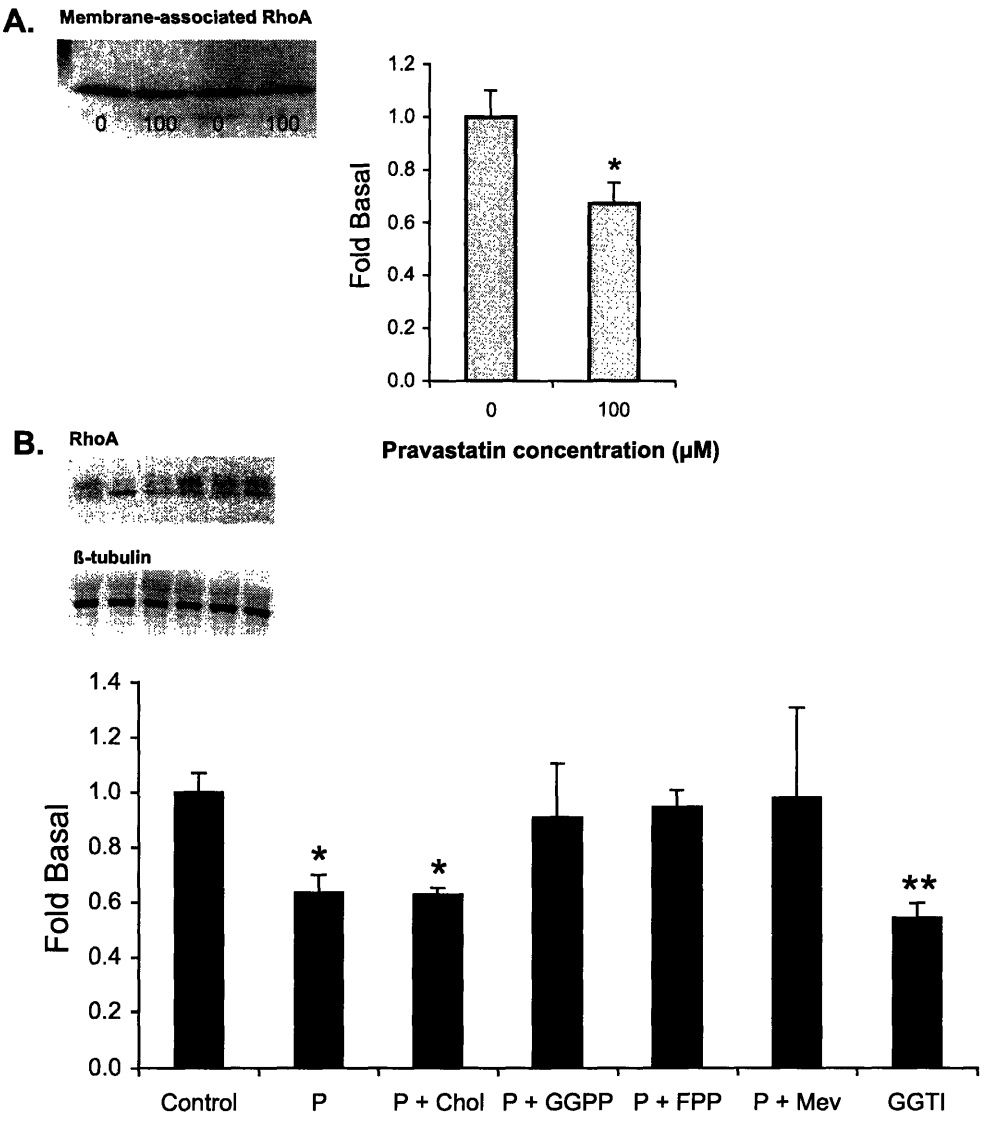

Figure 7. Pravastatin reduces membrane-asscociated RhoA levels and total RhoA expression. **A.** Following 24-hour pravastatin treatment, cell membranes were separated from total cell lysates by centrifugation. The membrane fraction was separated by SDS-PAGE and probed with an anti-RhoA antibody (left). Membrane-associated RhoA levels were decreased by pravastatin treatment (right). **B.** (Top) Representative Western blot of whole-cell lysates probed with antibodies directed against RhoA (top) or β-tubulin (bottom). Treatments are in the following order: control, pravastatin (100 μM), pravastatin plus cholesterol (20 μg/mL), pravastatin plus GGPP (20 μM), pravastatin plus FPP (20 μM), pravastatin plus mevalonate (1 mM). (Bottom) Pravastatin treatment (P; 100 μM) and GGTI-286 (GGTI; 50 μM) significantly inhibited RhoA expression. The effect of pravastatin was not prevented by co-incubation with cholesterol; however, mevalonate (Mev), GGPP and FPP abolished it.
Values represent means +/- S.E.M.; *$p<0.05$, **$p<0.001$ vs control.

DISCUSSION

These data show that the HMG-CoA reductase inhibitor pravastatin (50 – 100 μM) enhances neurite outgrowth (Figs. 2B and C), neurite length (Fig. 3A) and neurite branching (Fig. 3B) in cultured hippocampal neurons; this effect occurred as early as four hours after exposure and continued for up to two days (Fig. 2C). The increase in neurite outgrowth was accompanied by increased levels of the membrane phospholipid phosphatidylcholine (Fig. 2D). The stimulatory effect of pravastatin on neurite growth was not affected by cholesterol (Fig. 5A and B), whereas co-treatment with mevalonate (Fig. 5C and D) significantly inhibited this effect. These results suggest that pravastatin may stimulate neuritogenesis not by inhibiting cholesterol synthesis, but rather by a cholesterol-independent mechanism. To determine whether pravastatin might be stimulating neurite outgrowth by inhibiting formation of isoprenoids known to regulate Rho-GTPases, we co-treated neurons with pravastatin and either GGPP or FPP. Both GGPP and FPP completely prevented pravastatin's stimulation of neurite outgrowth (Fig. 6A). Furthermore, specific inhibition of geranylgeranylation by GGTI-286 mimicked the effect of pravastatin by significantly enhancing neurite number and neurite length, relative to the number and length of neurites observed in control cultures (Fig. 6B). By contrast, the farnesyltransferase inhibitor FTI-277 had no effect on neurite outgrowth, which suggests that geranylgeranylated proteins, but not farnesylated proteins, regulate neurite outgrowth. To investigate whether pravastatin treatment might affect levels of the Rho-GTPase RhoA, we measured RhoA in neurons treated with pravastatin, either alone or with cholesterol, mevalonate, GGPP or FPP. Pravastatin significantly reduced membrane-associated RhoA levels, as well as total RhoA expression. The effect of

pravastatin on total RhoA expression was prevented by mevalonate, GGPP and FPP, but not by cholesterol (Fig. 7). Taken together, our data suggest that pravastatin promotes neurite outgrowth by a cholesterol-independent mechanism, specifically by preventing isoprenylation of Rho-GTPases.

Here, we determined that the observed effect of pravastatin on neurite outgrowth was not due to cholesterol reduction, since enriching the media with cholesterol did not prevent pravastatin's stimulation of neuritogenesis. Furthermore, treatment of neurons with cholesterol in the absence of pravastatin had no effect on neurite growth, although the concentration of cholesterol used was similar a concentration reported to enhance synaptogenesis (Mauch et al., 2001). Previous studies present conflicting findings: different HMG-CoA reductase inhibitors have been shown to inhibit neurite outgrowth, either by reducing cholesterol levels (Fan et al., 2002) or by inhibition of isoprenylation (Schulz et al., 2004). However, these studies only observed neurite loss after 48 hours of statin (either compactin or atorvastatin, respectively) treatment, and started treatment within 24 hours following plating of primary neuronal cells. In the present study, we grew the neurons in culture for 7 days before starting treatment to better understand the effect that statin treatment might have on more developmentally mature neurons. The neurite loss observed in previous studies may therefore be due to different pharmacological profiles of the statins used (pravastatin vs. compactin or atorvastatin), or to increased sensitivity of immature neurons to toxicity induced by inhibition of mevalonate synthesis. Comparison of these results also suggests that statins may either inhibit or stimulate neuronal growth depending on the stage of neuronal development. Finally, our results are in agreement with earlier findings in PC12 and neuroblastoma

cells which suggest that HMG-CoA reductase inhibitors stimulate neurite growth (Maltese and Sheridan, 1985; Sato-Suzuki and Murota, 1996; Kumano et al., 2000).

Our data indicate that pravastatin's stimulation of neuritogenesis is due to inhibition of isoprenoid synthesis. Treatment with the isoprenoids GGPP or FPP completely prevented the effect of pravastatin on neurite outgrowth. Furthermore, neurite number and neurite length were significantly enhanced by treatment with GGTI-286, a transferase inhibitor which specifically blocks geranylgeranylation of small GTP binding proteins of the Rho family. This post-translational modification is required for activation of the Rho family of GTPases (Casey and Seabra, 1996). The monomeric G-protein RhoA, in particular, is involved in rearrangement of the actin cytoskeleton, and is activated by geranylgeranylation, but not farnesylation. Inhibition of RhoA signaling simulates neurite outgrowth in a variety of models (Lee et al., 2000; Scott and Luo, 2001; Bryan et al., 2004). Therefore, enhancement of neurite outgrowth by pravastatin is consistent with the hypothesis that reduction of RhoA signaling via inhibition of geranylgeranylation stimulates neuritogenesis.

HMG-CoA reductase inhibitors are either lipophilic or hydrophilic compounds. Lipophilic statins, such as pravastatin, enter cells via an ATP-dependent anion transport system (Nezasa et al., 2003). Pravastatin was not previously thought to cross the blood brain barrier, even though pravastatin use is associated with a reduced risk of AD (Wolozin et al., 2000; Rockwood et al., 2002). However, it was recently demonstrated in mice that oral pravastatin treatment results in measurable pravastatin levels in the brain (Johnson-Anuna et al., 2005). It will therefore be important to investigate the effects of pravastatin and other lipophilic statins on neuritic growth *in vivo*, since, as our data

suggest, pravastatin may be useful for preventing the neuritic dystrophy caused by AD.

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Chapter 6:

Conclusion

Alzheimer's disease (AD) is a multifactorial disorder, with an unknown underlying cause and numerous putative targets for treatment. Current treatments for AD utilize compounds (e.g. acetylcholinesterase inhibitors) which only temporarily amerliorate symptoms of the disease, without delaying disease progression. In order for drug treatments to either prevent or slow the onset of AD, they must target the underlying neuropathology. The studies presented in this thesis draw attention to various mechanisms by which AD might affect the brain, and suggest potential targets for treatments which might be useful for AD prevention.

Activation of various neurotransmitter receptors, such as muscarinic receptors (Buxbaum et al., 1992; Nitsch et al., 1992; Nitsch et al., 2000; Fisher et al., 2002) or metabotropic glutamate receptor (Lee et al., 1995; Lee and Wurtman, 1997; Ulus and Wurtman, 1997) can regulate amyloid precursor protein (APP) processing; however, expression patterns of these receptors in the brain and body suggest that drugs targeting them may have deleterious side effects (Kelly, 1999). Since expression of the serotonin receptor 5-HT_{2C} is restricted to the brain, stimulation of this receptor may not produce peripheral side effects. In Chapter 2, activation of serotonin receptors by the agonist dexnorfenfluramine was shown to affect APP processing *in vivo* by promoting formation of the soluble APP fragment and reducing Aβ production (Arjona et al., 2002). Subsequent studies support the hypothesis that serotonin receptors regulate APP processing in the brain (Lezoualc'h and Robert, 2003; Maillet et al., 2003; Robert et al., 2005); stimulation of these receptors might therefore prevent or reduce amyloid plaque formation.

Additional cell-surface receptors may also be involved in regulation of APP expression and processing. In Chapter 3, we found that, in microglia, stimulation of the prostaglandin EP2 receptor by the inflammatory mediator prostaglandin E2 (PGE_2) activated the cAMP signaling cascade and subsequently increased APP expression (Pooler et al., 2004). Overexpression of APP may lead to elevations in Aβ and subsequent formation of amyloid plaques. Brain inflammation or injury is a risk factor for developing AD (Fleminger et al., 2003), but the mechanism for this effect is unknown. Our data indicate that the inflammatory pathway might interact with APP production via the stimulation of the EP2 receptor by PGE_2. Recent studies have found that antiinflammatory compounds may be useful for minimizing risk of AD following brain inflammation (Calderon-Garciduenas et al., 2004; Yao et al., 2004); our findings suggest that EP2 receptor antagonists may constitute an additional target for preventative therapy.

The neuropathology associated with AD includes not just an abundance of amyloid plaques, but also loss of membrane phospholipids, neuritic dystrophy and neuronal degeneration (McKee et al., 1991; Calon et al., 2004; Moolman et al., 2004). Therefore, restoration of neuritic growth and repair of cell membranes may be important for treating AD. The principal constituents of mammalian cell membranes are phosphatides, the most abundant of which is phosphatidylcholine (PC). Since the rate at which cells form PC is affected by the availability of its precursors (Savci and Wurtman, 1995; Araki and Wurtman, 1997), stimulation of PC synthesis by increasing precursor levels may restore normal phospholipid metabolism in the AD-affected brain. Increasing cell membrane synthesis may also have morphological consequences for the cell, such as

promotion of neurite growth. In Chapter 4, we found that treatment of NGF-differentiated PC12 cells with the PC precursor uridine enhanced neurite outgrowth. Since uridine treatment increases cellular levels of the pyrimidine nucleotide UTP, we also tested whether UTP, by interacting with UTP-sensitive receptors, might also affect neurite outgrowth. We found that UTP directly stimulated neurite growth and, moreover, that antagonists of UTP-sensitive P2Y receptors blocked the stimulatory effect of uridine on neuritogenesis. Therefore, our data suggest uridine promotes neurite outgrowth by two distinct mechanisms: 1) by enhancing phosphatide biosynthesis, and 2) by stimulating a P2Y receptor-coupled signaling pathway (Pooler et al., 2005). In a related study, we found that, *in vivo*, oral uridine treatment can increase striatal dopamine release and neurofilament proteins; these results suggest that uridine treatment may be useful in humans (Wang et al., 2005). There is compelling evidence that the purinergic nucleotide ATP functions as a neurotransmitter (Fu and Poo, 1991; Zhang et al., 2003; Martin and Buno, 2005), but data are only to beginning accumulate in support of pyrimidine nucleotides like UTP stimulating cell-surface receptors and activating signaling pathways (Chorna et al., 2004). Our study suggests that pyrimidine nucleotides may be important for neurite growth and development, and moreover, that pyrimidine nucleotide-sensitive receptors may constitute a novel target for preventing neuritic degeneration caused by AD.

In Chapter 5, we investigated whether HMG-CoA reductase inhibitors (statins), might affect neurite growth in hippocampal neurons. Epidemiological studies suggested a link between statin usage and a reduced risk of AD, but the mechanism by which this might occur may not be related to statin-induced reduction in cholesterol levels (Wood et

al., 2003; Wolozin, 2004; Eckert et al., 2005). Statins inhibit not just cholesterol synthesis, but also synthesis of isoprenoids involved in activation of various signaling pathways (Rando, 1996). In our study, we found that the HMG-CoA reductase inhibitor pravastatin stimulated neurite outgrowth, and that this effect was likely due to inhibition of isoprenoid formation, not prevention of cholesterol synthesis. Our data suggest that pravastatin may reduce AD risk by averting neuritic dystrophy and degeneration. However, further studies must be conducted to determine whether statins can affect neuritic processes *in vivo*.

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