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Metabotropic Glutamate Receptor Subtype mGluR1α Stimulates the Secretion of the Amyloid β-Protein Precursor Ectodomain

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Abstract: To examine the effects of glutamatergic neurotransmission on amyloid processing, we stably expressed the metabotropic glutamate receptor subtype 1α (mGluR1α) in HEK 293 cells. Both glutamate and the selective metabotropic agonist 1-amino-1,3-cyclopentanedicarboxylic acid (ACPD) rapidly increased phosphatidylinositol (PI) turnover four- to fivefold compared with control cells that were transfected with the expression vector alone. Increased PI turnover was effectively blocked by the metabotropic antagonist α-methyl-4-carbophenylglycine (MCPG), indicating that heterologous expression of mGluR1α resulted in efficient coupling of the receptors to G protein and phospholipase C activation. Stimulation of mGluR1α with glutamate, quisqualate, or ACPD rapidly increased secretion of the APP ectodomain (APPs); these effects were blocked by MCPG. The metabotropic receptors were coupled to APP processing by protein kinases and by phospholipase A_2 (PLA_2), and melittin, a peptide that stimulates PLA_2, potently increased APPs secretion. These data indicate that mGluR1α can be involved in the regulation of APP processing. Together with previous findings that muscarinic and serotonergic receptor subtypes can increase the secretion of the APP ectodomain, these observations support the concept that proteolytic processing of APP is under the control of several major neurotransmitters. **Key Words:** Amyloid—Alzheimer's disease—Metabotropic glutamate receptor—α-Secretase processing.
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Glutamate is one of the major neurotransmitters in mammalian brain and modulates both pre- and postsynaptic neuronal activities through a variety of ionotropic and metabotropic receptors (Sugiyama et al., 1989; Nakanishi, 1992). Metabotropic glutamate receptors (mGluRs) are a family of G protein–coupled receptors with seven transmembrane domain topology but with no known sequence homology to other G protein–coupled receptors (Sugiyama et al., 1987; Houamed et al., 1991; Masu et al., 1991; Tanabe et al., 1992). Eight mGluR subtypes and five splice variants fall into three main groups that differ in signaling modes and in pharmacological characteristics (for review, see Nakanishi, 1994; Pin and Duvoisin, 1995). Group I receptors include mGluR1 and mGluR5, which activate phospholipases, phosphatidylinositol (PI) hydrolysis, and adenylyl cyclase, whereas groups II and III receptors inhibit adenylyl cyclase activity (Boss and Conn, 1992) but differ in agonist specificities. mGluR1 are widely distributed in the mammalian CNS and are expressed predominantly in hippocampal neurons, in cerebellar Purkinje cells, as well as in mitral and in tufted cells of the olfactory bulb (Shigemoto et al., 1992).

mGluR subtypes modulate both excitatory and inhibitory neurotransmission (McBain et al., 1994; Gereau and Conn, 1995), and they play a role in synaptic plasticity in as much as they promote long-term depression (LTD) both in cerebellum and in hippocampus (Bolshakov and Siegelbaum, 1994; Shigemoto et al., 1994; Kobayashi et al., 1996; Yokoi et al., 1996). Furthermore, activation of mGluR can induce long-term potentiation (LTP) in hippocampus (Bashir et al., 1993; Bortolotto et al., 1994). Both LTD and LTP are believed to be synaptic representations of memory processes (Bliss and Collingridge, 1993; Linden,

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Abbreviations used: Aβ, amyloid β-protein; ACPD, 1-amino-1,3-cyclopentanedicarboxylic acid; AD, Alzheimer's disease; APLP2, APP-like protein 2; APP, amyloid β-protein precursor; APPs, secreted APP ectodomain; DEDA, 7,7-dimethyleicosadienoic acid; DMEM/F12, Dulbecco's modified Eagle medium/F12 nutrient mixture; ECL, enhanced chemiluminescence; HBSS, Hanks' buffered saline solution; LTD, long-term depression; LTP, long-term potentiation; MCPG, α-methyl-4-carbophenylglycine; mGluR1α, metabotropic glutamate receptor subtype 1α; OPC, oleyloxyethyl phosphorylcholine; PI, phosphatidylinositol; PKC, protein kinase C; PLA_2, phospholipase A_2; PMA, phorbol myristate acetate.

1994), and they may be the electrophysiological basis for the observation that mGluR agonists improve memory-related behaviors in rats (Straubli et al., 1994). In addition to these physiological functions, mGluR may be involved in neuropathological mechanisms; pharmacological activation of mGluR can induce seizures and can also aggravate NMDA-induced excitotoxic damage by facilitating NMDA receptor responses to glutamate (McDonald and Schoepp, 1992; McDonald et al., 1993).

Reductions in glutamatergic neurotransmission are believed to contribute significantly to the cognitive impairment in Alzheimer's disease (AD) (Francis et al., 1993), a common neurodegenerative disorder that is characterized by the formation in brain of neurofibrillary tangles and amyloid plaques. Amyloid plaques are composed of amyloid β-protein (Aβ), a hydrophobic 39–43-amino acid peptide that is derived by proteolytic processing from the larger amyloid β-protein precursor (APP) (Kang et al., 1987). Proteolytic processing of APP follows several intracellular routes that involve cleavage events at diverse sites clustered around its Aβ domain (for review, see Selkoe, 1994). These proteolytic mechanisms are referred to as secretase processing events. α-Secretase processing involves cleavage within the Aβ domain (Sisodia et al., 1990), generating the secreted ectodomain (APPs) along with an ~10-kDa C-terminal fragment that is further degraded by γ-secretase processing at the C-terminus of the Aβ domain to produce p3 and an ~7-kDa C-terminus (Haass et al., 1993). In contrast, APP can be processed by β-secretase at the N-terminus, generating a truncated APPs molecule (Seubert et al., 1993), as well as a 100-amino acid C-terminal fragment that is the immediate precursor to generate the intact Aβ peptide by γ-secretase cleavage (Haass et al., 1993). α-Secretase processing occurs mostly in the trans-Golgi network as well as in secretory vesicles, and at the cell surface, whereas β-secretase processing involves recycling of APP from the cell surface to late endosomes or lysosomes. There are some indications that β-secretase processing can also occur in the secretory pathway (De Strooper et al., 1993; Thinakaran et al., 1996). Both α- and β-secretase processing are regulated by internal and external signals including G protein–coupled cell surface receptors. These include muscarinic m1 and m3, and serotonergic 5-HT_{2a} and 5-HT_{2c} receptors. (Buxbaum et al., 1992; Nitsch et al., 1992, 1996). Furthermore, α-secretase processing is modulated in an activity-dependent manner by action potentials in rat brain slice preparations (Nitsch et al., 1993; Farber et al., 1995). Evidence to date indicates that protein kinases and phospholipases are critical components in the signaling pathway that couples receptor activation to APP processing. To test whether mGluR subtypes that are coupled to this signaling pathway can also increase α-secretase processing of APP, we stably overexpressed mGluR1α in HEK 293 cells, treated them with metabotropic receptor ligands, and measured the amount of APPs released into the culture media. We show that secretion of the APP ectodomain was increased within minutes by stimulation of mGluR1 and that increased secretion was mediated by receptor-coupled activation of both protein kinase C (PKC) and phospholipase A_2 (PLA_2). These studies confirm and extend our prior report that glutamate influenced APP processing in primary cells (Lee et al., 1995).

MATERIALS AND METHODS

Constructs

The cDNA encoding the human mGluR subtype 1α (Masu et al., 1991) was generously provided by S. Nakanishi. A *Not*I/*Sal*I restriction fragment comprising the complete reading frame including the signal peptide was ligated into pBK-CMV (Stratagene). The intact reading frame of the resulting cDNA was confirmed by DNA sequencing. pBK-CMV without insert was used for control transfections.

Cell culture and stable transfection

Subconfluent monolayers of 293 cells were transfected with 10 μg plasmid DNA, precipitated with calcium phosphate, followed by 15% glycerin shock according to Sambrook et al. (1989). Stably transfected cells were selected with 500 μg/ml Geneticin (G418, GIBCO), and clonal lines were produced by two rounds of low density plating and collection of individual colonies with cloning cylinders. Individual clonal lines were screened for expression of functionally intact receptors by analyzing glutamate-induced stimulation of PI turnover; 53% of the screened clones effectively stimulated PI turnover. Experiments were repeated in three individual positive cell lines. Cells were maintained in Dulbecco's modified Eagle medium/F12 nutrient mixture (DMEM/F12) supplemented with 10% fetal calf serum, and with 500 μg/ml G418. To reduce glutamate concentrations in the culture media before the experiments, the growth and selection medium was replaced with serum-free DMEM/F12 without glutamine and without histidine. Experiments were also performed with this medium. Because G418 can interfere with G-protein coupling, it was removed from the media at least 2 days before the experiments.

Metabolic labeling and PI turnover analysis

Cells were labeled metabolically overnight with 1.25 μCi/dish of *myo*-[2-^{3}H]inositol (20.5 Ci/mmol; New England Nuclear) in inositol-deficient, serum- and glutamate-free DMEM/F12 medium, washed twice with Hanks' balanced salt solution (HBSS), and treated for 10 min with 10 m*M* lithium chloride in HBSS. Drugs were added in the presence of 10 m*M* lithium for 5–60 min at 37°C. Cells were lysed with ice-cold methanol, and lipids were removed by chloroform/methanol/water (2:2:1; by volume) extraction. Labeled water-soluble inositol phosphates were separated from free [^{3}H]inositol by ion-exchange chromatography, using AG 1-X8 columns (Bio-Rad), and 1 *M* ammonium formate and 0.1 *M* formic acid as eluent. Radioactivity was quantitated by liquid scintillation spectrometry.

Drugs

Glutamate (Sigma, St. Louis, MO, U.S.A.) was prepared freshly before each experiment and was used at 0–500 μ*M*. *trans*-(±)-1-Amino-1,3,-cyclopentanedicarboxylic acid

(ACPD; 10–500 μM), α-methyl-4-carboxyphenylglycine (MCPG; 400 μM), quisqualate (50–500 μM), chelerythrine chloride (1 μM), staurosporine (1 μM), melittin (0.1–1 mM), and thapsigargin (20 nM) were purchased from Research Biochemicals International (Natick, MA, U.S.A.). Phorbol myristate acetate (PMA; Sigma) was used at 100 nM to 1 μM for both stimulation and down-regulation of PKC. Manoalide (3.2–10 μM), 7,7-dimethyleicosadienoic acid (DEDA; 20–50 μM), and oleyloxyethyl phosphorylcholine (OPA; 0.1–1 mM) were purchased from Biomol (Plymouth Meeting, PA, U.S.A.).

Antibodies, western blotting, and densitometry

Cells were washed twice with histidine- and glutamine-free serum, DMEM/F12 medium, and were incubated at 37°C in the presence or absence of test substances freshly dissolved in the same medium. Antagonist and blockers were added 10 min before the coincubation with the agonist. After time intervals of 5–60 min, conditioned media were collected, cooled to 4°C, centrifuged at 300 *g*, and supernatant fluids were desalted by gel-filtration chromatography using G25 Sephadex (Pharmacia) columns, with water as eluent. Desalted proteins were dried by vacuum centrifugation, reconstituted in water followed by 2× Laemmli gel loading buffer, and boiled for 3 min. Total cell protein was extracted with 1% sodium dodecyl sulfate (SDS) in Tris-buffered saline and was quantitated by the bicinchoninic acid assay (Pierce). Equal volumes of reconstituted secretory protein solutions normalized to total cell protein were separated by SDS–polyacrylamide gel electrophoresis, electroblotted onto polyvinylidene difluoride (Immobilon P, Waters) membranes, and blocked with 5% nonfat dry milk (Carnation) in Tris-buffered saline containing 0.05% Tween 80. Membranes were probed with antibodies directed against various domains of APP and APP-like protein 2 (APLP2). Specifically, the monoclonal antibodies 22C11 (Boehringer Mannheim) against the APP ectodomain and 6E10 (Senetek) against residues 1–17 of the Aβ domain, as well as the polyclonal antiserum D2-1 raised against full-length APLP2 expressed in a baculovirus system (Slunt et al., 1994), were used. Secondary antibodies were visualized on preflashed x-ray films (Kodak) by enhanced chemiluminescence (ECL; Amersham). Immunoreactive bands were quantitated by laser scanning densitometry with an LKB Ultroscan densitometer set to 40-μm vertical interval size and 2.4-mm horizontal slit width. Areas under the optical density curves were expressed as arbitrary units (AU) and were normalized to areas generated by immunoreactive proteins secreted under control conditions determined on the same blot. Measurements were performed in the linear range of the ECL reaction as determined from serial dilution curves of secreted proteins. Control and stimulated conditions were always handled in parallel, processed identically, and run in parallel lanes on the same blot. All experiments were done in triplicate dishes and were repeated at least three or four times in three individual clonal lines. Statistical analysis was performed by ANOVA, using treatments as the independent variable.

RESULTS

After stable transfection and two rounds of cloning, we screened individual clones for intact coupling of mGluR1α to G-protein activation and stimulation of PI turnover. In 10 of 19 clones, both glutamate (Fig. 1A) and ACPD stimulated PI turnover four- to fivefold (Fig. 1B). In contrast, nontransfected parent cells and cells expressing the transfection vector alone, as well as nine of 19 clones stably expressing mGluR1α, failed to increase PI turnover in response to mGluR1α stimulation with either glutamate or ACPD. In the cell lines that expressed functionally intact receptors, agonist-induced PI was completely blocked by coincubation with MCPG, an mGluR antagonist (Fig. 1B). Similar results were obtained with two additional clonal 293 lines stably expressing mGluR1α. Three of the clonal lines that expressed functionally coupled mGluR1α were used for the further experiments. Under basal, unstimulated conditions, PI turnover in the lines that expressed functionally coupled receptor was consistently nine times higher compared with the wild-type, nontransfected cells (data not shown). This higher basal PI turnover may reflect receptor stimulation under basal conditions induced by glutamate, which may be derived from cellular synthesis, although both glutamine and histidine were absent in the serum-free labeling and chase media. Collectively, the data indicate that stable transfection of mGluR1α in 293 cells resulted in the expression of functionally active receptors that are coupled to, and activate, PI turnover.

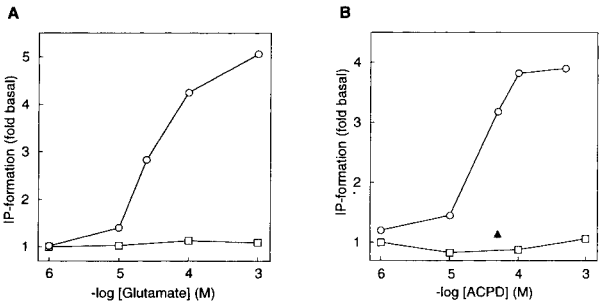

FIG. 1. Stimulation of PI turnover in 293 cells stably transfected with mGluR1α (○), and nontransfected 293 cells (□) by metabotropic receptor agonists. Glutamate (**A**); ACPD, a selective mGluR agonist (**B**). Both glutamate and ACPD increased PI turnover four- to fivefold in transfected cells but did not affect wild-type cells. Adding the mGluR1 antagonist MCPG (400 μM; ▲) effectively blocked the ACPD-induced accumulation of inositol phosphates (IP). Data are means from triplicate culture dishes.

To test whether agonist-induced increase in PI turnover in these cells was associated with changes in secretory APP processing, we analyzed conditioned media by western blotting and by using antibodies directed against the ectodomain of APP. Both the nonselective agonists glutamate and quisqualate, as well as the selective agonist ACPD, significantly increased the release of APPs by the cells that stably expressed mGluR1α (Fig. 2A). As a positive control, direct activation of PKC by PMA also increased APPs release. The increased secretion induced by ACPD was blocked by the metabotropic receptor antagonist MCPG (Fig. 2B). To show that APPs secreted in response to receptor activation, we used the monoclonal

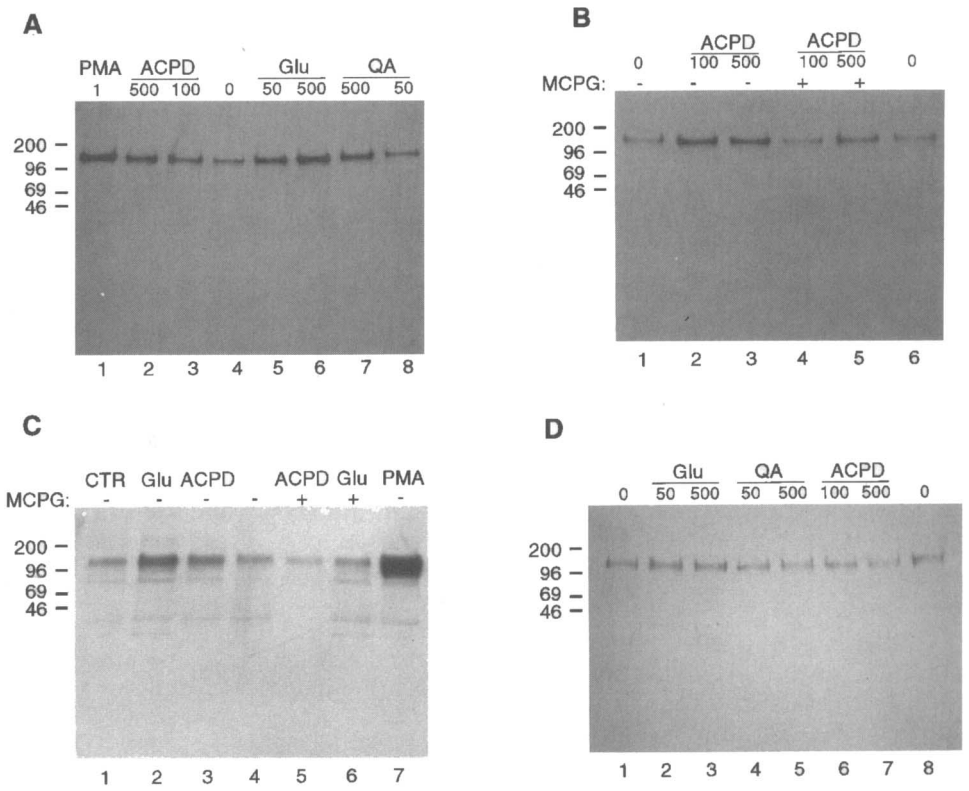

FIG. 2. Western blots of APPs secreted into the culture media. **A:** Both the nonselective mGluR1 agonists glutamate (Glu) and quisqualate (QA), as well as the selective agonist ACPD increased APPs secretion (detected by 22C11) from 293 cells stably transfected with mGluR1α. APPs was also stimulated by direct activation of PKC with PMA. **B:** MCPG (400 μ*M*) blocked ACPD-induced increase in APPs release (detected by 22C11) in 293 cells stably transfected with mGluR1α. **C:** Increased APPs secreted in response to mGluR1α stimulation was derived from α-secretase processing as indicated by the monoclonal antibody 6E10 directed against residues 1–17 of the human Aβ domain. **D:** In control experiments with 293 cells stably transfected with the vector only, glutamatergic agonists failed to increase APPs release. APPs was detected with the monoclonal antibody 22C11; concentrations are indicated in micromolar units and molecular masses in kilodaltons.

antibody 6E10 that is directed against the 16 N-terminal residues of the Aβ domain and, thus, selectively detects APPs derived from α-secretase cleavage. Stimulation of the mGluR clearly increased 6E10 immunoreactivity in western blots of secreted proteins (Fig. 2C). Control cell lines that expressed the vector only failed to respond to glutamate, to quisqualate, or to ACPD with changes in secretory APP processing. None of these treatments changed basal rates of APPs release (Fig. 2D). Time course experiments revealed that the major mGluR1α-induced increase in APPs secretion occurred between 30 and 60 min within receptor stimulation (Fig. 3), and a maximum response was attained after 120 min. Coincubation experiments with the protein synthesis inhibitor cycloheximide showed essentially similar time courses of mGluR1α-induced increases in APPs release. We quantitated these responses by densitometry and found that concentrations of APPs in stimulated cells expressing mGluR1α were three- to fourfold higher than the unstimulated, basal levels (Fig. 4). Dose–response analyses showed that glutamate concentrations of 50–1,000 μ*M* effectively increased APPs release (Fig. 4A). With the exception of the 1 m*M* concentration, this dose–response was equivalent to that of glutamate-induced stimulation of PI turnover. At 1 m*M*, the response to glutamate was lower than that at 500 μ*M*. It is currently unknown

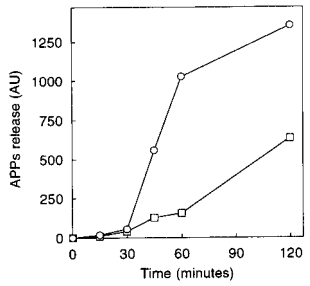

FIG. 3. Glutamate stimulated APPs release from 293 cells stably expressing mGluR1α within 60 min. ○, cells stimulated with 500 μ*M* glutamate; □, unstimulated, basal release of APPs by the same cell line. APPs in culture media was quantitated by densitometry after western blotting and immunoreaction with the monoclonal antibody 22C11. Data are mean values of triplicate culture dishes from a representative experiment. AU, arbitrary units.

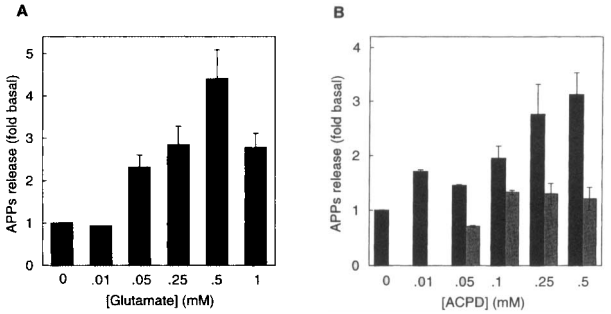

FIG. 4. Dose–response of APPs release induced by mGluR1 agonists applied for 60 min to 293 cells stably transfected with mGluR1α. The cells were treated with glutamate at concentrations of 10 μM to 1 mM (**A**), or with ACPD at concentrations of 10–500 μM (**B**) were significantly different from nontreated cells ($p \leq 0.01$ by ANOVA). The increase in APPs secretion induced by ACPD was blocked with 400 μM of the mGluR1 antagonist MCPG. Filled columns represent ACPD treatment alone; shaded columns represent coadministration of ACPD and MCPG. Data are mean ± SEM values of n = 3–5 experiments.

whether this effect is related to potential cytotoxic effects of high glutamate concentrations. Neither glutamate nor ACPD changed basal APPs secretion in wild-type 293 cells. In the presence of the mGluR antagonist MCPG, ACPD at various concentrations failed to increase basal APPs concentrations in stably transfected cells, indicating that ACPD increased APPs secretion specifically through the stimulation of the transfected mGluRs (Fig. 4B).

To identify the component steps in the signaling pathways that couple mGluR1α to APP-processing pathways, we reduced PKC activity both by pharmacological inhibitors and by down-regulation with PMA. Both the nonselective kinase inhibitor staurosporine and the more PKC-specific inhibitor chelerythrine chloride effectively inhibited the increase by glutamate in APPs release (Fig. 5A, lanes 1 and 3). Down-regulation of PKC by overnight incubation with PMA abolished the increase in APPs secretion by PMA and glutamate (Fig. 6A, lanes 6 and 7). Most, if not all, of the APPs released in response to stimulation of mGluR1α was processed by α-secretase cleavage, as indicated by the monoclonal antibody 6E10 that specifically detects residues 1–16 of the Aβ domain. The monoclonal antibody 22C11 recognizes both APPs and its homologue APLP2 (Slunt et al., 1994). To determine whether mGluR1α can also stimulate secretory processing of APLP2, we probed western blots with D2-1, a polyclonal antiserum that specifically recognizes APLP2 (Slunt et al., 1994). Glutamate stimulation clearly increased secretion of the ectodomain of APLP2 from cells stably transfected with mGluR1α (Fig. 6B).

To determine whether PLA_2 is involved in coupling mGluR1α to APP processing, we coadministered in separate experiments the PLA_2 inhibitors manoalide and DEDA along with glutamate to cells stably expressing mGluR1α. The PLA_2 inhibitors blunted glutamate-induced increase in APPs release (Fig. 6A). To investigate the possibility that the blocking effects of the inhibitors were due to nonspecific interaction with PI turnover, we stimulated PI turnover with various concentrations of glutamate in the presence and the absence of manoalide, DEDA, and OPC. We found that manoalide and OPC, but not DEDA, inhibited glutamate-induced PI turnover (Table 1). Thus, reduced PI turnover may explain in part the inhibitory effect of manoalide on APPs secretion. However, that DEDA did not block PI turnover but did block stimulated APPs secretion shows clearly that PLA_2 can be involved directly in coupling mGluR1α to increased APPs secretion. Furthermore, melittin, a peptide that stimulates PLA_2 activity, increased consistently APPs release more than twofold over the effect of glutamate or ACPD (Fig. 6B). Melittin's effect on APPs release was greater in 293 cells stably expressing mGluR1α than in wild-type 293 cells (Fig. 6B).

DISCUSSION

The results of this study show that glutamate, quisqualate, and the metabotropic agonist ACPD readily stimulate α-secretase processing of APP in cultured cells that stably express the metabotropic receptor subtype mGluR1α. This finding, along with the prior observation that glutamate increases the secretion of the

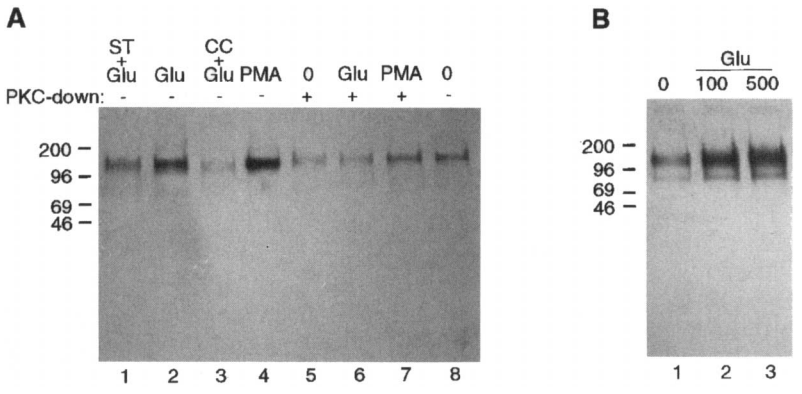

FIG. 5. PKC coupled mGluR1α to APPs secretion (**A**). Glutamate (Glu)-induced APPs secretion (60-min drug incubation) was blocked by the kinase inhibitor staurosporine (ST; 1 μM), as well as by the more specific PKC inhibitor chelerythrine chloride (CC; 1 μM). Direct activation of PKC with PMA (1 μM) also increased APPs release. After down-regulation of PKC by 15-h pretreatment with PMA (+), both glutamate and PMA failed to increase APPs release. Glutamate increased the release of the ectodomain of the APLP2 in 293 cells stably transfected with mGluR1α (**B**). Western blot of media proteins from cells stably expressing mGluR1α were probed with the APLP2-specific antibody D2-1.

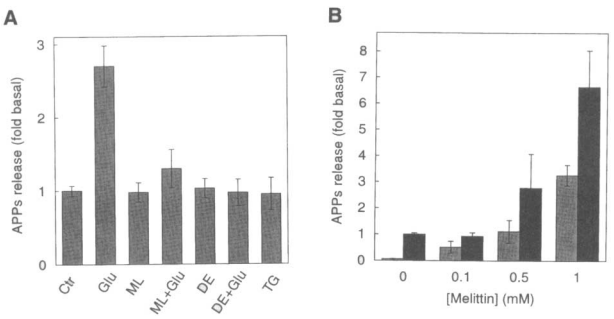

FIG. 6. The PLA_2 inhibitors manoalide (ML) and DEDA (DE) effectively blocked glutamate (Glu)-induced APPs release (**A**). Thapsigargin (TG), a compound that releases calcium from intracellular stores, failed to change APPs release in these cell lines (A). Stimulation of PLA_2 with melittin, a peptide that activates PLA_2, stimulated APPs release in 293 cells that stably overexpress mGluR1α (solid columns) as well as in wild-type 293 cells (shaded columns) (**B**). At 1 m*M* concentrations, APPs secretion was significantly greater in the transfected cells compared with the wild-type cells ($p \leq 0.05$, by ANOVA). Basal, unstimulated APPs secretion was also significantly higher in the transfected cells than in the wild-type cells ($p \leq 0.01$, by ANOVA).

APP ectodomain in primary neurons (Lee et al., 1995) indicates that glutamate receptors can regulate secretory APP processing. Together with the observation that other G protein–coupled receptors including muscarinic and serotonergic receptor subtypes (Nitsch et al., 1992, 1996), as well as the formation of action potentials in brain slices, can also increase α-secretase processing (Nitsch et al., 1993; Farber et al., 1995), the data reported here support the concept that several major neurotransmitters can regulate secretory APP processing.

Glutamate caused a rapid and dose-dependent increase in PI turnover in 293 cells stably transfected with mGluR1α, as evidenced by the accumulation of radiolabeled inositol phosphates in the presence of lithium after metabolic labeling of PI. This result demonstrates that our transfection protocol generated functionally intact and stably expressed surface receptors that couple to the expected signal transduction systems. Expression of functionally intact receptors was not consistently observed in all clones, because we found that ~47% of the stable clones failed to stimulate PI turnover despite the expression of mGluR1α message. In functionally positive clones, basal PI turnover was eightfold higher in cells transfected with mGluR1α. Similar increases of basal PI turnover by mGluR1α were previously obtained in transiently transfected cells (Gabellini et al., 1994; Lee et al., 1995), and we attribute such increases to constant, ligand-independent stimulation of the second messenger systems, or to endogenous glutamate synthesized and secreted by the cells. Despite the transfection-related increase in basal PI turnover, the heterologously expressed receptors retained the ability to respond to agonists with further increases in PI turnover; addition of mGluR1 receptor agonists including glutamate, ACPD, and quisqualate increased PI turnover ~37 times higher than basal PI turnover in untransfected, wild-type 293 cells.

Western-blotting analyses of secreted proteins revealed that metabotropic receptor agonists caused a dose-dependent increase in APPs secretion. The increase in APPs secretion was rapid, with the steepest rate of increase between 30 and 60 min after stimulation. These data are similar to the kinetics of serotonin- and carbachol-induced stimulation of APPs secretion (Nitsch et al., 1992, 1996) and support the view that preexisting APP holoprotein is the chief substrate for the increases in APPs in response to receptor stimulation. The effects reported here are specific to the metabotropic receptor, because the agonist-induced increases of APPs secretion were effectively blocked by the metabotropic receptor antagonist MCPG. That agonists failed to change basal rates of APPs secretion in cells transfected with the vector alone supports the specificity of the effect. These two lines of evidence indicate that increased APPs secretion was indeed caused by mGluR1α stimulation. Most of APPs secreted in response to receptor activation was derived from α-secretase processing, because it contained the 16 N-terminal residues of the Aβ domain that are recognized by the monoclonal antibody 6E10.

To dissect the second messenger signaling pathways that couple mGluR1α to increased α-secretase processing, we examined the independent effects of PKC and PLA_2. Both the kinase inhibitor staurosporine and the more selective PKC inhibitor chelerythrine chloride inhibited glutamate-induced increases in APPs secretion. In addition, down-regulation of PKC by chronic, 15-h incubation with PMA blunted the expected glutamate-induced increased in APPs secretion. These results demonstrate that PKC is critical in cou-

TABLE 1. *Effects of PLA_2 inhibitors on glutamate-induced PI turnover in 293 cells stably overexpressing mGluR1α*

Glutamate	Control	ML	DEDA	OPC
0	2.54 ± 0.29	1.97 ± 0.06	2.07 ± 0.10	1.96 ± 0.11
250 μ*M*	10.8 ± 0.50	6.34 ± 0.45	11.7 ± 1.72	11.1 ± 0.61
500 μ*M*	27.6 ± 3.45	9.33 ± 1.24	26.9 ± 4.38	19.8 ± 1.30

Data are expressed as cpm × 10^{-3} and are mean ± SD values from three independent assays. ML, manoalide.

pling mGluR1α to secretory APP processing. PLA_2 can also influence APP processing, as illustrated by increased APPs secretion in response to the PLA_2-stimulating agent melittin. Inhibiting PLA_2 activity had the expected opposite effect; i.e., the PLA_2 inhibitors manoalide, DEDA, and OPC blocked glutamate-induced APPs secretion. To control for potential nonspecific effects on other signaling pathways, we examined whether PLA_2 inhibitors interfere with agonist-induced PI turnover. Whereas one (manoalide) inhibited glutamate-induced PI turnover, two others (DEDA and OPC) did not, indicating that their effects on blocking agonist-induced increase of APPs secretion were specific to PLA_2 and not related to nonspecific interactions with PI turnover. Similar effects of PLA_2-mediated coupling of surface receptors to APP processing were reported for muscarinic receptors (Emmerling et al., 1993) and for serotonin receptors (Nitsch et al., 1996). Simply increasing intracellular concentrations of free calcium was not sufficient to change secretory APP processing in the cells we used; i.e., thapsigargin, which releases calcium from internal stores, failed to change basal increases in APPs secretion. Thus, the previously reported effects of thapsigargin on APPs secretion may be cell-type specific (Buxbaum et al., 1994; Querfurth and Selkoe, 1995).

APP is a member of a larger family of APP-like proteins (APLP). APLP2 is homologous to APP in both N- and C-terminal regions but lacks the Aβ domain. Proteolytic processing of APLP2 is similar to that of APP, and we have previously shown that stimulation of 5-HT_{2a} and 5-HT_{2c} serotonin receptors increases the secretion of the ectodomains of APLP2. Here, we demonstrate that mGluR1α can also increase secretory processing of APLP2 and accelerate the rate of secretion of its ectodomain. These findings strengthen the view that APP and APLP2 may be compete for similar processing pathways.

The cellular mechanism whereby external and internal signals regulate rates of APP and APLP2 processing are not completely understood. It was previously reported that PKC can accelerate the budding of secretory vesicles from the trans-Golgi network (Xu et al., 1995). Our data emphasizing the central importance of PKC in APP processing support the possibility that kinase activation influences the formation of APP-containing secretory vesicles and accelerates their transport to the cell surface. Another possibility is that receptor stimulation and the resultant second messenger signaling cascade stimulates directly the proteases involved in α-secretase processing. Testing this hypothesis must await the results of the search to identify these proteases.

The physiological relevance of having APP processing under neurotransmitter control is unclear because the function of APP remains uncertain. Many experimental data point to a role of APP as a neurotrophic and neuroprotective factor, possibly as an adhesion molecule with the ability to bind such extracellular matrix proteins as laminin, and the ability to promote neurite outgrowth (Milward et al., 1992). In contrast, deleting the APP gene in mice did not cause obvious brain or behavioral abnormalities (Zheng et al., 1995). Thus, the precise role of APP in mammalian brain remains to be determined. APP is involved, however, in the pathophysiology of AD. APP is the immediate precursor of Aβ peptides that can aggregate to form amyloid plaques, a major pathological hallmark of AD. The most direct evidence for the central importance of APP in causing AD comes from molecular genetic studies; i.e., mutations of the APP gene that cause misprocessing of APP cause familial forms of the disease. In a Swedish kindred, a double mutation at the N-terminus of the Aβ domain causes increases in Aβ levels; in other families, single-point mutations at codon 717 of the APP gene lead to the formation of longer Aβ molecules that may aggregate more readily. Furthermore, mutations of the presenilin 1 gene that cause some forms of early-onset familial AD are associated with increased formation of longer Aβ peptides in blood, fibroblasts (Scheuner et al., 1996), and brain (Mann et al., 1996). Thus, a unifying theme in early-onset autosomal dominantly inherited AD is an abnormality in APP processing that leads to increased amounts of $A\beta_{1-42}$ in blood and in brain. Whether Aβ deposition causes late-onset sporadic AD remains controversial, but amyloid plaque formation is an early and prominent feature of AD histopathology.

A rational strategy to treat AD would be to reduce the formation of potentially amyloidogenic APP derivatives (Nitsch and Growdon, 1994). Our findings suggest that one way to accomplish this goal would be to increase α-secretase cleavage of Aβ through mGluR1α receptor stimulation. However, increased α-secretase activity raises a theoretical concern because, in addition to APPs, the p3 fragment of APP may also increase. The p3 fragment is a hydrophobic APP derivative that may have the potential of aggregating into amyloid-like structures. Several lines of evidence, however, challenge a role for p3 in amyloid formation and neurodegeneration in AD brain; p3 is not a major constituent of neuritic plaques in AD brain. Amyloid plaques are stained with antibodies against the N-terminus of Aβ, and sequencing of peptides derived from senile plaques consistently identified the N-terminus of Aβ. In contrast, p3 was purified biochemically from AD brain with massive diffuse amyloid deposits (Gowing et al., 1994).

We were unable to measure either p3 or Aβ in the 293 cells used in this study. Because 293 cells secrete very small amounts of these derivatives, incubation times of 24 h and longer would have been necessary to detect measurable levels with immunoabsorbent assays or metabolic labeling and immunoprecipitation. Such long incubation times, however, preclude the study of receptor-coupled phenomena that are detectable within time periods of minutes, and that attain maximum responses within 1 or 2 h. Receptor-stimula-

tion paradigms that are longer than these time intervals cause internalization and down-regulation of the surface receptors and result in loss of the response. In general, there is a reciprocal relation in APP processing between APPs and Aβ; i.e., when APPs secretion increases, there is a concomitant decrease in Aβ (Buxbaum et al., 1993; Gabuzda et al., 1993; Hung et al., 1993; Wolf et al., 1995). Direct proof that this rule holds for mGluR1α receptor stimulation remains a goal for future research.

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REFERENCES

Bashir Z. I., Bortolotto Z. A., Davies C. H., Berratta N., Irving A. J., Seal A. J., Henley J. M., Jane D. E., Watkins J. C., and Collingridge G. L. (1993) Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. *Nature* **363,** 347–350.

Bliss T. V. P. and Collingridge G. L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361,** 31–39.

Bolshakov V. Y. and Siegelbaum S. A. (1994) Postsynaptic induction and presynaptic expression of hippocampal long-term depression. *Science* **264,** 1148–1152.

Bortolotto Z. A., Bashir Z. I., Davies C. H., and Collingridge G. L. (1994) A molecular switch activated by metabotropic glutamate receptors regulates induction of long-term potentiation. *Nature* **368,** 740–743.

Boss V. and Conn P. J. (1992) Metabotropic excitatory receptor activation stimulates phospholipase D in hippocampal slices. *J. Neurochem.* **59,** 2340–2343.

Buxbaum J., Ruefli A., Parker C., Cypess A., and Greengard P. (1994) Calcium regulates processing of the Alzheimer amyloid protein precursor in a protein kinase C-independent manner. *Proc. Natl. Acad. Sci. USA* **91,** 4489–4493.

Buxbaum J. D., Oishi M., Chen H. I., Pinkas-Kramarski R., Jaffe E. A., Gandy S. E., and Greengard P. (1992) Cholinergic agonists and interleukin 1 regulate processing and secretion of the Alzheimer β/A4 amyloid protein precursor. *Proc. Natl. Acad. Sci. USA* **89,** 10075–10078.

Buxbaum J. D., Koo E. H., and Greengard P. (1993) Protein phosphorylation inhibits production of Alzheimer amyloid β/A4 peptide. *Proc. Natl. Acad. Sci. USA* **90,** 9195–9198.

Caporaso G. L., Gandy S. E., Buxbaum J. D., Ramabhadran T. V., and Greengard P. (1992) Protein phosphorylation regulates secretion of Alzheimer β/A4 amyloid precursor protein. *Proc. Natl. Acad. Sci. USA* **89,** 3055–3059.

De Strooper B., Umans L., Vanleuven F., and Vandenberghe H. (1993) Study of the synthesis and secretion of normal and artificial mutants of murine amyloid precursor protein (APP)—cleavage of APP occurs in a late compartment of the default secretion pathway. *J. Cell Biol.* **121,** 295–304.

Emmerling M. R., Moore C. J., Doyle P. D., Carroll R. T., and Davis R. E. (1993) Phospholipase A2 activation influences the processing and secretion of the amyloid precursor protein. *Biochem. Biophys. Res. Commun.* **197,** 292–297.

Farber S. A., Nitsch R. M., Schulz J. G., and Wurtman R. J. (1995) Regulated secretion of β-amyloid precursor protein in rat brain. *J. Neurosci.* **15,** 7442–7450.

Francis P. T., Sims N. R., Procter A. W., and Bowen D. M. (1993) Cortical pyramidal neurone loss may cause glutamatergic hypoactivity and cognitive impairment in Alzheimer's disease: investigative and therapeutic perspectives. *J. Neurochem.* **60,** 1589–1604.

Gabellini N., Manev R. M., and Manev H. (1994) Is the heterologous expression of metabotropic glutamate receptors (mGluRs) an appropriate method to study the mGluR function? Experience with human embryonic kidney 293 cells transfected with mGluR1. *Neurochem. Int.* **24,** 533–536.

Gabuzda D., Busciglio J., and Yankner B. A. (1993) Inhibition of β-amyloid production by activation of protein kinase C. *J. Neurochem.* **61,** 2326–2329.

Gereau R. W. and Conn P. J. (1995) Multiple presynaptic metabotropic glutamate receptors modulate excitatory and inhibitory synaptic transmission in hippocampal area CA1. *J. Neurosci.* **15,** 6879–6889.

Gowing E., Roher A. E., Woods A. S., Cotter R. J., Chaney M., Little S. P., and Ball M. J. (1994) Chemical characterization of A beta 17-42 peptide, a component of diffuse amyloid deposits of Alzheimer's disease. *J. Biol. Chem.* **269,** 10987–10990.

Haass C., Hung A. Y., Schlossmacher M. G., Teplow D. B., and Selkoe D. J. (1993) β-Amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. *J. Biol. Chem.* **268,** 3021–3024.

Houamed K. M., Kuijper J. L., Gilbert T. L., Haldeman B. A., O'Hara P. J., Mulvihill E. R., Almers W., and Hagen F. S. (1991) Cloning, expression, and gene structure of a G protein-coupled glutamate receptor from the rat brain. *Science* **252,** 1318–1321.

Hung A. Y., Haass C., Nitsch R. M., Qiao Qiu W., Citron M., Wurtman R. J., Growdon J. H., and Selkoe D. J. (1993) Activation of protein kinase C inhibits cellular production of the amyloid β-protein. *J. Biol. Chem.* **268,** 22959–22962.

Kang J., Lemaire H.-G., Unterbeck A., Salbaum J. M., Masters C. L., Grzeschik K. H., Multhaup G., Beyreuther K., and Miller-Hill B. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325,** 733–736.

Kobayashi K., Manabe T., and Takahashi T. (1996) Presynaptic long-term depression at the hippocampal mossy fiber-CA3 synapse. *Science* **273,** 648–650.

Lee R. K. K., Wurtman R. J., Slack B. E., Cox A. J., and Nitsch R. M. (1995) Amyloid precursor protein processing is stimulated by metabotropic glutamate receptors. *Proc. Natl. Acad. Sci. USA* **92,** 8083–8087.

Linden D. J. (1994) Long-term depression in the mammalian brain. *Neuron* **12,** 457–472.

Mann D. M., Iwatsubo T., Cairns N. J., Lantos P. L., Nochlin D., Sumi S. M., Bird T. D., Poorkaj P., Hardy J., Hutton M., Crook R., Rossor M. N., and Haltia M. (1996) Amyloid β protein (Aβ) deposition in chromosome 14-linked Alzheimer's disease: predominance of Aβ42(43). *Ann. Neurol.* **40,** 149–156.

Masu M., Tanabe Y., Shigemoto R., and Nakanishi S. (1991) Sequence and expression of a metabotropic glutamate receptor. *Nature* **349,** 760–765.

McBain C. J., DiChiara T. J., and Kauer J. A. (1994) Activation of metabotropic glutamate receptors differentially affects two classes of hippocampal interneurons and potentiates excitatory synaptic transmission. *J. Neurosci.* **14,** 4433–4445.

McDonald J. W. and Schoepp D. D. (1992) The metabotropic excitatory amino acid receptor agonists 1*S*,3*R*-ACPD selectively potentiates *N*-methyl-D-aspartate-induced brain injury. *Eur. J. Pharmacol.* **215,** 353–354.

McDonald J. W., Fix A. S., Tizzano J. P., and Schoepp D. D. (1993) Seizures and brain injury in neonatal rats induced by 1*S*,3*R*-ACPD, a metabotropic glutamate receptor agonist. *J. Neurosci.* **13,** 4445–4455.

Milward E. A., Papadopoulos R., Fuller S. J., Moir R. D., Small D., Beyreuther K., and Masters C. L. (1992) The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth. *Neuron* **9,** 129–137.

Nakanishi S. (1992) Molecular diversity of glutamate receptors and implications for brain function. *Science* **258,** 597–603.

Nakanishi S. (1994) Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. *Neuron* **13,** 1031–1037.

Nitsch R. M. and Growdon J. H. (1994) Role of neurotransmission in the regulation of amyloid β-protein precursor processing. *Biochem. Pharmacol.* **47,** 1275–1284.

Nitsch R. M., Slack B. E., Wurtman R. J., and Growdon J. H. (1992) Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. *Science* **258,** 304–307.

Nitsch R. M., Farber S. A., Growdon J. H., and Wurtman R. J. (1993) Release of amyloid β-protein precursor derivatives from hippocampal slices by electrical depolarization. *Proc. Natl. Acad. Sci. USA* **90,** 5191–5193.

Nitsch R. M., Deng M., Growdon J. H., and Wurtman R. J. (1996) Serotonin 5-HT_{2a} and 5-HT_{2c} receptors stimulate amyloid precursor protein ectodomain secretion. *J. Biol. Chem.* **271,** 4188–4194.

Pin J. P. and Duvoisin R. (1995) The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* **34,** 1–26.

Querfurth H. W. and Selkoe D. J. (1995) Calcium ionophore increases amyloid beta peptide production by cultured cells. *Biochemistry* **33,** 4550–4561.

Sambrook J., Fritsch E. F., and Maniatis T. (1989) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scheuner D., Eckman C., Jensen M., Song X., Citron M., Suzuki N., Bird T. D., Hardy J., Hutton M., Kukull W., Larson E., Levy-Lahad E., Viitanan M., Peskind E., Poorkaj P., Schellenberg G., Tanzi R., Wasco W., Lannfelt L., Selkoe D., and Younkin S. (1996) Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat. Med.* **2,** 850–852.

Selkoe D. J. (1994) Cell biology of the amyloid β-protein precursor and the mechanism of Alzheimer's disease. *Annu. Rev. Cell Biol.* **10,** 373–403.

Seubert P., Oltersdorf T., Lee M., Barbour R., Blomquist C., Davis D., Bryant K., Fritz L., Galasko D., Thal L., Lieberburg I., and Schenk D. (1993) Secretion of β-amyloid precursor protein cleaved at the amino terminus of the β-amyloid peptide. *Nature* **361,** 260–263.

Shigemoto R., Nakanishi S., and Mizuno N. (1992) Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat. *J. Comp. Neurol.* **322,** 121–135.

Shigemoto R., Abe T., Nomura S., Nakanishi S., and Hirano T. (1994) Antibodies inactivating mGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells. *Neuron* **12,** 1245–1255.

Sisodia S. S., Koo E. H., Beyreuther K., Unterbeck A., and Price D. L. (1990) Evidence that β-amyloid protein of Alzheimer's disease is not derived by normal processing. *Science* **248,** 492–495.

Slack B. E., Nitsch R. M., Livneh E., Kunz G. M. Jr., Breu J., Eldar H., and Wurtman R. J. (1993) Regulation by phorbol esters of amyloid precursor protein release from Swiss 3T3 fibroblasts overexpressing protein kinase Ca. *J. Biol. Chem.* **268,** 21097–21101.

Slunt H. H., Thinakaran G., Von Koch C., Lo A. C. Y., Tanzi R. E., and Sisodia S. S. (1994) Expression of a ubiquitous, cross-reactive homologue of the mouse beta-amyloid precursor protein (APP). *J. Biol. Chem.* **269,** 2637–2644.

Straubli U., Rogers G., and Lynch G. (1994) Facilitation of metabotropic receptors enhances memory. *Proc. Natl. Acad. Sci. USA* **91,** 777–781.

Sugiyama H., Ito I., and Hirono C. (1987) A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature* **325,** 531–533.

Sugiyama H., Ito I., and Watanabe M. (1989) Glutamate receptor subtypes may be classified into two major categories: a study on *Xenopus* oocytes injected with rat brain mRNA. *Neuron* **3,** 129–132.

Tanabe Y., Masu M., Ishii T., Shigemoto R., and Nakanishi S. (1992) A family of metabotropic glutamate receptors. *Neuron* **8,** 169–179.

Thinakaran G., Teplow D. B., Siman R., Greenberg B., and Sisodia S. S. (1996) Metabolism of the "Swedish" amyloid precursor protein variant in Neuro2a (N2a) cells—evidence that cleavage at the "beta-secretase" site occurs in the Golgi apparatus. *J. Biol. Chem.* **271,** 9390–9397.

Wolf B. A., Wertkin A. M., Jolly Y. C., Yasuda R. P., Wolfe B. B., Konrad R. J., Manning D., Ravi S., Williamson J. R., and Lee V. M. Y. (1995) Muscarinic regulation of Alzheimer's disease amyloid precursor protein secretion and amyloid beta-protein production in human neuronal NT2N cells. *J. Biol. Chem.* **270,** 4916–4922.

Xu H., Greengard P., and Gandy S. (1995) Regulated formation of Golgi secretory vesicles containing Alzheimer beta-amyloid precursor protein. *J. Biol. Chem.* **270,** 23243–23245.

Yokoi M., Kobayashi K., Manabe T., Takahashi T., Sakaguchi I., Katsuura G., Shigemoto R., Ohishi H., Nomura S., Nakamura K., Nakao K., Katsuki M., and Nakanishi S. (1996) Impairment of hippocampal mossy fiber LTD in mice lacking mGluR2. *Science* **273,** 645–647.

Zheng H., Jiang M. H., Trumbauer M. E., Sirinathsinghji D. J. S., Hopkins R., Smith D. W., Heavens R. P., Dawson G. R., Boyce S., Conner M. W., Stevens K. A., Slunt H. H., Sisodia S. S., Chen H. Y., and Van der Ploeg L. H. T. (1995) Beta-amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. *Cell* **81,** 525–531.