The calpain inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) has been reported to have complex effects on the production of the β-amylloid peptide (Aβ). In this study, the effects of ALLN on the processing of the amyloid precursor protein (APP) to Aβ were examined in 293 cells expressing APP or the C-terminal 100 amino acids of APP (C100). In cells expressing APP or low levels of C100, ALLN increased Aβ40 and Aβ42 secretion at low concentrations, decreased Aβ40 and Aβ42 secretion at high concentrations, and increased cellular levels of C100 in a concentration-dependent manner by inhibiting C100 degradation. Low concentrations of ALLN increased Aβ42 secretion more dramatically than Aβ40 secretion. ALLN treatment of cells expressing high levels of C100 did not alter cellular C100 levels and inhibited Aβ40 and Aβ42 secretion with similar IC50 values. These results suggest that C100 can be processed both by γ-secretase and by a degradation pathway that is inhibited by low concentrations of ALLN. The data are consistent with inhibition of γ-secretase by high concentrations of ALLN but do not support previous assertions that ALLN is a selective inhibitor of the γ-secretase producing Aβ40. Rather, Aβ42 secretion may be more dependent on C100 substrate concentration than Aβ40 secretion.

The β-amylloid peptide (Aβ) is the major protein component of the senile plaques found in the brain of Alzheimer’s disease (AD) patients. Aβ is produced by proteolysis of a single transmembrane domain protein known as the amyloid precursor protein (APP) (reviewed in Refs. 1 and 2). The first step in Aβ production involves the cleavage of APP by an uncharacterized protease termed β-secretase. Cleavage of APP by β-secretase produces a large ectodomain protein known as APPsβ, which is ultimately secreted, and a C-terminal 14-kDa membrane-bound fragment known as C100 (also termed C99 in some references). C100 is subsequently cleaved by γ-secretase, another uncharacterized protease that cleaves within the transmembrane domain of C100 and produces the 39–43-amino acid Aβ peptides. Aβ40 is the dominant species of Aβ secreted from cultured cells and is also more abundant in cerebrospinal fluid of normal and AD patients. Aβ42, which comprises about 5–10% of total Aβ secreted from cultured cells, is more amyloidogenic and is the major species of Aβ that is deposited at the early stage of senile plaques formation.

Familial AD has thus far been associated with autosomal dominant mutations in the genes encoding APP, presenilin 1 (PS1), and presenilin 2 (PS2, 3). Multiple mutations in these three genes are associated with increased Aβ42 production (4–6). Collectively, these data suggest that excessive Aβ42 production is critical for the development of AD. Whereas the locations of mutations in the APP gene suggest that the mutations lead to increased Aβ42 production by increasing cleavage of APP by β- or γ-secretase, the mechanism by which presenilin mutations increase Aβ42 production remains unclear. Primary neuronal cultures derived from PS1 knock-out mice exhibit marked reduction of Aβ secretion (7), suggesting an essential role of PS1 in generating Aβ. Understanding the cellular mechanisms that regulate Aβ production will be a key step to unraveling the pathogenesis of AD.

Aβ production can also be modulated by peptide aldehyde protease inhibitors such as N-acetyl-leucyl-leucyl-norleucinal (ALLN, also known as calpain inhibitor I or LLnL) (5, 8–11). ALLN was first identified as a cysteine protease inhibitor (12), but at high concentrations it can also inhibit proteasome-associated activities (13). It has been reported that ALLN inhibits Aβ40 production at concentrations that have little effect on or even increase Aβ42 production (5, 9). These data are interpreted as evidence suggesting that Aβ40 and Aβ42 are produced by distinct γ-secretases. In contrast, a recent study demonstrates that ALLN increased Aβ40 and Aβ42 production at low concentrations and decreased Aβ40 and Aβ42 production at higher concentrations (10). Thus, the reported effects of ALLN on Aβ production are conflicting, and the mechanism(s) by which ALLN modulates Aβ production are not clear. Nevertheless, ALLN may serve as an important tool to investigate the regulation of Aβ biosynthesis.

In this study, the effects of ALLN on Aβ40 and Aβ42 secretion are examined in detail and the mechanism by which ALLN modulates Aβ secretion is further defined. The data provide the novel insights that substrate availability plays a major role in regulating Aβ40 and Aβ42 production by γ-secretase.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies W02, G2-10, and G2-11 were obtained from Dr. Konrad Beyreuther (University of Heidelberg, Heidelberg, Ger-
many). W02 recognizes an epitope at amino acids 5–8 of the Aβ peptide, and G2-10 and G2-11 specifically recognize the C terminus of Aβ40 and Aβ42, respectively (14). Antibody 54 was obtained from Dr. Barry Greenberg (Cephalon, Inc., West Chester, PA) and recognizes the secreted APP ectoprotein formed after β-secretase cleavage (APPs) (15).

Antibody 14, which recognizes an N-terminal domain of PS1(16), was obtained from Dr. Samuel Gandy (Cornell University, New York). N-Acetyl-leucyl-leucyl-norleucinal (ALLN) was purchased from Boehringer Mannheim. All tissue culture reagents used in this work were from Life Technologies, Inc.

cDNA Constructs, Cell Culture, and Transfection of Cultured Cells—A human APP695 cDNA clone with the Swedish mutation (APPsw) and a cDNA encoding the the C-terminal 99 amino acids of APP plus an N-terminal methionine (hereafter referred to as C100) were obtained from Dr. Barry Greenberg. C100 was cloned into the expression vector pcDNA3.1 (Invitrogen, San Diego, CA). The SPC100 construct consists of the N-terminal 18 amino acids of APP appended to the N terminus of C99 as described by others (17). To prepare the SPC100 construct, residues 19–596 of the APP695 cDNA were deleted by using the Seamless Cloning Kit (Stratagene, La Jolla, CA). The resulting SPC100 construct was cloned into the pcDNA3.1 vector. The APP London mutation (18) was introduced into C100 and SPC100 constructs using the QuickChange™ site-directed mutagenesis kit (Stratagene). The human cDNAs encoding wild type PS1 and mutant PS1 with the exon 9 deletion (PS1ΔE9) (19) were obtained from Dr. Peter St. George-Hyslop (University of Toronto, Toronto, Canada). PS1ΔE9 sometimes is referred to as exon 10 deletion (20), as an alternate 5'-untranslated region exon was missed in the initial characterization of PS1 genomic structure (19).

Human embryonic kidney 293 cells were purchased from American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin. For transient expression of C100 and SPC100, 293 cells were seeded into 6-well plates, and transfection was conducted 2 days later when the cells reached 60–70% confluence. Cells were transfected by means of Lipo- fectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's instructions. To prepare 293 cells stably expressing APPsw, 293 cells were transfected as described above. About 24 h after transfection, cells were passed to media containing 0.4 mg/ml G418, and G418-resistant clones were analyzed for Aβ secretion by ELISA (see below). Clones secreting high levels of Aβ were expanded and maintained in media supplemented with 0.2 mg/ml G418.

ALLN Treatment—APPsw cells and cells transiently expressing C100 or SPC100 were treated with various concentrations of ALLN for 16 h. The conditioned media were then collected, centrifuged at 10,000 × g for 5 min to remove cell debris, and stored at −20 °C prior to ELISA and Western blot analysis. The data shown are from one transfection and are representative of more than five transfections.

**Fig. 1.** Protein expression and Aβ secretion in cells expressing C100 and SPC100. A, schematic diagram of the C100 and SPC100 expression constructs. B, Western blot analysis of lysates prepared from cells expressing C100 and SPC100. The Western blot was probed with antibody W02 as described under “Experimental Procedures.” C and D, concentration of Aβ40 (C) and Aβ42 (D) in the conditioned media from cells expressing C100 and SPC100. Aβ40 and Aβ42 were quantitated by ELISA assay as described under “Experimental Procedures.” The data shown are from one transfection and are representative of more than five transfections.
Western Blot—APPsβ was detected in conditioned media by Western blot analysis with antibody 54. C100 and SPC100 were identified in cell lysates with antibody W02. Visualization was performed with an ECL kit (Amersham Pharmacia Biotech) according to the manufacturer’s procedure.

ELISA Analysis of Aβ Peptides—Sandwich ELISA assays were developed to measure Aβ40 and Aβ42 using the combination of antibodies G2-10/W02 and G2-11/W02, respectively. Both antibody G2-10 and G2-11 are more than 100-fold selective for Aβ40 and Aβ42, respectively (14), and the sensitivity of these assays are about 50–100 pg/ml. Briefly, Nunc MaxiSorb immunoassay plates were coated overnight at 4 °C with 0.4 μg/well G2-10 in 100 mM NaHCO3 (pH 9.5) or with 1 μg/well G2-11 in 100 mM Tris-HCl (pH 7.4). The wells were then washed with TTBS and were stored at 4 °C for up to 6 months. Conditioned media were diluted with 10% bovine serum albumin in TBS to yield a final concentration of 2% bovine serum albumin, and 100 μl of diluted media was added to each well along with 40 ng/well biotinylated W02. Biotinylation of W02 was performed with the EZ-Link™ Sulfo-NHS-LC-Biotinylation kit (Pierce) according to the manufacturer’s instructions. The plate was incubated at 4 °C with gentle shaking either overnight (for Aβ40 measurement) or for at least 24 h (for Aβ42 measurement). The plate was then washed five times with TTBS, and 100 μl of 0.5 μg/ml horseradish peroxidase-conjugated NeutrAvidin (Pierce) was added to each well and incubated at room temperature for 1 h. The color was developed with the TMB-H2O2 system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer’s instructions, and absorption at 450 nm was measured on a plate reader.

Metabolic Labeling of C100 with [35S]Methionine—293 cells were seeded in 60-mm dishes and transiently transfected with C100 as described above. About 40 h after transfection, cells were incubated in methionine- and cysteine-free DMEM medium for 1 h and were then labeled for 1 h with 150 μCi/ml [35S]methionine. The cells were subsequently washed and either kept frozen at −20 °C (pulse) or incubated with fresh complete DMEM in the absence and presence of 25 μM ALLN. The chase media and cell monolayers were collected at different time points and kept frozen until further analysis. C100 and Aβ peptide were immunoprecipitated with antibody W02 from radiolabeled cells and chased media, respectively. The cells were solubilized with 0.6 ml/dish of 1× RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS), and 0.5 ml of cell lysate was used for each immunoprecipitation assay with 3 μg of anti-
Expression of C100 and SPC100—In addition to full-length APP with the Swedish mutation, two truncated APP constructs were used in this study to examine the processing of APP by γ-secretase (see Fig. 1A). The construct designated as C100 consists of the methionine initiation codon plus the C-terminal 99 amino acids of APP. The N terminus of C100 corresponds to the β-secretase cleavage site, i.e. the N terminus of the Aβ peptide. The construct designated SPC100 consists of the methionine initiation codon, the 16 amino acid signal peptide of APP, and the first two amino acids of APP appended to the N terminus of C100. C100 and SPC100 are similar to the constructs previously reported by Dykes et al. (17) except that the expression vector pcDNA3.1 was used instead of pCEP4. Western blot analysis of extracts from 293 cells transiently expressing SPC100 detected a 14-kDa band that co-migrated with native C100 (Fig. 1B). These data confirm that the signal peptide of SPC100 was removed. As reported previously (21), the level of expression of C100 was much higher in cells transiently expressing SPC100 than in cells transiently expressing C100 (Fig. 1B).

293 cells transiently expressing SPC100 secreted 2–3-fold more Aβ40 and Aβ42 than cells transiently expressing C100 (Fig. 1, C and D), consistent with a previous report (21). The increased Aβ secretion from cells expressing SPC100 relative to cells expressing C100 is probably due to the higher level of C100 in these cells (Fig. 1B). Despite the quantitative differences in Aβ secretion from cells expressing C100 and SPC100, the relative amounts of Aβ42 and Aβ40 secreted by cells expressing the two constructs (i.e. the Aβ42:Aβ40 ratios) were similar (Fig. 2A). The effects of APP and PS1 FAD mutations on Aβ secretion from cells expressing C100 and SPC100 were also tested. As reported previously (16), extracts from 293 cells expressing wild type PS1 displayed a 48-kDa protein corresponding to full-length PS1 as well as a 33-kDa N-terminal fragment of PS1 (Fig. 2B). Extracts from 293 cells expressing the PS1 mutant PS1ΔE9 displayed a full-length PS1 protein with slightly greater mobility than that of full-length wild type PS1 due to the deletion of exon 9 in this mutant (Fig. 2B). The 33-kDa N-terminal fragment of PS1, which is derived primarily from endogenous PS1, was not increased significantly by overexpression of PS1wt or PS1ΔE9. As previously reported for full-length APP (4), the secretion of Aβ42 from cells expressing either C100 or SPC100 was selectively increased by co-expression of PS1ΔE9 or by introduction of the London mutation into C100 or SPC100 (Fig. 2A). Co-expression of PS1ΔE9 did not alter Aβ40 secretion from cells expressing either C100 or SPC100, nor did co-expression of wild type PS1 affect Aβ secretion from cells expressing either construct (data not shown). Co-expression of wild type PS1 or PS1ΔE9 did not affect the levels of C100 protein in cells expressing either C100, SPC100, C100-London, or SPC100-London (Fig. 2C and data not shown).

Thus, γ-secretase processing of C100 derived from either the C100 or SPC100 constructs is qualitatively similar as evidenced by the similar relative secretion of Aβ40 and Aβ42 (i.e. the Aβ42:Aβ40 ratio) and the similar effect of APP and PS1 FAD mutations on Aβ secretion.

**RESULTS**

**Expression of C100 and SPC100**—In addition to full-length APP with the Swedish mutation, two truncated APP constructs were used in this study to examine the processing of APP by γ-secretase (see Fig. 1A). The construct designated as C100 consists of the methionine initiation codon plus the C-terminal 99 amino acids of APP. The N terminus of C100 corresponds to the β-secretase cleavage site, i.e. the N terminus of the Aβ peptide. The construct designated SPC100 consists of the methionine initiation codon, the 16 amino acid signal peptide of APP, and the first two amino acids of APP appended to the N terminus of C100. C100 and SPC100 are similar to the constructs previously reported by Dykes et al. (17) except that the expression vector pcDNA3.1 was used instead of pCEP4. Western blot analysis of extracts from 293 cells transiently expressing SPC100 detected a 14-kDa band that co-migrated with native C100 (Fig. 1B). These data confirm that the signal peptide of SPC100 was removed. As reported previously (21), the level of expression of C100 was much higher in cells transiently expressing SPC100 than in cells transiently expressing C100 (Fig. 1B).

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Thus, γ-secretase processing of C100 derived from either the C100 or SPC100 constructs is qualitatively similar as evidenced by the similar relative secretion of Aβ40 and Aβ42 (i.e. the Aβ42:Aβ40 ratio) and the similar effect of APP and PS1 FAD mutations on Aβ secretion.
Modulation of Aβ Production by ALLN—ALLN and related peptide aldehyde protease inhibitors have multiple effects on APP processing. In addition to modulating Aβ production, it has been observed that these inhibitors can also potentiate the α- and β-secretase processing. In cells expressing C100 or APPsw, both Aβ40 and Aβ42 secretions were increased by treatment with low concentrations of ALLN and decreased by treatment with high concentrations of ALLN (Fig. 3, A and C). Aβ42 secretion from cells expressing C100 or APPsw was increased much more dramatically by low concentrations of ALLN than was Aβ40 secretion (Fig. 3, A and C). In contrast, ALLN had only concentration-dependent inhibitory effects on Aβ40 and Aβ42 secretion from cells expressing SPC100 (Fig. 6B).

Examination of cell lysates revealed that ALLN increased the cellular level of C100 in a concentration-dependent manner in cells expressing C100 and APPsw, whereas it did not affect C100 protein levels in cells expressing SPC100 (Figs. 4 and 5A). The ALLN-induced increase in C100 in APPsw cells was not due to increased β-secretase activity since ALLN did not potentiate the secretion of APPβ in these cells (Fig. 5B). The increase in C100 protein level induced by ALLN in cells expressing C100 or APPsw was not a result of inhibition of γ-secretase since concentrations of ALLN that increased cellular C100 protein levels also increased both Aβ40 and Aβ42 production. In addition, pulse-chase experiments demonstrated that at concentrations of ALLN that increased Aβ production (data not shown), this compound decreased the rate of C100 turnover in cells expressing C100 (Fig. 6B), suggesting that ALLN increases C100 protein levels by inhibiting C100 degradation.

DISCUSSION

In cells expressing APP, the protease inhibitor ALLN has recently been shown to inhibit selectively the production of Aβ40 at concentrations that have little effect on the production of Aβ42 (5, 9). These data were interpreted as indicating that
distinct γ-secretases are responsible for Aβ40 and Aβ42 production. Although these data are intriguing, a recent study suggests that the effects of ALLN on Aβ secretion from cells expressing APP are complex (10). To elucidate the mechanisms by which ALLN modulates Aβ production and, by inference, γ-secretase activity, this study examined the effects of ALLN on Aβ production in more detail.

The γ-secretase reaction was studied in 293 cells expressing either APPsw or various amounts of C100, the C-terminal fragment of APP that represents the immediate substrate of this enzyme. C100 was produced in 293 cells by expression of two constructs designated as C100 and SPC100 (Fig. 1). As reported previously, much higher levels of C100 were produced in cells expressing SPC100 than in cells producing C100 (21), presumably because the signal peptide present in SPC100 permits more efficient processing or sorting of the protein. The characteristics of Aβ production in cells expressing APPsw, C100 or SPC100 cells were similar. The relative amounts of Aβ40 and Aβ42 secreted by cells expressing C100 or SPC100 were similar as reflected by the similar Aβ42:Aβ40 ratios, and like the APP expression cells (4, 18), the secretion of Aβ42 was specifically increased by co-expression of mutant PS1 or by introduction of the London mutation into the constructs (Fig. 2). These observations argue that the same γ-secretase(s) is(are) responsible for Aβ production in cells expressing all three constructs.

Treatment of cells expressing C100 or APPsw with low concentrations of ALLN resulted in increased secretion of both Aβ40 and Aβ42 (Fig. 3, A and D), which is consistent with results reported by others (10). Interestingly, treatment of cells expressing either C100 or APPsw with low concentrations of ALLN also increased cellular C100 protein accumulation (Figs. 4 and 5A). Pulse-chase experiments in cells expressing C100 demonstrated that a low concentration of ALLN decreased the rate of C100 turnover (Fig. 6), whereas Aβ secretion was increased during the same period (data not shown). Taken together, these data suggest that low concentrations of ALLN increase Aβ production by inhibiting C100 turnover and, hence, the amount of C100 substrate available for γ-secretase cleavage (Fig. 7). This suggestion is supported by the observation that ALLN did not affect cellular C100 levels in cells expressing SPC100 and correspondingly did not increase Aβ secretion from these cells at any concentration. One implication of these results is that in addition to cleavage by γ-secretase, C100 is normally degraded by a distinct ALLN-sensitive pathway (Fig. 7). Channeling of C100 into this alternative, ALLN-sensitive degradation pathway would prevent Aβ production (Fig. 7). The fact that ALLN did not increase cellular C100 levels in cells expressing SPC100 may be due to the fact that the ALLN-sensitive degradation pathways is overwhelmed by the much higher levels of cellular levels of C100 present in these cells. Low concentrations of ALLN also increase Aβ secretion from primary hippocampal cultures where only endogenous APP is expressed,2 suggesting that the ALLN-sensitive degradation of this APP intermediate represents a normal metabolic process and is not merely an artifact of overexpressing C100 in cultured cells. The presence of an ALLN-sensitive catalytic pathway for C100 may provide a mechanism by which cells regulate substrate availability for γ-secretase and thus regulate cellular Aβ production. In this regard, the regulation of C100 metabolism may play an important role in AD pathogenesis.

An alternative mechanism has been proposed to explain the ability of ALLN to increase Aβ secretion (22). Several protease inhibitors, including ALLN, are known to prevent the proteasome-mediated degradation of PS1 (22, 23). Based on this observation and the fact that presenilin mutations are associated with increases in Aβ42 secretion, it has been suggested that the stabilization of presenilins by protease inhibitors like ALLN may directly potentiate Aβ42 production in cells (22). Our data do not support this model for two reasons. First, the effects of ALLN and PS1 mutations are additive, i.e. low concentrations ALLN further increase the Aβ42:Aβ40 ratio in cells expressing mutant PS1 (22). Second, the fact that Aβ42 production in cells expressing SPC100 can be increased by co-expression of a mutant PS1 (Fig. 2A), but not by ALLN (Fig. 3B), provides additional evidence that PS1 mutations and ALLN regulate Aβ42 production by independent mechanisms.

High concentrations of ALLN inhibited Aβ40 and Aβ42 production by cells expressing either APPsw or C100. High concentrations of ALLN may directly inhibit γ-secretase and, hence, decrease Aβ production, but other effects of ALLN on cellular metabolism could also be responsible for this effect. In cells expressing SPC100, which gives rise to much higher cellular C100 levels than in cells expressing APPsw or C100, ALLN inhibited Aβ40 and Aβ42 production at all concentrations and the IC50 values of ALLN for inhibition of Aβ40 and Aβ42 production were very similar. This observation does not support the conclusion that ALLN selectively inhibits Aβ40 production (5, 9), a finding that was interpreted as implying the existence of distinct γ-secretases responsible for Aβ40 and Aβ42 production. Since ALLN modulates Aβ production by multiple mechanisms, as documented in this study and Ref. 10, it is difficult to use this compound to pharmacologically distinguish multiple γ-secretases.

Whereas low concentrations of ALLN and other calpain/proteasome inhibitors increase Aβ40 and Aβ42 production, the increase in Aβ42 production induced by these protease inhibitors is much more pronounced (Ref. 10, Fig. 3, A and D). Although a definitive explanation for this phenomenon cannot be discerned from the existing data, a reasonable model can be proposed. As discussed above and illustrated in the model diagrammed in Fig. 7, ALLN increases Aβ production by increasing the availability of the γ-secretase substrate C100. Since ALLN increases Aβ42 secretion more dramatically than Aβ40 secretion, this model implies that the γ-secretase cleavage reaction producing Aβ42 is more dependent on substrate concentration than the reaction producing Aβ40. In other words, the γ-secretase that cleaves C100 to produce Aβ42 has a higher $K_m$ for the substrate C100 than the γ-secretase that

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2 P. Fraser and L. Zhang, unpublished data.

3 L. Zhang and L. Song, unpublished data.
cleaves C100 to produce Aβ40. Thus, the ALLN-induced increase in the cellular concentration of the γ-secretase substrate C100 will increase Aβ42 secretion more than Aβ40 secretion. The fact that Aβ40 secretion was only slightly increased by ALLN would suggest that the γ-secretase that produces Aβ40 is nearly saturated with C100 under the conditions used in these experiments. This model would explain why Aβ40 is the dominant Aβ species produced during normal physiologic processing of APP. It should be pointed out that this model and the experimental data that support it do not necessarily require the processing of APP. It should be pointed out that this model and the experimental data that support it do not necessarily require the existence of two distinct γ-secretase enzymes that independently produce Aβ40 and Aβ42. The major weakness of this model is the observation that the higher cellular levels of C100 seen in cells expressing SPC100 relative to cells expressing C100 is associated with similar increases in Aβ40 and Aβ42 secretion rather than a more selective increase in Aβ42 secretion as would be predicted by the model. It is possible that the model is fundamentally correct but that increasing cellular C100 levels by expressing SPC100 rather than C100 is somehow biochemically or mechanistically different from increasing cellular C100 levels with ALLN. In any case, the hypothesis that substrate concentration is a more important determinant of Aβ42 production than Aβ40 production provides a novel framework for further experiments aimed at understanding the mechanisms regulating Aβ production.

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REFERENCES
1. Selkoe, D. (1994) Annu. Rev. Cell Biol. 10, 373–403
2. Selkoe, D. J. (1996) J. Biol. Chem. 271, 18285–18288
3. Hardy, J. (1997) Trends Neurosci. 20, 154–159
4. Borchelt, D., Thinakaran, G., Eckman, C., Lee, M., Davenport, F., Ratovitsky, M., Prada, C., Kim, G., Seekins, S., Yager, D., Hust, H., Wang, R., Steiger, M., Levey, A., Gandy, S., Copeland, N., Jenkins, N., Price, D., Younkin, S., and Sisodia, S. (1996) Neuron 17, 1005–1013
5. Citron, M., Diehl, T., Gordon, G., Bierie, A., Seubert, P., and Selkoe, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13170–13175
6. Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C., Perez-tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., Morgan, D., Gordon, M., Holcomb, L., Refolo, L., Zenk, B., Hardy, J., and Younkin, S. (1996) Nature 383, 710–713
7. De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Ghede, G., Annaert, W., Von Figura, K., and Van Leuven, F. (1998) Nature 391, 397–399
8. Higaki, J., Quon, D., Zhong, Z., and Cordell, B. (1995) Neuron 14, 651–659
9. Klafki, H.-W., Abramowski, D., Sweeney, P., Pagani, P. A., and Staufner, M. (1996) J. Biol. Chem. 271, 28655–28659
10. Yamazaki, T., Haass, C., Saito, T. C., Omura, S., and Ihara, Y. (1997) Biochemistry 36, 8377–8383
11. Klafki, H.-W., Paganetti, P. A., Sommer, B., and Staufner, M. (1995) Neuron 15, 1245–1252
12. Hiwasa, T., Sawada, T., and Sakiyama, S. (1990) Carcinogenesis 11, 37–42
13. Viniski, A., Michaud, C., Powers, J., and Orlowski, M. (1992) Biochemistry 31, 9421–9426
14. Ida, N., Hartmann, T., Pantel, J., Schroder, J., Zerfass, R., Fostil, H., Sandbrink, R., Masters, C. L., and Beyreuther, K. (1996) J. Biol. Chem. 271, 22908–22914
15. Savage, M. J., Trusko, S. P., Howland, D. S., Pinsky, L. R., Mistretta, S., Reaume, A. G., Greenberg, B. D., Siman, R., and Scott, R. W. (1998) J. Neurosci. 18, 1743–1752
16. Thinakaran, G., Borghelt, D., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A. I., Gandy, S. E., Jenkins, N. A., Copeland, N. G., Price, D. L., and Sisodia, S. S. (1996) Neuron 17, 181–190
17. Dykes, T., Dykes, E., Masters, C., and Beyreuther, K. (1992) FEBS Lett. 309, 20–24
18. Suzuki, N., Cheung, T., Cai, X., Oda, A., Otos, L., Jr., Eckman, C., Gerl, H., and Younkin, S. (1994) Science 264, 1336–1340
19. Rogaev, E. I., Sherrington, R., Wu, C., Levesque, G., Liang, Y., Rogaeva, E. A., Ikeda, M., Holman, K., Lin, C., Lukiw, W. J., de Jong, P. J., Fraser, P. E., Rommens, J. M., and St George-Hyslop, P. (1997) Genomics 40, 415–424
20. Clark, R. F., and The Alzheimer's Disease Collaboration Group (1995) Nat. Genet. 11, 219–222
21. Dykes, T., Dykes, E., Fonnum, U., rnemone, B., Turner, J., and Beyreuther, K. (1993) FEBS Lett. 335, 89–93
22. Marambaud, P., Ancolio, K., Lopez-Perez, E., and Checler, F. (1998) Mol. Med. 4, 147–157
23. Fraser, P. E., Levesque, G., Yu, G., Mills, L. R., Thirwell, J., Frantsvea, M., Gandy, S. E., Seeger, M., Carlen, P. L., and St. George-Hyslop, P. (1998) Neurobiol. Aging 19, 19–21