Mutations in the presenilin genes (PS-1 and PS-2) are linked to early onset familial Alzheimer’s disease (AD), but the mechanisms by which these mutations cause the cognitive impairment characteristic of AD are unknown. Basal forebrain cholinergic neurons are involved in learning and memory processes, and reductions in choline acetyltransferase (ChAT) activity are a characteristic feature of AD brain. We therefore hypothesized that presenilin mutations suppress expression of the cholinergic phenotype. In rat PC12 cells stably transfected with the human PS-1 gene containing the Leu → Val mutation at codon 286 (L286V), we observed a drastic reduction (>99%) in basal ChAT activity compared with cells transfected with vector alone. By immunocytochemistry, a similar decrease in ChAT protein levels was found in the mutant transfectants. In cells differentiated with nerve growth factor, ChAT activity was again markedly lower in L286V-expressing cells than in control cells. We also observed reductions in ChAT activity in PC12 cells expressing the wild-type human PS-1 gene but to a lesser extent than in L286V-expressing cells. The viability of cells transfected with either the wild-type or the mutant PS-1 gene was not compromised. Our results suggest that PS-1 mutations may contribute to the cognitive impairment in AD by causing a nontoxic suppression of the cholinergic phenotype.

Understanding the pathogenesis of Alzheimer’s disease (AD) is complicated by the facts that several genes have been linked to this disorder and that familial forms of AD account for only about 10% of the total number of cases (reviewed in Ref. 1). However, irrespective of the underlying cause, the brains of all AD patients have common pathological end points, namely, neuritic plaques and neurofibrillary tangles (reviewed in Ref. 2). The neuritic plaque is an extracellular deposit composed of the amyloid-β protein (Aβ), a 39–43-amino acid peptide derived from the 110–130-kDa transmembrane glycoprotein known as the amyloid precursor protein (APP). Thus, studies aimed at elucidating the pathogenesis of AD have focussed on the mechanisms by which Aβ is produced and deposited in AD brain. Mutations in genes located on chromosomes 14 and 1, designated presenilin-1 (PS-1) and presenilin-2 (PS-2), respectively, are responsible for the majority of early onset familial AD cases (3, 4). Patients carrying mutations in the PS-1, PS-2, or APP genes have increased plasma Aβ 1–42 levels versus controls (5). Furthermore, in the medium of N2a mouse neuroblastoma cells and in the brains of transgenic mice co-expressing wild-type human APP and mutant human PS-1, elevations in the ratio of Aβ 1–42 to Aβ 1–40 were observed (6). Thus, a consequence of PS mutations appears to be altered processing of APP in favor of production of Aβ 1–42, which would promote neuritic plaque formation.

Basal forebrain cholinergic neurons, whose cell bodies reside within the medial septum, Broca’s diagonal band, and the nucleus basalis magnocellularis and which project to the neocortex and hippocampus, are involved in learning and memory processes (7, 8). A “cholinergic” hypothesis for the memory impairment that is characteristic of AD was proposed (9) based on the observation of a profound loss of cholinergic neurons in the nucleus basalis magnocellularis in AD brain (10). For unknown reasons, the hippocampus and certain regions of the cortex are vulnerable to deposition of Aβ, and numerous studies carried out in vitro and in vivo have shown that Aβ is toxic to neurons (reviewed in Refs. 11 and 12). Recently, it was demonstrated in vitro that either mutant PS-1 or PS-2 can potentiate Aβ-induced apoptosis (13, 14), which is relevant given that markers of apoptosis have been observed in AD brain (15, 16). However, apart from the neurodegenerative effects of Aβ, there is now evidence to support a contribution of nontoxic mechanisms to the cholinergic deficit in AD. Under conditions where there was no cell death, exposure of either a septal cell line or primary sepal cultures to Aβ 1–42 reduced the levels of certain cholinergic markers (17, 18), and exposure of hippocampal slices to Aβ 1–42 impaired potassium-evoked ACh release (19). Thus, we sought to determine if PS mutations have adverse effects on cholinergic systems that do not involve degenerative mechanisms.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—PC12 cells were maintained at 37°C in an atmosphere of 95% air/5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, and 10 μg/ml of gentamyces. The cells were subcultured by mechanically removing them from the substrate with squirts of fresh medium. We cloned the cDNA encoding full-length human PS-1, with and without the L86V mutation, into the pBluescript vector as described in our previous studies (13, 20). Transfection of the PC12 cells was carried out with Lipofectamine (Life Technologies, Inc.), and G418-resistant clones were isolated. Transfectants were cultured continuously in the presence of G418 (0.5 mg/ml). Because ChAT activity is decreased as a function of the number of generations of the cells (21), experiments were carried out using control cells and transfectants that had been passaged equal numbers of times.

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For studies involving treatment with NGF (2.5 S; Boehringer Mannheim), cells were cultured as described but with reduced serum content (1% total). The Aβ peptides were synthesized by standard Fmoc procedures as described (22). Stock solutions of the peptides were prepared in dimethyl sulfoxide to a final concentration of 1 mM and stored at −20 °C. Immediately prior to use, an aliquot of the peptide solution was diluted in medium, and treatments were carried out in medium with normal serum content.

**Immunocytochemistry—**Cells were grown to subconfluence on glass in 35 × 10-mm dishes. Following washes with phosphate-buffered saline (PBS), cells were fixed for 30 min with 4% paraformaldehyde and then exposed for 5 min to 0.2% Triton X-100 in PBS to permeabilize the membranes. Blocking was carried out for 30 min with 0.5% horse serum. The cells were then incubated overnight at 4 °C with a mouse monoclonal anti-ChAT antibody (23) at a final concentration of 2 ng/ml. The antibodies were from Vector Laboratories (Burlingame, CA). Immunofluorescence images were obtained with a confocal laser scanning microscope (Molecular Dynamics) using a 60 ×, n.a. 1.3 oil immersion lens. The excitation wavelength was 488 nm, and emitted fluorescence passed through a 510 nm barrier.

**ChAT Activity Assay—**The measurement of ChAT activity was carried out by the radioenzymatic assay of Fonnum (24). Cells were grown to subconfluence in 60 × 15-mm dishes. For preparation of homogenates, cells were washed with 1 × 2 ml ice-cold PBS, scraped into 1 ml of a 50 mM sodium phosphate buffer, pH 7.4 (“buffer A”), and transferred to a microcentrifuge tube. The suspension was centrifuged, the supernatant removed by aspiration, and the pellet was resuspended in buffer A and sonicated. Protein content was determined using the bicinchoninic acid assay with bovine serum albumin as the standard (Pierce). For the measurement of ChAT activity, 100–200 μg of homogenate was used in each assay. Each aliquot of homogenate was diluted to an appropriate volume with buffer A. Samples were incubated for 30 min at 37 °C following addition of 200 μl of buffer A containing: 150 mM NaCl, 5 mM EDTA, 5 mM choline, 0.1 mM eserine, 0.2 mM acetyl-CoA, and 0.25 μCi of [3H]acetyl-CoA. The reaction was terminated by the addition of 200 μl of 1.5% triphenylboron in 3-heptanone. The mixtures were vortexed, 75 μl was taken from the upper phase (which contained [3H]ACh), and the radioactivity was measured using a Packard 2500 TR Liquid Scintillation Analyzer (Downers Grove, IL) using 5 ml of ScintiVerse II as the scintillator.

**RESULTS AND DISCUSSION**

One of the neurotransmitters synthesized by rat pheochromocytoma PC12 cells is acetylcholine (ACh). The enzyme responsible for ACh synthesis is choline acetyltransferase (ChAT), which utilizes the precursors choline and acetyl-CoA. Although not derived from a true cholinergic cell population, PC12 cells represent a useful model for investigating factors that regulate ChAT activity and ACh synthesis. As a cell model to determine if mutations in the PS-1 gene alter cholinergic properties, we generated stable transfectants of PC12 cells with a DNA construct encoding human PS-1 with the Leu-Val mutation at position 286 (L286V). Although mutations are found throughout the PS-1 protein, the majority of them are in exon 8, which encodes part of transmembrane domain 6 and most of the hairpin loop comprising domain 7 (reviewed in Ref. 25). It is proposed that exon 8 is a functional domain, and elucidating the biological consequences of mutations in this domain may therefore be critical in understanding the pathogenesis of early onset familial AD. Thus, we chose to investigate the effects of the L286V mutation, which is found in the hairpin loop domain 7, on ChAT activity in PC12 cells. We limited our studies to the effects of L286V on ChAT activity, because this cholinergic marker has been reported to provide the best biochemical index of the severity of dementia in AD (26).

The PC12 cells expressing L286V displayed a marked reduction in ChAT activity versus untransfected cells (Fig. 1). Consistently, we observed reductions in ChAT activity in the range of 50–90% in PC12 cells expressing this mutation. Transfection of the cells with the expression vector alone did not alter the ChAT activity of the cells (Fig. 1). We also observed a reduction in ChAT activity in PC12 cells transfected with wild-type human PS-1, although it was to a lesser extent than in cells that express the L286V mutation (Fig. 1). The differential effects on ChAT activity between wild-type and mutant PS-1 were not due to differences in protein expression levels, which were found to be similar in both transfectants by Western blotting analysis (13). We observed the suppressive effects on ChAT activity in two other clonal lines expressing either wild-type or mutant human PS-1 (data not shown). Importantly, by measurement of lactate dehydrogenase release and intracellular calcium content, the viability of none of the transfectants was altered relative to control cells under basal culture conditions (Ref. 20 and data not shown).

The regulation of ChAT activity appears to predominantly involve transcriptional mechanisms but also may occur at the level of RNA processing, transport, turnover, or translation (reviewed in Ref. 27). However, there is no convincing evidence in support of a role for post-translational modifications in the regulation of ChAT activity. Thus, to begin to elucidate the mechanisms by which mutant PS-1 suppresses ChAT activity,
we determined if there were differences in the levels of ChAT protein in L286V-expressing versus control PC12 cells. We carried out immunostaining with a mouse monoclonal antibody that specifically recognizes ChAT. In control cells, there was intense ChAT immunoreactivity localized to the cytoplasm (Fig. 2). The levels of ChAT protein in L286V-expressing PC12 cells were virtually undetectable (Fig. 2), consistent with the results obtained from ChAT activity measurements in these transfectants. These data indicate that reductions in ChAT activity in PC12 cells caused by mutant PS-1 are due primarily to decreased levels of the ChAT protein rather than direct effects on the activity of the enzyme.

An important feature of PC12 cells is their ability to differentiate into a neuronal phenotype in the presence of nerve growth factor (NGF), i.e., extension of neurite-like processes. Concomitantly, NGF increases the transcription of the ChAT gene, which appears to be mediated by the AP-1 transcriptional response element (reviewed in Ref. 27), thereby enhancing the cholinergic state of the cell. Thus, we tested the hypothesis that the NGF-induced differentiation of PC12 cells expressing L286V can reverse or attenuate the suppressive effects of the mutation on ChAT activity. In the presence of 50 ng/ml of NGF for 7 days, both control and L286V-expressing cells became more flattened and substrate-adherent and extended processes (data not shown). There was a statistically significant increase in ChAT activity in both control and L286V-expressing cells treated with NGF (Fig. 3), which demonstrates that overexpressing the human PS-1 gene does not interfere with the ability of NGF to induce ChAT activity in PC12 cells. However, the level of ChAT activity in NGF-treated cells containing mutant PS-1 were still suppressed below that observed in NGF-treated control cells. Note that the differentiation experiments were carried out in the presence of medium containing 1% serum, which caused a decrease in the basal levels of ChAT activity. These results demonstrate that the reduction in ChAT activity caused by mutant PS-1 is not limited to undifferentiated cells, which leaves open the possibility that the effect is relevant to differentiated, post-mitotic cholinergic neurons.

Evidence from studies of Down's Syndrome patients suggests that Aβ 1–42 is the species initially deposited in the brain during the formation of the neuritic plaque (28). Given that fibrils formed from Aβ 1–42 are directly toxic to neurons (reviewed in Refs. 11 and 12), it is believed that the degeneration of basal forebrain cholinergic neurons caused by deposition of the peptide is responsible for the cognitive impairment in AD. However, new evidence from in vitro studies suggests that soluble, nontoxic Aβ 1–42 induces cholinergic hypoaqitivity (17–19), which may also contribute to the cognitive impairment in AD. Because expression of a mutant human PS-1 gene appears to alter APP processing such that there is increased production of Aβ 1–42 (5, 6) and based on the finding that nontoxic Aβ 1–42 peptide derived from either the rodent or human sequence, and cells were processed for ChAT activity measurements as before. There is clearly no difference in ChAT activity between control and Aβ-treated cells.

We have demonstrated that rat cholinergic cells expressing mutant human PS-1 have reduced ChAT activity. The mechanism(s) by which the mutant PS-1 protein exerts this suppressive effect is unclear. However, the parallel decreases in ChAT activity and ChAT protein levels in L286V-expressing cells indicate that the mutant PS-1 protein does not have a direct effect on the activity of the enzyme. Interestingly, expression of the mutant PS-1 gene in PC12 cells did not prevent NGF inducibility of ChAT activity. It has been shown, using human neuroblastoma SH-SY5Y cells, that activation of muscarinic ACh receptors by carbachol increases the DNA binding of AP-1 (29). This effect was found to be sensitive to oxidative stress imposed on the cells by treatment with H₂O₂. In our mutant PS-1 transfectants, however, the basal intracellular levels of peroxides (measured using the dye 2,7-dichlorofluorescein) were found to be the same as in control cells (13). Thus, mutant PS-1 does not appear to alter the levels of ChAT activity in PC12 cells by targeting the AP-1 transcriptional machinery, and the mechanism by which it exerts its suppressive effect is suggested to be independent of that by which NGF induces ChAT activity. Moreover, because NGF treatment caused an increase in ChAT activity in L286V-expressing cells, it appears that our selection process resulted in a population of cells that have an intact AP-1 response pathway.

Although the physiological roles of the PS proteins are unknown, some understanding has been gained regarding the pathological consequences of mutations in the PS genes. It has
been shown that NGF-treated PC12 cells expressing a mutated human PS-1 gene are more sensitive to apoptosis induced by withdrawal of NGF or by exposure to Aβ1–42 (13). Although less effective than the mutant, it was observed that expression of wild-type human PS-2 in NGF-treated PC12 cells potentiates Aβ-induced apoptosis (14). Thus, a gain-of-function with respect to pro-apoptotic actions appears to be conferred upon PS proteins with familial AD mutations. In the current study, we have found that expression of the wild-type human PS-1 gene suppressed ChAT activity in PC12 cells, yet to a lesser extent than expression of the L286V mutation. Mutant PS proteins appear, therefore, to have gained anti-cholinergic functions relative to the wild-type proteins. Our results suggest that PS proteins may have a role in regulating expression of the cholinergic phenotype, at least at the level of modulating ChAT protein levels and overall cellular activity of the enzyme.

A number of neurotransmitter systems are altered in AD brain, but evidence suggests that disruption of the cholinergic system may have a particular importance for the cognitive impairment in AD. In a study where the levels of several neurochemical indices were measured in ten neocortical brain regions from a large number of AD cases and age-matched controls, it was concluded that ChAT activity provides the best biochemical correlate of the severity of dementia in AD (26). Hence, these results support the original proposal that basal forebrain cholinergic neurons are selectively vulnerable in AD brain (10). Indeed, basal forebrain cholinergic neurons appear to be more vulnerable than γ-amino butyric acid-expression neurons, for instance, to the toxic effects of Aβ peptides, apparently due to the differences in the abundance of calcium buffering proteins (30, 31). It is possible that basal forebrain cholinergic neurons are more vulnerable to the proapoptotic actions of mutant PS proteins. However, the results presented here suggest that PS mutations may have other adverse consequences in addition to potentiation of degenerative processes. In summary, our results provide further support for the hypothesis that nondegenerative mechanisms contribute to the cholinergic deficit and cognitive impairment characteristic of AD.

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REFERENCES

1. Yankner, B. A. (1996) Neuron 16, 921–932
2. Selkoe, D. J. (1994) Annu. Rev. Neurosci. 17, 489–517
3. Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, C., Lin, G., Helman, K., Tsuda, T., Mar, L., Foncin, J. F., Bruni, A. C., Montesi, M. P., Sorbi, S., Rainiero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinski, R. J., Wasco, W., Da Silva, H. A. R., Haines, J. L., Pericak-Vance, M. A., Tanzi, R. E., Roses, A. D., Fraser, P. E., Rommens, J. M., and St. George-Hyslop, P. H. (1995) Nature 375, 754–760
4. Levy-Lahad E., Wasco, W., Poorkaj, P. Romano, D. M., Oshima, J., Pettingell, W. H., Yu, C.-E., Jondro, P. D., Schmidt, S. D., Wang, R., Crowley, A. C. Fu, Y.-H., Guenette, S. Y., Galas, D., Nemens, E., Wijman, E. M., Bird, T. D., Schellenberg, G. D., and Tanzi, R. E. (1995) Science 269, 973–977
5. 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., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Nat. Med. 2, 864–870
6. Borchart, D. B., Thinakaran, G., Eckman, C. B., Lee, M. K., Davenport, P., Ratovitsky, T., Prada, C.-M., Kim, G., Seekins, S., Yager, D., Shunt, H. H., Wang, R., Seeger, M., Levey, A. I., Gandy, S. E., Copeland, N. G., Jenkins, N. A., Price, D. L., Younkin, S. G., and Sisodia, S. S. (1996) Neuron 17, 1005–1013
7. Dutar, P., Bassant, M.-H., Senut, M.-C., and Lamour, Y. (1995) Physiol. Rev. 75, 393–427
8. Winkler, J., Stuh, S. T., Gage, F. H., Thal, L. J., and Fisher, L. J. (1995) Nature 375, 484–487
9. Bartus, R. T., Dean, R. L., Beer, B., and Lippa, A. S. (1982) Science 217, 408–417
10. Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T., and DeLong, M. R. (1982) Science 215, 1237–1239
11. Iversen, L. L., Mortishire-Smith, R. J., Pollack, S. J., and Shearn, M. S. (1995) Biochem. J. 311, 1–18
12. Mattson, M. P., Furukawa, K., Bruce, A. J., Mark, R. J., and Blanc, E. (1996) in Molecular Mechanisms of Dementia (Wasco, W., and Tanzi, R. E., eds) pp. 103–143, Humana Press, Totowa, New Jersey
13. Gao, Q., Sopher, B. L., Furukawa, K., Pham, D. G., Robinson, N., Martin, G. M., and Mattson, M. P. (1997) J. Neurosci. 17, 4212–4222
14. Wolozin, B., Iwasaki, K., Vito, P., Ganjei, J. K., Lasona, E., Sunderland, T., Zhao, B., Kusiak, J. W., Wasco, W., and D’Adamo, L. (1996) Science 274, 1710–1713
15. Su, J. H., Anderson, A. J., Cummings, B., and Cotman, C. W. (1994) Neuron 5, 2529–2533
16. Smale, G., Nichols, N. R., and Brady, D. R. (1995) Exp. Neural. 133, 225–230
17. Kat, S., Seto, D., Gaudreau, P., and Quirion, R. (1996) J. Neurosci. 16, 1034–1040
18. Pedersen, W. A., Kloczewiak, M. A., and Bluszta, J. K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8068–8071
19. Hoshi, M., Takashima, A., Murayama, N., Yasutake, K., Yoshida, N., Ishiguro, K., and Imahori, K. (1997) J. Biol. Chem. 272, 2038–2041
20. Gao, Q., Furukawa, K., Sopher, B. L., Pham, D. G., Xie, J., Robinson, N., Martin, G. M., and Mattson, M. (1996) Neuron Report 8, 379–383
21. Yamada, M., Ikeuchi, T., Tsukui, H., Aimoto, S., and Hatanaka, H. (1994) Brain Res. 661, 137–146
22. Mattson, M. P., Cheng, B., Davis, G., Bryant, K., Lieberburg, I., and Rydell, R. E. (1995) J. Neurosci. 15, 375–389
23. Oakman, S. A., Faris, P. L., Kerr, P. E., Cozzari, C., and Hartman, B. K. (1995) J. Neurosci. 15, 5859–5869
24. Fornoan, P. (1979) J. Neurochem. 4, 407–409
25. Hardy, J. (1997) Trend. Neurosci. 20, 154–159
26. Brierer, L. M., Haroutunian, V., Gabriel, S., Knott, P. J., Carlin, L. S., Purshut, D. P., Perl, D. P., Schmidt, J., Kanof, P., and Davis, K. L. (1995) J. Neurochem. 64, 749–760
27. Du, D., and Hersh, L. B. (1994) J. Neurochem. 62, 1653–1663
28. Iwatsubo, T., Mann, D. M., Odaka, A., Suzuki, N., and Ihara, Y. (1995) Ann. Neurol. 39, 294–299
29. Li, X., Song, L., and Jope, R. S. (1996) J. Neurosci. 16, 5914–5922
30. Harkany, T., De Jong, G. I., Soos, K., Penke, B., Luiten, P. G. M., and Gulya, K. (1995) Brain Res. 698, 270–274
31. Pike, C. J., and Cotman, C. W. (1993) Neurosci. 56, 269–274