were obtained at autopsy. Subjects had no neurological disease, except for one man and two women who had been diagnosed clinically and pathologically as suffering from senile dementia of the Alzheimer's type. One 46-year-old woman had suffered during at least 1 year from a tumor of the adrenal cortex, which induced high concentrations of androstenedione and testosterone in the blood. At autopsy, male brains weighed 1467.9 ± 66.3 g (mean ± standard error of the mean) and female brains weighed 1201.7 ± 41.5 g (18.1 percent smaller) (Table 1). Brains were fixed in 10 percent formaldehyde at room temperature generally for 30 days. The hypothalamic area was subsequently dissected, dehydrated, and embedded in paraffin. Serial 6-μm frontal sections were cut on a Leitz microtome, mounted on chromium aluminum sulfate–coated slides, hydrated, brought to phosphate-buffered saline, and stained with thionin.

8. D. F. Swaab *et al.*, in *Topics in Aging Research*, D. L. Knook *et al.*, Eds. (Eurage, Brussels, 1984), vol. 2, pp. 71–78.
9. The rostrocaudal length of the SDN-POA was determined by staining every 25th section from the lamina terminalis to the caudal end of the optic chiasm with thionin. The rostral and caudal borders of the SDN-POA were assessed by subsequent staining of every fifth section in the most rostral and the most caudal parts and by determining the sections in which the first and last SDN-POA cells were present. Area measurements of the cross-sectional SDN-POA and the cell nuclei were performed by means of a Calcomp digitizer, through the use of a Zeiss microscope with ×10 and ×40 (plan) objectives, respectively, and ×12.5 plan oculars. The maximum cross-sectional SDN-POA area is presented as a separate measure. The volume of the SDN-POA was determined by integrating area measurements from the first to the last SDN sections [C. G. Van Eden *et al.*, *Dev. Brain Res.* **12**, 146 (1984)], 11 ± 3 sections (mean ± standard error of the mean) being measured per subject.
10. The number of SDN cells per (unit) volume (cell density) was estimated through the use of a discrete "unfolding" procedure [E. R. Weibel, *Stereological Methods*, vol. 1, *Practical Methods for Biological Morphometry* (Academic Press, New York, 1979)], which included the modification for classification proposed by L. M. Cruz-Orive [*J. Microsc.* **112**, 153 (1978)] and a correction for section thickness (6 μm). The nuclear profiles of 132 ± 6 cells (mean ± standard error of the mean) per SDN-POA were measured per subject for this procedure in the section containing the maximum SDN-POA area. The computer program for these procedures was developed in our institute by R. W. H. Verwer.
11. The influence of age and sex on SDN-POA length, maximum area, cell density, and cell number were tested by means of two-way analysis of variance ($\alpha = 0.05$). Significant sex (main) effects were found for maximum area [$F(1, 25) = 18.21$; $P < 0.001$], volume [$F(1, 25) = 12.17$; $P = 0.002$], cell number [$F(1, 25) = 15.97$; $P = 0.001$], and the ratio of SDN-POA volume to brain weight [$F(1, 25) = 11.63$; $P = 0.002$]. Significant age (main) effects were found for the decline in maximum area [$F(2, 25) = 6.61$; $P = 0.005$], volume [$F(2, 25) = 5.52$; $P = 0.001$], cell number [$F(2, 25) = 9.44$; $P = 0.001$], and the ratio of SDN-POA volume to brain weight [$F(1, 25) = 5.29$; $P = 0.012$], while no significant interactions between the effects of age and sex on these variables were found ($P > 0.25$). As in the rat (*12*), there was no statistically significant sex difference either in the length of the rostrocaudal axis ($P = 0.162$) or in cell density ($P = 0.937$) of the SDN-POA. In addition, no significant effects of postmortem delay ($P > 0.05$) or duration of fixation ($P > 0.05$) were found.
12. R. A. Gorski *et al.*, *J. Comp. Neurol.* **193**, 529 (1980).
13. R. A. Gorski, in (*2*), pp. 129–146.
14. D. M. Ayoub, W. T. Greenough, J. M. Juraska, *Science* **219**, 197 (1983); N. J. Bean *et al.*, *Brain Res. Bull.* **6**, 109 (1981); B. L. Hart, *J. Comp. Physiol. Psychol.* **86**, 328 (1974); N. E. Van de Poll and H. Van Dis, *Brain Res. Bull.* **4**, 505 (1979); J. F. Rodriguez-Sierra and E. Terasawa, *ibid.* **4**, 513 (1979); D. Shander and C. A. Barraclough, *Exp. Brain Res.* **40**, 123 (1980); S. J. Wiegand *et al.*, *Endocrinology* **102**, 1645 (1978).
15. G. W. Arendash and R. A. Gorski, *Science* **217**, 1276 (1982).
16. Y. Oomura *et al.*, *Brain Res.* **266**, 340 (1983).
17. D. A. Keefer and W. E. Stumpf, in *Anatomical Neuroendocrinology*, W. E. Stumpf and L. D. Grant, Eds. (Karger, Basel, 1975), pp. 153–165; P. J. Sheridan *et al.*, *ibid.*, pp. 134–141; W. E. Stumpf and M. Sar, *ibid.*, pp. 82–103; D. Pfaff and M. Keiner, *J. Comp. Neurol.* **151**, 121 (1973).
18. G. W. Arendash and R. A. Gorski, *Brain Res. Bull.* **10**, 147 (1983).
19. R. Bleier, W. Byne, I. Siggelkow, *J. Comp. Neurol.* **212**, 118 (1982); D. Commins and P. Yahr, *ibid.* **224**, 132 (1984); M. Hines *et al.*, *Biol. Reprod. Suppl.* **26** 49a (1982); S. A. Tobet *et al.*, *Endocrinology* **112** (Suppl.) (1983).
20. R. B. Simerley *et al.*, *J. Comp. Neurol.* **225**, 151 (1984); R. E. Watson, Jr., and G. E. Hoffman, *Anat. Rec.* **205**, 210A (1983).
21. We thank B. Fisser, P. J. van Nieuwkoop, G. v.d. Meulen, and H. Stoffels for their assistance, and F. C. Stam, W. Kamphorst, J. Jöbsis, J. M. Wigboldus, and H. Bakker-Winnubst for supplying the brain material. Supported by the Foundation for Medical Research (FUNGO; grant 13-51-30).

18 September 1984; accepted 14 February 1985

Loss of M2 Muscarine Receptors in the Cerebral Cortex in Alzheimer's Disease and Experimental Cholinergic Denervation

Abstract. *Cerebral cortex samples from patients with Alzheimer's disease and from rats after experimental cholinergic denervation of the cerebral cortex exhibited reductions in the presynaptic marker choline acetyltransferase activity and in the number of M2 muscarine receptors, with no change in the number of M1 receptors. These results are in keeping with evidence that M2 receptors function in cholinergic nerve terminals to regulate the release of acetylcholine, whereas M1 receptors are located on postsynaptic cells and facilitate cellular excitation. New M1-selective agonists and M2-selective antagonists directed at post- or presynaptic sites deserve consideration as potential agents for the treatment of the disease.*

Large reductions in choline acetyltransferase (CAT) activity are found in postmortem samples from the cerebral cortices of patients with senile dementia of the Alzheimer's type (SDAT) (*1–5*). Cortical cholinergic deficits result from the degeneration of cell bodies located in the nucleus basalis of Meynert (*6*). However, all but two (*7, 8*) of many studies have indicated no change in the numbers of receptors for acetylcholine ("muscarine" receptors) in samples of SDAT cerebral cortex and hippocampus (*9–11*). The absence of major changes in the number of receptors has been taken as evidence that muscarine receptors are located postsynaptically. We have reexamined this situation because of evidence that there are two basic subtypes of muscarine receptors in the brain, M1 and M2 (*12*), and that the M2 receptors may function primarily to regulate the release of acetylcholine from cholinergic nerve terminals (*13*). We report that M2 receptors are lost in advanced SDAT and are diminished (in proportion to the reduction of CAT activity) after experimental cholinergic denervation of the cerebral cortex in rats. The number and affinity of postsynaptic M1 receptors remain unchanged.

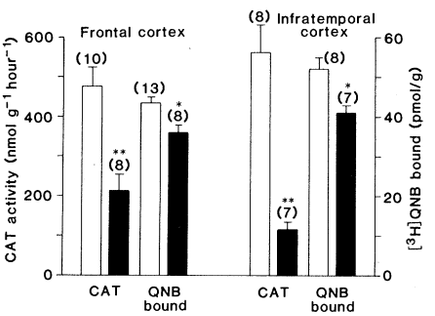

Fig. 1. Changes in CAT activity and in the total number of muscarine receptors in cortical samples from patients with SDAT (filled bars) as compared with controls (open bars). The numbers in parentheses are the numbers of tissue samples assayed as described in the text. Data are expressed per gram of wet tissue weight, and values are means ± the standard errors of the mean. Statistical significance of the differences was judged by Student's *t*-test: $*P < 0.05$; $**P < 0.001$.

Brain tissue was obtained from deceased persons with and without SDAT and from rats with nucleus basalis lesions (*14*). Frontal and infratemporal cortical samples were obtained at autopsy from neurologically normal individuals (mean age, 71 years) and patients with SDAT (mean age, 74 years) within 12 hours of death (mean delay, 8 hours). One SDAT patient had been receiving low doses of phenobarbital, and two SDAT patients had received Thorazine; except for those, none of the patients had been receiving drugs that affect the central nervous system. Phenobarbital and Thorazine are not known to affect muscarine receptors or CAT activity. The diagnosis of SDAT was made histologically on the basis of the widespread distribution of neurofibrillary tangles and neuritic plaques. No brain showed other significant neuropathology. For the animal experiments, male Sprague-Dawley rats weighing 200 g each were injected with 10 μg of ibotenic acid unilaterally into the ventral and medial aspects of the globus pallidus 72 hours before neurochemical assays of the frontoparietal cortex.

CAT activity was estimated by the

technique of Fonnum (*15*), which involves measurement of the transfer of [^{3}H]acetate from coenzyme A to choline. Total numbers of muscarine receptors were measured with sufficient (−)[^{3}H]quinuclidinyl benzilate (QNB) to saturate 99 percent of all receptor sites (*12*); in this procedure of Potter *et al.*, 1.0 m*M* EDTA and 1.0 m*M* *N*-ethylmaleimide (NEM) are used to uncouple endogenous agonist-receptor complexes that can prevent binding of the labeled ligand. We estimated the proportions of total sites that were M1 and M2 in the same media by measuring the ability of carbachol to inhibit binding of QNB to the lowest affinity state of each of these receptors (*12*). The data were fitted to a two-site model according to a nonlinear, iterative, least-squares computer program. We estimated the number of M2 receptors in their highest affinity state by subsaturating these receptors with [^{3}H]oxotremorine-M (*12*). Each of these techniques with agonists derives from pioneer work by Birdsall and colleagues (*16*).

In brain samples from patients with SDAT the CAT activity decreased 55 to 80 percent (Fig. 1), in keeping with earlier findings (*1–5*). There was also a 20 to 25 percent decrease in the total number

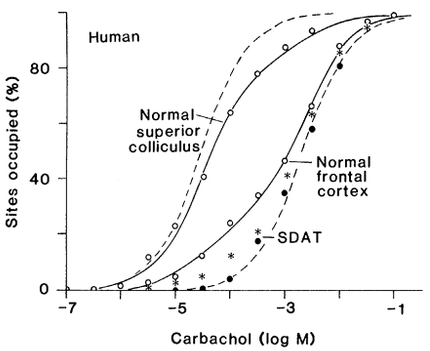

Fig. 2. Prevalences of M1 and M2 receptors in normal and SDAT cortex. The ordinate measures the percent of inhibition of binding of [^{3}H]QNB by carbachol. The dashed lines show binding isotherms for pure M2 (left) and M1 (right) receptors, in their lowest affinity states. The IC_{50} (inhibitory concentration 50 percent) values for these isotherms are $10^{-4.5}$ and $10^{-2.7}M$, respectively. The points are experimental data, and the solid lines are computer fits (two-site model) of the experimental data showing mixed proportions of M1 and M2 receptors. The normal superior colliculus showed 82 percent M2 and 18 percent M1, whereas the normal frontal cortex showed 23 percent M2 and 76 percent M1 (n = 3; open circles). Selected frontal cortex samples from patients with SDAT having a 50 percent loss in CAT activity (n = 3; asterisks) showed roughly half the usual number of M2 receptors, whereas samples with negligible CAT activity (n = 2; filled circles) had an almost pure population of M1 receptors. Thus M2 receptors are lost in SDAT.

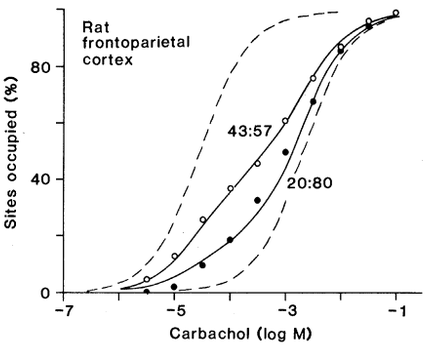

Fig. 3. Proportions of M1 and M2 receptors in normal (○) and denervated (●) rat frontoparietal cortex. Isotherms are as in Fig. 2. The points are from five animals studied 3 days after the production of a lesion of the Ch4 cell group. The proportion of M2 receptors was reduced from 43 to 20 percent of the total number of receptors, in parallel with a 50 to 60 percent loss in CAT activity.

of muscarine receptors; this result has been observed in the cortex in only one study (*8*).

Figure 2 shows the results of competition experiments designed to demonstrate the proportions of M1 and M2 receptors in the normal and SDAT cortex; the normal superior colliculus was included as an example of an M2-rich region. In samples of normal frontal cortex, 23 percent of the total muscarine receptors were of the high-affinity M2 type. This fraction corresponds closely to the fraction of total receptors lost in severe SDAT (Fig. 1). In cortical samples selected for almost complete loss of CAT activity the fraction of M2 was negligible, and in samples where the CAT activity was reduced by 50 percent the fraction of M2 receptors was reduced by half. The loss in total muscarine receptors in SDAT appears to be due to a selective reduction in M2 receptors.

We attempted to model cholinergic denervation of the cortex in rats by producing lesions in cell bodies located within the Ch4 group of the basal forebrain (*17*). In five animals, the average total number of muscarine receptors in samples of the frontoparietal cortex was 75 pmol per gram of fresh tissue and the normal proportion of M2 receptors was 43 percent (Fig. 3). Partial destruction of extrinsic cholinergic input to the cortex resulted in a 50 to 60 percent loss in both CAT activity and number of M2 receptors but only a 23 percent loss in the total number of receptors. Thus excitotoxic lesions of extrinsic cholinergic inputs to the cortex in rats resulted in proportionate reductions in CAT activity and in the number of M2 receptors, with no change in the number of M1 receptors.

Figure 4 shows the relation between CAT activity and the binding of the agonist [^{3}H]oxotremorine-M to the high-affinity M2 receptors in normal and SDAT infratemporal cortices. There were concomitant reductions in these markers.

These results are in agreement with other evidence that M2 receptors function in cholinergic terminals and suggest that this is their primary location in the tissues studied. Autoradiographs (*12*) show that M1 receptors are widespread in the neocortex, whereas M2 receptors are most apparent in cortical laminae having the highest CAT activity (*18, 19*). In the hippocampus, there is a correspondence between the location of septal afferents, acetylcholinesterase-positive sites (*20*), and M2 receptors (*12*). Muscarine receptors of the high-affinity M2 type undergo axonal transport (*12*). There is also evidence that lesions of cholinergic neurons in the basal forebrain of the rat give rise in the short term to cortical reductions of these higher affinity receptors (*14*). It is well known that acetylcholine inhibits its own release (*13, 22, 23*) and that several antagonists, but not M1-selective antagonists such as pirenzepine (*13, 23*), diminish this action of acetylcholine. The mechanism of M2 receptors is believed to involve attenuation of the activation of adenylate cyclase (*24*). The evidence favors the view that M2 receptors function in cholinergic nerve terminals as autoreceptors to modulate the release of acetylcholine.

In contrast, electrophysiological studies demonstrate that exogenously applied cholinergic agonists act via muscarine receptors to modify the firing patterns of intrinsic cortical neurons (*25*). Firing due to the activity of other neurotransmitters is converted from a few

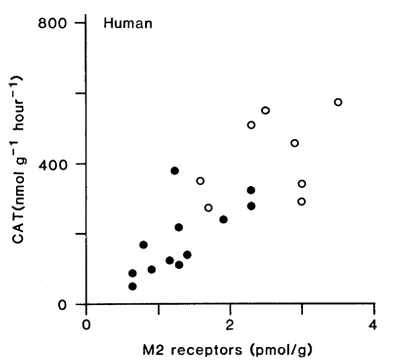

Fig. 4. Relation between losses of CAT activity in SDAT and losses of high affinity binding of (−)-[^{3}H]oxotremorine-M to M2 receptors: (●) SDAT samples, (○) samples from age-matched controls. There was a parallel reduction in both markers (correlation coefficient r = 0.75, P = 0.05).

postsynaptic events into bursts of impulses; the mechanism involves the closing of special potassium channels. We have demonstrated that the number of M1 receptors remains unchanged in patients with SDAT and after experimental cortical cholinergic denervation in rats; these results suggest a postsynaptic location for these sites. Taken together, these data indicate that M1 and M2 receptors are differently disposed at cholinergic synapses in the cerebral cortex and have different mechanisms and functions.

The selective reduction in the number of M2 receptors in patients with SDAT has therapeutic implications. When cholinergic nerve terminals are gone, there is a need to replace the action of acetylcholine on postsynaptic M1 receptors. Replacement therapy is being attempted directly with acetylcholine analogs (*26*) and indirectly with esterase inhibitors (*27*). It is also necessary to avoid M1 antagonists, such as atropine, some drugs used to treat Parkinson's disease, certain antihistamines and neuroleptics, and possibly quinidine. We hypothesize that early in the course of Alzheimer's disease, an M2-selective antagonist might be useful in addition to an esterase inhibitor, to prevent acetylcholine from inhibiting its own release. Part of the reason why physostigmine has not been very useful by itself in early SDAT (*27*) may be that as it maintains the presence of acetylcholine, which is highly M2-selective, further transmitter release is decreased. Thus both M1 agonists and M2 antagonists deserve clinical trials.

DEBORAH C. MASH*
DONNA D. FLYNN
LINCOLN T. POTTER

Department of Pharmacology, University of Miami School of Medicine, Post Office Box 016189, Miami, Florida 33101

References and Notes

1. E. K. Perry *et al.*, *Lancet* **1977-I**, 189 (1977).
2. P. Davies, *Brain Res.* **171**, 319 (1979).
3. M. W. Rossor, *Lancet* **1982-II**, 1200 (1983).
4. T. D. Bird *et al.*, *Ann. Neurol.* **14**, 284 (1983).
5. E. K. Perry *et al.*, *J. Neurochem.* **42**, 1402 (1984).
6. J. T. Coyle, D. L. Price, M. R. DeLong, *Science* **219**, 1184 (1983).
7. T. D. Reisine *et al.*, *Brain Res.* **159**, 477 (1978).
8. P. L. Wood *et al.*, *J. Neurol. Sci.* **62**, 211 (1983).
9. P. Davies and A. Verth, *Brain Res.* **138**, 385 (1978).
10. M. P. Caulfield *et al.*, *Lancet* **1982-II**, 1277 (1982).
11. H. Henke and W. Lang, *Brain Res.* **267**, 281 (1983).
12. L. T. Potter *et al.*, *Trends Pharmacol. Sci. (Suppl.)* (1984), p. 22. M1 receptors may be distinguished from M2 receptors on the basis of higher affinity of the former for the antagonist pirenzepine or by the higher affinity of the latter for the agonist carbachol (*12*). An essential feature of the assays used is stabilization of each receptor in its lowest affinity state, with NEM and EDTA, which permits quantitative separation of receptor subtypes. The results of earlier studies of receptors showing "high and low affinity" for agonists (*10, 14, 16*) are complicated by overlap between the low affinity state of M2 receptors and the high affinity state of M1 receptors. Improvement in the separation and assay of receptor subtypes may account for the new results in the present study.
13. M. Raiterai, R. Leardi, M. Marchi, *J. Pharmacol.* **228**, 209 (1984).
14. M. McKinney and J. T. Coyle, *J. Neurosci.* **2**, 97 (1982). To destroy extrinsic cholinergic inputs to the frontoparietal cortex, we injected ibotenic acid at the following coordinates: 0.8 mm caudal to the bregma, 2.5 mm lateral to the midline, and 7.0 mm ventral to the pial surface. To minimize transsynaptic changes, animals were killed 72 hours after the injection. In subsequent studies, six rats were killed 7 days after the lesion had been produced. Carbachol-[^{3}H]QNB competition assays revealed significantly fewer M2 sites remaining, and the concentration of CAT was decreased 80 to 90 percent from control values. M1 sites remained unchanged.
15. F. Fonnum, *Biochem. J.* **115**, 465 (1969).
16. N. J. M. Birdsall, E. E. Hulme, A. J. V. Burgen, *Proc. R. Soc. London Ser. B* **207**, 1 (1980).
17. M-M. Mesulam *et al.*, *Neuroscience* **10**, 1185 (1983).
18. M. V. Johnston, A. C. Young, J. T. Coyle, *J. Neurosci. Res.* **6**, 597 (1981).
19. H. C. Fibiger, *Brain Res. Rev.* **4**, 327 (1982).
20. S. Mosko, F. Lynch, C. W. Cotman, *J. Comp. Neurol.* **152**, 163 (1973).
21. M. A. Zarbin, J. K. Wamsley, M. J. Kuhar, *Brain Res.* **211**, 129 (1982).
22. P. C. Molenaar and R. L. Polak, *Prog. Pharmacol.* **314**, 39 (1980).
23. E. M. Meyer and D. J. Otero, *J. Neurosci.*, in press.
24. L. E. Limbird, *Biochem. J.* **195**, 1 (1983). This reference refers to the action of muscarine receptors in the heart, which are known to be of the M2 type (*12*).
25. D. A. Brown, *Trends Neurosci.* **6**, 302 (1983).
26. R. E. Harbaugh *et al.*, *Neurosurgery* **15**, 514 (1984).
27. A. Weinstein, *Ann. Neurol.* **13**, 210 (1983).
28. This work would not have been possible without the cooperation of M. Norenberg, who performed the postmortem and neuropathological examinations. The technical assistance of H. Hanchett and J. Vega is also acknowledged. Supported in part by grants from the National Institute of Health (BNS 8307665 and HLO-7188) and the National Science Foundation (BNS-8307665).

* Present address: Neurological Unit, Beth Israel Hospital, 330 Brookline Avenue, Boston, Mass. 02215.

23 August 1984; accepted 16 January 1985

Circulating Autoantibodies to the 200,000-Dalton Protein of Neurofilaments in the Serum of Healthy Individuals

Abstract. *There is substantial evidence that human serum contains antibodies to many autoantigens. For example, all healthy people have autoantibodies (immunoglobulin M) to some undefined brain antigens. In this study immunoblots and immunohistochemical staining were used to detect antibodies to neural tissues in serum samples from 200 healthy people and 200 patients with various neurological diseases. Ninety-nine percent of the 400 subjects had serum immunoglobulin M and 95 percent had immunoglobulin G that bound to a 200-kilodalton protein in homogenates of neural tissues. In most cases there were no antibodies to anything else in the homogenates. The 200-kilodalton protein was the heaviest of the neurofilament triplet proteins. These observations do not support a role for antibodies to the 200-kilodalton protein of neurofilaments in the pathogenesis of neurological diseases.*

In his clonal selection theory, Burnet (*1*) proposed that lymphocytes that respond to antigenic stimulation during development are killed. This would mean that lymphocytes that respond to autoantigens present during development are also extinguished. Therefore, autoantibodies should only appear in pathologic conditions, either from emergence of new clones of lymphocytes or from exposure of the immune system to autoantigens that either were not expressed or were sequestered during development. According to the clonal selection theory, the presence of autoantibodies signifies disease.

There has been considerable recent evidence against the clonal selection theory (*2–6*). It is, for example, probable that most people have serum antibodies to certain autoantigens (*3*). It is even possible that most people have in their serum antibodies to virtually all autoantigens. However, most of the self-reactive antibodies in the serum of healthy people are present at low titers, and they may be less specific than induced antibodies (*5*). The existence of these autoantibodies in the serum of healthy people makes unclear the implications of finding autoantibodies against components of the affected organs in the serum of patients.

Doar and Fabre (*3*) reported that healthy people have in their serum organ-specific immunoglobulin M (IgM) autoantibodies to liver, heart, and brain. We attempted to determine whether the serum IgM antibodies to brain tissue in healthy people bind to specific antigens and, if so, to which. Using immunoblots containing electrophoresed proteins from homogenates of various neural tissues and isolated neurofilaments, we found that most healthy people have in their serum both IgM and immunoglobulin G (IgG) antibodies that are directed against the 200-kD neurofilament protein (NFP) and that only a few people have a high enough titer of serum antibodies to other proteins (including other NFP's) in the neural homogenates to be detected by this method.