Parvalbumin-Immunoreactive Neurons in the Neocortex are Resistant to Degeneration in Alzheimer's Disease

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Abstract. Recent studies have stressed the fact that specific neuronal subtypes may display a differential sensitivity to degeneration in Alzheimer's disease. For example, large pyramidal neurons have been shown to be vulnerable, whereas smaller neurons are resistant to pathology. Using a monoclonal antibody against the calcium-binding protein parvalbumin, we investigated the possible changes in a subpopulation of interneurons in two cortical areas known to be strongly damaged in Alzheimer's disease. In the prefrontal cortex as well as in the inferior temporal cortex, we observed no differences in parvalbumin-immunoreactive cell counts or cell size in Alzheimer's disease brains as compared to control cases. Moreover, the general cellular morphology of these neurons was preserved in the Alzheimer's disease cases, in that their perikarya and dendritic arborizations were intact. These results suggest that parvalbumin-immunoreactive cells represent a neuronal subset resistant to degeneration, and further support the hypothesis that the pathological process in Alzheimer's disease involves specific neuronal subtypes with particular morphological and molecular characteristics.

Key Words: Alzheimer's disease; Calcium-binding proteins; \( \gamma \)-aminobutyric acid; Interneurons; Neocortex, human; Quantitative neuroanatomy.

INTRODUCTION

Parvalbumin (PV) is a low molecular weight \( \text{Ca}^{2+} \)-binding protein that is present primarily in certain populations of muscle and nerve cells (1, 2), as well as in other tissues (3). The precise physiological function of PV remains unclear, although it acts as a relaxing factor in muscle (4, 5). Parvalbumin, in addition to other \( \text{Ca}^{2+} \)-binding proteins, also may play a role in \( \text{Ca}^{2+} \) translocation and/or \( \text{Ca}^{2+} \) buffering in neurons (6). Parvalbumin has been observed in the central nervous system of different species including human (7–21), and has been claimed to be present within most of the \( \gamma \)-aminobutyric acid- (GABA) containing interneurons (12, 15, 16). Recently, PV has been associated with two specific subclasses of GABAergic inhibitory interneurons, chandelier cells (17) and basket cells (18). In fact, \( \text{Ca}^{2+} \)-binding
proteins have been demonstrated to be reliable markers for the differentiation of specific subtypes of GABAergic neurons (18, 19).

Interneurons have been shown to remain mostly unaffected in Alzheimer's disease (AD), whereas the large pyramidal cells are likely to be more sensitive to degeneration (22–26). Furthermore, one of the histopathological markers of the disease, neurofibrillary tangles (NFT), are located within pyramidal cells and are not observed within interneurons in the neocortex (27). Several studies suggested that a global disruption of long corticocortical projections occurs in AD, resulting clinically in the dementia observed in AD patients (24, 26, 28–35). Moreover, the severity of dementia is correlated with NFT and senile plaque (SP) density (36, 37), although recent studies suggested that there is a stronger correlation of the degree of dementia with NFT than with SP, since the brains of non-demented aged patients may contain large amounts of diffuse amyloid deposits and SP (38). Thus, NFT formation may be more clearly linked to the neuronal degeneration that underlies dementia than is amyloid deposition. Recently, we demonstrated that a subset of pyramidal neurons containing a high somatodendritic concentration of neurofilament proteins was highly vulnerable in AD (24, 26). Thus, it was of interest to determine if the PV-immunoreactive (PV-ir) neuron population was damaged during the course of the degenerative process, and the possible susceptibility of specific neuronal subsets in AD (21, 24, 26). As described below, a detailed quantitative analysis of the PV-ir neuron distribution in the superior frontal and inferior temporal cortex revealed that this particular subpopulation is resistant to degeneration in AD.

METHODS

The brains of eight patients with Alzheimer's disease (81.0 ± 6.1 years old) and four patients with no history of neurologic or psychiatric disorders (75.5 ± 4.4 years old) were obtained at autopsy. The postmortem delay ranged from two to six hours (h). The clinical and neuropathological data were obtained from the records of the Institute for Biogerontology Research, Sun City, AZ, and the Department of Psychiatry, University of Geneva, Switzerland. Dementia was clinically documented and AD was neuropathologically confirmed by the presence of high densities of SP and NFT in the hippocampal formation and neocortex of the AD cases. In these cases, NFT and SP counts always met the requirements for a diagnosis of AD (39). These same cases were used in our studies of the distribution of non-phosphorylated neurofilament protein-containing neurons in AD (26), and a detailed quantitative analysis of the distribution of NFT and SP in the same neocortical areas has been included in that manuscript (26). All control cases displayed no NFT and very rare SP in the neocortex. In the oldest cases, isolated NFT were occasionally observed in the hippocampal formation. There was no evidence of traumatic lesions, infections, gross area of infarction, primary tumor or metastatic process in any of the AD or control brains. Minimal atheromatosis was present within the major cerebral arteries, which never occluded the vessels.

The brains were prepared as previously described by Campbell and Morrison (40). Briefly, the brains were perfused ex situ through the internal carotid and basilar arteries with cold 4% paraformaldehyde (1.0–1.5 liters of the perfusion solution per vessel). The brains were subsequently suspended by the basilar artery in the same fixative for 12 h, cut into 1 cm-thick coronal blocks and postfixed for 48 to 72 h. After postfixation, the blocks were washed in a series of graded sucrose solutions (12%, 16%, and 18%) in cold phosphate-buffered saline (PBS), frozen and cut at 40 μm on a cryostat. Adjacent sections were collected for immunohistochemical purposes as well as for thioflavine S and cresyl violet staining. Prior to immunohistochemistry, the 40 μm-thick sections were pretreated with a solution of absolute methanol and 3% hydrogen peroxide (80/20 volume to volume ratio) for 15 minutes (min) to avoid visualizing endogenous peroxidase activity, followed by several five min rinses in PBS. Then, they were incubated overnight at 4°C with a mouse monoclonal antibody raised...
against the calcium-binding protein parvalbumin (41) at a working dilution of 1:3,000 in PBS containing 0.3% Triton X-100, and 0.5 mg/ml bovine serum albumin. This monoclonal antibody has been raised against purified parvalbumin from carp muscle and its ability to recognize primate brain parvalbumin has been previously demonstrated (41, 42). Following incubation, the sections were processed by the avidin–biotin method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB). Finally, the immunoreactivity was intensified in serial baths of 0.005% osmium tetroxide for six to seven min, 0.5% thioacetamide for ten min, and 0.005% osmium tetroxide for three min. Adjacent sections were stained with cresyl violet in order to clarify the cytoarchitecture. Some sections incubated with the parvalbumin antibody were also counterstained with the Giemsa stain as previously described (43). These sections were not intensified with osmium, since Giemsa stain simultaneously intensifies the DAB product. In addition, two to four sections per area in each brain were stained with 1% thioflavine S (a fluorescent stain that visualizes extracellular amyloid and paired helical filaments) in order to identify NFT and SP.

The areas investigated in this study were (according to Brodmann’s nomenclature) area 9’ in the superior frontal gyrus (prefrontal cortex) and area 20 in the inferior temporal gyrus (a visual association area). Parvalbumin-ir neurons were counted under three 1 mm-wide cortical traverses in layers II–IV and V–VI separately of both areas in all cases at a magnification of ×200. The analyses were done on cortical traverses so that the confounding effect of tissue shrinkage in terms of cortical thickness is minimized. However, atrophy in the horizontal plane may still be a factor and might lead to a slight overestimation of the cell counts in the AD cases. Cross-sectional perikaryal areas of PV-ir neurons were assessed at a magnification of ×400, on all neurons within two to three fields in layers II–IV and V–VI separately, in both areas 9 and 20 in all cases. Thus, a total of 320 PV-ir neurons were analyzed for size in the control cases and 415 in the AD cases. According to their perikaryal areas, the PV-ir cells were subsequently divided into seven size categories (i.e. <49, 50–89, 90–129, 130–169, 170–209, 210–249, >250 μm²). Then, the mean perikaryal area within each size category and the relative frequency of each size category were calculated in control and AD brains, in layers II–IV and V–VI of both areas.

All quantitative analyses were performed on a computer-assisted microscopy system consisting of a Zeiss Axiophot photomicroscope equipped with a Martzhäuser motorized stage, a high resolution video scanner, a Megavision XM1024 image processing system, a Microvax II computer and morphometry software developed at the Research Institute of Scripps Clinic, La Jolla, CA. The statistical analysis was performed separately in layers II–IV and V–VI of both areas using a two-sided Mann-Whitney U-test to compare the PV-ir neuron counts, perikaryal areas, and frequencies of PV-ir cell size categories between the control and AD brains.

RESULTS

Parvalbumin-ir cells could be detected in all layers except layer I and displayed a very similar distribution in both areas 9 and 20 (Figs. 1, 2). The majority of PV-ir neurons was located within layers III and IV, and a few were present in layer II. Parvalbumin-ir neurons were also concentrated within layers V–VI, although they were less numerous than in layers II–IV (Figs. 1, 2). Very few PV-ir cells were present in the subcortical white matter. A denseplexus of immunoreactive fibers was present in the upper part of layer I and in layers III and IV, and the density of these fibers and terminals was not diminished in the AD cases (Figs. 1, 2).

The PV-ir neuron population displayed some diversity in size and shape (Fig. 3). Some neurons were characterized by a small and ovoid perikaryon with restricted arborizations, whereas large multipolar neurons with a much more extensive dendritic and axonal field were present as well. The general morphology of PV-ir neurons was similar in AD and control cases (Fig. 3). The vast majority of the PV-ir neuron population had an intermediate size ranging between 60 and 160 μm² of perikaryal

cross-sectional area. Cells with a cross-sectional area below 50 \(\mu m^2\) and over 170 \(\mu m^2\) were less frequently encountered.

No statistically significant differences in the distribution of PV-ir profiles were observed in AD brains as compared to control cases (Figs. 1, 2). Cellular densities calculated in layers II–IV and V–VI were similar in AD and control cases in both areas (values were: area 9, layers II–IV, controls, 151.6 ± 19.9 [range: 114–191]; AD, 145.4 ± 15.2 [range: 103–188]; layers V–VI, controls, 64.3 ± 11.1 [range: 42–86]; AD, 50.9 ± 6.8 [range: 32–69]; area 20, layers II–IV, controls, 121.3 ± 14.7 [range: 92–150]; AD, 116.7 ± 10.1 [range: 88–144]; layers V–VI, controls, 47.8 ± 7.6 [range: 33–63]; AD, 52.0 ± 8.2 [range: 29–75]; see Fig. 4). There were no differences in PV-ir neuron size between the two brain groups (Fig. 3). For instance, the overall mean PV-ir perikaryal area in area 9 was 123.9 ± 18.3 \(\mu m^2\) in the control
and $123.0 \pm 17.1 \, \mu m^2$ in the AD brains (means $\pm$ SEM). In area 20, these values were $134.8 \pm 16.6 \, \mu m^2$ and $127.5 \pm 17.3 \, \mu m^2$, respectively. In order to refine the quantitative analysis, since subtle differences in cell size could have occurred in view of the rather large distribution of PV-ir cell sizes (from $37 \, \mu m^2$ to $222 \, \mu m^2$ in area 20, and $38 \, \mu m^2$ to $228 \, \mu m^2$ in area 9), PV-ir neurons were further classified into seven size categories (see Methods). The mean cell size within each size category was not different in AD cases as compared to control brains (Table 1). Furthermore, the relative frequency of each size category in layers II–IV and V–VI of both areas also was not different in AD cases (Table 2). Also, there were no statistically significant differences in cell size between layers II–IV and V–VI, and between the two areas within each brain group. Finally, it should be noted that the staining intensity of the PV-ir profiles was qualitatively comparable in AD and control cases, and that PV-ir neurons showed an intact morphology in the AD cases (Figs. 1–3).

**DISCUSSION**

The results presented in this article can be briefly summarized as follows. In the normal human frontal and temporal neocortex PV-ir neurons were distributed in
Fig. 3. Intermediate magnification of PV-ir neurons in layer III of inferior temporal gyrus from a control brain (A) and an AD brain (B). Note that in both cases there are numerous labeled neurons with an intact, extensive dendritic tree. Bar = 100 μm.

all layers except layer I, and were more numerous in layers II–IV than in layers V–VI. In both areas all PV-ir cells displayed the morphological characteristics of interneurons. The perikaryal area of PV-ir cells was similar in layers II–IV and V–VI, with a mean value slightly above 120 μm². It should also be noted that in the control brains there were no differences in PV-ir cell counts and cell size between areas 9 and 20. No statistically significant differences in labeled cell counts or size were observed between the control and AD cases. However, it should be noted that since no correction for the cortical atrophy that exists in AD was applied, our figures might be slightly overestimated in the AD cases and that a marginal decrease of PV-ir neuron counts may have occurred. Finally, the general morphology and staining pattern of PV-ir neurons was preserved in the AD brains.

Morphologic properties such as cell size have been correlated with vulnerability in AD (22, 26). For example, Terry et al demonstrated an increased vulnerability of neurons with a cross-sectional perikaryal area greater than 90 μm² (22). These authors made their measurements on conventional Nissl-stained materials with different fixation and tissue preparation protocols than used in the present immunohistochemical analysis, such that direct size comparisons across the studies are difficult. However, our data demonstrate that PV-ir cells in the size range of 150–250 μm² were no more susceptible than cells in the 60–150 μm² size range (see Tables 1, 2). In addition, we have reported that a positive correlation exists between vulnerability and cell size among a selective group of pyramidal cells in AD (26, and see below). Although pyramidal cells tend to be larger than non-pyramidal cells, the present data show that a significant proportion of non-pyramidal PV-ir cells are larger than 100 μm². Thus, although the data of Terry et al and Hof et al (22, 26) point to a correlation between neuron size and vulnerability, we would suggest that size is not the only determinant of vulnerability, but that other cellular characteristics might also be involved.

Contrasting with our results, Arai et al (13) showed that PV-ir neuron density in areas 9 and 21 were significantly reduced in AD cases by as much as 56%. The PV-ir cell size was also found to be reduced, with statistically significant differences only in the mid-temporal cortex. Several methodological factors may explain these dis-
TABLE 1
Mean Perikaryal Area (µm²) of PV-ir Neurons in Areas 9 and 20

<table>
<thead>
<tr>
<th>Categories</th>
<th>Layers II-IV</th>
<th></th>
<th>Layers V-VI</th>
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<tr>
<td>&lt;49</td>
<td>48.5 ± 1.5</td>
<td>50.0</td>
<td>44.3 ± 1.8</td>
<td>46.8 ± 2.6</td>
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<tr>
<td>50-89</td>
<td>77.7 ± 2.2</td>
<td>77.2 ± 1.8</td>
<td>68.9 ± 1.7</td>
<td>71.8 ± 1.7</td>
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<tr>
<td>90-129</td>
<td>106.8 ± 2.3</td>
<td>109.6 ± 2.8</td>
<td>106.4 ± 1.8</td>
<td>107.8 ± 2.4</td>
</tr>
<tr>
<td>130-169</td>
<td>139.2 ± 2.8</td>
<td>148.0 ± 2.4</td>
<td>146.6 ± 3.9</td>
<td>151.0 ± 2.7</td>
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<tr>
<td>170-209</td>
<td>190.8 ± 9.4</td>
<td>181.3 ± 5.6</td>
<td>202.3 ± 3.8</td>
<td>182.0 ± 7.5</td>
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<tr>
<td>&gt;250</td>
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<td>228.0</td>
<td>—</td>
<td>231.5</td>
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<td>Area 20</td>
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<tr>
<td>&lt;49</td>
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<td>43.0 ± 4.2</td>
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<td>45.3 ± 3.4</td>
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<tr>
<td>50-89</td>
<td>72.6 ± 1.7</td>
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<td>107.2 ± 2.2</td>
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<td>130-169</td>
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<tr>
<td>170-209</td>
<td>174.7 ± 3.8</td>
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<tr>
<td>&gt;250</td>
<td>—</td>
<td>217.7 ± 4.8</td>
<td>218.5 ± 7.0</td>
<td>217.8 ± 4.6</td>
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Results represent means ± SEM and were calculated as described under Methods. There are no statistically significant differences (two-sided Mann-Whitney U-test). A missing figure means that no PV-ir cells in this size category were observed in our sample. Cells below 50 µm² and over 220 µm² in size were very rare in our materials.

crepancies. It appears that these authors used different antisera and fixation parameters in their study. Moreover, the postmortem intervals of their material was likely to be generally over 30 h, whereas in the present study we never used brains with postmortem delays over six hours. However, it is not clear whether postmortem delay differentially affects tissue preservation in control and AD brain. In addition, Rossor et al reported less severe neurochemical changes in brains from very old AD patients as compared to a younger AD population (44). The fact that our AD sample was slightly older than that of Arai et al may account for part of the differences between the two studies. Authors from the same laboratory also reported that another subpopulation of interneurons that contains the Ca²⁺-binding protein calbindin (CB) is also vulnerable in AD (45), and similarly, we and other investigators could not demonstrate changes in the density of these CB-ir interneurons in AD (21, 46).

Finally, it is interesting to note that PV-ir neurons have also been reported to be resistant to degeneration in Huntington’s disease (47).

Recent observations on the cellular distribution of Ca²⁺-binding proteins parvalbumin and calbindin have demonstrated that both are present within GABAergic interneurons in the cerebral cortex as well as in subcortical structures (12, 15-20, 48, 49). In the neocortex, these two proteins subdivide the GABAergic neurons in non-overlapping classes that together account for virtually all of the GABAergic cells (18). These subclasses of Ca²⁺-binding proteins/GABA-containing neurons further display morphological specificity since PV/GABA cells tend to be basket and chandelier cells, while CB/GABA cells are mostly double bouquet cells (17, 18, 48). There is further evidence for cellular specialization at the neurotransmitter level since somatostatin appears to be colocalized within CB/GABA neurons (49). Such a colocalization has not been directly demonstrated for PV/GABA cells, however it is possible that some of them contain corticotropin releasing factor (50). Decreases in
Fig. 4. Parvalbumin-immunoreactive neuron density in layer II-IV and V-VI of areas 9 and 20. Results represent means ± SEM and were calculated as described under Methods. There are no statistically significant differences between the AD and control cases (two-sided Mann-Whitney U-test). See text for details.

GABA content have been described in AD (44, 51), but these biochemical data may reflect a particular vulnerability of terminals as opposed to perikarya, or of one selective subpopulation of GABAergic interneurons. We demonstrated that somatostatin-containing fibers were present in SP and degenerating neurites in spite of persistent immunoreactivity in perikarya (52). In addition, certain GABAergic neurons that contain somatostatin are vulnerable in AD, whereas others are not (53). Nakamura and Vincent reported no loss of somatostatin- or neuropeptide Y-ir perikarya in AD; however, immunoreactive fibers were swollen and observed within SP, suggesting that a degeneration of terminals may have occurred (54). Kowall and Beal showed that somatostatin- and neuropeptide Y-ir fibers were distorted in AD and that both peptides were colocalized in SP (55). The degree to which PV-ir neurites contribute to SP formation has not been determined, however the continued use of antisera of Ca²⁺-binding proteins to further characterize subtypes of GABAergic cells may help to clarify the nature of differential susceptibility among cortical interneurons.

The classification of cortical cells into specific subtypes involves several criteria that eventually lead to the detailed characterization of a given neuronal class. For
### TABLE 2

<table>
<thead>
<tr>
<th>Categories</th>
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<th>Layers V-VI</th>
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Results were calculated as described under Methods. There are no statistically significant differences between the AD and control groups (two-sided Mann-Whitney U-test). A missing figure means that no PV-ir cells in this size category were observed in our sample.

instance, neurons can be classified following their morphological features, laminar and regional distribution, connectivity, and molecular phenotype. In addition to Ca²⁺-binding proteins, we are currently investigating in the monkey neocortex a subpopulation of pyramidal neurons that furnishes long corticocortical projections (40, 56, 57). The pyramidal cells that project from the superior temporal sulcus to dorsal prefrontal cortex display clear morphological heterogeneity, are distributed within both supra- and infragranular layers with no overlap of their dendritic arborization between the two groups, and are all characterized by a high somatodendritic content of non-phosphorylated neurofilament proteins (40, 56, 57). Interestingly, in the human neocortex a comparable pyramidal cell subset is highly vulnerable in AD (24, 26), and this selective vulnerability is likely to be related to one or more of the criteria listed above. We have recently shown that up to 90% of the large pyramidal cells containing high concentration of non-phosphorylated neurofilament proteins are lost in AD, using the same brain series and in the same neocortical areas as in the present study (26). Furthermore, data from our laboratory demonstrated that while the CB-ir interneurons in the supragranular layers of the prefrontal cortex remain unchanged in AD, a previously unreported subset of CB-ir pyramidal neurons, restricted to layer III and exhibiting specific staining and distribution patterns, is severely affected by the degenerative process (21, 58).

There are several additional examples of the relationships between cellular characteristics and heightened vulnerability in neurodegenerative disorders. Thus, Gaspar et al (53) reported that subpopulations of somatostatin-ir neurons characterized by a specific neocortical distribution and biochemical profile displayed a differential vulnerability in AD. Furthermore, it is worth noting that the layer II cells in the entorhinal cortex that project to the hippocampus through the perforant path and contain glutamate as their main neurotransmitter are consistently affected in AD (59). McKee et al (60) recently demonstrated that hippocampal pyramidal cells that
are prone to NFT formation are selectively enriched in type II Ca\(^{2+}\)/calmodulin-dependent protein kinase. Finally, Cudkowicz and Kowall showed that in the neocortex of Huntington's disease patients, the large pyramidal cells that contain high concentrations of neurofilament proteins are severely damaged (61), an observation that parallels our findings in AD (24, 26). Further examples of a biochemical characteristic being linked to resistance or to degeneration have also been demonstrated. For instance, in the case of Ca\(^{2+}\)-binding protein-containing neurons, the presence of high concentrations of these proteins within rat hippocampal interneurons seems to protect them against seizure-induced damages (62). Similarly, GABAergic interneurons are preserved in human epileptic hippocampus (63). Therefore, the combination of factors such as high cytoplasmic levels of Ca\(^{2+}\)-binding proteins and GABA, and morphological features of locally-projecting interneurons may confer to these cells a heightened resistance.

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