Functional Alterations in Alzheimer's Disease: Diminution of Cytochrome Oxidase in the Hippocampal Formation

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Abstract. In Alzheimer's disease, the relationship between structural alterations such as neurofibrillary tangles and senile plaques and neuronal function is unknown. Cytochrome oxidase, the terminal enzyme of the electron transport, is a marker of neuronal functional activity. Its activity is diminished in experimentally deafferented neurons. Based on evidence that the molecular layer of the dentate gyrus is deafferented in the brains of individuals with Alzheimer's disease, we hypothesized that cytochrome oxidase activity would be diminished in this region secondary to reduced glutamatergic input. Using cytochrome oxidase histochemistry, we found a change in the distribution of cytochrome oxidase in the molecular layer of the dentate gyrus and a decrease in activity in both the dentate gyrus and hippocampal subfields in Alzheimer's disease. In contrast, we found relatively little structural pathology in the dentate gyrus, CA4, and CA3 in these individuals. These results suggest that neurons that remain structurally intact in Alzheimer's disease may nonetheless undergo changes in metabolic function as neural systems fail.

Key Words: Alzheimer's disease; Cytochrome oxidase; Deafferentation; Mitochondria.

INTRODUCTION

Neurons in layer II of the entorhinal cortex are among those affected earliest and most severely by neurofibrillary tangles (NFT) in Alzheimer's disease (AD) (1). These neurons provide the major afferent input to the hippocampal formation. Their projections via the perforant pathway terminate on the outer two-thirds of dendrites of the dentate granule cells. In contrast to the cell loss and NFT that occur in layer II of the entorhinal cortex in AD, granule cells are structurally intact and rarely develop NFT (2). Several lines of evidence, however, suggest that these neurons are deafferented. Loss of synaptic markers (3) and glutamate (4) and sprouting of cholinergic terminals in the terminal zone of the perforant pathway occur (5, 6), but the physiologic effect of these changes on neuronal function in AD is not known. We sought to determine whether neurons in the dentate gyrus and in hippocampal subfields receiving projections from the dentate gyrus have normal functional activity.

Cytochrome oxidase activity has been proposed to be a marker of neuronal functional activity (7). Cytochrome oxidase, or complex IV, is the terminal enzyme of the electron transport chain and is an integral transmembrane protein of the inner mitochondrial membrane. This enzyme catalyzes the transfer of electrons to generate adenosine triphosphate (ATP) via the coupled process of oxidative phosphorylation. Cytochrome oxidase activity is closely related to the level of functional activity of a neuron. Unilateral silencing of the cochlea in adult cats (8) and monocular deprivation of monkeys (9) and cats (10) results in decreased cytochrome oxidase activity in structurally intact neurons in the auditory nucleus and ocular dominance columns, respectively. In contrast, electrical stimulation of a chronically silenced cochlear nerve increases cytochrome oxidase activity in the auditory nucleus (11). Cytochrome oxidase histochemistry allows precise localization of the enzyme at the regional, laminar, cellular and subcellular levels (12). The linear relationship between the density of diaminobenzidine reaction product and cytochrome oxidase activity measured spectrophotometrically in brain homogenates (13) allows quantitative comparisons of enzyme activity by histochemical methods. Based on evidence of synaptic and glutamatergic loss in the dentate gyrus in AD, we hypothesized that cytochrome oxidase activity would be diminished in the dentate gyrus despite the fact that the dentate gyrus is structurally intact. We also hypothesized that cytochrome oxidase activity in all subfields of the Alzheimer hippocampus would be lower than in control hippocampus due to diminished afferent volley's in the first step of the entorhinal–dentate–Ammonic projection.

MATERIALS AND METHODS

Temporal lobes from ten patients with the clinical and neuropathological diagnosis of AD (mean age 77 years old, range 62–84) and seven age control subjects (mean age 71 years old, range 51–94) were obtained from the Alzheimer's Disease Research Center (ADRC) Brain Bank. The mean postmortem interval (PMI) for the AD cases was 9.4 hours (range 4–18) and for the control subjects 17.7 hours (range 4–48). Occipital lobes containing primary visual cortex from eight patients with AD (mean age 76.5 years old, range 51–96) and four aged control subjects (mean age 79.6 years, range 70–94) were also obtained. Layer IV of primary visual cortex was used as a control because the neurons in the lateral geniculate nucleus that project to this lamina are structurally intact in AD (14). The mean PMI for the AD cases was 12.1 hours (range 4–24) and control cases

S80
11.5 hours (range 6-16). In every AD case, the antemortem diagnosis of AD was confirmed neuropathologically (15); each case was free of coincident lesions such as infarcts or Lewy bodies. The control subjects were individuals older than 50 years of age in whom routine neuropathologic study showed that the number of senile plaques (SP) did not meet CERAD criteria for possible AD (15). Neuropathologic diagnoses were made independently by neuropathologists in the ADRC on examination of paraffin-embedded material using Bielchowsky silver stain for NFT and SP.

Tissue used in this study was fixed in paraformaldehyde-metaperiodate at 4°C for 24-48 hours and then cryoprotected in 15% glycerol in 0.1 M phosphate buffered saline, pH 7.4, for 24-48 hours until processing. Tissue blocks from the temporal lobe containing the hippocampal formation and from the occipital lobe containing area 17 were cut at 50 μm with a freezing sledge microtome and individually stored in glycerol buffer in microfuge tubes at -80°C until use.

Cytochrome oxidase activity was determined using the Wong-Riley method (16). The reaction mixture contained 50 mg of diminobenzidine, 30 mg cytochrome C and 20 mg catalase (SIGMA, St. Louis, MO) in 100 ml of 0.1 M phosphate buffered saline at pH 7.4. Free-floating tissue sections were incubated in the reaction mixture overnight at 4°C. As a control, some tissue sections were incubated in the reaction mixture containing 10 μM potassium cyanide, a specific inhibitor of cytochrome oxidase, and no staining was seen.

Using the Bioquant Image Analysis System, the optical density of cytochrome oxidase histochemical product was measured in the outer and inner molecular layers of the dentate gyrus, CA4, CA3 and CA1 of the hippocampal formation, and in layer 1Vc of area 17 using a 16× objective. Three measurements were made in the inner and outer portions of dentate molecular layer and layer 1Vc of area 17 and ten measurements in CA4, CA3, and CA1, and the means of the measurements were recorded. Measurements were made under the identical optical conditions. An image of a blank area of each slide was captured and subtracted from each area in which measurements were made to correct for background. The operator was unaware of the diagnostic category. A translucent strip with increasing steps of grey density (Kodak, Rochester, NY) was used as an external standard to quantitate optical density measurements. Optical density was converted to diffuse density units (a negative log function of the optical density) which are then directly proportional to the optical density of the histochemical product.

Comparisons between AD and control hippocampal subfields were made using a mixed between–within subject ANOVA (SAS program, Statistica Analytical Systems, Inc., Cary, NJ). The Student t-test was used to compare staining density between AD and control densities in layer 1Vc of area 17.

Fluorescence microscopy was used to visualize NFT and SP in thioflavine S-stained sections. For the thioflavine S procedure, sections were pretreated with a 2:1 chloroform/ethanol mixture, rehydrated in water, stained with 0.1% thioflavine S for 5 minutes, differentiated briefly in 80% ethanol, and counterstained with Gmelin’s coverslipping media. Senile plaque and NFT counts were made in the dentate gyrus, CA4, CA3, CA1 and layer 1V of area 17 using a semiquantitative degree of severity according to the following scheme: + = 1-10 NFT or SP/1.2 mm² (10× microscopic field), + + = 11-25 NFT or SP/1.2 mm², + +++ = 26-50 NFT or SP/1.2 mm², and +++++ = >50 NFT or SP/1.2 mm².

RESULTS

Distribution of Neurofibrillary Tangles and Senile Plaques

The distribution of NFT and SP in the hippocampal formation was similar in all AD samples. The greatest number of NFT was seen in CA1, with an average of >50 NFT/1.2 mm². In contrast, only 1-10 NFT/1.2 mm² were seen in CA3 and CA4 and none in the dentate granule cells. Senile plaques were more evenly distributed across hippocampal subfields with an average of 1-10 SP/1.2 mm² in the dentate gyrus, CA4, CA3 and CA1. One control sample had 1-10 NFT/1.2 mm² in CA1 only and the remainder of the controls were without NFT or SP in the hippocampal formation.

In layer 1Vc of area 17, 1-10 NFT/1.2 mm² were seen in two of the eight AD samples. The remainder of AD samples and all control samples had no NFT. Senile plaques were seen more frequently than NFT, with an average of 11-25 SP/1.2 mm² in the majority of AD and half of the control samples.

Cytochrome Oxidase Histochemistry in the Hippocampal Formation

In the hippocampal formation of control individuals, cytochrome oxidase activity was highest in the outer molecular layer of the dentate gyrus (Figs. 1, 2A). The lowest activity was seen in the inner dentate gyrus molecular layer. In all control samples, there was a clear demarcation between the inner and outer molecular layers (Fig. 3A). In CA4, CA3 and CA1, cytochrome oxidase activity was seen throughout the neuropil and in cell bodies and dendrites of pyramidal neurons. Cytochrome oxidase was not present in cell bodies of the granule cells.

In all AD brains the distribution of cytochrome oxidase activity in the molecular layer of the dentate gyrus was different than controls. The dark band of cytochrome oxidase activity in the outer molecular layer seen in controls was not present, and activity in the inner portion was equal to or greater than the activity in the outer (Figs. 1, 3B). This difference in the distribution of cytochrome oxidase activity in the dentate gyrus was highly significant, F(1,15) = 6.79, p < 0.02. In the molecular layer of the dentate gyrus and in the hippocampal subfields CA4, CA3 and CA1, the mean cytochrome oxidase activity as measured by the diffuse density of histochemical staining was also significantly lower in AD than in control individuals, F(1,15) = 5.92, p < 0.03 (Figs. 1, 2B), though the rank order of activity, with CA1 the highest and CA4 the lowest, was not different from control individuals. The greatest difference in cytochrome oxidase activity...
Fig. 1. Mean diffuse density of cytochrome oxidase histochemical staining in the hippocampal formation (seven aged control and ten AD subjects) and primary visual cortex (four aged controls and eight AD subjects). The scale of the vertical axis is diffuse density units which are directly proportional to the darkness of histochemical product. The horizontal axis is area where the reaction product was measured. DG out = outer molecular layer of dentate gyrus. DG in = inner molecular layer of dentate gyrus. Area 17 = layer 1Vc, area 17. * Statistical significance p < 0.05; error bar = standard error of the mean.

was in the outer molecular layer of the dentate gyrus where there was a 32% reduction in the diffuse density of staining. There was no correlation between cytochrome oxidase activity and age or postmortem interval in either AD or control patients.

Cytochrome Oxidase
Histochemistry in Visual Cortex

In control individuals, a dark band of cytochrome oxidase activity was seen in the neuropil in layer IV of area 17 with a sharp demarcation at the 17/18 border (Fig. 4A). In AD brains, the distribution of histochemical staining in primary visual cortex was the same (Fig. 4B). Unlike the hippocampal formation, there was no significant difference in cytochrome oxidase activity in layer 1Vc of area 17 between AD and control samples (Fig. 1).

DISCUSSION

The main findings of this study are that the distribution of cytochrome oxidase activity in the dendrites of dentate granule cells of individuals with AD differs from the pattern in aged controls, and the overall activity in the dentate gyrus and hippocampal subfields is decreased. The sharp demarcation between the dense staining in the outer molecular layer of the dentate gyrus and the light staining in the inner portion seen in normal control subjects is lost in individuals with AD. Significant cell loss in the dentate gyrus does not occur in AD (17, 18) and we saw no NFT and only occasional SP in the dentate gyrus of our samples. The alteration in cytochrome oxidase activity observed in the dendrites of the dentate granule cells is thus occurring in neurons that are structurally intact.

The same alteration in the pattern of cytochrome oxidase activity in the dentate gyrus that we observed in AD has been reported to occur in rats after lesions of the entorhinal cortex (19). This similarity suggests that a common pathophysiologic mechanism may exist. In AD, neurons in layer II of the entorhinal cortex, the origin of the perforant pathway, have large numbers of NFT (1). The perforant pathway terminal zone in the outer portion of the molecular layer of the dentate gyrus undergoes synaptic (3) and glutamatergic loss (4). We have now shown diminished cytochrome oxidase activity in the recipient neurons of the perforant pathway, the granule cells. These results suggest that neuronal dysfunction in
AD may in part be a result of decreased excitatory input to neurons in specific neural pathways.

Diminished cytochrome oxidase activity was also found in all hippocampal subfields. This decrease in the hippocampal formation was seen both in an area that contained a substantial number of NFT, CA1, and areas relatively spared of both NFT and SP, CA3 and CA4. Because the perforant pathway initiates a series of excitatory projections around Ammon's horn, functional deafferentation of the dentate gyrus from excitatory cortical input could cause diminished functional activity polysynaptically in projections from dentate gyrus to CA4 and CA3, and subsequently of the Schaffer collaterals from CA3 to CA1. In support of this hypothesis, a polysynaptic decrease in cytochrome oxidase activity has been shown in human primary visual cortex after monocular deprivation (20).

In contrast to the hippocampal formation, we found no difference in cytochrome oxidase activity in layer IV of primary visual cortex between AD and control samples. In comparison to the hippocampal formation, NFT and SP were much less common in this region. Furthermore, in contrast to the entorhinal cortex, the lateral geniculate nucleus from which the major input to layer IV of area 17 originates is without structural changes (14).

This is the first report of cytochrome oxidase histochemistry in human hippocampal formation and in Alzheimer's disease. Our findings complement the work of Chandrasekaran et al (21) who used *in situ* hybridization to detect a decrease in cytochrome oxidase subunit II mRNA in hippocampal subfields and in the dentate gyrus in AD. The only other study of cytochrome oxidase activity in AD measured cytochrome oxidase activity in frozen brain homogenates and found reduced activity in frontal and temporal cortex, but no difference in the hippocampus in AD (22). Cytochrome oxidase activity in tissue homogenates could appear to be unchanged if the homogenates represent several anatomical regions, some of which are unaffected. However, our data show a decrease in cytochrome oxidase activity both in the hippocampal formation and in the surrounding parahippocampal gyrus (data not shown), thus the basis for the discrepancy between our results is not clear.

In broader terms, there is growing evidence that alterations in energy metabolism may exist in AD (23). Hypometabolism of higher order association cortices has been demonstrated by positron emission tomography (24), and mitochondrial dysfunction has been postulated to occur in AD patients (25). Our results support the idea that brain hypometabolism in AD may be due in part to
deafferentation of components of neural systems linked to memory and cognition. We have recently suggested that reduced NADPH-diaphorase (nitric oxide synthase) activity in the perforant pathway terminal zone in AD may be similar to downregulation of granule cell functional activity (26). Together, these results reinforce the idea that neurons that remain structurally intact in AD may nonetheless undergo substantial changes in chemical and metabolic function as neural systems fail.

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